# PPARs: A Double-Edged Sword in Cancer Therapy?

Guest Editor: Dipak Panigrahy, Arja Kaipainen, Mark Kieran, Judah Folkman, and Sui Huang
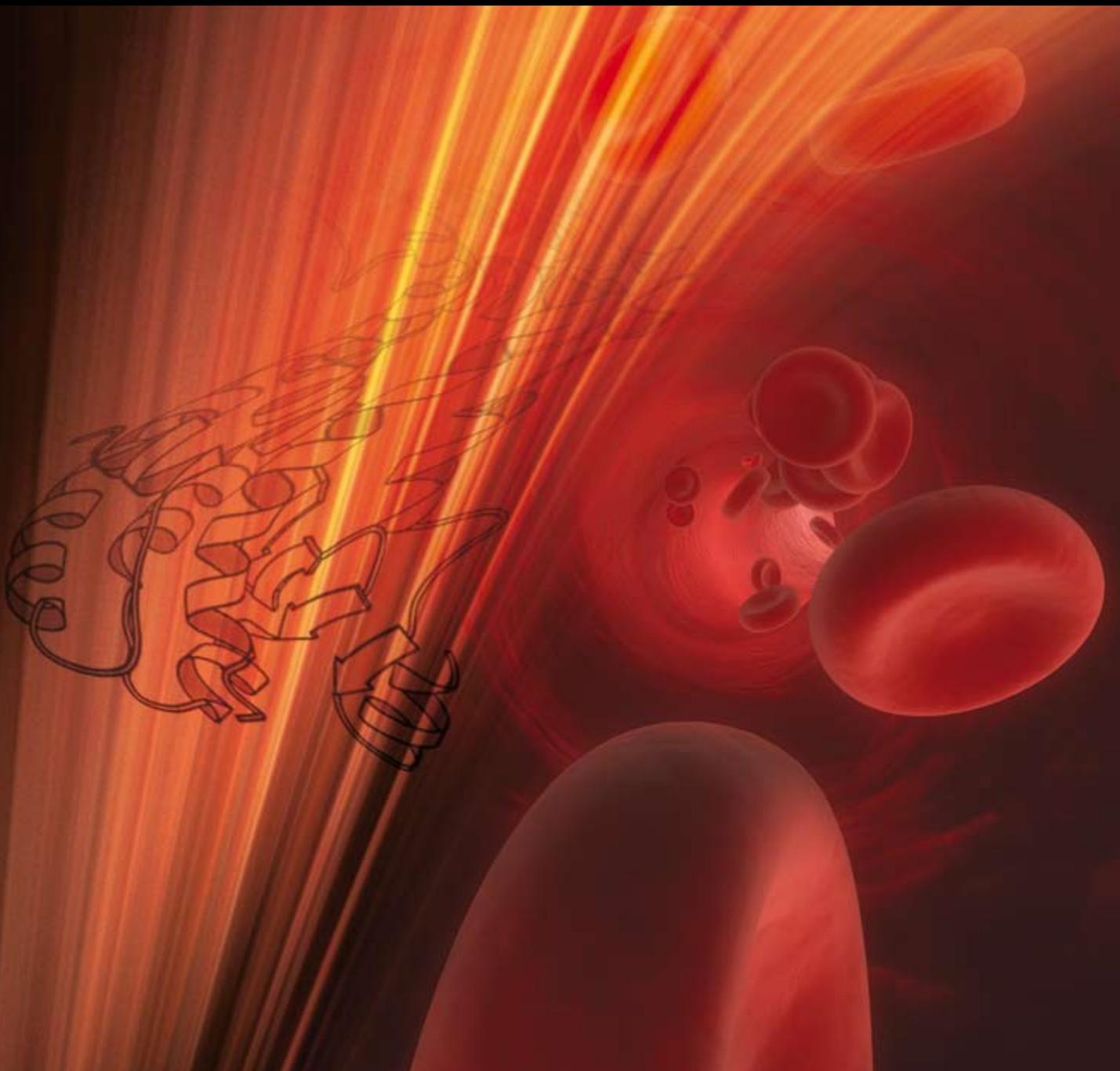
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Editorial

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Welcome to this special issue of PPAR Research, PPARs: A Double-Edged Sword in Cancer Therapy. Peroxisome Proliferator-Activated Receptors (PPARs) are a family of pleiotropic transcription factors that play central roles in cell metabolism and regulation of inflammation. Cancer is thought to be the uncontrolled clonal evolution and expansion of a mutated cell [1]. What is the connection between PPARs and cancer? The theme of this special issue reflects the impressive confluence of two originally separate streams of investigation: PPARs and cancer research. Despite the multitude of points of intersection between PPARs and neoplasia, and associated unresolved paradoxes, the link between PPARs and tumors is not yet widely appreciated among specialists in either field. However, over the past years, investigators who cross these fields have unearthed a myriad of remarkable connections.

In view of the multiple links between PPARs and cancer, perhaps epitomizing the pleiotropy of the biological effects of PPARs, this special issue contains an unusually large number of excellent contributions. This large volume may also reflect the increasing recognition of PPARs as a key player in cancer. To help guide the readers, we have organized the articles, in a departure from tradition, not according to the subtypes PPARα, δ/β and γ, but instead, broadly in Sections 1–6. Section 1 contains reviews that offer a comprehensive overview on PPARs' effects in cancer and on the more established role of PPARγ in cancer therapy. This is followed by Sections 2, 3, and 4 which have articles that discuss the following three key questions.

   (i) *Are PPARs friends or foes of tumors?*

   (ii) *Do PPARs modulate tumor cell-autonomous or noncell autonomous processes?*

   (iii) *Are the cancer-related effects of PPAR ligands mediated by a PPAR-dependent or independent mechanism?*

We close our special issue with Sections 5 and 6, which focus on PPAR ligand-based cancer therapies and the molecular mechanisms through which these ligands may act.

(1) We start with five reviews that provide the necessary background on structure and physiology of PPARs with an emphasis on their role in cancer. One of these reviews focuses on PPARγ, which historically has been studied most intensely in conjunction with cancer. Notably, the therapeutic potential of PPARγ agonists has been evaluated in clinical trials for liposarcoma and prostate cancer. In fact, 38 out of a total of 56 articles in this issue focus on PPARγ.

(2) We continue with the first key question: *Do PPARs promote or inhibit cancer?* The opposite directions of observed PPAR effects on cancer—which gave this special issue its title—cannot be addressed in a straightforward manner. This is not because of ambiguous observations but (what makes it interesting) because the observed effects of PPAR on tumors have been clear—cut and powerful in either direction—either stimulating or suppressing tumors. That PPARs act as a "double-edged sword" may not come as a surprise to veterans of PPAR research who appreciate their pleiotropic effects. While PPARα was the first PPAR to be associated with tumorigenesis, the emerging awareness of the PPARγ-cancer connection is evidenced by the fact that 4 out of 7 reviews in this issue focus on PPARγ.

(3) While much attention has been devoted to determining whether PPARs are friend or foe of tumors, our second question is also of fundamental significance: *Is PPAR's role in tumor growth mediated by cell autonomous or by noncell autonomous mechanisms?* From the perspective

of PPAR investigators, this question may arise naturally because PPARs regulate intracellular processes, including proliferation, apoptosis, and differentiation as well as inflammatory processes through the control of mediators in cell-cell communication. In cancer biology this dualism has deeper roots. It is the subject of a major paradigm shift that has occurred over the past decade in cancer research. The simple notion, unquestioned for decades, that cancer is a cell-autonomous disease, driven by mutation and selection for fast growing and increasingly malignant cell clones, has yielded to the more encompassing view that cancer is also a nonautonomous disease, requiring the support from the "tissue microenvironment" in the "tumor bed".

It took many years to overcome the picture of cancer cell autonomy afforded by cellular oncogenes. It began with a simple idea that had far-reaching consequences. Judah Folkman proposed in 1972, against all conventional wisdom, that tumor growth required neovascularization, and that such "tumor angiogenesis" was induced by soluble factors produced by the tumor. We dedicate this special issue to Dr. Folkman (1933–2008), our teacher and mentor, who has opened the world's eye to the tissue context of tumors. His arduous uphill battle against the established paradigm of cell-autonomous growth, although focused on angiogenesis, has shined the first beam of light on the role of the host microenvironment which was hidden in the shadow of the quest for mutations that establish the oncogenic pathways in the cancer cell. Dr. Folkman's persistence paved the path to the acceptance of the active role of nonneoplastic, "host" cells in the tumor microenvironment. In this generalization of the concept of tumor angiogenesis, it is now firmly established that the tumor stroma is comprised a variety of cells that are essential for tumor growth, including "tumor associated fibroblasts", various inflammatory cells, and the pericytes around the tumor endothelium.

Much as cancer research was initially focused on the tumor parenchyma, the first connection between PPARα and tumorigenesis was also directed at understanding how prolonged PPARα activation by its ligands induces hepatocarcinogenesis in rodents by altering liver cell function [2]. However, mirroring the development in tumor biology, attention soon turned toward the effects of PPAR on the tumor microenvironment. In this issue, ten articles discuss the modulation of the tumor stroma by PPARs. Five of these reviews discuss their effects on the tumor endothelium, while the other five focus on the inflammatory compartment.

(4) The third major question addressed in this issue refers to the tumor-inducing or inhibiting effects of PPAR ligands: are their activities on tumors mediated by their nominal targets, the nuclear receptors, or do they act in a PPAR-independent manner? This matter is complicated by the fact that both PPAR agonists and antagonists can inhibit tumor progression. Six reviews provide an overview of the use of PPAR agonists and their "off-target" effects in various cancer therapies. We have also included one original research article on how rosiglitazone inhibits both tumor and endothelial cells via receptor dependent and independent mechanisms.

(5) The vast majority of PPAR research in the context of cancer focuses on the use of ligands in anticancer therapies.

Thus, we dedicate the next section to articles that review preclinical and clinical studies of the use of PPARα and PPARγ ligands in a variety of cancer models, including combinatorial therapy.

(6) The last section of this special issue contains articles that review the molecular mechanisms through which PPARs, or their ligands, modulate tumor growth. There is an additional original research article in this section on how rosiglitazone inhibits tumor cell proliferation by interfering with IGF-IR signaling.

We hope you will find these articles informative. Clearly, much work lies ahead if we are to unravel the mysteries behind the double edged-sword nature of PPARs. This special issue describes the problem from many angles, and in doing so it reveals the gaps in our knowledge. Thus, rather than providing a unifying answer, it may hopefully inspire you to further research.

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Review Article

PPARs Mediate Lipid Signaling in Inflammation and Cancer

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Lipid mediators can trigger physiological responses by activating nuclear hormone receptors, such as the peroxisome proliferator-activated receptors (PPARs). PPARs, in turn, control the expression of networks of genes encoding proteins involved in all aspects of lipid metabolism. In addition, PPARs are tumor growth modifiers, via the regulation of cancer cell apoptosis, proliferation, and differentiation, and through their action on the tumor cell environment, namely, angiogenesis, inflammation, and immune cell functions. Epidemiological studies have established that tumor progression may be exacerbated by chronic inflammation. Here, we describe the production of the lipids that act as activators of PPARs, and we review the roles of these receptors in inflammation and cancer. Finally, we consider emerging strategies for therapeutic intervention.

1. INTRODUCTION

Signal lipids are known to trigger systemic physiological responses, to control inflammatory reactions, and to regulate key cellular processes, such as cellular energy metabolism, cell survival, proliferation, migration, and differentiation [1]. Among these lipids, fatty acids, diverse fatty acid derivatives, some eicosanoids, and sterol derivatives are modulators of gene expression via binding and activation of the nuclear hormone receptors (NHRs) peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), and farnesoid X receptor (FXR) [2]. These transcription factors control genes that regulate lipid homeostasis [2] and, for PPARs in particular, inflammatory responses [3]. Disturbance of lipid signaling and/or NHR pathways promotes the progression of a long list of imbalances and diseases, such as obesity, type 2 diabetes, chronic inflammation, cardiovascular diseases, cancer, hypertension, degenerative diseases, autoimmune diseases, and a few others [1, 2]. Important cross-regulation exists between lipid signaling and NHR pathways, which generates a variety of responses dependent on signaling networks that are often tissue-specific [1].

In this paper, we propose an integrated view of the production of the lipids that activate PPARs, and of the functions of these receptors in inflammation and cancer. We conclude with comments on therapeutic opportunities.

The three PPAR isotypes (PPARα or NR1C1, PPARβ/δ or NR1C2, and PPARγ or NR1C3) share a high degree of structural similarity with all members of the nuclear hormone receptor superfamily [4–6]. The cellular and systemic roles that have been attributed to PPARs extend far beyond the control of hepatic peroxisome proliferation in the rodents after which they were initially named [2, 3, 7]. PPARs exhibit isotype-specific tissue expression patterns, with PPARα expressed at high levels in organs with a significant catabolism of fatty acids, PPARβ/δ in all cell types analyzed so far with levels depending on the extent of cell proliferation and differentiation, and with PPARγ found at high levels in the adipose tissues and lower levels in colon, immune cells, and other tissues [8]. Transcriptional regulation by PPARs requires heterodimerization with the retinoid X receptor (RXR), and interactions with coregulator complexes [9–11]. When activated by a ligand, the PPAR:RXR dimer controls transcription via binding to the peroxisome proliferator response element (PPRE) in the regulatory region of target genes [9]. The selective action of PPARs in different tissues results from the combination, at a given time point, between expression levels of each of the three PPAR and RXR isotypes, affinity for a specific regulatory PPRE, ligand production

by lipid-modifying enzymes, and cofactor availabilities [12].

2. PRODUCTION OF ENDOGENOUS PPAR LIGANDS

The prevalent point of view today is that the three PPAR isotypes function, in a broad sense, as lipid sensors that translate lipid signals from different origins into responses whose aim is to maintain energy homeostasis, in response to the different physiologic challenges to which the body is exposed. However, the connection between lipid metabolism pathways and PPAR responses was only recently unveiled. The production and nature of the endogenous ligands or mediators of PPAR activation have not been well characterized although it is known that many lipid-modifying enzymes are involved. The pathways that generate these lipid signals from fatty acids, which also serve as PPAR ligands, are recapitulated in Figure 1.

ω-3 and ω-6 polyunsaturated fatty acids are stored in membrane phospholipids and lipid bodies, and are released by cytosolic phospholipase A2 (cPLA2) [13]. ω-6 fatty acids, predominantly arachidonic acids, are abundant in the western diet and they are often converted to leukotrienes, prostaglandins, and other cyclooxygenase or lipoxygenase products [13]. They regulate cellular functions with inflammatory, atherogenic, and prothrombotic effects [13]. The ω-3 fatty acids, such as docosahexaenoic acid and eicosapentaenoic acid, are also substrates for cyclooxygenases and lipoxygenases. Interestingly, ω-3 fatty acid-derived eicosanoids antagonize the proinflammatory effects of ω-6 fatty acids by downregulating inflammatory and lipid synthesis genes, and by stimulating fatty acid degradation [13]. Many eicosanoids bind to PPARs and control tissue homeostasis and inflammation [3, 14].

The epoxygenases are a group of microsomal cytochrome P450s (CYP) enzymes that convert arachidonic acid to epoxyeicosatrienoic acids (EETs), which function primarily as autocrine and paracrine mediators in the cardiovascular and renal systems [15]. These mediators, which are unstable and are rapidly metabolized in most tissues, have important roles in cellular migration and proliferation, and in inflammation. Although their mechanism(s) of action is not fully understood, the epoxygenase pathway can generate potent ligands for the PPARs, which participate in antiatherogenic, antithrombotic, and cardioprotective processes that may be targeted by new therapeutic developments in vascular and inflammatory disorders [16].

The various lipases have unique pattern of expression, distinct biological actions, and preferred substrate from which they release diverse products [17]. They preferentially hydrolyze triglycerides versus phospholipids, and use lipoproteins, such as very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs), as substrates [17]. Hydrolysis of triglycerides within triglyceride-rich lipoproteins by the lipoprotein lipase (LPL) results in the transfer of lipids and apolipoproteins to HDLs. In turn, hepatic lipase (HL) hydrolyzes HDL triglyceride and phospholipids, generating smaller lipid-depleted HDL particles. Finally, endothelial lipase (EL)

might hydrolyze HDL phospholipids, thus promoting HDL catabolism [17]. Lipases generate various lipolytic products such as fatty acids with different chain lengths and degrees of saturation, as well as other molecules such as monoacylglycerol. While fatty acids can be oxidized in order to gain energy, or alternatively stored in fat, they can also direct transcriptional responses. PPAR activation, as a consequence of lipolysis, underscores a key role of the functional interplay between lipases and lipoproteins. It was reported that LPL acts on circulating lipoproteins to generate PPARα ligands that induce endothelial vascular cell adhesion molecule 1 (VCAM1) [18]. LPL can release HODEs, which are known as PPARα agonists, from electronegative LDL, thereby reversing the proinflammatory responses of this lipoprotein. Similarly, HDL hydrolysis, and to a lesser extent hydrolysis of LDL and VLDL, by EL can also activate PPARα [19, 20]. In macrophages, VLDL regulates gene expression through activation of PPARβ/δ, an activation that depends on the release of the VLDL triglycerides by LPL [21]. An additional lipase, named as adipose triglyceride lipase, desnutrin, iPLA2ζ, or transport secretion protein 2, was identified more recently. It increases the availability of fatty acids from VLDL, resulting in increased PPARβ/δ activity [22–24]. Obviously, the combination of a variety of lipases and lipoproteins and the resulting distribution in the organism of fatty acids and their often short-lived derivatives did not enable a precise characterization of their impact on PPAR functions as a whole. Furthermore, activation of PPARs by ligands produced by the different lipid signaling enzymes can lead to a feedback stimulation or inhibition of the expression of these enzymes (see Section 3).

3. GUIDING LIGANDS TO PPARs: ROLES OF FABPs

Both fatty acid binding proteins (FABPs) and retinoic acid binding proteins (CRABPs) belong to an evolutionarily conserved family of intracellular proteins [25]. Various functions have been attributed to these proteins, including cellular uptake and transport of fatty acids, the targeting of fatty acids to specific metabolic pathways, and the regulation of gene expression and cell growth [26]. Interestingly, FABPs are thought to deliver ligands to the PPARs. For instance, specific interactions with fatty acid-loaded adipocyte FABP (FABP4) and keratinocyte FABP (FABP5) selectively enhance the activity of PPARγ and PPARβ/δ, respectively [27]. In this function, FABPs relocate to the nucleus when bound to ligands that are selective for the PPAR isotype they activate, and thus FABPs mediate the transcriptional activities of their own ligands. Retinoic acid receptors (RARs) belong to the same type-2 class of receptors as PPARs in the nuclear receptor superfamily [12]. A coevolution between the fatty acid and retinoid-binding protein families and the RAR and PPAR families can be postulated, which has promoted the emergence of a mechanism for directing a ligand to the appropriate receptor. The two associated systems, FABPs-PPARs and CRABPs-RAR, show some promiscuity at the expense of specificity, but in favor of an increased diversity in transcriptional responses. Depending on the ratio of FABP5 to CRABP-II, RA activates RAR or PPARβ/δ. Surprisingly,
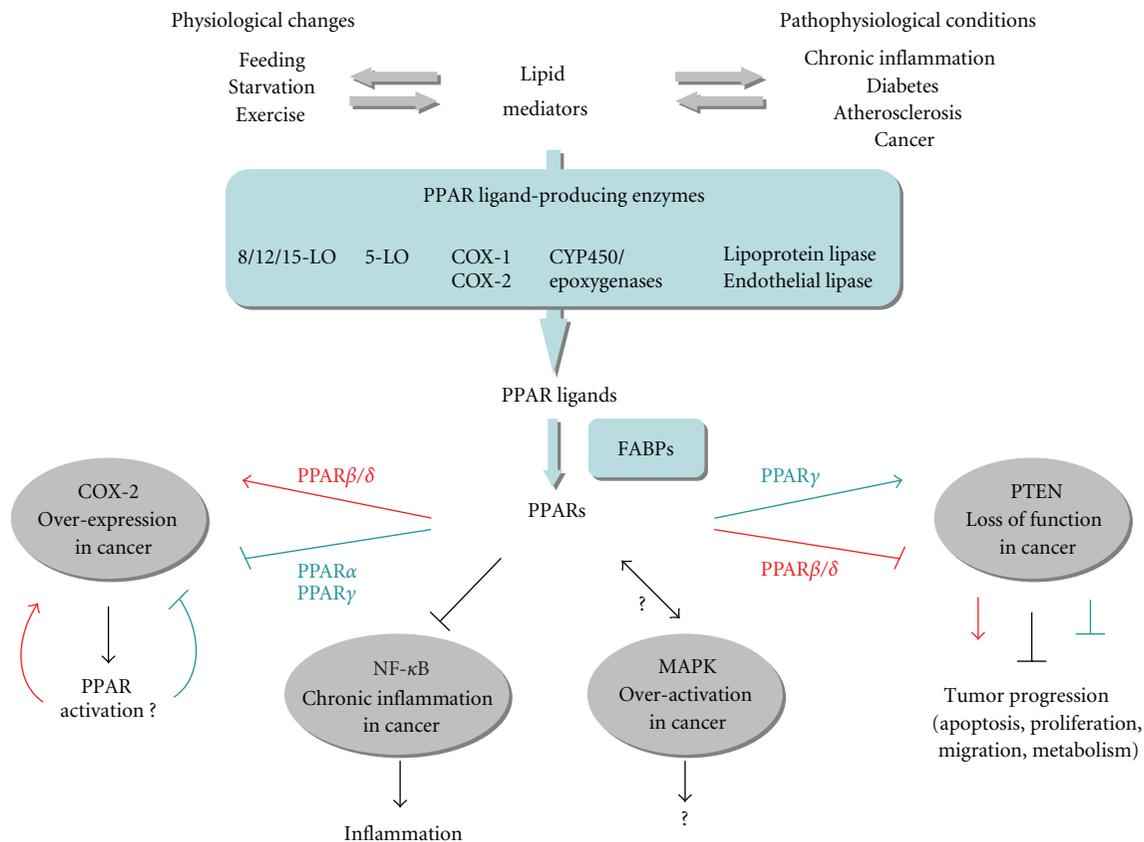
FIGURE 1: PPARs are mediators of lipid signaling in inflammation and cancer. Lipid mediators originate from and participate in the control of physiological and pathophysiological situations. Many lipid-modifying enzymes are involved in the production of PPAR ligands. The cyclooxygenases (COX), lipoxygenases (LO), epoxygenases/cytochrome (CYP)/P450s enzymes, and the lipases use either fatty acids, triglycerides, or phospholipids as substrates to generate PPAR ligands, which are guided to their receptors by the cytoplasmic fatty acid binding proteins (FABPs). PPARs translate these lipid signals into responses, which maintain energy homeostasis, regulate inflammation and modify tumor growth. Among the pathways involved in inflammation and cancer, PPARs interact with COX2, NF-κB, MAPKs, and PTEN. PPARα and γ inhibit COX2 expression, thereby reducing the production of their own ligands. Conversely, PPARβ/δ is thought to activate COX2 expression, generating a positive feedback loop by increasing the production of PPAR ligands. PPARs reduce inflammation by inhibiting NF-κB, a major pathway that links chronic inflammation to cancer promotion. Several modes of interactions between PPARs and MAPKs have been reported, but the relevance and consequences of such crosstalks are unclear. Finally, PPARβ/δ and γ decrease and increase the expression of the tumor suppressor PTEN (phosphatase and tensin homologue deleted from chromosome 10), respectively. PPARγ activation of PTEN is thought to potentiate its tumor suppressor function, whereas PPARβ/δ would have the opposite effect.

when the FABP5-to-CRABP-II ratio is high, RA serves as a physiological ligand for PPARβ/δ, which broadens the spectrum of physiological regulation due to the activity of this receptor in an unexpected way [28, 29]. The key issue raised by these studies concerns the importance of the role of directed ligand transport in nuclear receptor activation, and ligand-dependent crosstalk between different receptor types [29]. Overruling ligand selectivity between receptor categories by this mechanism might promote a promiscuity that may contribute significantly to the pleiotropic effects of key members of the nuclear receptor superfamily [28, 29].

Similarly to the genes encoding lipid-signaling enzymes, the expression of FABPs is controlled by PPARs in specific situations. L-FABP is highly expressed in the liver and small

intestine, where it plays an essential role in controlling cellular fatty acid flux. Its expression is increased by both the fibrate hypolipidemic drugs and LCFAs. The different PPAR isotypes (α, β/δ, and γ) promote the upregulation by FAs of the gene encoding L-FABP in vitro, while PPARα is an important regulator of L-FABP in the liver, but not in the intestine [30, 31]. In contrast, only PPARβ/δ is able to upregulate the gene encoding L-FABP in the intestine of PPARα-null mice. Thus, PPARβ/δ contributes to metabolic adaptation of the small intestine to changes in the lipid content of the diet [30, 31]. In summary, FABPs bind PPAR ligands within the cytoplasm, channel this cargo to the respective nuclear receptors, and by so doing influence their activation, which sometimes regulates their own expression [32].

4.   PPARs IN INFLAMMATION AND CANCER

Although acute inflammation is a necessary process aimed at protecting the organism after an injury or an infection, unresolved chronic inflammation may promote cancer formation by providing an appropriate environment for tumor growth [33, 34]. Mechanisms that link inflammation and cancer have only recently been studied, but epidemiological studies show a convincing association between them (see [33–36] and references therein). For example, hepatitis is often followed by the development of hepatocarcinoma, ulcerative colitis is a risk factor for colon cancer, and inflammation due to infection by *Helicobacter pylori* precedes the majority of gastric cancers [34]. In the lungs also, the risk of developing lung cancer is higher in patients suffering from asthma or from chronic bronchitis [37, 38].

The role of immune cells in tumor development is not yet fully understood. Although inflammatory mediators may promote cancer development, immune cells can also secrete cytokines that can limit tumor progression [33–35]. Data collected from mouse models suggest that the role of the immune system in cancer is likely to depend on the profile of cytokines secreted by the immune cells. Modifying this profile may contribute to the development of new treatments [33]. Based on present knowledge, the NF-κB and COX2 pathways have emerged as important links between inflammation and cancer (reviewed in [36, 39–42]). Consistent with inflammation and COX2 favoring the development of tumors, long-term use of NSAIDs, albeit at relatively high doses, prevents colorectal tumor development [43].

The roles of PPARs in tumor development are still unclear and their pro- or anticarcinogenic effects remain open to discussion (reviewed in [7, 44]). PPAR activity has been associated with numerous cancer types in organs such as the liver, colon, skin, prostate, breast, and lung (reviewed in [7, 45]). The mechanisms reported so far suggest that the anticarcinogenic activity of PPARs is due to direct effects in the cancer cells themselves, such as inhibition of the cell cycle, activation of cell differentiation, or cell death (reviewed in [7, 45]). But in addition to these functions, one can speculate that PPARs may have non-cell autonomous effects by acting on the tumor environment. In fact, PPARs regulate inflammatory processes [3, 46, 47], and they fulfill vital regulatory functions in cells that are important components of the tumor stroma, such as immune or endothelial cells [35, 48–51]. In line with the link between inflammation and cancer promotion, we provide below an overview of PPARs' involvement in organs in which inflammatory pathways and cancer development are known to have been connected, namely, the skin and the digestive tract.

4.1.   Skin, inflammation, and cancer

An analysis of various models of PPAR activation or invalidation shows that PPARs are not absolutely indispensable for normal epidermal maturation and renewal, but that they accelerate mouse and human keratinocyte differentiation, as well as mouse epidermal barrier recovery after disruption

(reviewed in [52, 53]). In addition, PPARα and PPARβ/δ activation regulates human hair follicle survival and mouse hair follicle growth, respectively, whereas the roles of PPARs in the sebaceous glands remain unclear (reviewed in [52]).

After an injury, skin repair involves the recruitment of inflammatory cells, the migration and proliferation of keratinocytes, activation of dermal fibroblasts, and angiogenesis [54]. Though undetectable in the interfollicular epidermis of healthy rodent skin, the expression of PPARα and PPARβ/δ is reactivated in the epidermis at the edges of skin wounds [55]. The expression of PPARα is upregulated early after the injury, but the signal involved is unknown. The study of genetically modified mice showed that no, or low, PPARα activity results in impaired inflammatory reaction, which causes a transient delay in healing [55, 56]. The upregulation of PPARβ/δ expression, as well as the production of an unknown endogenous agonist, is triggered by proinflammatory cytokines, such as TNF-α [57], whereas TGFβ-1 signaling is responsible for the repression of inflammatory-induced PPARβ/δ expression at the end of the healing process [58]. The completion of skin healing in the PPARβ/δ-null animals is delayed, mostly because of impaired epithelialization due to apoptosis and defects in keratinocyte adhesion and migration [55, 59, 60]. Consistent with decreased healing efficiency in its absence, prolonged expression of PPARβ/δ accelerated wound closure [61, 62], whereas premature downregulation of PPARβ/δ expression temporarily delayed wound closure [62]. In summary, PPARα and PPARβ/δ both promote the healing of skin wounds. PPARα prevents exacerbated early inflammation, while PPARβ/δ, whose expression and activity are increased by inflammatory cytokines, enhances keratinocyte survival and migration.

Inflammatory skin disorders are usually characterized by keratinocyte hyperproliferation and aberrant differentiation, as observed in psoriasis [63, 64]. Moreover, numerous lipid molecules, which are potent activators of PPARs, are produced in the psoriatic lesions where they accumulate [65]. Consistent with stimulated expression by inflammatory cytokines after skin injury in the mouse [57], the PPARβ/δ levels are particularly high in the hyperproliferative lesional skin of psoriatic patients [66], while those of PPARα and PPARγ remain unchanged, or even decrease [65, 66]. Overall, PPAR activation reduces inflammation in skin disorders [53]. It is well documented that PPARα activation is beneficial in mouse models of hyperproliferative epidermis [67], in models of irritant and allergic dermatitis [68], and in a model of atopic dermatitis [69]. Interestingly, PPARα may be the molecular target of the antiallergic and anti-inflammatory effects of palmitoylethanolamide, a natural fatty acid derivative present in murine skin [70]. PPARγ activation also has beneficial consequences in various models of psoriatic skin, such as in organ cultures, in a model of human psoriatic skin transplant, and in murine models of keratinocyte hyperproliferation [71, 72]. Despite these promising studies in models of psoriatic skin, PPARα, PPARβ/δ, or PPARγ activation did not improve skin homeostasis when locally applied on psoriatic plaques [73, 74]. However, PPARγ agonists thiazolidinediones efficiently

normalized skin homeostasis when orally administered to patients suffering from psoriasis (reviewed in [75, 76]), suggesting that their beneficial effects are most likely due to systemic anti-inflammatory functions of PPARγ.

The skin is constantly exposed to many types of aggression, including carcinogens such as xenobiotics or UV. Much remains to be explored regarding PPAR functions in skin cancers, either squamous or basal cell carcinomas (tumors of keratinocyte origin) or melanomas (tumors of melanocyte origin) (reviewed in [52]). Activation of PPARα and PPARγ reduces proliferation and stimulates differentiation of cultured melanocytes [77, 78]. Several PPARγ agonists inhibit the proliferation of human malignant melanomas [79], and the PPARα agonist fenofibrate has antimetastatic effects on melanoma tumors in vivo in a hamster model [80]. Interestingly, combined treatment with the PPARγ agonist pioglitazone, the COX2 inhibitor rofecoxib, and angiostatic chemotherapy stabilized or even reversed chemorefractory melanoma progression, though in only 11% of the treated patients [81]. In a search for genetic factors that may increase melanoma risk, correlation between PPARγ variants and melanoma development in a Caucasian population indicated that PPARγ polymorphisms are an unlikely risk factor for melanoma development in this population [82]. In tumors of keratinocyte origin, increased expression of PPARβ/δ was reported in head and neck squamous carcinoma [83]. In a mouse model of DMBA/TPA-induced skin tumors, PPARβ/δ-null animals showed enhanced tumor formation, suggesting that PPARβ/δ attenuates tumor development. A possible mechanism of this effect is that, by activating the expression of ubiquitin C, PPARβ/δ activates the ubiquitin degradation pathway that is critical for the breakdown of many proteins involved in cell cycle progression [84]. Another proposed mechanism is the downregulation by PPARβ/δ of protein kinase Cα (PKCα) activity, thereby also inhibiting keratinocyte proliferation [85]. However, the selective ablation of PPARβ/δ in keratinocytes did not have any incidence on the development of DMBA/TPA-induced skin tumors, suggesting that PPARβ/δ may exert its tumor modifier activity by acting on the tumor environment [49, 86]. It is worth noting that PPARα activators prevented DMBA/TPA-induced skin tumors when locally applied to mouse skin [87], and reduced UV-induced inflammation in human skin, which is a risk factor for further development of UV-induced skin cancers [88]. On the contrary, the activation of PPARγ did not prevent the development of UV- or DMBA/TPA-induced skin tumors [89], despite increased susceptibility of PPARγ+/− and keratinocyte-selective PPARγ-null mice to DMBA-mediated carcinogenesis [86, 90]. Finally, UV treatment of a human keratinocyte cell line induced the production of an unknown PPARγ activator [91], but the relevance of this observation remains unclear.

Taken together, these many observations underscore the implications of PPARs in inflammatory skin disorders, UV-induced inflammation, and tumor development. So far, PPARγ activation in patients has proven efficient to treat psoriasis, but other therapeutical applications remain to be explored and defined, particularly in the field of carcinogenesis.

4.2. Digestive tract inflammation

Inflammatory bowel diseases (IBDs) are inflammatory diseases affecting the small or the large intestine [92]. Crohn's disease and ulcerative colitis are the best known forms of IBDs although their causes remain unclear. In their acute phase, IBDs are characterized by acute inflammation, involving the recruitment of immune cells and an elevated production of cytokines. Under chronic conditions, abnormal intestinal epithelium morphology and scarring develop. In various animal models of IBD, the activation of PPARα or PPARγ has anti-inflammatory effects in the intestine, resulting in decreased production of inflammatory markers and slower progression of colitis [93–96]. In these models, PPARγ is the best studied isotype. With the exception of one contradictory study showing that long-term pretreatment with a PPARγ agonist aggravated colitis [97], the preventive activation of PPARγ was efficient, whereas the efficacy of ligand administration after the onset of the disease was dependent on the levels of PPARγ [95, 98–100]. PPARγ activation also prevented colon damage caused by immobilization-induced stress [101]. Conversely, enhanced susceptibility to colitis was observed in mice with reduced PPARγ levels or activity [95, 102–105]. The bases of the protective action of PPARγ in colitis are reduced proinflammatory cytokine production, attenuated expression of ICAM-1 and COX-2, inhibition of NF-κB and JNK/p38 MAPK, and modification of immune cell activity [44, 95, 98, 99, 102, 105–107]. In patients suffering from active ulcerative colitis, a twelve-week treatment with the PPARγ agonist rosiglitazone efficiently cured four out of fifteen patients [108]. Furthermore PPARγ is thought to be one of the molecular targets underlying the beneficial anti-inflammatory effect of 5-aminosalicylic acid, a drug widely used to treat inflammatory bowel diseases (IBDs) [109]. Together, these treatments confirm PPARγ as a potential target in IBDs. The beneficial role of PPARγ activation in inflammatory diseases of the digestive tract may not be limited to the intestine, but seems to extend to gastritis and pancreatitis, an inflammation of the gastric mucosa and pancreas, respectively. In several models of gastritis or gastric ulcers, activation of PPARγ attenuates mucosa damage and accelerates healing, via reduction of inflammation, apoptosis, and lipid peroxidation [110–115]. As in the stomach, PPARγ activity is beneficial in various animal models of pancreatitis, reducing inflammation, restoring exocrine pancreas functions, and limiting chronic pancreatitis development [116–121].

In addition to its already mentioned anti-inflammatory effects, PPARα protects the intestine from colitis-induced permeability [122]. So far, the benefits of PPARβ/δ activation in colitis are poorly documented [44]. One report suggested that PPARβ/δ-null mice exhibit more severe damage in a model of DSS-induced colitis, whereas a PPARβ/δ agonist had no protective or deleterious effect when administrated to PPARβ/δ-wt or -null animals [123]. This observation suggests not only that PPARβ/δ protects wt animals against

DSS-colitis, but also that this protective effect may be ligand-independent or triggered by a so far nonidentified ligand.

The liver is an additional target organ of PPARs for the control of inflammation. Prolonged liver inflammation, which is deleterious, usually activates hepatic stellate cells (HSCs), also known as Ito cells or lipocytes, which proliferate, transdifferentiate into myofibroblasts, and produce excess extracellular matrix, finally leading to severe fibrosis and end-stage cirrhosis [124]. Animal models suggest that limiting, or even reversing, fibrosis may be possible by reducing inflammation, enhancing HSC apoptosis, blocking HSC transdifferentiation, or stimulating ECM degradation [124]. Although PPARβ/δ activation seems to enhance fibrosis via activation of HSC [125], increasing PPARα or PPARγ activity appears to have antifibrotic effects. PPARα reduces inflammation and oxidative stress [126, 127], and PPARγ decreases HSC proliferation, reverses their profibrotic activity, and counteracts the TGFβ1-induced production of collagen [128–136]. Recently, PPARγ activity in human hepatic stellate cells has been shown to be inhibited by acetaldehyde, the major product of ethanol oxidation and one of the main mediators of alcohol-induced liver fibrosis [137].

In conclusion, manipulating the balance of PPAR isotype activities is an interesting therapeutic concept when used to control inflammation of the digestive tract and associated glands.

4.3. Digestive tract and cancer

As the literature includes extensive recent reviews on the interaction between PPARs and Wnt/Apc, known to play a major role in colorectal cancer progression [7, 138], this paragraph will focus on data dealing with chronic inflammation as a risk factor for colon carcinogenesis. Inflammatory bowel diseases, particularly ulcerative colitis, increase the risk of colorectal cancer in patients [139]. As discussed above, PPARγ activation has protective effects in animal models of ulcerative colitis (reviewed in [140]). Moreover, activation of PPARα and PPARγ in rodents reduced the formation of aberrant crypt foci, a risk factor for colon cancer [94]. However, the PPARγ agonists pioglitazone and rosiglitazone had no effect on the development of tumors in a mouse model of azoxymethane/dextran sodium sulfate-induced colon cancer, whereas in the same study the anti-inflammatory 5-ASA reduced the number and the size of the tumors [141], showing that PPARγ is certainly not the only target of 5-ASA. However, in a different study, COX2 inhibitors, the PPARγ agonist troglitazone and, to a lesser extent, the PPARα agonist bezafibrate, reduced the development of adenocarcinoma in a mouse model of azoxymethane/dextran sodium sulfate-induced colon cancer [142, 143].

Chronic inflammation finally leading to cancer may also arise from infections, as in the stomach where infection by *Helicobacter pylori* is a common risk factor for gastric cancer [144]. PPARγ expression is increased in gastric epithelia infected by *Helicobacter pylori*. The consequences of upregulated PPARγ expression are unknown, but it may

contribute to reducing inflammation [145]. The treatment of gastric cancer patients with the COX2 inhibitor rofecoxib correlated with increased levels of PPARγ in the tumor [146]. An epidemiological study performed in a restricted region of Japan suggested that the Pro12Ala variant of PPARγ, which is less active than the wt protein, might be associated with increased risk of gastric cancer [147].

Pancreatic cancer is still lethal in most cases, due to the lack of early markers and specific symptoms and because of aggressive tumor growth and resistance to treatments [148]. While PPARγ activation shows beneficial anti-inflammatory effects in the pancreas, the consequences of such activation in patients with pancreatic cancer are unknown. In vitro data show that PPARγ inhibits pancreatic cell proliferation, which would be beneficial, but also suggest that PPARγ may activate angiogenesis through induced VEGF expression, which would be detrimental (reviewed in [148]). In one in vivo study, however, the PPARγ agonist pioglitazone prevented cancer in a hamster model [149]. In human patients, a high level of PPARγ expression correlated with high-grade pancreatic carcinoma [150]. The mechanism responsible for this effect remains unknown.

4.4. Age-related diseases

Oxidative stress and inflammation increase with age, and further enhancement by environmental factors is thought to favor the development of age-related diseases and cancers. Although this is not fully clear in human, slight caloric restriction diet may retard these processes. The roles of PPARs in age-related inflammation and associated diseases have been reviewed recently in [151–153]. In short, PPARs are thought to be involved in age-related inflammation, caloric restriction physiology, and longevity. Increased inflammation levels during aging are correlated to decreased PPAR activity. Conversely, administration of the PPARα activator Wy14,643 improved the redox balance and reduced inflammation in aged mice [154, 155]. A similar inhibition of age-related inflammation was observed in rat kidney after feeding with a PPARγ agonist [156]. Interestingly, among flavonoids found in fruits and vegetables, which have been associated with decreased risk of inflammation-mediated diseases, some are PPARγ agonists that are known to decrease proinflammatory mediator production. For instance, curcumin, a naturally occurring compound in turmeric, has been used in India for centuries as an anti-inflammatory agent. It is thought to be a PPARγ activator and was suggested to have beneficial effect on colorectal cancer when taken on a daily basis [152, 157].

5. CROSSTALK BETWEEN PPARs AND PATHWAYS RELEVANT TO CANCER AND INFLAMMATION

It is obvious from the above that PPARs interact with numerous pathways involved in cancer development (reviewed in [7, 45, 158]). For instance, PPARα regulates the expression of miRNA let-7C in hepatocytes, a tumor suppressor gene that regulates cancer cell proliferation. PPARβ/δ is a downstream

target of two pathways often involved in colon cancer development, namely, the Ras and the APC-β-catenin pathways. PPARβ/δ also controls the PTEN/Pi3K/Akt pathway, whose actors are often associated with cancer, and promotes cell migration via activation of the Rho-GTPases [60]. Finally, PPARγ activation can induce growth arrest, differentiation, or apoptosis in many cancer cells [7].

In the next sections, we summarize the interaction of PPARs with the main pathways involved in the control of inflammatory responses and cancer development [3, 46].

5.1. COX2 as a link to lipid mediators

Cyclooxygenases (COX) are the enzymes that catalyze the first steps of the production of prostaglandins from arachidonic acid. The COX1 isoform is constitutively expressed in most tissues, whereas the expression of COX2 is induced in inflamed tissues and in tumors. Genetic, epidemiological, and pharmacological evidence supports the hypothesis that elevated COX2 activity is involved in tumor progression (reviewed in [159–161]). Laboratory experiments as well as clinical studies have shown that COX2 inhibitors are promising antitumoral compounds to combine with other anticancer treatments. However, there is a need to develop new compounds with reduced risk of cardiovascular side effects (reviewed in [40, 159, 161, 162]). Antitumoral activity of COX2 inhibitors most probably results from a combination of effects on angiogenesis, apoptosis, tumor cell invasiveness, and inflammation. Interestingly, PPARα and γ activation may help in inhibiting the activity of COX2 by reducing its expression. PPARα agonists prevented PMA-induced expression of COX2 and VEGF [163], and the PPARγ agonist ciglitazone decreased the expression of COX2 and cJun in a colorectal cancer cell line [164]. COX2 can also modify PPAR activity since some of the COX-2-produced fatty acid derivatives are PPAR activators. COX2 has been proposed to modify the activity of PPARβ/δ in colorectal cancer by producing activators such as PGI2 [165–167] or PGE2, which indirectly increase PPARβ/δ activity [168]. In human cholangiocarcinoma cell lines, activation of PPARβ/δ was shown to increase cell proliferation by increasing the expression of COX2 and thus the production of PGE2 [169]. In this model, PGE2 is meant to subsequently activate PPARβ/δ indirectly via cPLA2α, thereby triggering a positive feedback loop controlling cholangiocarcinoma cell proliferation. Inhibiting COX2 is likely to result in decreased PPAR activity. This was in fact demonstrated in hair follicle growth of murine skin, during which inhibition of COX2 replicates the phenotype of PPARβ/δ-null animals [170]. However, increased PPARγ activity by COX2 inhibitors was also reported, although the mechanism remains unknown (reviewed in [148]). The COX2 and PPAR pathways are certainly interconnected, but to what extent the PPAR activity contributes to the COX2 cancer promotion function is unclear. However, drug-combined modification of PPAR activity in inflammation and cancer is an interesting therapeutic prospect.

5.2. NF-κB links inflammation to cancer

The NF-κB pathway is an important link between inflammation and cancer (see [41]; reviewed in [36, 42]). The three PPARs are able to antagonize this pathway, via their transactivation or transrepression activities, thereby leading to the repression of several genes involved in inflammation [3, 44, 47]. In colon cancer cell lines, the PPARγ agonist 15d-PGJ$_2$ attenuated the production of IL-1β-induced IL-8 and MCP-1 by inhibition of NF-κB activity [96], and induced apoptosis via NF-κB and Bcl-2 [171]. In the liver, the disruption of NF-κB signaling resulted in the suppression of PPARα-increased expression during a high-fat diet, whereas, in parallel, an increase in PPARγ expression was observed. In these mice, liver steatosis (a consequence of decreased FA oxidation and increased expression of genes involved in lipogenesis), inflammation, and development of liver cancer were aggravated [172]. Animal and preclinical studies showed that an ω-3 fatty acid supplement to the diet should provide a useful complement to cancer therapy, slowing down progression of various tumors and improving patients' quality of life [173]. Among the mechanisms proposed for these beneficial effects, ω-3 fatty acids repress the NF-κB function and Bcl-2 expression, which in turn leads to decreased COX2 expression and restoration of functional apoptosis [173]. In addition to PPARs regulating the activity of NF-κB, the p65 subunit of the latter was shown to inhibit the transcriptional activity of PPARγ on adipocyte gene expression [174] and of the three PPARs in transfected keratinocytes [65], suggesting that a reciprocal regulation between the two pathways exists.

5.3. MAPK pathway as a major player in carcinogenesis

The MAPK pathway is activated by cytokines, and its overactivation is found in the vast majority of cancer cells and tumors (reviewed in [175]). Phosphorylation of PPARα and PPARγ by this pathway increases or decreases their transcriptional activity, respectively (reviewed in [9, 176]). The physiological impact of the regulation of PPAR activity through phosphorylation has mostly been addressed for PPARα and γ regarding insulin signaling and fatty acid metabolism, but the impact of this modification on inflammation or cancer is currently not documented [9, 176]. Nevertheless, PPAR and MAPK crosstalk has been described in immune or cancer cells. In its unliganded form, PPARα suppressed p38 MAPK phosphorylation in CD4(+) T cells. Ligand activation reversed this inhibition, resulting in the expression of the transcription factor of T cells (T-bet), a marker of Th1 inflammatory responses [177]. The PPARγ agonist rosiglitazone attenuated TNBS-induced colitis via inhibition of the activity of the MAPKs p38 and the c-Jun N-terminal kinase (JNK), and of NF-κB, thereby limiting the expression of proinflammatory genes [95]. In a human colon cancer cell line, PPARγ activation was reported to increase the expression of caveolin1, a protein that is linked to cancer development [178]. This induction seemed to result from an activation of the MAPK

pathway by PPARγ. In another study, the activation of PPARγ in turn activated the Rho-GTPase/MEK1/ERK1/2 cascade, resulting in morphological changes and increased motility in rat intestinal epithelial cells [179]. In lung cancer cell lines, the PPARγ agonist troglitazone induced cell differentiation, probably via activation of Erk1/2 [180, 181]. In addition, the Erk5-dependent activation of PPARγ seemed to be responsible for the antitumorigenic effect of the Wnt signaling pathway [182]. PPARβ/δ also interacts with the MAPK pathway. When activated by TNFα, the MAPK pathway induced the expression of the PPARβ/δ gene in inflamed keratinocytes [57]. Once activated by a ligand produced in parallel, PPARβ/δ facilitates keratinocyte survival and migration. Interestingly, both the expression of PPARβ/δ and the activity of the MAPK pathway are elevated in many tumors [7, 175]. Whether the expression of PPARβ/δ is stimulated by this pathway in cancers remains to be investigated. Finally, anti-inflammatory effects of the MEK5/Erk5 pathway in a muscle cell line are due to inhibition of NF-κB and are thought to involve PPARβ/δ activation [183].

Crosstalk between PPARs and MEKs, the upstream regulators of the MAPK, has also been described [184]. It has been suggested that MEK1 interacts with PPARγ, thereby causing PPARγ delocalization from the nucleus to the cytoplasm [185]. Interestingly, PPARγ was described as mainly cytoplasmic in human biopsies of salivary duct carcinoma and breast cancer [186, 187]. Although the significance of this shuttling is unclear, it should decrease PPARγ transactivation functions.

5.4. PTEN/Pi3K pathway and its target mTOR

The phosphatase and tensin homologue deleted from chromosome 10 (PTEN) is a tumor suppressor whose activity is lost in many human cancers. PTEN is a lipid and protein phosphatase whose main substrate is the PIP3 produced by the Pi3K. Through its phosphatase activity, PTEN antagonizes PiK3 activity and inhibits the Pi3K/Akt pathway involved in the regulation of apoptosis, cell proliferation and growth, and metabolism [188, 189]. The mammalian target of rapamycin (mTOR), one of the targets of the PTEN/Pi3K pathway, is a conserved kinase that regulates central cellular functions in response to environmental signals, such as transcription and translation, mRNA and protein turnover, or autophagy (reviewed in [190, 191]). Impaired mTOR pathway is often associated with tumorigenesis [1]. PPARβ/δ was shown to indirectly inhibit the expression of PTEN in keratinocytes, thereby activating the Pi3K/Akt pathway, which enabled keratinocyte survival [59]. In lung carcinoma cells, the activation of PPARβ/δ stimulated cell proliferation, via decreased expression of PTEN and activation of NF-κB and Pi3K/Akt [192, 193]. While PPARβ/δ decreases PTEN expression, PPARγ activation has the opposite effect. In a model of allergic inflammation in mouse lung, PPARγ agonists decreased inflammation, most probably via increased PTEN expression, and reduced PiP3 levels as well as Akt and NFκB activities [194]. Treatment of lung carcinoma cell

lines with rosiglitazone decreased proliferation via PPARγ-dependent upregulation of PTEN and inhibition of Akt activity, and also via PPARγ-independent inhibition of the mTOR pathway [195, 196]. PPARγ-independent inhibition of mTOR by TZD was also reported in keratinocytes [197]. In this model, TZD inhibited the mitogenic effect of IGF via indirect inhibition of mTOR, a mechanism which may be involved in TZD-mediated inhibition of skin tumor development in transgenic mice overexpressing IGF.

In a hepatocarcinoma cell line, PPARγ activation by rosiglitazone inhibited cell migration through increased expression of PTEN [198]. Rosiglitazone also had important anticarcinogenic effects in some highly aggressive anaplastic thyroid cancer cell lines. In these cells, rosiglitazone induced apoptosis, cell cycle inhibition, differentiation, and decreased anchorage-independent growth and migration. This was at least partially due to upregulation of PTEN and inhibition of Akt activity, which antagonized IGF-1 effects necessary for the progression of thyroid cancers [199].

In summary, PPARβ/δ and γ are both regulators of the expression of PTEN, and interact with the mTOR pathway. PPARβ/δ decreases PTEN expression, whereas PPARγ activates this tumor suppressor gene.

6. CONCLUSIONS

In numerous cancer types, PPARs regulate autonomous processes in tumor cells, such as apoptosis, proliferation, and differentiation, by interacting with major pathways involved in carcinogenesis. They also act on the tumor cell environment, modifying angiogenesis, inflammation, and immune cell functions (reviewed in [3, 7, 45, 48–51]). Not surprisingly, their activation has complex consequences, in which the contribution of tumor cell-autonomous versus nonautonomous mechanisms remains to be evaluated. Whether PPARs are pro- or anticarcinogenic actors is still open to discussion, and may depend not only on the origin and genetics of the tumor cell, but also on the nature of the host tissue and inflammation levels. Although the possible carcinogenic or toxic effects of PPAR activation remain an unresolved issue, PPARs nevertheless constitute valuable therapeutic targets (reviewed in [7, 200]). The use of PPARα and PPARγ agonists is increasing in the treatment of a constantly expanding number of diseases related to the metabolic syndrome. In this context, although their supposedly carcinogenic or toxic effects have to be carefully monitored, PPARs are important therapeutic targets. Many valuable approaches are now under investigation in order to better understand the mechanisms of adverse effects, and to develop better compounds. In vivo models, such as tissue or cell-type selective PPAR knock-out mice, as well as humanized animals carrying the human PPAR genes, will certainly help in sorting out the various actions of PPARs in inflammation and cancer. In addition, the development of selective PPAR modulators (SPPARMs), rather than PPAR full agonists, which would retain most of the benefits while reducing the adverse effects of PPAR activation, is a promising approach. For all these reasons, PPARs are

certainly useful pharmaceutical targets to be explored further in the context of inflammation and/or cancer therapy.

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Review Article

The Role of PPARs in Cancer

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPARα is mainly expressed in the liver, where it activates fatty acid catabolism. PPARα activators have been used to treat dyslipidemia, causing a reduction in plasma triglyceride and elevation of high-density lipoprotein cholesterol. PPARδ is expressed ubiquitously and is implicated in fatty acid oxidation and keratinocyte differentiation. PPARδ activators have been proposed for the treatment of metabolic disease. PPARγ2 is expressed exclusively in adipose tissue and plays a pivotal role in adipocyte differentiation. PPARγ is involved in glucose metabolism through the improvement of insulin sensitivity and represents a potential therapeutic target of type 2 diabetes. Thus PPARs are molecular targets for the development of drugs treating metabolic syndrome. However, PPARs also play a role in the regulation of cancer cell growth. Here, we review the function of PPARs in tumor growth.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily [1]. PPARs bind to a direct repeat of two hexanucleotides, spaced by one or two nucleotides (the DR1 or DR2 motif) as heterodimers with the retinoid X receptor (RXR), and activate several target genes [2–4]. These peroxisome proliferator responsive elements (PPREs) are found in various genes that are involved in lipid metabolism and energy homeostasis, including lipid storage or catabolism, and fatty acid transport, uptake, and intracellular binding [5]. Three subtypes, PPARα, PPARδ (also known as PPARβ), and PPARγ, have been identified and these subtypes with a high degree of sequence conservation of each subtype across various species have been characterized. The DNA-binding domains of the three subtypes are 80% identical, while their ligand-binding domains exhibit a lower degree (approximately 65%) of identity (Figure 1). Consistent with this relatively high divergence among the subtype-specific ligand-binding domains, differ-

ential activation of PPARs by endogenous and exogenous compounds may account for the specific biological activity of the three PPAR subtypes [6, 7].

PPARα is expressed in the liver, kidney, small intestine, heart, and muscle, where it activates fatty acid catabolism and is involved in the control of lipoprotein assembly [8]. PPARα is activated by several molecules, such as long chain unsaturated fatty acids, eicosanoids, and hypolipidemic drugs (e.g., fenofibrate) [9–12]. PPARα activators have been used to treat dyslipidemia, causing a reduction in plasma triglyceride and elevation of high-density lipoprotein (HDL) cholesterol [13, 14]. PPARδ is expressed ubiquitously and is implicated in fatty acid oxidation, keratinocyte differentiation, wound healing, and the response of macrophages for very low-density lipoprotein [15–19]. PPARδ activators have been proposed for the treatment of metabolic disease and are under clinical trial [20, 21]. There are two PPARγ isoforms: PPARγ1 and γ2 [22, 23]. PPARγ2, which is generated by alternative splicing, contains an additional 28 amino acids at the N-terminal compared to PPARγ1. PPARγ3 is a splicing variant of PPARγ1 and gives rise to the same protein [24].
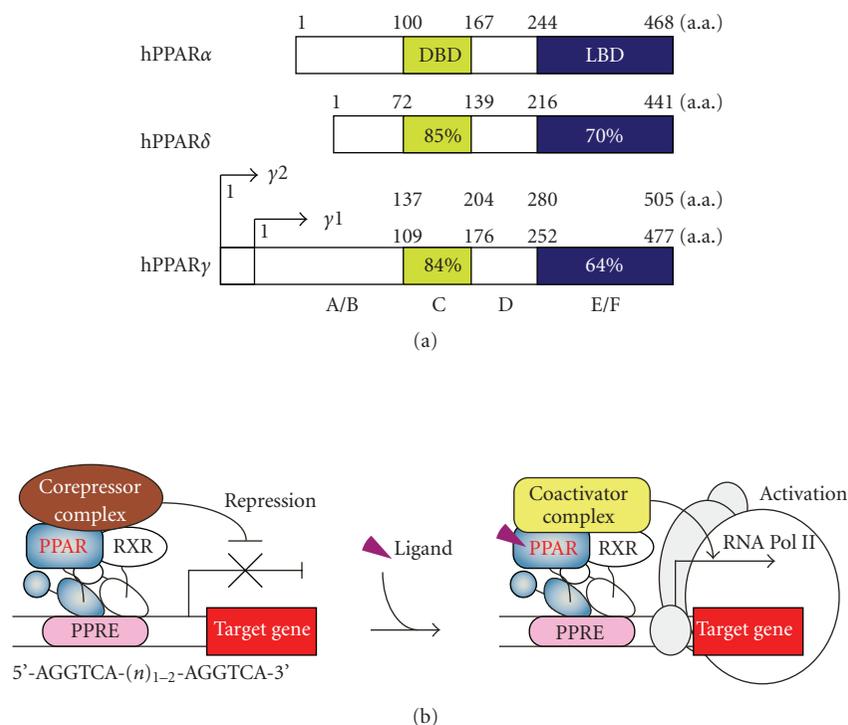
FIGURE 1: The general features of human PPARs. (a) Structure and functional domain of human PPARs. A/B, C, D, and E/F indicate N-terminal A/B domain containing a ligand-independent activation function 1, DNA-binding domain (DBD), hinge region, and C-terminal ligand-binding domain (LBD), respectively. The number inside each domain corresponds to the percentage of amino acid sequence identity of human PPARδ and PPARγ relative to PPARα. (b) PPAR/RXR heterodimers bind to a PPRE located in the promoter of target genes through the DBD. Unliganded PPAR associates with the corepressor complex. In the presence of ligand, the ligand-bound LBD associates with the coactivator complex.

PPARγ2 is expressed exclusively in adipose tissue and plays a pivotal role in adipocyte differentiation, lipid storage in the white adipose tissue, and energy dissipation in the brown adipose tissue [22, 25]. On the other hand, PPARγ1 is expressed in the colon, the immune system (e.g., monocytes and macrophages), and others. Except for the function of PPARγ2 in adipose tissue, PPARγ also participates in inflammation, cell cycle regulation, and other functions [26]. PPARγ is involved in glucose metabolism through the improvement of insulin sensitivity and represents a potential therapeutic target of type 2 diabetes [26]. Indeed, insulin-sensitizing thiazolidinedione (TZD) drugs are PPARγ ligands [27]. Thus PPARs are molecular targets for the development of drugs to treat type 2 diabetes and metabolic syndrome. On the other hand, PPARs also play a role in the regulation of cancer cell growth.

2. PPARα AND CANCER

Fibrates, which are relatively weak PPARα ligands, are useful for the treatment of dyslipidemia [7, 9–11]. Fibrates lower serum triglyceride levels and increase HDL levels through the activation of PPARα [5]. PPARα induces lipoprotein lipase (LPL) expression, reduces the expression levels of apolipoprotein C-III (ApoC-III), a natural LPL inhibitor,

and stimulates the uptake of cellular fatty acids and the conversion of fatty acids to acyl-CoA derivatives [5, 28, 29]. These catabolism functions are mediated by upregulating the expression of a series of genes-related carbohydrate and lipid metabolism [5, 30]. In addition, PPARα increases the expressions of ApoA-I and ApoA-II, resulting in raising HDL cholesterol levels in humans [31, 32]. Thus PPARα plays a central role in the control of fatty acid and lipoprotein metabolism, and improves plasma lipid profiles. Although peroxisome proliferators have carcinogenic consequences in the liver of rodents, epidemiological studies suggest that similar effects are unlikely to occur in humans [10, 33–36].

Several mechanisms have been proposed to explain the carcinogenesis of peroxisome proliferators in rodents. Peters et al. reported that wild-type mice treated with the Wy-14,643 showed increase of replicative DNA synthesis in hepatic cells and developing liver tumors with 100% incidence, whereas PPARα-null mice were refractory to this effect [37]. Peroxisome proliferators increase the peroxisome volume and number and result in an increase in hydrogen peroxide (H_2O_2) levels [38–40]. These effects may be mediated in part by the increased expression of peroxisomal enzymes that produce H_2O_2, such as acyl CoA oxidase (ACO) [39–41]. PPARα upregulates the expression levels of ACO via PPRE in the promoter region [42, 43]. A stably transfected African

green monkey kidney cells (CV-1) overexpressing rat ACO increased H_2O_2 production, formed transformed foci, and grew efficiently in soft agar when the cells were treated with linoleic acid [44]. Furthermore, when these cells were transplanted into nude mice, these cells formed solid tumors [44]. An increase of intracellular levels of H_2O_2 could lead to DNA damage via a variety of mechanisms [45]. Any reduced iron present can catalyze the cleavage of H_2O_2, via the Fenton reaction, to produce hydroxyl radicals (HO•) [46]. The HO• attacks guanine residues, producing residues of 8-oxo-7,8-dihydroguanine (8-oxoguanine). When DNA synthesis occurs before the 8-oxoguanine is repaired, this damaged base will have a chance to pair with adenine nucleotide, resulting in a mutation in the daughter cells [47]. In addition, antioxidants inhibit ciprofibrate-induced hepatic tumorigenesis by scavenging active oxygen [48]. Thus oxidative stress by peroxisome proliferators acts as a driving force to malignancy. The activation of PPARα also leads to increased hepatocellular proliferation and inhibition of apoptosis. Chronic administration of nafenopin, PPARα agonist, to mice causes significant increase in the liver weight, hepatic DNA synthesis, and the development of hepatocellular carcinomas [49]. Nafenopin treatment of primary cultures of adult rat hepatocytes also stimulated DNA synthesis [50]. Indeed, Peters et al. showed that mRNAs encoding cyclin-dependent kinase (CDK) 1, CDK4, cyclin D1, and c-*myc* and their proteins, which induce cell proliferation, increased in wild-type mice fed by Wy-14,643 but not in PPARα-null mice [51]. Increase of the average liver weight and the levels of mRNAs encoding cell cycle regulation, such as CDK4, proliferating cell nuclear antigen (PCNA) and cyclin B1, were also found in wild-type mice fed by bezafibrate, the less specific PPARα agonist, and these effects were not found in PPARα-null mice [52]. Moreover, the treatment of the primary culture of rat hepatocytes and the rat hepatoma cell line, FaO, with nafenopin suppressed apoptosis [53, 54]. Thus the activation of PPARα leads to the increase of oxidative stress, induction of cell proliferation and inhibition of apoptosis, indicating that PPARα increases hepatocarcinogenesis in mice.

A number of experimental observations suggest that there is a species difference between rodents and humans in the response to PPARα agonists, although the functional differences of PPARα derived from species are not clear (Table 1). One possible explanation for the difference is the expression levels of PPARα in the liver. The expression levels of PPARα in human liver are approximately one order less than that observed in mouse liver [55]. Small expression levels of PPARα could allow PPREs to be occupied by other members of the nuclear receptor superfamily, such as RXR, the chicken ovalbumin upstream promoter transcription factor I (COUP-TFI), COUP-TFII, hepatocyte nuclear factor-4 (HNF4), retinoic acid receptor (RAR), and thyroid hormone receptor (TR), and affect responsiveness to peroxisome proliferators [56–62]. We and others have shown that elevated expression of PPARα in HepG2 cells dramatically increased the expression of several target genes, such as 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial) (HMGCS2), carnitine palmitoyltransferase 1A (CPT1A), and long chain fatty acyl-CoA synthetase (ACS) [30, 63, 64]. In this way,

TABLE 1: Summary of the species differences of PPARα.

	Human	Rodent
PPARα expression levels	+	++
PPARα variants	Yes	?
Peroxisome proliferation	+/−	+
Fatty acid metabolism	+	+
Expression of cell cycle regulator genes	+/−	+
Expression of miRNA (let-7C)	+	−
Hepatocellular proliferation	+/−	+
Apoptosis	+	−
Liver tumor	+/−	++

the lower expression levels of PPARα in human liver might contribute to holding down peroxisome proliferation and subsequent pathologic effects. Another explanation is that several PPARα variants, which lack the entire exon 6 or contain mutations, are detected in human cells and these variants act as a dominant negative regulator of PPAR-mediated gene transcription [55, 65, 66]. But this has not been found in rodents yet. One PPARα variant containing the mutation prevents the suppression of hepatocyte apoptosis by nafenopin [55, 65, 66]. Thus the expression levels of PPARα variants might affect the response to peroxisome proliferators. Next, there appears to be sequence differences in the PPRE found in the promoter region of ACO. Osumi et al. identified ACO to be a direct PPARα target gene and a functional PPRE located in the proximal promoter of the rat ACO gene [42]. In contrast to the rodent ACO gene promoter, the human ACO gene promoter differs at three bases within the PPRE from the rat ACO promoter and appears refractory to PPARα [42, 67, 68]. This human PPRE was unable to drive peroxisome proliferators-induced gene transcription in cell-based assays [67–69]. Indeed, human liver cell lines and primary hepatocytes did not induce ACO mRNA by treatment with fibrates or other PPARα agonists [63, 64]. A similar pattern, such differences between human and other species, was observed in the expression of ApoA-I gene [31]. Fibrates influence the ApoA-I gene expression, raising it in humans, and lowering it in rodents. These differences are due to a combination of two distinct mechanisms implicating the nuclear receptors PPARα and Rev-erbα, a negative regulator of gene transcription [31]. The species-distinct regulation is due to sequence differences in *cis*-acting elements in their respective promoters leading to repression by Rev-erbα of rat ApoA-I and activation by PPARα of human ApoA-I. There is a positive PPRE in the human ApoA-I promoter but not in rats. The expression of Rev-erbα is induced by fibrates [3, 31]. In the case of rat, this induction leads to the repression of the ApoA-I gene expression through an Rev-erbα response element. On the other hand, there is no Rev-erbα response element in the human ApoA-I gene [31]. Thus the sequence differences in *cis*-acting elements cause the species-distinct regulation of target genes expression by peroxisome proliferators. However, the mechanism of the species differences is not known in detail.

To determine the mechanism of species difference in response to peroxisome proliferators, Gonzalez et al. generated a liver-specific PPARα humanized mouse line (hPPARα^{TetOff} mice) in which the human PPARα was expressed in the liver in a PPARα-null background under the control of the tetracycline (Tet) responsive regulatory system [70–72]. The expression of several target genes encoding peroxisomal and mitochondrial fatty acid metabolizing enzymes were elevated in hPPARα^{TetOff} mice fed Wy-14,643 or fenofibrate, resulting in the decrease of serum triglycerides [70, 73]. However, the expressions of various genes involved in cell cycle regulation (PCNA, c-*myc*, CDK1, CDK4, and cyclins) in the liver were unaffected by Wy-14,643. In addition, hPPARα^{TetOff} mice were resistant to Wy-14,643-induced hepatocarcinogenesis [70, 73]. Recently, Shah et al. showed that Wy-14,643 regulated mice hepatic MicroRNA (miRNA) expression via a PPARα-dependent pathway [74]. miRNAs are a class of nonprotein-coding, endogenous, small RNAs, and regulate gene expression by translational repression and mRNA cleavage [75]. Some miRNAs regulate cell proliferation and apoptosis processes that are important in cancer formation [76]. The activation of PPARα with Wy-14,643 inhibits the expression of miRNA let-7C, which functions as a tumor suppressor gene [74]. let-7C degrades c-*myc* mRNA by binding to 3' untranslated region (UTR) of the c-*myc* gene. Treatment of mice with Wy-14,643 showed that let-7C expression was decreased and a subsequent increase in c-*myc* was observed. Following an increase in c-*myc*, the levels of the oncogenic mir-17 miRNA cluster were increased [74]. In this way, inhibition of the let-7C signaling cascade may lead to increased hepatocellular proliferation and tumorigenesis. In contrast, hPPARα^{TetOff} mice do not exhibit downregulation of let-7C and induced c-*myc* and mir-17 expression [74]. Furthermore, Yang et al. generated another type of PPARα humanized mice, hPPARα^{PAC} mice, that has the complete human PPARα gene sequence including 5' and 3' flanking sequences on a P1 phage artificial chromosome (PAC) genomic clone, introduced onto the mouse PPARα-null background [71]. Upon treatment with the peroxisome proliferators (Wy-14,643 or fenofibrate), hPPARα^{PAC} mice exhibited peroxisome proliferation, lowering of serum triglycerides, and induction of PPARα target genes encoding enzymes involved in fatty acid metabolism. However, let-7C expression was not decreased and the expression levels of c-*myc*, cyclin D1 and CDK4 were not increased [71]. Thus these observations suggest that the species differences in response to peroxisome proliferators could be due in part to a differential ability of the mouse and human PPARα to suppress let-7C gene expression [74]. However, the mechanism involved in PPARα-dependent repression of let-7C is unclear. The differences between the wild-type mice and PPARα humanized mice could be caused by the structural differences between human and mouse PPARα and differential coactivator recruitment. However, additional investigation is required to better understand and clarify the mechanism of action of PPARα in causing hepatocarcinogenesis.

3. PPARδ AND CANCER

The role of PPARδ in oncogenesis is controversial, especially in colon cancer. Some reports show that PPARδ promotes tumorigenesis by increasing cell proliferation. Indeed, the levels of PPARδ mRNA are increased in both human and rodent colorectal carcinomas [77, 78]. PPARδ is a potential downstream target gene of the adenomatous polyposis coli (APC)/β-catenin/T cell factor-4 (TCF-4) pathway [77]. APC is a tumor suppressor gene and is mutated in familial adenomatous polyposis (FAP) and most sporadic colorectal tumors [79–83]. β-catenin, which binds to APC and axin in a large protein complex, can be phosphorylated by glycogen synthase kinase-3β (GSK3β) and is followed by ubiquitination and degradation. Mutation of APC results in the accumulation of β-catenin, which in turn translocates to the nucleus and associates with the transcription factor TCF-4 [84]. The β-catenin-TCF-4 transcription complex increases the transcription of growth-promoting genes, such as c-*myc* and cyclin D1 [85, 86]. The β-catenin-TCF-4 transcription complex also activates the human PPARδ promoter activity via TCF-4 binding sites, namely, APC suppresses the PPARδ expression through the degradation of β-catenin [77]. K-Ras mutation is found in colorectal cancer [80, 87]. Activation mutations in Ras result in the activation of the mitogen-activated protein kinase (MAPK) pathway and induce tumor growth and progression [88]. The expression levels and activity of PPARδ were increased by the induction of mutated K-Ras in conditionally K-Ras-transformed rat intestinal epithelial cells [89]. Thus PPARδ is also a downstream target gene of Ras/Raf/MAPK and extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathway [89]. In this way, PPARδ may play a role in colon cancer.

Epidemiological studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, indomethacin, and sulindac, reduce the overall number and size of adenomas in patients with FAP. Healthy individuals using NSAIDs regularly can lead to a 40–50% reduction in the relative risk of developing colon cancer [90]. NSAIDs inhibit cyclooxygenase (COX) activity and thereby reduce prostaglandin synthesis [91]. COX is a key enzyme in arachidonic acid metabolism and prostaglandin production. COX catalyzes a two-step reaction that converts arachidonic acid to prostaglandin H$_2$ (PGH$_2$), which in turn serves as the precursor for the synthesis of all biologically active prostaglandins, including PGD$_2$, PGE$_2$, PGF$_2\alpha$, prostacyclin (PGI$_2$), and thromboxane A$_2$ (TXA$_2$) [92]. COX exists in two isoforms that are encoded by two separate genes. COX-1 is constitutively expressed in most tissues, on the other hand, the expression of COX-2 is normally low or absent in most tissues but is rapidly upregulated by proinflammatory cytokines [93]. Expression of COX-2 is also elevated in colorectal cancer and in a subset of adenomas [94]. Moreover, since both the introduction of the knockout mutation of the COX-2 gene into $Apc^{\Delta 716}$ mice, a model of human FAP, and treating $Apc^{\Delta 716}$ mice with NSAIDs reduce the development of intestinal tumors, COX-2 inhibitors have been considered as therapeutic agents for colorectal polyposis and cancer [95]. He et al. reported that NSAIDs

inhibited the transcriptional activity of PPARδ by disruption of the DNA binding ability of PPARδ/RXR heterodimers, and ectopic expression of PPARδ in the human colorectal cancer cell line, HCT116, protected the cells from sulindac-induced apoptosis [77]. PPARδ and COX-2 mRNA are expressed in similar regions in human colon cancer, and the stable PGI$_2$ analog, carbaprostacyclin (cPGI), acts as a PPARδ ligand [11, 78]. Indeed, ectopic expression of COX-2 and PGI synthase (PGIS) in the human osteosarcoma cell line, U2OS, produced high levels of endogenous PGI$_2$ and transactivation of PPARδ [78]. PGE$_2$ levels are also elevated in human colorectal cancers and adenomas, and PGE$_2$ increases the growth and motility of colorectal carcinoma cells [96, 97]. D. Wang et al. showed that PGE$_2$ promoted resistance to serum starvation-induced apoptosis of cultured human colon carcinoma cells, LS-174T, through indirectly upregulation PPARδ transcriptional activity via a phosphotidylinositol-3-kinase (PI3K)-Akt pathway [98]. Furthermore, PGE$_2$ accelerates intestinal adenoma growth of $Apc^{\min}$ mice, a model of human FAP that harbors a mutation in the apc gene, via PPARδ [98]. Xu et al. showed that PGE$_2$ activated cytosolic phospholipase A$_2\alpha$ (cPLA$_2\alpha$) through PI3K or MAPK pathway, and subsequently cPLA$_2\alpha$ enhanced PPARδ activity in the human cholangiocarcinoma cells [99]. They also showed that PPARδ enhanced COX2 expression and PGE$_2$ production. This positive feedback loop may play an important role in cholangiocarcinoma cell growth, although it is not known whether this kind of positive feedback loop exists in the colorectal cancer cells [99]. Thus PPARδ induces the cell proliferation through the inhibition of apoptosis. However, sulindac sulfide induces apoptosis not only in wild-type HCT116, but also in HCT116 PPARδ-null cell lines [100]. On the basis of these observations, although NSAIDs may reduce tumorigenesis through the inhibition of PPARδ activity, PPARδ is not a major mediator of sulindac-mediated apoptosis.

Recent evidence supports the hypothesis that PPARδ promotes tumor progression. HCT116 PPARδ-null cell lines grew slightly more slowly than wild-type HCT116 cells, and exhibited a decreased ability to form tumors compared with wild-type mice when inoculated as xenografts in nude mice [100]. Gupta et al. showed that exposure of $Apc^{\min}$ mice to 10 mg/kg of GW501516, a high-affinity PPARδ-selective agonist, led to a two-fold increase in polyp number in the small intestine [101]. The most prominent effect was on polyp size, mice treated with the PPARδ activator had a five-fold increase in the number of polyps larger than 2 mm, suggesting that PPARδ activation primarily affected the rate of polyp growth rather than initiating polyp formation. Pretreatment of wild-type HCT116 cells with GW501516 significantly suppressed serum starvation-induced apoptosis in a dose-dependent manner, but not HCT116 PPARδ-null cells [101]. Furthermore, D. Wang et al. showed that $PPARd^{-/-}/Apc^{\min}$ mice decreased intestinal adenoma growth and inhibited the tumor-promoting effect of GW501516 [102]. They also showed that PPARδ activation with GW501516 upregulated vascular endothelial growth factor (VEGF) transcription, expression, and peptide release in intestinal epithelial tumor cells, and subsequently activated PI3K-Akt signaling [102]. Similar results were obtained in the human endothelial cells [103, 104]. Piqueras et al. showed that GW501516 induced VEGF mRNA and peptide release, and thus PPARδ induced endothelial cell proliferation and angiogenesis [103]. Stephen et al. showed that the activation of PPARδ resulted in increased expression of VEGF and its receptor fms-related tyrosine kinase 1 (FLT-1), and they suggested that PPARδ might initiate an autocrine loop for cellular proliferation and possibly angiogenesis [104]. These results demonstrate that VEGF mediates the antiapoptotic effects of PPARδ in intestinal epithelial tumor cells by activating the PI3K-Akt cell survival pathway, and the VEGF autocrine loop plays an important role in cell survival. Diminished apoptosis is also linked to downregulated 15-lipoxygenase-1 (15-LOX-1) expression in colorectal cancer cells. 13-S-hydroxyoctadecadienoic acid (13-S-HODE), which is the primary product of 15-LOX-1 metabolism of linoleic acid, inhibits cell proliferation and induces cell cycle arrest and apoptosis in transformed colonic epithelial cells [105]. 15-LOX-1 protein expression and 13-S-HODE intracellular levels are decreased in human colonic tumors [105]. Shureiqi et al. showed that 13-S-HODE bound to PPARδ and then downregulated PPARδ expression and activation in colorectal cancer cells, DLD-1 and RKO, and that the loss of PPARδ expression in HCT116 markedly suppressed 13-S-HODE-mediated apoptosis [106]. 15-LOX-1 expression also downregulated PPARδ expression and transcriptional activity in these colorectal cancer cells [106]. Furthermore, NSAIDs increase 15-LOX-1 protein expression and its product 13-S-HODE levels and downregulate PPARδ expression in association with subsequent growth inhibition and apoptosis [106, 107]. Thus it is considered possible that PPARδ promotes the growth of colon cancers.

On the contrary, other reports suggest that ligand activation of PPARδ promotes the induction of terminal differentiation and inhibition of cell growth. PPARδ was found in intestinal epithelial cells in both the normal intestine and adenomas of $Apc^{\min}$ mice [101]. Reed et al. reported that targeted deletion of the APC alleles in mouse intestines decreased the expression levels of PPARδ mRNA and protein, although β-catenin and c-myc were increased [108]. Marin et al. showed that PPARδ expression was reduced in both the $Apc^{\min}$ mouse colon polyps and azoxymethane (AOM)-treated wild-type mouse polyps, though the expression levels of PPARδ mRNA in colonic epithelium were not different between $Apc^{\min}$ mice and wild-type mice with or without AOM-treatment [109]. Several reports identified that the transcription factor binding sites for AP-1, CCAAT/enhancer-binding proteins, vitamin D receptor, and others were found in human or mouse PPARδ promoter, and these transcription factors regulated PPARδ expression [16, 110, 111]. However, further investigation is required to certify the regulation of PPARδ expression in cancer.

Hollingshead et al. reported that GW501516 and GW0742, highly specific PPARδ ligands, did not increase the growth of human colon cancer cell lines (HT-29, HCT116, and LS-174T) and liver cancer cell lines (HepG2 and HuH7) cultured in the presence or absence of serum [112]. In

addition, treatment of these cell lines with either GW501516 or GW0742 did not change the phosphorylation of Akt, and no increase in the expression levels of COX2 or VEGF were detected [112]. Similar results were observed in the colon or liver of Apc^{min} mice treated with GW501516 or GW0742 [109, 112]. Barak et al. showed that the average number of intestinal polyps was not significantly different between $PPARd^{+/+}/Apc^{min}$, $PPARd^{+/-}/Apc^{min}$, and $PPARd^{-/-}/Apc^{min}$ mice, although this study was limited to a small number [113]. On the other hand, several studies showed that colon polyp formation was enhanced in the absence of PPARδ expression in both $PPARd^{-/-}/Apc^{min}$ and AOM-treated $PPARd^{-/-}$ mice [108, 109, 114]. Moreover, Marin et al. showed that the administration of GW0742 had no effect on colon or small intestinal tumorigenesis in either $PPARd^{-/-}/Apc^{min}$ or $PPARd^{+/+}/Apc^{min}$ mice as compared with controls [109]. In addition, decrease of colon polyp multiplicity was observed in $PPARd^{+/+}$ AOM-treated mice administrated with GW0742 compared with control wild-type mice. This effect was likely due in part to PPARδ-dependent induction of colonocyte differentiation and enhancement of apoptosis [109]. Indeed, PPARδ induces keratinocyte terminal differentiation, which normally opposes cell proliferation [115, 116]. Hatae et al. also showed that intracellular PGI$_2$, an endogenous PPARδ ligand, formed by expressing PGIS in human embryonic kidney 293 (HEK293) cells, promoted apoptosis by activating PPARδ [117]. In this way, PPARδ inhibits tumor growth by inducing apoptosis or differentiation.

Thus the conflicting reports in the literature suggest that PPARδ either potentiates or attenuates colon cancer. Similar discrepancies were observed in other tissues. Di-Poï et al. showed that the activation of PPARδ inhibited apoptosis in keratinocyte [118]. The activation of PPARδ by L-165041, one type of PPARδ ligand, upregulates 3-phosphoinositide-dependent kinase-1 (PDK1) and integrin-linked kinase (ILK) gene expression via PPRE and downregulates phosphatase and tensin homolog (PTEN) protein expression, and subsequently leads to the activation of Akt1 in a PI3K-dependent manner in mouse primary keratinocytes and human keratinocyte HaCaT cells [118]. Yin et al. showed that PPARδ ligand GW501516 accelerated progestin- and carcinogen-induced mouse mammary carcinogenesis [119]. Stephen et al. reported that PPARδ selective agonists stimulated the proliferation of human breast and prostate cancer cell lines and primary endothelial cells [104]. On the other hand, Burdick et al. reported that ligand activation of PPARδ with GW0742 inhibited the cell growth of either human keratinocyte cell line N/TERT1 or mouse primary keratinocytes [120]. In these cells, ligand activation of PPARδ by GW0742 did not alter expression and/or modulation of the PTEN/PDK1/ILK1/Akt pathway [120]. Girroir et al. reported that both GW0742 and GW501516 inhibited the growth of the human breast cancer cell line, MCF7, and human melanoma cell line, UACC903 [121].

To date, however, the reason for the contradiction in these observations is unclear. One explanation for these conflicting results may be the ability of PPARδ to repress the transcription of target genes. We and others observed that unliganded PPARδ repressed target gene expression, though ligand-activated PPARδ induced these genes [30, 122–124]. It has been reported that unliganded PPARδ bound to PPRE and recruited corepressors, such as B-cell lymphoma 6 (BCL-6), silencing mediator for retinoid and thyroid hormone receptor (SMRT), nuclear receptor corepressor (NCoR), and others. On the other hand, liganded PPARδ is thought to release the corepressor and form a complex with coactivators [122–124]. Furthermore, binding of ligand to the PPARδ or deletion of PPARδ expression may lead to the release of BCL-6. Subsequently, BCL-6 represses the transcription of a number of inflammatory cytokine genes [124]. Thus the PPARδ activity may be influenced by the cellular environment, such as the existence of PPARδ ligands, cofactors, and others. From this viewpoint, the conflicting results may be due to differences in the condition of cell cultures or the genetic background of animal models. Secondly, prostaglandins, some of which act as PPAR ligands, have a variety of biological activities. Prostaglandins, synthesized via the COX pathway from arachidonic acid, are released outside the cells and lead to changes in the cellular levels of cyclic AMP and Ca^{2+} through binding to G-protein-coupled receptors on the plasma membrane [90]. Indeed, Hatae et al. suggested that cAMP produced by the PGI$_2$-PGI receptor (IP)-cAMP pathway might protect vascular endothelial cells from intracellular PGI$_2$-PPARδ-mediated apoptosis [117]. On the other hand, Fauti et al. showed that the ectopic expression of COX-2 and PGIS in HEK293 cells results in a dramatic induction of PGI$_2$ synthesis, but no increase in PPARδ transcriptional activity is observed [125]. Thus they suggest that PGI$_2$ lacks agonistic activity for PPARδ. Since PGI$_2$ is unstable and rapidly hydrolyzed to 6-keto-PGF$_{1\alpha}$ within minutes and increases the production of intracellular cAMP via stimulation of adenylyl cyclase through the cell surface IP receptor, further investigation is necessary to certify the mechanism of the effect of the PGI$_2$ on PPARδ activity in detail. Therefore, additional analyses are necessary to define the PPARδ functions in cancer (Figure 2).

4. PPARγ AND CANCER

Cancer cells represent dysregulaton of the cell cycle and lead to cell proliferation. In this viewpoint, modulators of the cell cycle and/or apoptosis are useful as chemotherapeutic agents for cancer [126, 127]. A number of investigators have shown that PPARγ was expressed in a variety of tumor cells, and the activation of PPARγ by ligands led to either inhibition of cell proliferation or induction of apoptosis (Table 2) [128, 129]. PPARγ is expressed in colonic tumors, normal colonic mucosa, and colon cancer cell lines [130–135]. Kitamura et al. showed that TZDs, such as troglitazone and rosiglitazone, inhibited the cell growth and induced G1 cell cycle arrest of rat intestinal epithelial cells [136]. The cell growth inhibition by TZDs was caused by the decrease of the expression of cyclin D1, critical for entering the S phase of the cell cycle. TZDs suppressed the cyclin D1 promoter activity through inhibition of the transcriptional activities of AP-1 and Ets [136]. Shao et al. demonstrated that treatment
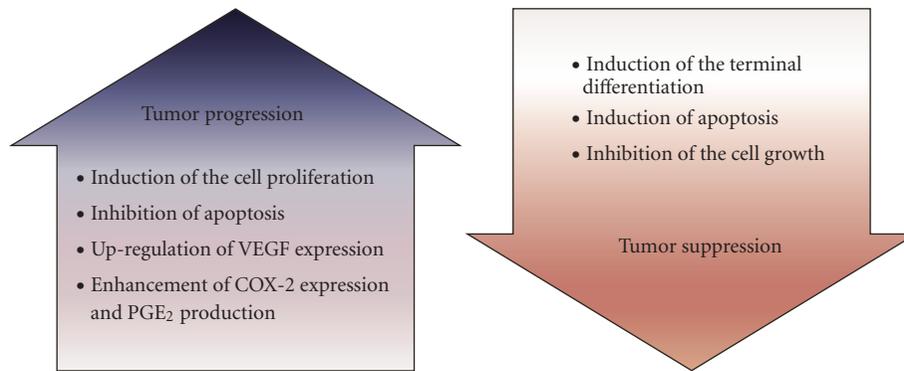
Figure 2: Does PPARδ progress or suppress tumor growth?

Table 2: The expression of PPARγ in cancer.

	References
Colonic tumor	[135]
Breast tumor	[137]
Esophageal tumor	[138]
Gastric cancer	[139]
Pancreatic cancer	[140]
Hepatocellular carcinoma	[141]
Adrenocortical carcinoma	[142]
Lung tumor	[143]
Prostate cancer	[144]
Liposarcoma	[145]
Thyroid carcinoma	[146]
Bladder cancer	[147]
Renal cell carcinoma	[148]
Melanoma	[149]
Squamous cell carcinoma	[150]
Cervical carcinoma	[151]
Testicular cancer	[152]
Neuroblastoma	[153]
Pituitary tumor	[154]

with rosiglitazone inhibited the K-Ras-induced elevation of the expression levels of cyclin D1 by inhibition of the K-Ras-induced phosphorylation of Akt, resulting in the G1 cell cycle arrest [89]. Furthermore, J.-A. Kim et al. showed that treatment of the human colorectal cell line, HCT15, with troglitazone induced the expression of p21$^{Cip1/Waf1}$, that is, a CDK inhibitor (CKI) and negatively regulates the cell cycle progression, through the ERK pathway, and inhibited HCT15 cell growth [155]. PPARγ ligands also induce apoptosis in human colon cancer cells [156]. Chen et al. showed that PPARγ ligands, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15dPGJ$_2$), or ciglitazone, induced apoptosis in HT-29 by inhibiting nuclear factor kappa B (NF-κB) activity, which upregulates various antiapoptotic genes, and suppressing the expression of BCL-2, which protects cells against apoptosis

[133]. Furthermore, using the in vivo mouse model, the administration of TZD to mice reduced AOM and/or dextran sodium sulfate-induced formation of aberrant crypts foci and colon carcinogenesis [131, 157]. In addition, PPARγ ligands also inhibit the cell growth of several breast cancer cell lines and mammary gland tumor development [137, 158–162]. Elstner et al. showed that PPARγ ligands, troglitazone, 15dPGJ$_2$, and indomethacin, caused inhibition of proliferation in several human breast cancer cell lines, such as MCF7, MDA-MB-231, BT474, and T47D [162]. Troglitazone also inhibited MCF7 tumor growth in triple-immunodeficient BNX nude mice [162]. Clay et al. reported that 15dPGJ$_2$ and troglitzazone attenuated cellular proliferation of MDA-MB-231 by blocking cell cycle progression and inducing apoptosis [160]. Pretreatment of MDA-MB-231 cells with 15dPGJ$_2$ attenuated the capacity of these cells to induce tumors in nude mice [160]. Yin et al. showed that treatment of MCF7 with troglitazone also decreased the expression of several regulators of pRb phosphorylation, such as cyclin D1, CDK4, CDK6, and CDK2 [158]. pRB is a retinoblastoma tumor suppressor gene product, and phosphorylated pRB induces cell cycle progression [163]. Troglitazone induced the G1 cell cycle arrest by attenuation of pRb phosphorylation, resulting in inhibition of cell proliferation [158]. Suh et al. showed that GW7845, synthetic PPARγ ligand, prevented mammary carcinogenesis in the rat model that used nitrosomethylurea as the carcinogen [159]. Mehta et al. also reported that troglitazone prevented the induction of preneoplastic lesions by 7, 12-dimethylbenz[a]anthracene in a mouse mammary gland organ culture model [161]. Moreover, PPARγ ligands inhibit the cell proliferation in other types of cancer. PPARγ ligands inhibited the growth of esophageal squamous carcinoma cell lines by inducing G1 arrest associated with an increased level of several CKIs, such as p27^{Kip1}, p21$^{Cip1/Waf1}$, and p18^{Ink4c} [138]. PPARγ ligands also induced apoptosis and G1 cell cycle arrest in human gastric cancer cells, and that inhibited cell proliferation [139, 164]. In human pancreatic cancer cells, PPARγ ligands induced apoptosis and growth inhibition associated with G1 cell cycle arrest through increasing p27^{Kip1} protein expression [140, 165–167]. In human hepatocellular carcinoma cell lines, PPARγ ligands induced cell cycle arrest through increased expression

of p21$^{Cip1/Waf1}$, p27^{Kip1}, and p18^{Ink4c} protein levels [141, 168]. Troglitazone also induced the activation of the cell death protease, caspase 3, and that induced apoptosis of human liver cancer cell lines [169]. PPARγ is abundantly expressed in human adrenal tumors including adrenocortical carcinomas and normal adrenal tissues. PPARγ agonists suppress adrenocortical tumor cell proliferation, increase apoptosis, and induce adrenal differentiation [142, 170]. Moreover, PPARγ ligand showed antitumor effect against human prostate cancer cells and human lung cancer cells [143, 144, 171–173]. Thus PPARγ ligands could suppress the tumorigenesis. Therefore, PPARγ ligands could be used as antineoplastic drugs.

In contrast, both troglitazone and rosiglitazone treatment increased the frequency and size of colon tumors in Apc^{min} mice [174, 175]. Treatment with rosiglitazone also increased the expression levels of β-catenin, a protein involved in Wnt signaling and correlating with enhanced cell proliferation, in the colon of Apc^{min} mice and HT-29 cells [174]. To investigate the basis for this contradiction, Girnun et al. used mice heterozygous for PPARγ with both chemical and genetic models of human colon cancer [176]. Heterozygous loss of PPARγ caused a greater incidence of colon cancer when these mice were treated with AOM. Although there was no difference in β-catenin expression levels in colorectal tumors between AOM-treated $PPARg^{+/-}$ and wild-type mice, β-catenin expression levels in the colonic epithelium of untreated $PPARg^{+/-}$ mice were greater than that of untreated wild-type mice. When crossing to Apc^{1638N} mice, the mouse model for FAP, there were also no difference in β-catenin levels between $PPARg^{+/-}/Apc^{1638N}$ and $PPARg^{+/+}/Apc^{1638N}$ mice before polyp formation. Survival and the number of tumors formed in the colon also showed no difference in both mice. Thus although PPARγ has the potential to suppress β-catenin levels and colon carcinogenesis, PPARγ has no effect on β-catenin levels or tumorigenesis in the presence of APC signaling dysfunction [176]. Furthermore, PPARγ mutations, some of which show the loss of the transactivation ability, are found in colon cancers in humans, and that PPARγ may be considered as a tumor suppressor gene [134]. On the other hand, to evaluate the contribution of PPARγ to breast cancer, Saez et al. generated transgenic mice, MMTV-VpPPARγ mice, that express a constitutively active form of PPARγ in mammary gland [177]. MMTV-VpPPARγ mice showed normal development of mammary gland and no increased tendency to develop tumors. To assess the influence of increased PPARγ signaling on mammary gland neoplasia, MMTV-VpPPARγ mice were crossed to mice that express a polyoma virus middle T antigen (PyV-MT) in mammary tissue, MMTV-PyV mice, which rapidly develop tumors. These mice that expressed both activated PPARγ and PyV-MT showed accelerated development of mammary tumors. Therefore, although increased PPARγ activation does not initiate tumor formation in normal mammary tissue, once a tumor-initiating event occurs, PPARγ signaling serves as a tumor promoter in the mammary gland. Furthermore, there is no difference in tumor development between MMTV-PyV mice and the mice, generated by crossing $PPARg^{+/-}$ mice to MMTV-PyV mice [177]. Thus in this model, PPARγ does not act as a tumor suppressor gene.

Furthermore, PPARγ ligands exert their biological effects through a PPARγ-independent pathway. Palakurthi et al. reported that troglitazone and ciglitazone induced G1 arrest by inhibiting translation initiation in both $PPARg^{-/-}$ and $PPARg^{+/+}$ mouse embryonic stem cells. Thus TZDs inhibit cell proliferation and tumor growth in a PPARγ-independent manner [178]. Therefore, although PPARγ ligands are used as insulin sensitizers, further investigation is needed to clarify whether PPARγ ligands are effective chemotherapeutic agents for cancer in humans.

5. SUMMARY

PPARs are linked to metabolic disorders and are interesting pharmaceutical targets. Among the synthetic ligands, fibrates are hypolipidemic compounds that activate PPARα, and TZDs, which selectively activate PPARγ, are hypoglycaemic molecules that are used to treat type 2 diabetes. PPARδ agonists might form effective drugs for obesity, diabetes, and cardiovascular disease. Moreover, recent evidence suggests that PPAR modulators may have beneficial effects as chemopreventive agents [179]. However, as mentioned above, it remains unclear whether PPARs act as oncogenes or as tumor suppressors. From this viewpoint, current strategies are aimed at reducing the side effects and improving the efficacy and safety profile of PPAR agonists, termed selective PPAR modulators (SPPARMs) [180, 181]. This model proposes that SPPARMs bind in distinct manners to the ligand binding pocket of PPAR and induce distinct conformational changes of the receptor, resulting in differential interactions with cofactors according to the combination of their expression levels in different organs. Thus each SPPARM leads to differential gene expression and biological response. However, what kinds of cofactors are recruited to PPAR by each SPPARM is still unknown. Thus it is important to identify the cofactor complex for PPAR with each SPPARM and the expression patterns of cofactors in various tissues. Furthermore, recent evidence suggests that the ligand binding protein in the cytosol that transports ligands into the nucleus is important to modulate the action of nuclear receptors. Long-chain fatty acids, endogenous PPAR ligands, are highly hydrophobic and fatty acids are bound to fatty acid binding proteins (FABPs) in the aqueous intracellular compartment [182]. FABPs also bind to PPAR ligands and transport them from the cytosol into the nucleus [183–191]. In the nucleus, FABPs interact directly with PPARs and deliver ligands to PPARs, and the activity of PPARs is modulated [186, 187, 190–192]. Recently, Schug et al. showed that when the cellular retinoic acid binding protein-II (CRABP-II) expression levels were higher than FABP5 in the cells, retinoic acid (RA) bound to CRABP-II. Subsequently, CRABP-II relocated to the nucleus and delivered RA to RAR, resulting in inhibition of cell proliferation and induction of apoptosis [187, 193]. On the contrary, when the FABP5 to CRABP-II ratio is high, RA serves as a physiological ligand for PPARδ, which induces cell survival and proliferation [187, 194]. Therefore, it is

important to identify the cytosolic ligand binding proteins and the expression levels of the proteins for defining the physiological effects of ligands. Furthermore, several ligands exert their biological effects through a PPAR-independent pathway [195]. Thus further studies are required to elucidate the role of PPARs for developing new efficiently and safety chemotherapeutic agents for cancer.

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Review Article

Screening for PPAR Responsive Regulatory Modules in Cancer

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Peroxisome proliferator-activated receptors (PPARs) have via their large set of target genes a critical impact on numerous diseases including cancer. Cancer development involves numerous regulatory cascades that drive the progression of the malignancy of the cells. On a genomic level, these pathways converge on regulatory modules, some of which contain colocalizing PPAR binding sites (PPREs). We developed an in silico screening method that incorporates experiment- and informatics-derived evidence for a more reliable prediction of PPREs and PPAR target genes. This method is based on DNA-binding data of PPAR subtypes to a panel of DR1-type PPREs and tracking the enrichment of binding sites from multiple species. The ability of PPARγ to induce cellular differentiation and the existence of FDA-approved PPARγ agonists encourage the exploration of possibilities to activate or inactivate PPRE containing modules to arrest cancer progression. Recent advances in genomic techniques combined with computational analysis of binding modules are discussed in the review with the example of our recent screen for PPREs on human chromosome 19.

1. INTRODUCTION

Cellular proliferation and differentiation are controlled by transcriptional regulation of a large subset of the human genome. The transcriptomes of normal and tumor cells as revealed by microarray analysis show significant differences [1] suggesting that in cancer the precise transcriptional control got lost due to overactive oncogenes and loss of function of tumor suppressor genes, many of which are coding for transcription factors. For a molecular insight into cancer, the transcriptional regulation of probably thousands of genes has to be uncovered in detail by integrating expression array data with regulatory site location data [2]. Although the understanding of the regulation of a couple of key genes, like the cyclin-dependent kinase inhibitor $p21^{WAFI/CIPI}$ [3], is already quite advanced, for the majority of the cancer-associated genes such detailed analyses have not been performed. Even "big biology" projects, such as ENCODE [4], have focused only on 1% of the human genome sequence so far, while other genome-wide scans, for example, for histone modifications [5–7] or transcription factor binding [8, 9], had to concentrate on only a subset of modifications and factors under limited experimental conditions. Databases, such as oncomine [1] for gene

expression data and the UCSC genome browser [10] for visualization of genome-wide chromatin immunoprecipitation data and transcription factor binding site location data, allow the combination of data from various projects. Together, these data resources may provide sufficient insight to understand the regulation of an individual gene in a complex disease state, such as cancer. In addition, efforts to improve bioinformatics methods predicting the binding and interaction of transcription factors together with more extensive experimental datasets will fill important gaps [11].

Each individual gene is under the control of a large set of transcription factors that can bind upstream and downstream of its transcription start site (TSS) [12]. These sites typically arrange into collections of neighboring sites, the so-called modules or enhancers. Modules of transcription factors that act on focused genomic regions have been shown to be far more effective than individual factors on isolated locations and can act from large distances up to hundreds of thousands of base pairs. In an ideal case such transcription factor modules can be identified by parallel and comparative analysis of their binding sites. Here, bioinformatics approaches can be of great help, in case they can predict the actions of the transcription factors precisely enough [13].

PPARs are transcription factors that have the special property to be ligand-inducible, which they share with most other members of the nuclear receptor superfamily [14]. This property has attracted a lot of interest in the nuclear receptor family as possible therapeutical targets in context of cancer. PPARs were initially described as the nuclear receptors for compounds that induce peroxisome proliferation in rodents [15], but now they are know to be important sensors of cellular levels of fatty acids and fatty-acid derivatives that are mainly derived from the lipoxygenase and cyclooxygenase pathways [16]. Polyunsaturated fatty acids activate the three PPAR subtypes with relatively low affinity, whereas fatty acid derivatives show more binding selectivity [17]. PPARs are prominent players in the metabolic syndrome because of their role as important regulators of lipid storage and catabolism [18], but they also regulate cellular growth and differentiation and therefore have an impact on hyperproliferative diseases, such as cancer [19]. Bioinformatic approaches to identify genomic targets of PPARs and important cancer regulatory modules with colocalizing PPREs, as they will be described below, should have a major impact on understanding the role and potential therapeutic value of PPARs in cancer.

2. THE PPAR SUBFAMILY

The three PPAR subtypes α (NR1C1), β/δ (NR1C2), and γ (NR1C3) are coexpressed in numerous cell types from either ectodermal, mesodermal, or endodermal origin, although their concentration relative to each other varies widely [20, 21]. Importantly, most tumor cells express at least one PPAR subtype at higher levels suggesting that PPAR ligands may modulate the transcription of many PPAR target genes in a beneficial way.

PPARα is highly expressed in cells that have active fatty acid oxidation capacity including hepatocytes, cardiomyocytes, enterocytes, and the proximal tubule cells of the kidney [22]. This PPAR subtype is a central regulator of hepatic fatty acid catabolism and glucose metabolism. Furthermore, it potently represses the hepatic inflammatory response by downregulating the expression of numerous genes, such as various acute-phase proteins. PPARα is the molecular target for the hypolipidemic fibrates, a group of drugs that are prescribed for their ability to lower plasma triacylglycerols and elevate plasma HDL (high-density lipoprotein) levels.

PPARβ/δ is expressed ubiquitously and often displays at higher expression levels than PPARα and γ. It stimulates fatty acid oxidation in both adipose tissue and skeletal muscle, regulates hepatic VLDL (very low-density lipoprotein) production and catabolism and is involved in wound healing by governing keratinocyte differentiation [23].

PPARγ is expressed predominantly in adipose tissue and the immune system and exists as two distinct protein forms $\gamma 1$ and $\gamma 2$, which arise by differential TSSs and alternative splicing [22]. PPARγ is the master regulator of adipogenesis and regulates cell-cycle withdrawal, as well as induction of fat-specific target genes that are involved in adipocyte metabolism [24]. PPARγ stimulates the expression of numerous genes that are involved in lipogenesis, including

those for adipocyte fatty acid-binding protein, lipoprotein lipase, and fatty acid translocase (CD36). The general role for PPARγ in the regulation of lipid metabolism is underlined by the therapeutic utilization of the PPARγ ligands thiazolidinediones in obesity-linked type II diabetes [25].

3. PPARs AND THE TRANSCRIPTIONAL MACHINERY

An essential prerequisite for the direct modulation of transcription by PPAR ligands is the location of at least one activated PPAR protein close to the TSS of the respective primary PPAR target gene. This is commonly achieved through the specific binding of PPARs to a DNA binding site, the so-called PPRE, and DNA-looping towards the TSS [26]. In detail, the DNA-binding domain of PPARs contact the major groove of a double-stranded hexameric DNA sequence with the optimal AGGTCA core binding sequence. PPARs bind to DNA as heterodimers with the nuclear receptor retinoid X-receptor (RXR) [27]. PPREs are therefore formed by two hexameric core binding motifs in a direct repeat orientation with an optimal spacing of one nucleotide (DR1), where PPAR occupies the 5′-motif [28]. However, characterization of PPREs from regulated gene promoters has resulted in a large collection of PPREs that deviate significantly from this consensus sequence. An extensive binding data collection for PPARs was recently published [29], where more critical deviations and well-tolerated deviations from the consensus were identified as will be further explained in the following chapters.

When a nuclear receptor, such as PPAR, is bound to PPREs in the regulatory regions of its target genes, it recruits positive and negative coregulatory proteins, referred to as coactivators [30] and corepressors [31], respectively. In consequence, the transcriptional output is dependent on cell- and time-specific expression patterns of these coregulators and can produce distinct modulations of transcription factors, such as PPARs, due to differences in the relative corepressor and coactivator protein levels. This aspect has diagnostic and therapeutic value and can be extracted from expression level data in different types of cancer [32]. Most unliganded nuclear receptors preferentially interact with corepressors to mediate repression, but PPARs have been found to show a reasonable level of constitutive activity [33], that is, in the absence of ligand coactivator proteins can compete for binding. Most coregulators are not exclusive to PPARs and even not specific to nuclear receptors, but are also used in a similar manner by other transcription factors [34]. One group of coregulators covalently modifies histone proteins, which are as nucleosome constituents the main chromatin proteins. This acetylation/deacetylation and methylation/demethylation follows a precise and combinatorial code, the so-called histone code [35]. The second group of coregulators includes ATP-dependent chromatin remodeling factors that modulate the accessibility of genomic regions to transcription factors and to the basal transcriptional machinery [36]. Recently, their actions have been monitored on genome-wide level to reveal common patterns of transcriptionally active regions and regulatory

sites [5, 7, 9]. These snap-shots have provided important insights to common regulatory code, whereas more detailed studies have explored the dynamics of these processes as described below.

Repression and activation are more likely achieved by a series of sequential events that are mediated by multiple enzymatic activities that are promoter and cell-type specific. Transcriptional regulation is a highly dynamic event of rapid association and dissociation of proteins and their modification, including degradation and de novo synthesis. A pattern of recruitment and release of cohorts of coregulatory complexes was demonstrated on a single region of the *trefoil factor-1* promoter in breast cancer cells [37]. This study revealed detailed and coordinated patterns of coregulator recruitment and preferential selectivity for factors that have similar enzymatic activities. Similar cycling was also observed for the recruitment of PPARβ/δ to the TSS of the *pyruvate dehydrogenase kinase* 4 (Degenhardt et al., unpublished). Understanding the events that lead to the disturbance of such coordinated action of regulatory proteins in cancer progression could help finding means to reinitiate the coordinated regulation. Partial restoration of regulation was demonstrated on the *trefoil factor-1* promoter by removal of methylation in an unresponsive cell line [38].

4. PPARs IN CANCER

The rapid growth of tumor cells is highly dependent on the availability of macronutrients and their metabolism. In their role as master regulators of lipid metabolism, all three PPAR subtypes have at least an indirect function in controlling cellular growth [26]. Moreover, the dominant function of PPARγ in adipocyte differentiation and the suppression of apoptosis in keratinocytes by PPARβ/δ suggest a direct role of PPARs in the control of cellular growth and death [19]. As a consequence, a number of prominent PPAR target genes, such as *angiopoietin-like 4*, *lipoprotein lipase*, *LDL-receptor-related protein 1*, and *caveolin-1*, were described to be involved in the control of tumor cell growth [39–42]. Furthermore, there is a strong physiological link between chronic inflammation and the onset of cancer [43]. In this way, the anti-inflammatory actions of PPARs [44] provide an additional argument for their control function on cellular proliferation, differentiation, and apoptosis.

However, there is also evidence to state that PPARs may in some cases promote cancer progression. PPARβ/δ has been implicated in colorectal carcinogenesis [45], its mRNA is often upregulated in tumors and the deletion of the PPARβ/δ gene results in a profound loss of tumorigenicity in nude mice [46]. Moreover, PPARβ/δ was found to have an essential role in constraining tumor endothelial cell proliferation to allow the formation of functional tumor microvessels, that is, the receptor is important for angiogenesis [47].

As a general argument, we can propose that the main role of PPARs, the control of metabolism or inflammation, may also contribute to the regulation of cellular growth. How that translates (via transcriptional regulation) into interference in cancer progression or change to a more benign phenotype, may be highly dependent on cancer type and state. In fact,

the net effect of the activation of some PPAR target genes may rather result in the stimulation of cellular proliferation than in its inhibition, when examined alone. Data on gene expression, on regulatory modules, on their accessibility, and on the binding of PPARs to those modules need to be joined, in order to get a handle on the pleiotropic effects of PPARs in cancer.

5. METHODS FOR IN SILICO SCREENING OF TRANSCRIPTION FACTOR BINDING SITES

The specificity of PPARs for their binding sites allows constructing a model to describe the PPRE properties that can be used to predict potential binding sites in genomic sequences. For this, the PPAR binding preference, often expressed as position weight matrix (PWM), has to be described on the basis of experimental data, such as series of gel shift assays with a large number of natural binding sites [48–51]. However, PPAR-RXR heterodimers do not only recognize a pair of the consensus binding motifs AGGTCA, but also a number of variations to it. Dependent of the individual PWM description, this leads to a prediction of PPREs every 1000 to 10000 bp of genomic sequence. This probably contains many false positive predictions, which is mainly due to scoring methodology and the limitations that are imposed by the available experimental data. For example, the quantitative characteristics of a transcription factor, that is, its relative binding strength to a number of different binding sites, is neglected in a position frequency matrix, where simply the total number of observations of each nucleotide is recorded for each position. Moreover, in the past there was a positional bias of transcription factor binding sites upstream in close vicinity to the TSS. This would be apparent from the collection of identified PPREs, but is in contrast with a multigenome comparison of nuclear receptor binding site distribution [52] and other reports on wide-range associations of distal regulatory sites [7].

Internet-based software tools, such as TRANSFAC [53], screen DNA sequences with databases of matrix models. One approach used PWMs to describe the binding preferences of PPARs using all published PPREs [54]. The accuracy of such methods can be improved by taking the evolutionary conservation of the binding site and that of the flanking genomic region into account. Moreover, cooperative interactions between transcription factors, that is, regulatory modules, can be taken into account by screening for binding site clusters. The combination of phylogenetic footprinting and PWM searches applied to orthologous human and mouse gene sequences reduces the rate of false predictions by an order of magnitude, but leads to some reduction in sensitivity [11]. Recent studies suggest that a surprisingly large fraction of regulatory sites may not be conserved but yet are functional, which suggests that sequence conservation revealed by alignments may not capture some relevant regulatory regions [55].

In effect, these approaches and tools are still insufficient and there has to be a focus on the creation of bioinformatics resources that include more directly the biochemical restrains to regulate gene transcription. One important

aspect is that most putative transcription factor binding sites are covered by nucleosomes, so that they are not accessible to the transcription factor. This repressive environment is found in particular for those sequences that are either contained within interspersed sequences, are located isolated from transcription factor modules, or lie outside of insulator sequences marking the border of chromatin loops [56]. This perspective strongly discourages the idea that isolated, simple PPREs may be functional in vivo. In turn, this idea implies that the more transcription factor binding sites a given promoter region contains and the more of these transcription factors are expressed, the higher is the chance that this area of the promoter becomes locally decondensed.

The PAZAR information mall [57] is a tertiary database that is build on the resource of a multitude of secondary databases and provides a computing infrastructure for the creation, maintenance, and dissemination of regulatory sequence annotation. The unambiguous identification of the chromosome location for any given transcription factor binding site using genomic coordinates allows to link the results from "big biology" projects, such as ENCODE [4], and other whole genome scans for histone modification and transcription factor association. Unfortunately, so far only a few boutiques have been opened inside the PAZAR framework. In order to benefit from binding site predictions, it is still necessary to explore dedicated resources. For example, the well-known regulator of cell cycle progression, the transcription factor p53, has an own dedicated database (p53FamTaG) for integration of gene expression and binding site data [58].

The concept of cancer-specific regulatory modules has raised increasing attention recently. Genome-wide prediction of enhancers based on analysis of transcription factor binding affinity by a computational tool, called enhancer element locator [8], was shown effective to dissect which types of cancer can be targeted by a given transcription factor. Predictions validated in transgenic mouse embryos revealed the presence of multiple tissue-specific enhancers in mouse *c-myc* and *N-myc* genes, which has implications for organ-specific growth control and tumor-type specificity of oncogenes.

6. THE CLASSIFIER METHOD FOR PPREs

Approaches for PPRE predictions have been based on a collection of disparate binding data. To combine evidence from several publications for an efficient binding model has challenges thus creating a demand for a coherent binding dataset. The recently published classifier method [29] used the in vitro binding preferences of the three PPAR subtypes on a panel of 39 systematic single nucleotide variations of the consensus DR1-type PPRE (**AGGTCAAAGGTCA**) [59] as an experimental dataset. The single nucleotide variants were sorted into three classes, where in class I the PPAR subtypes are able to bind the sequence with a strength of 75 ± 15% of that of the consensus PPRE, in class II with 45 ± 15%, and in class III with 15 ± 15%. Although the overall binding pattern of the three PPAR subtypes showed no major differences, some variations gave rise to
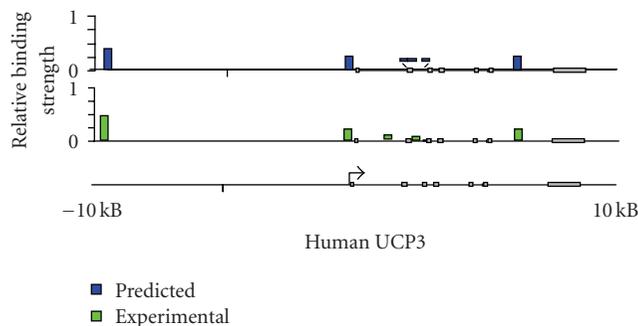
Figure 1: Comparison of in silico and experimental analysis of PPAR target genes. Overview of the genomic organization of the *UCP3* gene; 10 kB upstream and downstream of its TSS are shown (horizontal black line). Putative PPREs were identified using the classifier method performing in silico screening of the genomic sequences. For each predicted PPRE, the calculated binding strength of PPARγ is represented by column height. The average in vitro DNA binding strength of PPARγ-RXR heterodimers was also determined by gel shift experiments.

a PPAR subtype-specific classification. Additional 130 DR1-type PPREs were sorted on the basis of counting increasing number of variations from the consensus and taking into account the single nucleotide variant binding strength. Those variants that alone decrease the binding only modestly (class I) could be combined with even three deviations from consensus still resulting in more than 20% binding relative to consensus. Other combinations resulted in faster loss of binding detailed in 11 categories, where such combinations still resulted in more than 1% relative binding.

The in silico binding strength predictions of PPAR-RXR heterodimers were confirmed by gel shift assays for the six PPREs of the *uncoupling protein 3* (*UCP3*) gene and showed a deviation of less than 15% (Figure 1). Moreover, from 23 investigated genomic regions that were selected from eight genes, 17 regions display significant inducibility in the presence of PPAR ligands and in living cells. PPARα and RXRα associated with 16 of these regions. For the *UCP3* gene, for which previously no regulatory regions had been described to account for the effect of PPAR ligands on its mRNA transcription, three functional areas were identified [29].

The main advantage, when comparing the classifier to PWM methods, is a clear separation between weak PPREs and those of medium and strong strength [29]. For the discovery of potential binding sites, this is extra information that could be especially of interest in processes considered context dependent, for example, for PPREs that reside in genomic context of transcription factor modules. Predicting the strength of PPAR binding can be a predictor of how prominent effect this receptor can have on a target gene. For example, if binding is easily competed by other transcription factors, the effect may not manifest in most tissues or it may manifest only in tissues expressing all transcription factors of a module containing the PPRE. As an example of the latter case, the *insulin-like growth factor binding protein 1* gene has a weak PPRE located inside a well-conserved area
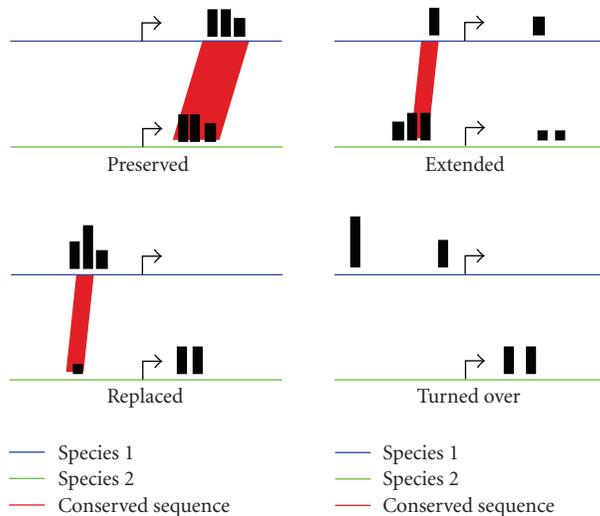
FIGURE 2: Possible evolutionary changes to PPRE location, strength, and conservation. Hypothetical genes from two different species (e.g., human and mouse) were compared for their PPREs (black bars, their height indicates relative strength). When the PPRE pattern is preserved, the genes will be sorted into cluster I, when extended in cluster II, when replaced in cluster III and when not at all conserved (e.g., when turned-over) in cluster IV.

(suggesting presence of other transcription factor binding sites) and was only in liver responsive to PPAR ligands [59]. In contrast, genes with strong PPREs, such as *carnitine palmitoyltransferase 1A* and *angiopoietin-like 4*, are PPAR responsive in many tissues (Heinäniemi et al., unpublished data).

7. CLUSTERING OF KNOWN PPAR TARGET GENES

The data added by binding strength analysis and by covering a larger regulatory region (± 10 kB) was examined with all 38 human genes that are known to be primary PPAR targets together with their mouse ortholog. The clustering by predicted binding strength and evolutionary conservation of their PPREs resulted in four groups [29]. In general, clusters I to II contain genes that are well conserved between human and mouse. Cluster I contains genes that carry multiple conserved PPREs, while genes in cluster II have only one or two strong or medium conserved PPRE in human, which are found in comparable strength and location in the mouse. Cluster III contains genes that have strong or medium PPREs in one species that are conserved only as weak PPREs in the other species. Finally, cluster IV contains more than 25% of all tested genes, which have the common property that they carry one or more PPREs, but none of them is conserved. These examples suggest that regulation of target gene can survive turnover of binding sites and might even benefit from it as indicated in Figure 2.

The clustering analysis indicated some useful features for whole genome PPRE screens. Either the presence of at least one strong PPRE or more than two medium PPREs within the 20 kB surrounding the annotated TSS of a gene is a strong

indication for a PPAR target gene. In this way, 28 out of the 38 the human genes would have been identified as PPAR targets. Similarly, for 29 of these 38 genes the analysis of their murine ortholog would have come to the same conclusion. A combination of these two criteria (passing the threshold in either the human or mouse ortholog) would have identified 37 of the 38 genes as PPAR targets.

7.1. A look at PPREs in their genomic context: putative target genes and binding modules

In the paper described above, the gene-dense human chromosome 19 (63.8 MB, 1445 known genes) and its syntenic mouse regions (956 genes have known orthologs) were selected for an in silico screening based on the above explained criteria; that is, both species were investigated for medium and strong PPREs (based on a PPARγ prediction) [29]. Interestingly, 20% of genes of chromosome 19 contain a colocalizing strong PPRE and additional 4% have more than two medium PPREs or a proximal medium PPRE. These numbers suggest a total of 4000 to 5000 targets for PPARs in the human genome, if no false positives are assumed. Certainly, not all sites will be accessible and the human genome also contains weak binding sites that could gain function via interaction with other transcription factors. The latter can also be screened with the acquired knowledge on PPAR binding preferences down to 1% relative to the consensus PPRE. Experimentally, a complete evaluation of the selectivity of any such screen is complicated by the restricted expression profiles of the predicted genes, which prevents simple readouts from individual target tissues. When requiring the detection in human and mouse, 12.1% of genes from chromosome 19 were predicted as PPAR targets. In this approach, full alignment was not required, just preservation of what could be called PPAR binding potential. The more strong PPREs a gene has accumulated, the smaller the chances are that given all 250 human tissues none of these sites would get accessible or be built into a regulatory module with other transcription factor binding sites. Of relevance to cancer several cell cycle regulating genes were found by the screen, some of which have been reported as PPAR targets by others, such as *G1/S-specific cyclin E* [60], $p19^{INK4d}$ [61], *prostate tumor overexpressed gene*, *serine protease hepsin* [62] and the serine/threonine kinases associated with cell cycle regulation *p21-activated kinase 4* (*PAK4*), and *homeodomain-interacting protein kinase 4*. In addition, the prostate tumor marker *kallikrein-3* [63] and several other *kallikrein* gene family members were detected. From novel targets, the regulatory regions of a ceramide synthesis regulator, *LASS1*, were experimentally confirmed [29]. Overexpression of this protein has been shown to restore normal ceramide levels and inhibit the growth of head and neck squamous carcinomas [64].

The complete list of putative PPAR target genes in chromosome 19 [29] offers interesting candidates representing physiological functions connected to PPARs. It will gain more power, when it can be integrated with other genomic screens, both experimental and bioinformatics, as has been outlined in the previous discussion. A vision for future of
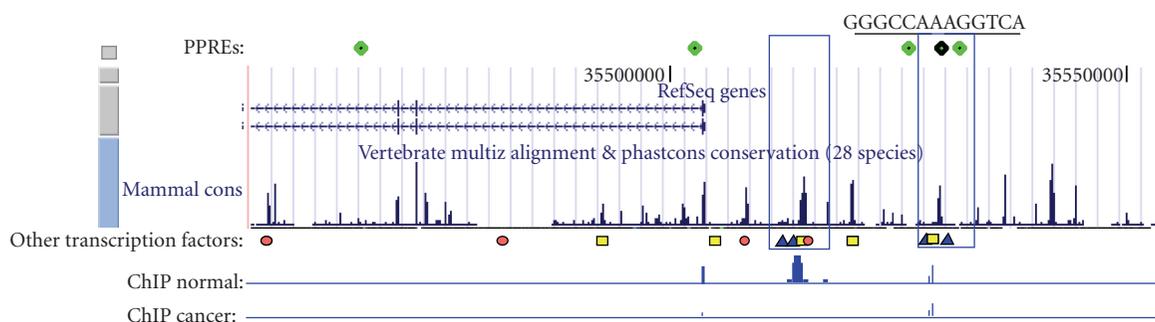
FIGURE 3: A gene module map compiled from bioinformatics data and experimental datasets. The superimposition of the PPRE track (in green on top) on other genome-wide datasets can reveal promising PPRE-containing binding modules for targeted therapy via PPAR activation. In this imaginary setting, transcription factor 1 (in blue) is known to be one main regulator of the hypothetical gene X and this regulation is altered in cancer. Transcription factor 2 (in yellow) synergistically activates gene X, but is lost in cancer cells. Chromatin immunoprecipitation data comparing normal and cancer binding profiles for this transcription factor reveal two main regulatory modules under normal conditions and a weaker binding in cancer samples due to loss of transcription factor 2. A colocalizing PPRE in module 2 could enable PPARs to replace transcription factor 2 in this module and to restore strong activation of this gene.

targeting cancer regulatory modules with colocalizing PPREs is depicted in Figure 3. A PPRE track (for simplicity binding strength was not indicated) provided by bioinformatics approaches can be compared against evidence of other regulatory modules provided by conservation analysis and screens for other transcription factors. Experimental data comparing regulation in a specific cancer type versus normal cells can be visualized in the same context to detect overlap in functional binding sites. Given the high interest of the scientific community to better characterize binding profiles of different transcription factors and the improved experimental techniques to detect genome-wide binding events, such additional tracks combined with a PPRE binding track could be available in near future.

8. CONCLUSION

The identification of genes showing a primary response to PPARs and their ligands, the so-called PPAR regulome, can be used as a prediction of their therapeutic potential as well as their possible side effects. Methods incorporating both experimental- and informatics-derived evidence to arrive at a more reliable prediction of PPAR targets and binding modules can bring all available data together with the aim to predict outcome in specific context. Taking the chromosome 19 in silico screening trial as an example and extrapolating the results to the whole human genome, we suggest that approximately 10% of all human genes (an estimate of 2000 to 2500 genes) have the potential to be directly regulated by PPARs by their PPRE content within 10 kB distance to their TSS. Translated to regulatory modules that colocalize with PPREs, an even larger number of genomic regions could be targeted by PPARs. In conclusion, in this review we have addressed the identification of direct targets using genomic sequences and binding data. In parallel, we have discussed the potential of looking for PPREs inside regulatory modules foreseeing that in future, very likely the emphasis will shift from target genes to target regulatory modules to alter a physiological response and from individual genes to whole genome response.

LIST OF ABBREVIATIONS

DR1:   Direct repeat spaced by one nucleotide
PPAR: Peroxisome proliferator-activated receptor
PPRE: PPAR response element
PWM: Position weight matrix
RXR: Retinoid X-receptor
TSS: Transcription start site
UCP3: Uncoupling protein 3.

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Review Article

A Role for the PPARγ in Cancer Therapy

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In 1997, the first published reports highlighted PPARγ as a novel cancer therapeutic target regulating differentiation of cancer cells. A subsequent flurry of papers described these activities more widely and fuelled further enthusiasm for differentiation therapy, as the ligands for the PPARγ were seen as well tolerated and in several cases well-established in other therapeutic contexts. This initial enthusiasm and promise was somewhat tempered by contradictory findings in several murine cancer models and equivocal trial findings. As more understanding has emerged in recent years, a renaissance has occurred in targeting PPARγ within the context of either chemoprevention or chemotherapy. This clarity has arisen in part through a clearer understanding of PPARγ biology, how the receptor interacts with other proteins and signaling events, and the mechanisms that modulate its transcriptional actions. Equally greater translational understanding of this target has arisen from a clearer understanding of in vivo murine cancer models. Clinical exploitation will most likely require precise and quantifiable description of PPARγ actions, and resolution of which targets are the most beneficial to target combined with an understanding of the mechanisms that limits its anticancer effectiveness.

1. CURRENT UNDERSTANDING OF PPARγ BIOLOGY

1.1. PPARγ is a transcription factor

The human PPARγ was cloned in 1994 and subsequently two murine isoforms were identified in mouse: gamma-1 and gamma-2, resulting from the use of different initiator methionines [1, 2]. Subsequently, at least three isoforms have been identified in humans with common expression in adipocytes and the large intestine and more restricted isoform expression in other tissues [3]. PPARγ plays a key role in energy metabolism and differentiation (reviewed in [4–7]); and reflecting this, the murine $Ppar\gamma^{-/-}$ is embryonically lethal, and if rescued, the animal lacks normal adipocytes [8].

PPARγ is a phylogenetic member of subfamily 1 the nuclear receptor (NR) superfamily and shares a number of generic mechanistic features in common with other subgroup members, including the retinoic acid receptors (RARs), vitamin D receptor (VDR), farnesoid X receptor (FXR), and liver X receptors (LXRs). These receptors are most commonly located in the nucleus and heterodimerize with one of three retinoid X receptor (RXR) subtypes, to bind specific response elements in target gene regulatory regions. Crystallization studies of PPARγ bound with RXRα proved pivotal for deciphering the basis for heterodimerization with RXR for multiple NRs [9]. The presence of ligand changes the receptor confirmation and also influences choice of association with either coactivator (CoA) or corepressor (CoR) complexes. In the absence of ligand, NR heterodimers are contained within multimeric complexes (∼2.0 MDa) containing CoRs (e.g., NCOR1) [10]. Also, within these complexes is a range of enzymes, which act to modify the posttranslational status of histone tails and maintain a locally closed repressive chromatin environment, for example, histone deacetylases (HDAC), such as HDAC3 and SIRT1 [10–15].

Ligand activation shifts receptor conformation and distribution to enhance interaction with CoA complexes. A large number of interacting CoA proteins have been

described, which can be divided into multiple families including the NCOA/SRC family and members of the large bridging mediator complex including PPARγ binding protein (PBP/MED1) complex [16, 17]. Through the latter, the NR receptor complex links to the cointegrators CBP/p300 and basal transcriptional machinery. For example, PPARγ is known to associate with proteins, such as SRC-1, PGC1-α, CARM1, and a battery of histone modifying enzymes, such as histone acetyltransferases (HAT), which together initiate and promote transactivation [18–22].

The complex choreography of these events is a very active area of research, being at a crossroads of several important areas in contemporary biology, such as multimeric protein complex assembly and chromatin remodeling. Transcription involves cyclical rounds of promoter-specific complex assembly, gene transactivation, complex disassembly, and proteosome-mediated receptor degradation [23–25].

1.2. Newly characterized and unique features of PPARγ

Outside of these general characteristics, uncertainty and ambiguity remain in constructing a predicative schema for understanding PPARγ function and signaling in cancer biology. Some of the uncertainties arise due to a number of structural and regulatory variations of PPARγ outside the core features of NRs, thereby leading to apparently pleiotropic actions. Compounding these difficulties is the issue of studying PPARγ signaling in cancer biology, which is intrinsically an unstable and evolving disease environment.

By contrast to a high-affinity receptor, such as estrogen receptor α (ERα), the members of the subfamily 1 of the NR superfamily are typified by their large ligand-binding domain and may therefore accept different ligands. The PPARγ ligand-binding pocket has a volume of more than $1400 \, \text{Å}^3$ and therefore can bind a wide range of different lipophilic molecules (see Figure 1). As shown in Figure 1, free fatty acids are metabolized to arachidonic acid, and then through either lipooxygenase (LO) or cyclooxygenase (COX) activities to give rise to a range of natural ligands for PPARγ. Many of these reactions are tightly controlled such that a ligand metabolite is enzymatically generated and cleared.

Circulating and cellular fatty acids give rise to the majority of the natural ligands for PPARγ; therefore, the PPARs in general and PPARγ specifically form a sensing mechanism to maintain homeostasis in changing physiological circumstances such as feeding and exercise. This capacity, as discussed later, is implicated in a range of disease settings including cancer. The omega 6 fatty acid, linoleic acid, is highly inflammatory and therefore carefully controlled *in vivo*. It is a PPARγ ligand and, through subsequent desaturase and elongase activities, is metabolized to arachidonic acid. A wide range of natural ligands for PPARγ is subsequently derived through arachidonic acid metabolism. LO activity (e.g., arachidonate 5-LO and 15-LO) generates oxidized lipids which act as PPARγ ligands, such as 8 (S)-hydroxyeicosatetraenoic acid (8-(S)-HETE), 15-(S)-HETE, 9-hydroxy-10,12-octadecadienoicacid (9HODE), and 13-HODE. Subsequent dehydrogenase activity, for example,

of 13-HODE by 13-HODE dehydrogenase, can result in a further series of PPARγ ligands prior to their subsequent conversion to leuktrienes [26–28].

In parallel, arachidonic acid can be metabolized through cyclooxygenase activity (through COX-1 and -2) to prostaglandins such as PGH_2 and subsequently PGD_2, PGE_2, PGF_2, and PGI_2. These compounds exert a diverse range of cellular actions, but key metabolites in these cascades appear to exert potent PPARγ activation. PGD_2, the product of prostaglandin D synthase (encoded by *PGDS*), is able to undergo nonenzymatic degradation to a J series prostaglandin, 15-deoxy-^{12,14}-prostaglandin J_2 (15d-PGJ_2), which is a potent PPARγ ligand [26, 29–33]. Similarly, metabolites of PGE_2 can activate PPARγ, and their generation is controlled during differentiation, for example, of adipocytes [34]. Many of these reactions appear to be regulated through classical feedback loops, thus, the regulation of arachidonic acid metabolism to provide prostaglandins and leukotrienes is regulated at multiples levels by the actions of PPARγ, for example, regulation of LOs and of COX-2 activity and several of the downstream enzymes [26, 29–35].

The discovery of synthetic ligands for this receptor has been driven by the identification of a number of significant disease settings, in which PPARγ signaling is implicated (inflammation, metabolic disorders, and cancer). A goal of this research is the identification of novel pharmacological compound that display gene- and cell-selective actions [36]. The diversity of cell function, and presumably the relatively large ligand-binding pocket, has encouraged investigators to undertake rational screening approaches to identify a diverse panel of ligands [31, 37–51]. Indeed, novel selective compounds frequently display differential ligand-binding pocket docking sites. Implicit, within these discoveries is that the subtly different induced receptor conformations allow for the different spatiotemporal associations of CoA and ancillary proteins thereby deriving target gene specificity [40, 41, 52–55]. Thiazolidinediones (TZDs) were the first synthetic compounds investigated as PPARγ ligands [56]; this class also includes rosiglitazone, pioglitazone, and troglitazone. The latter caused a severe idiosyncratic liver problem and thus has been discontinued. The TZDs have proven to be a breakthrough in the therapy of type II diabetes because they decrease insulin resistance by promoting glucose uptake, mitochondrial biogenesis and fatty acid absorption by increasingly differentiated adipocytes (reviewed in [57]).

This focus at the level of the PPARγ ligand may be too exclusive. For example, the RXR member of this complex can also bind simultaneously with its ligand, which can result in enhanced transcriptional activity (6). Perhaps more importantly, the receptor structure allows it to influence both the basal and regulated transcription levels of target genes independent of ligand. That is, the unliganded structure of PPARγ also exposes a number of critical amino acids on helix 12 that allows CoA binding and may explain the high basal expression levels of PPARγ target genes in the absence of ligand. In this regard, PPARγ most closely resembles another xenobiotic metabolizing NR, constitutive androstane receptor (CAR) [58]. These findings may also

Fatty acids
e.g. linoleic acid

Arachidonic acid → LO → Oxidized AA metabolites
e.g. 8/15-HETEs   9/13-HODEs

COX −1 and −2

Secondary metabolism
e.g. 13-HODE dehydrogenase

Prostaglandin H_2 (PGH_2)

Intermediary metabolites
e.g. 13-Oxo-ODE

AKR1C3

$11\beta PFD_{2\alpha}$ ← PGD_2 PGE_2

15-hydroxyprostaglandin
dehydrogenase

Leukotrienes

PGJ_2 PGF_2

15-keto-PGE_2

15Δ12PGJ_2

15-oxoprostaglandin-Δ13-reductase

FIGURE 1: Generation of natural PPARγ ligands (solid arrows = direct conversion, broken arrows = multiple step conversion. Metabolites are indicated and some of the key regulating enzymes are shown in boxes).

suggest that the expression of CoA and CoR proteins are actually more important for regulating gene targets than either the levels or specificity of ligands.

The biology of PPARγ is intimately associated with that of the PGC-1α CoA and a number of other cofactors. The actions of these proteins have most clearly been described in well-established PPARγ systems, such as adipocyte differentiation and regulation of energy metabolism. The Pgc-1α murine knockout displays abnormal metabolic rates, temperature fluctuations, and a lethal cardiac defect [59, 60]. Reflecting its importance for regulating PPARγ function, levels of PGC-1α are tightly regulated by ubiquitination [61].

PPARγ receptor activity is also regulated by a cohort of posttranslational mechanisms, such as small ubiquitin-related modifier (SUMO) process. Sumoylation of the ligand-binding domain, in the presence of ligand, prevents the release and subsequent ubiquitination of NCOR1, and therefore sustains the repressive action, leading to the so-called ligand-dependent transrepression [62, 63]. This process is antagonized, by the removal of the SUMO modification by the SUSP-1 enzyme [64] thus establishing a dynamic level of regulation to modify the actual impact of ligand. Furthermore, PPARγ is serine phosphorylated, for example, in response to MAPK signaling leading to nuclear export and attenuation of transcriptional ability [65–67]. By contrast, PBP/MED1 is regulated at multiple sites by phosphorylation to enhance signaling by PPARγ [68].

To place the expression and regulation of PPARγ within the broader context of NR biology, several scientists have proposed and utilized system level approaches to dissect NR function including PPARγ. One of the most significant examples of this approach has been the spatiotemporal profiling of all 49 murine NRs in multiple tissues at different time points during the circadian rhythm [69, 70]. These approaches have revealed a number of provocative findings. In terms of tissue expression, *Pparγ* most closely follows *Lxrα* and *Gr*, and forms a triumvirate that is intimately implicated in the control of inflammation. The expression of PPARγ was shown also to follow circadian rhythm expression in white adipose tissue and the liver, but not other tissues [69, 70]. Similarly, others have shown that *Pgc-1α* follows a circadian rhythm in the liver and skeletal muscle of mice [20], and it cooperates with other NRs to regulate additional members of the clock family.

1.3. Transcriptional targets of PPARγ

One approach to defining PPARγ specificity has been to describe the cohort of target genes regulated by its actions; generally, these studies involve microarray studies in a range of cell types including adipocytes [71] and macrophages [72]. Commonly, a range of gene targets has been identified associated with metabolism and transport of lipids, including lipoprotein lipase, fatty acid binding, and transport proteins and acyl-CoA synthase. Similar approaches have been used to study the impact of PPARγ signaling on proliferation and differentiation. For example, in chondorosarcoma and ovarian cancer cells, PPARγ actions were associated with changes in the ratio of BAX to BCL-2, induction of programmed cell death [73], and upregulation of cyclin-dependent kinase inhibitors (CDKIs), such as *CDKN1A* (encodes p21[(waf1/cip1)]) [74]. In MCF-7 breast cancer cells

PPARγ upregulated a similar spectrum of CDKIs [75]. A number of studies have identified the IGF axis as a target of PPARγ signaling. For example, in bone marrow cells [76], and *in silico* and in vitro studies have characterized a range of PPAR response elements (PPREs) in several insulin-like growth factor binding protein (*IGFBPs*) genes [77]. Other scientists have attempted to increase the accuracy of gene target identification by using selective ligands, for example, in colorectal cells, and identified gene targets associated with mitotic restraint and cell adhesion [78–82]. Complimentary approaches have utilized adenoviral transfection of receptor subtypes to identify differentially expressed genes, confirmed with chromatin immunoprecipitation (ChIP) approaches [83].

The accurate prediction of target genes is compounded by the highly integrated nature of PPARγ signaling with other NR family members. For example, its activities are mutually antagonized with ERα signaling, and appear to be cooperative with both VDR and RAR, in part by increased retinol synthesis [84–86]. To investigate this apparent transcriptome plasticity will require the integrations of *in silico* response element identification protocols combined with ChIP-sequencing approaches to establish specificity and redundancy; comparable approaches have been undertaken for ERα [87]. Building towards this goal, we have undertaken a meta-analyses of PPRE sequences to generate an algorithm to predict PPAR subtype binding and screened chromosome 19, as a test set, to identify and confirm a number of novel genes [88].

Together, these findings suggest that ligand is just one of a number of mechanisms to regulate receptor function. Other regulatory contributions are determined by PPARγ expression level, isoform, posttranslational modification, location, crosstalk with functionally related receptors and cofactor expression. Together, these components combine with wider transcriptional programs, such as energy utilization, circadian rhythm, and the control of inflammation to drive and specify the timing of transcriptional outputs.

2. CONTROL OF SELF-RENEWING TISSUES

2.1. Common cancers and leukemia arise in self-renewing tissues

The weighted contribution of the underlying forces, acting at the levels of genes, chromosomes, signaling cascades and tissue organization, that drive cancer initiation and progression remain poorly understood. Historically, a paradigm of exclusive genetic causality was the basis for investigating cancer etiology and it identified certain key nodal points of cellular control, such as p53. In the postgenomic era, other strong penetrance genes have not been readily identified. The sporadic, multistage acquisition of a cancer phenotype requires disruption of multiple mechanisms of cellular restraint and tissue organization (reviewed in [89]). Reflecting a sporadic multifactorial cancer phenotype, the single greatest risk factor for most cancers is age, with the average age of onset of breast, prostate, and colon cancer in the sixth and seventh decades of life.

Further understanding of transformation processes has arisen through appreciation of the diverse cell types present at the sites of high-profile malignances. Epithelial linings of the prostate and mammary glands, the gastrointestinal tract and hematological systems all typify self-renewing tissues containing stem cell populations [90–94]. These cells give rise to committed progenitors, and in turn the multiple-cell lineages required for tissue function. Stem cells are relatively rare and long-lived, but frequently quiescent. They are uniquely able to undergo asymmetric division, to give rise to both other stem cells and transiently amplifying populations of progenitor cells, that in turn give rise to the differentiated cell types. The differentiated epithelial cells are functional but short-lived and lost through programmed cell death processes, to be replaced by newly differentiated transiently amplifying cells. Cellular control of the intricate balance of the processes of division, differentiation, and programmed cell death include common roles for Wnt, Hedgehog, and other developmental signal transduction processes. Convergent targets for these signals include key regulators of cellular proliferation, such as Myc and p21$^{(waf1/cip1)}$.

As a result of their long life cycle and high proliferative capacity, stem cells, rather than their short-lived terminally differentiated daughter cells, are the candidates for transformation. However, a range of mechanisms is in place to maintain stem cell genomic integrity, perhaps including retention of the so-called "immortal" DNA strand and enhanced protection mechanisms [95–103]. These controls notwithstanding, the transformation of stem cells has given rise to the concept of cancer stem cells. Such cancer stem cells are well established in leukemia and accumulating evidence supports the presence of these cells in prostate, breast, and colon cancers [104–108].

2.2. Restoration of controlled self-renewal as a therapeutic goal

Members of the NR superfamily play a number of well-established roles in the control of self-renewal and the process of normal differentiation. For example, the AR and ERα receptors play pivotal roles in prostate and breast tissue development and maintenance. Distortion of some of these actions is, in turn, central to the development of cancer in these tissues and is targeted therapeutically though antagonism, either completely in the case of the AR, or selectively in the case of the ERα. Agonism of other receptors has been pursued to induce differentiation and inhibit proliferation of cancer cells. The best example of this paradigm is the induction of remission of patients with acute promyelocytic leukemia using the RAR ligand, all-*trans* retinoic acid, and also to prevent recurrence of head and neck cancers.

As a consequence of the induced terminal differentiation of normal preadipocytes by ligands for PPARγ [1, 2], investigators were encouraged to use TZDs to attempt to induce differentiation of human liposarcoma cells *in vivo* [109]. Successes in vitro encouraged these same physician-scientists to give troglitazone to a series of patients with

liposarcoma, which resulted in a retardation of growth and induction of differentiation of these tumor cells. The long-term effect of TZD on liposarcomas requires further study; nevertheless, these pioneer studies spurred the examination of the effect of TZDs on a number of cancers both in vitro and *in vivo* in colon, breast, prostate, myeloid leukemia, neuroblastoma, glioblastoma, lymphoma, lung, cervical, bladder, head and neck, esophageal, gastric, pancreatic, and choriocarcinoma cancers [21, 81, 110–140]. The multiple findings from studies illustrate the promise and failings of targeted therapies toward PPARγ to restore mitotic restraint and induce differentiation.

3. PPARγ SIGNALING IN CANCER

3.1. Colon cancer

To establish a role for PPARγ to protect against the development of colon cancer, investigators have used a range of *in vivo* and in vitro approaches. In murine models, the expression of *Pparγ* has been manipulated in either an environmental or a genetic background that displays enhanced susceptibility to colonic cancer. For example, mice with heterozygous germ-line deletions of *Pparγ* have an increased proclivity to develop *N*-methyl-*N*-nitrosourea carcinogen-induced colon cancer compared with wild-type mice, supporting a growth inhibitory role for *Pparγ*. Significantly, troglitazone reduced the tumor incidence in wild-type but not heterozygote mice [122]. By contrast, other scientists have utilized the well-established APC$_{min}$ model of colon cancer with apparently contradictory findings. These mice have a germ-line mutation of the APC gene resulting in deregulated β-catenin signaling, and a very significantly increased frequency of small and large intestinal adenocarcinomas. Surprisingly, administration of TZD to APC$_{min}$ mice resulted in increased frequency of colon cancers compared to control animals [141]. Subsequently, however, generation of the intestinal specific *Pparγ*$^{-/-}$ and APC$_{min}$ bigenic mouse demonstrated an unequivocal effect of *Pparγ* to suppress tumor formation and suggests that significant off-target effects of TZD occur in mice, especially in the APC$_{min}$ mouse colon cancer model [142]. Off-target effects of TZD generally appear to also have broad anticancer properties; therefore, the findings in this model appear quite unusual. For example, *Pparγ* inactive analogs of TZD initiate the proteosomic degradation of β-catenin [143] and cyclin D1, as well as, interfering with BAX family member interactions to bring about apoptosis [144, 145]. Nevertheless, why APC$_{min}$ mice receiving a TZD have more colon cancers still is not fully elucidated. APC$_{min}$ mice have high levels of *Pparγ* in the colonic cells and are inappropriately sequestrated by β-catenin to a unique set of gene targets [146]. Interestingly, PPARα ligands inhibit polyp formation in the APC$_{min}$ model [118] re-enforcing the concept that the TZD-driven enhanced tumor formation in the APC$_{min}$ mouse is a model artifact, or at least not general phenomena.

In humans, multiple lines of evidence support an unequivocal function for PPARγ signaling in colon cancer.

Mutations of the receptor have been reported, although rare [147], and polymorphisms are functionally linked with an increased incidence of this cancer [148]. A range of natural and synthetic PPARγ ligands inhibit proliferation, induce programmed cell death and exert prodifferentiation actions in vitro and *in vivo*, for example, when tested in human xenografts [149–151]. The potency of the ligand actions can be significantly enhanced further by combining the treatment with RXR ligands [124, 152]. Furthermore, this signaling capacity is integrated with the control of other proliferative signals, such as gastrin [153] (reviewed by [154]).

3.2. Breast cancer

The findings on breast cancer support the broad anti-cancer activities of PPARγ signaling, and also reflect the studies in colon cancer. That is, generally in vitro and *in vivo* studies support a clear role for this receptor to suppress proliferation, induce differentiation and programmed cell death. In rodent models, the PPARγ agonists block *N*-nitroso-*N*-methylurea-induced breast cancer in Sprague-Dawley rats [155] and DMBA-induced breast cancer in mice [114]. Similarly, *Pparγ*$^{+/-}$ mice have a greater susceptibility to develop breast and ovarian cancers after their exposure to 7,12-dimethylbenz(a)anthracene [156].

By contrast, transgenic mice having a constitutively active PPARγ in their breast tissue crossed with the MMTV-neu mouse model of breast cancer displayed accelerated kinetics of breast cancer development, although the authors noted that the tumors surprisingly were more secretory and differentiated in nature [157]. Similar to the APC$_{min}$ model, this tumor model depends on deregulated Wnt activity, and the authors suggested that the effects may also reflect aberrant interplay between PPARγ and Wnt signaling.

Human breast cancer cells express PPARγ [158] and can be targeted, for example, with TZD, and a range of other PPARγ ligands to induce differentiation and inhibition of cell growth both in vitro and in xenograft models, effects which can be enhanced by cotreatment with either retinoids, TGFβ or TNFα [110, 111, 113, 114, 130, 158–163]. For example, PPARγ ligands plus selective retinoid ligands converge on targets, such as RARβ, which is known to act as a tumor suppressor and is commonly silenced in malignancy [164]. Similarly, PPARγ activation results in upregulation of E-cadherin and thereby redistribution of β-catenin [130]. Natural ligands, such as dietary fatty acids, change expression in syndecan-1 with an impact on cytoskeleton structure and the induction of apoptosis [165]. Furthermore, 15d-PGJ$_2$ inhibits ERα signaling in a PPARγ-independent manner by covalent modification of the receptor [166]. PPARγ expression is a favorable prognostic factor [167] and associates with ERα positive disease [75]. A note of caution, however, phase II trials of TZDs in women with hormone refractory metastatic breast cancer were equivocal [168].

3.3. Prostate cancer

The biology of the prostate is intimately associated with the synthesis of prostaglandins, as suggested by the name. These growth regulatory factors are readily secreted by the gland [169] and give rise to the H and D series prostaglandins and 15d-PGJ$_2$. Equally, the biology of the prostate is associated with the metabolism of fatty acids 15S-HETE [33]. Therefore, the prostate seems a tissue where PPARγ may play a strong role in governing cell growth and differentiation. For example, signals derived from *PGDS* activity in the adjacent stroma, such as PGD$_2$, activate PPARγ, and control epithelial proliferation [170].

PPARγ actions in prostate cancer cell lines [171] and primary cancer models [120] are well documented and include the induction of type II programmed cell death also known as autophagy [112]. These studies encouraged several groups to undertake clinical trials with PPARγ ligands and disease stabilization was reported [115]. Again in this disease setting, PPARγ-independent actions of TZDs were apparently identified, which were nonetheless potent anticancer signals [172, 173].

Set against these findings, the Evans team used a prostate cancer, the TRAMP model, to demonstrate that *Pparγ* heterozygote mice have no change in disease progression compared to wild-type litter mates [174].

3.4. Leukemia and lymphoma

Previously, we showed that human myeloid and lymphoid leukemia cells express PPARα and PPARγ; ligands, such as troglitazone, inhibited their cell growth [139, 175]. This antiproliferative effect was markedly enhanced in the presence of various retinoids. Also, macrophages and myelomonocytic leukemic cells express abundant PPARγ (73), and PPARγ ligands can induce acute myelomonocytic leukemic cells (THP-1) to differentiate toward macrophages with an increased expression of the CD36 scavenger receptors, as well as other surface markers associated with differentiation including CD11b, CD14, and CD18 (73). Studies by others and us have also shown that PPARγ ligands can inhibit growth and/or induce apoptosis of Hodgkin's disease [139] and multiple myeloma cells [176, 177]. The mechanism, by which PPARγ ligand inhibits the proliferation of malignant hematopoietic cells, is not totally clear. Some of the antileukemic effects of PPARγ may be independent of the PPARγ receptor. Furthermore, we have found that a dual PPARα/γ ligand (TZD18) has the ability to induce marked apoptosis and to inhibit growth of lymphoid leukemia cells [178]. In general, the effect of PPARγ ligands on myeloid leukemic growth and differentiation is modest (74).

3.5. Mechanisms of resistance

Genetically, the PPARγ generally appears to retain its integrity. Rare mutations have been reported and more recently dominant negative variants of the receptor were identified although the biological impact remains to be established firmly [179]. Similarly, altered isoforms may be overexpressed in cancer [180–183]. Cytogenetic rearrangement has been identified in follicular thyroid cancer fusing the PAX-8 transcription factor to PPARγ. In vitro studies suggest PAX-8-PPARγ acts in a dominant negative fashion toward wild-type PPARγ [184] (Figure 2).

In parallel to these genetic changes, the actions of PPARγ appear to be attenuated by changes in receptor expression and known cofactors. The range of interactions with partner proteins of PPARγ appears to be altered. Interactions with PGC1-α are reduced in several cancers [21, 185, 186]; and oppositely the known CoRs associated with PPARγ are overexpressed and the transcriptional actions of PPARγ are repressed by epigenetic mechanisms involving HDAC3 [187–189]. Equally, the control of posttranslational modifications appears to be altered. *SUSP-1* [64], which removes the SUMO mark (required for ligand-dependent transrepression) appears to be downregulated in a number of breast and prostate cancers [190]. Within the NR network, PPARγ is coexpressed and interacts both positively and negatively with a cohort of other receptors. For example, the ERα and Cyclin D1, (itself a well-known ERα target gene and CoA) can both repress the PPARγ gene promoter [191, 192].

The natural ligands for PPARγ are diverse and it is more challenging to make definitive statements concerning their altered generation in malignancy. Equally, the ability for PPARγ to act in a significant and ligand-independent manner also reduces, to an extent, the significance of ligand levels. These considerations aside, the patterns of ligand generation for PPARγ appear to be altered in malignancy. The balance between LO and COX-2 is dysregualted to favor generation of PGH production [193] and accompanied by downregulation of PPARγ [194]. This causes an elevation of PGH$_2$, which in turn is converted to protumorigenic prostaglandins, such as PGE$_2$, through other synthases. The levels of PGD$_2$, which gives rise to 15-PGJ$_2$, are closely regulated by an aldo-ketoredcutase (AKR1C3) that is upregulated in malignancy [195–199].

An emergent area of distortion is the extent to which PPARγ signaling is at the mercy of more dominant signal transduction and transcriptional programs. The two tumor promotion models associated with signaling by PPARγ involved elevated levels of signaling by the Wnt pathway. These findings combined with observations on the diversity of genes regulated by the receptor suggest that PPARγ signaling displays plasticity in terms of exact promoter choice. Gene regulatory options are distilled by the combination of receptor-associating cofactors and other signal transduction events. For example, overwhelming Wnt signaling pulls *Pparγ* to β-catenin gene targets [146]. This plasticity of signaling is probably reflected by the fact that complete loss or mutation of PPARγ in malignancy is relatively rare. Rather, expression is retained but probably sequestrated and distorted by more dominant signaling events. Resolving these interactions will require a quantitative and hierarchical understanding of the signaling paths through which PPARγ combines with other NRs and signal transduction events to regulate cell fates.

4. IS PPARγ A LIGAND-ACTIVATED TUMOR SUPPRESSOR?

A tumor suppressor can be characterized as a protein that reduces the probability that a cell in a metazoan will undergo transformation. Initiation and progression of cancer are associated with attenuation, corruption, expression, and protein function of tumor suppressor genes, increasing the likelihood of tumor formation.

Approximately 10 years have past since the first few reports of PPARγ exerting anticancer cellular effects [109, 111]. Taken together the overwhelming body of data suggests that PPARγ can behave as a ligand-activated tumor suppressor.

(1) PPARγ ligands through activating PPARγ can inhibit proliferation and induce differentiation and apoptosis of a wide range of neoplastic cell types in vitro and in murine xenograft tumor models.

(2) *Pparγ*$^{-/-}$ mice are more susceptible than wild-type mice to mammary, colon, ovarian, and skin tumors after exposure to carcinogens and enhance tumor formation in some genetic models of cancer, for example, APC$_{min}$ model of colon cancer.

(3) The actions of these receptors are attenuated in malignancy by genetic, cytogenetic, and epigenetic mechanisms, and ligand generation is compromised.

Set against, these data are two findings of enhanced tumor formation related to PPARγ in murine cancer models. TZD enhances tumor formation in the APC$_{min}$ model [141] and the bigenic mice overexpressing PPARγ in the MMTV-neu breast cancer model have more, highly differentiated tumors [157]. In retrospect, these high-profile studies perhaps reveal important facts of the dominant relationship between Wnt signaling over PPARγ in the mouse. This understanding may have important implications for the necessary molecular diagnostics required to target PPARγ therapies most effectively.

5. FUTURE DIRECTIONS

5.1. Exploiting dietary understanding from chemoprevention

Recently, the appreciation of the impact of diet on either the initiation or progression of cancer has come significantly to the fore. The World Health Organization has now stated that after smoking diet forms the most preventable cause of cancer. Aspects of these relationships are found in breast, prostate, and colon cancer, where the rate of initiation and progression of disease may be influenced both positively and negatively by the cumulative impact of dietary factors over an individual's lifetime. Beyond the specific micro and macronutrient constituents, the energetic status of an individual is emerging as a risk factor with increased calorific intake and decreased energy expenditure, both contributing deleteriously to cancer initiation and progression (reviewed in [200]).

The NR network has emerged as a systemic sensor of lipid and energetic status [201]. This capacity includes components for sensing carbohydrates [202, 203], cholesterol homeostasis through LXRs and FXR, regulation of metabolic rate through TRs, and sensing of diverse lipids by PPARs. Crosstalk within the superfamily ensures that these sensing and regulatory functions integrate with other receptors such as those for sex steroids. Multiple aspects of these relationships are observed in cancer. For example, fatty acids, such as those present in fish oil and a range of other dietary factors, can activate PPARγ and are associated with *in vivo* prevention of colon cancer in mouse models [165, 204–206] and in human trials in breast cancer [207]. Equally, convergence on PPARs and VDR to regulate IGFBPs and other negative regulatory components of the AKT signaling cascade [208] provides attractive targets for therapeutic intervention.

To exploit this, understanding in either dietary guidelines for the general population or as a chemoprevention strategy for groups defined at risk (e.g., by age or molecular diagnostic) is highly demanding. Despite the significance and potential clinical benefit of these relationships, it remains unclear the critical time frame and dose range when dietary factors may be protective against cancer development, for example, during embryogenesis, childhood, or adult life. By comparison, considerable resources were required to elucidate what is now established as a clear causal relationship between cigarette smoke and lung cancer [209]. There are reasons to be encouraged in targeting PPARγ in a chemoprevention context as studies on the consequences of long-term usage TZDs in diabetes patients have revealed a protective benefit against lung cancer [210].

To address the impact of diet on disease, the emerging field of nutrigenomics aims to dissect the impact of dietary factors on genomic regulation, and thereby physiology and pathophysiology, utilizing a range of postgenomic technologies [211, 212]. This level of integration is emerging. For instance, PPARγ polymorphisms recently have been shown to play a role in determining cancer susceptibility only when patients are above a certain body mass index threshold [213]. Exploitation of such understanding will require modeling of these functions in a network context (reviewed in [214, 215]). Most likely, the application of such rational approaches will resolve the significance of PPARs to mediate anticancer actions of potent dietary factors, such as conjugated linoleic acid [130, 216].

5.2. PPARγ and the regulation of cellular energetics

A number of deleterious side effects occur through the use of fatty acids as an energy store, including the generation of reactive oxygen species as a result of lipid peroxidation. The PPAR family combines roles in lipid sensing and utilization with cellular protection against lipid excess. Specifically, PPARγ plays a role in fatty acid uptake and transport (e.g., by adipocytes) and acts to control inflammation that can arise from increased adipocyte differentiation and proliferation (reviewed in [217, 218]). These actions are all altered in malignancy. As proposed by Otto Warburg in the 1930s
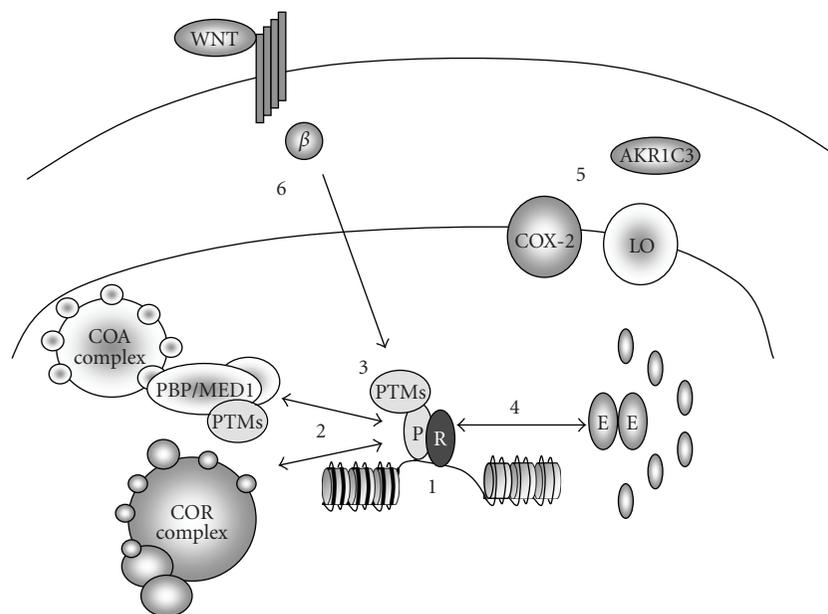
FIGURE 2: The actions of the PPARγ to regulate target genes are highly choreographed, being influenced by many factors. This is reflected by the multiple mechanisms that distort PPARγ signaling in cancer. PPARγ-RXR heterodimer binds to specific response elements contained within upstream, intronic, and downstream sequences of target genes. The ability of this heterodimer to participate in either transactivation or transrepression is disrupted by multiple mechanisms in cancer cells. (1) *Genetic mechanisms*; although relatively rare, mutations to the PPARG gene occur, as do cytogenetic rearrangements, notably in thyroid cancer with the generation of the PAX-8-PPARγ fusion product. (2) *Epigenetic mechanisms*; the PPARγ receptor normally exists in a dynamic equilibrium with each of two large complexes, namely, coactivator (CoA) and corepressor (CoR) complexes to regulate genes targets. Central components of these complexes are a cohort of ancillary proteins that act to regulate a cohort of posttranslational modifications (PTMs) to histone tails and thereby determine local chromatin organization. In cancer, the stochiometry of this equilibrium is disrupted with downregulation of CoA components such as PGC1-α and upregulation of CoR components such as NCOR1. The net result is the distortion of gene regulation abilities, most likely in a promoter specific manner. (3) *Posttranslational mechanisms*; PPARγ is regulated by a number of posttranslational modification including sumoylation, which can allow the liganded receptor to retain associations with the CoR complex and bring about ligand-dependent transrepression. The enzymes responsible for this activity appear altered in malignancy suggesting that the levels of sumoylated PPARγ are in turn distorted. In parallel, associated cofactors, such as PBP/Med1, are also regulated by PTMs and further manipulate and PPARγ signaling. (4) *Nuclear receptor network dynamics*; the PPARγ is a member of a highly interactive network of receptors and in malignancy these interactions appear distorted. For example, the ERα (E) homodimer is able to repress the PPARG promoter, and equally PPARγ is both coexpressed with, and regulates expression of other receptors such as PPARα, LXRs, FXR, and VDR to coordinate transcriptional programs. (5) *Ligand generation*; PPARγ senses a wide panel of lipophilic ligands many of which are derived from and catabolized downstream of metabolism of arachidonic acid. Key steps include generation of fatty acids, which are PPARγ ligands, through lipooxygenase (LO) activity (e.g., 5-LO). To counterbalance these activities, the generation of prostaglandins is mediated in large part through the actions of cyclooxygenase (COX) activity (e.g., COX-2). While this can also give rise to PPARγ ligands, these effects are protected further by the clearance of potent prostaglandin PPARγ ligands by the actions of enzymes, such as AKR1C3. In malignancy, an inversion of COX-2 to 5-LO occurs, and further protection from generation of potent prostaglandin ligands occurs, for example, through upregulation of AKR1C3. (6) *Dominant transcriptional programs*; the actions of the PPARγ appear to be distorted as a consequence of deregulated dominant transcriptional programs, such as Wnt signaling. These effects are mediated by enhanced β-catenin (β) levels and include sequestration of PPARγ to β-catenin responsive genomic regions. Implicit within this is that there is a high degree of plasticity of PPARγ signaling and that transcriptional signals can be placed within a quantifiable hierarchy.

(and summarized later [219]), cancer cells derive their energy increasingly from anaerobic glycolysis; this concept has received renewed support in recent years [220–222]. The altered energetics of cancer cells are common events, and cancer patients frequently display symptoms which in many ways mimic type II diabetes [223]. Associated with many of these events is an increased propensity for local inflammation.

PPARγ therapeutics have been explored within these separate arenas in different disease settings. That is, to regulate fatty acid metabolism and insulin resistance within the metabolic syndrome, to suppress inflammation, for example, in colitis models [224], and to promote mitotic restraint and induce differentiation within cancer cells. These functions are not separated, but rather all distorted within malignancy. The fact that PPARs, in general, and PPARγ specifically play an integrated regulatory role in these processes suggests that new avenues of exploitation will require a more detailed and quantitative understanding of the contribution of PPAR signaling against a tissue and whole body background of inflammation and altered cellular energetics.

5.3. Ongoing questions

The current challenges in PPARγ cancer biology include the following.

(1) Determine at which stage PPARγ can influence normal tissue self-renewal.

(2) Understand in cancer systems which combination of critical cellular processes to exploit: exert mitotic restraint, induce differentiation, regulate local inflammation, and impact on cellular energetic processes.

(3) Define to what extent conformationally restricted synthetic ligands (the so-called SPARMS [225]) can regulate target of these cellular processes through selective cohorts of PPARγ target genes.

(4) Identify the mechanisms that attenuate, manipulate, dissociate, and redirect PPARγ signaling in cancer cells and address to what extent the proteins involved in these processes are drugable therapeutic targets.

(5) Reveal whether this understanding can be best exploited in the setting of either chemoprevention and/or chemotherapy.

(6) Quantify, model, and predict to what extent PPARγ is a nodal point within the NR network and other signal transduction process. Establish hierarchies that place PPARγ specifically, and NRs generally, in the context of other signal processes that collectively maintain homeostasis.

ABBREVIATIONS

APC:	Adenomatous polyposis coli
AR:	Androgen receptor
CAR:	Constitutive androstane receptor
CDKI:	Cyclin-dependent kinase inhibitors
ChIP:	Chromatin immunoprecipitation
CoA:	Coactivator
CoR:	Corepressor
COX:	Cyclooxygenase
DMBA:	7,12-dimethylbenz[a] anthracene
ERα:	Estrogen receptor α
FXR:	Farnesoid X receptor
GR:	Glucocorticoid receptor
HAT:	Histone acetyltransferase
HDAC:	Histone deacetylase
HETE:	Hydroxyeicosatetraenoic acid
HODE:	Hydroxyoctadecadienoic acid
IGFBP:	Insulin-like growth factor binding protein
LXR:	Liver X receptor
NCOA/SRC:	nNuclear receptor coactivator/steroid receptor coactivator
NCOR1:	Nuclear corepressor
NR:	Nuclear receptor
P:	Prostaglandin
PBP/MED1:	PPARγ binding protein/mediator 1
PGC1-α:	Peroxisome proliferator-activated receptor γ coactivator 1 α
PPARγ:	Peroxisome proliferator-activated receptor γ
PSA:	Prostate specific antigen
RAR:	Retinoic acid receptor
RXR:	Retinoid X receptor
SIRT:	Sirtuin 1
SUMO:	Small ubiquitin-related modifier
TCF:	T-cell factor
TNF:	Tumor necrosis factor
TR:	Thyroid receptor
TZD:	Thiazolidinedione
VDR:	Vitamin D receptor
15d-PGJ$_2$:	15-deoxy-^{12,14}-prostaglandin J$_2$.

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Review Article

PPAR Ligands as Potential Modifiers of Breast Carcinoma Outcomes

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Chemically synthesized ligands for nuclear receptors of the PPAR family modulate a number of physiological functions, particularly insulin resistance in the context of energy homeostasis and the metabolic syndrome. Additionally, these compounds may treat or prevent the development of many secondary consequences of the metabolic syndrome. Many PPAR agonists are also known to influence the proliferation and apoptosis of breast carcinoma cells though the experiments were carried out at suprapharmacological doses of PPAR ligands. It is possible that the breast epithelium of diabetics exposed to PPAR agonists will experience perturbation of the corresponding signaling pathway. Consequently, these patients' lifetime breast carcinoma risks could be modified, as their breast lesion incidence or the rates of the conversion of these lesions to carcinomas might vary upward or downward. PPAR activating treatment may also influence the progression of existing, undiagnosed invasive lesions. In this review, we attempt to summarize the possible influence of chemical PPAR ligands on the molecular pathways involved in the initiation and progression of breast carcinoma, with a major emphasis on PPARγ agonists thiazolidinediones (TZDs).

1. INTRODUCTION

Breast carcinoma is the most common nonskin cancer among women worldwide, responsible for about 375 000 deaths per year [1]. The probability of the development of breast carcinoma increases before menopause (ages 40–50) and then gradually decreases, possibly due to diminishing levels of circulating estrogens [2]. In developed countries, the prevalence of breast carcinoma is higher due to the frequency of known risk factors for the disease, including early age at menarche, nulliparity, late age at first birth, late menopause, and brief duration of breastfeeding [2]. All of these risk factors are tightly linked to hormonal background, particularly to lifelong exposure of breast tissue to endogenous estrogens [3]. Exogenous factors influencing breast carcinoma development include the use of oral contraceptives [4] and hormone replacement therapy [5, 6] as well as dietary or lifestyle-related variables. The latter category is rather vague, as it includes many factors detrimental to general health, such as high body-mass index [7], high fat intake [8], high red meat consumption [9], excessive alcohol consumption [10], and reduced physical activity [11].

A number of chemoprevention strategies for breast carcinoma are developed or under development. The noteworthy example is a tamoxifen chemoprevention in high-risk premenopausal women, which heralded the success of selective estrogen receptor modulators (SERMs) [12]. A new agent, raloxifene (Evista, Eli Lilly, IN, USA) also competes with endogenous estrogen for ER binding and shows similar promises with fewer side effects [13]. Interestingly, many potential breast carcinoma preventive agents studied earlier are also available over-the-counter and widely used by target populations. Examples of this kind include aspirin [14], soy isoflavones [15], and Vitamin D [16].

Recently, the universe of chemical compounds commonly encountered by current and future breast carcinoma patients has been enriched by a number of pharmacotherapeutic agents being prescribed as a lifelong support for common chronic diseases. Depending on the particular molecular pathways which these agents modulate, they may contribute to initial immortalization of breast epithelia, stimulate proliferation and invasion of existing tumor cells, or on the contrary, prevent the tumor's development. For example, type II diabetes patients are routinely treated with

chemically synthesized ligands for PPARγ, thiazolidine-dione (TZD), namely pioglitazone (Actos, Takeda/Lilly), and rosiglitazone (Avandia, GlaxoSmithKline). The glucose-lowering effects of these compounds are mediated primarily by decreasing insulin resistance and increasing glucose uptake by the skeletal muscles [17]. In addition, TZDs suppress glucose production in the liver [17]. These and other beneficial effects rapidly made TZDs a mainstream diabetes therapy [18].

In addition to their antidiabetic effects, TZDs are known to suppress the proliferation and induce apoptosis of breast carcinoma cells in vitro [19, 20]. It is likely that the breast epithelium of diabetics exposed to TZDs will also experience perturbation of the PPAR signaling pathway. Consequently, current or past TZD users' lifetime breast carcinoma risks may be modified, as their breast lesion incidence or rates of the conversion of these lesions to carcinomas might change upward or downward. TZD treatment may also influence the progression of existing undiagnosed invasive lesions.

In addition to PPARγ ligands, PPARα [21] and PPARδ [22] are currently being explored as potential cardiovascular therapeutics and metabolic syndrome alleviation agents. If these agents will be approved by FDA, it is very possible that in the next two or three decades the number of women exposed to one or another type of PPAR ligands may reach 10–15 million in the USA alone. Possible modifications of the breast carcinoma incidence and outcomes resulted by the chronic exposure to these compounds might translate into statistically significant changes visible in epidemiological survey data, similar to those seen in cohorts taking hormone replacement therapy [5, 6].

In this review, we attempt to summarize the possible influence of chemical PPAR ligands on the molecular pathways involved in the initiation and progression of breast carcinoma. Major emphasis will be on PPARγ, as small molecular agonists of this nuclear receptor are widely used in the treatment of type II diabetes all over the world.

2. PPARγ LIGANDS

A gene encoding nuclear hormone receptor, PPARγ, expresses as two different mRNA isoforms derived from the alternative promoters, ubiquitous PPARγ1 and adipose-specific PPARγ2 [23]. Both isoforms stimulate adipogenesis; however, PPARγ2 can be activated by lower concentrations of ligands [23]. Activated PPARγ heterodimerizes with various coactivators [24, 25], which modulate the expression of genes with promoters containing bi-hexametric PPRE elements. These elements are widespread in the human genome, being present in both fatty acid metabolism and cell cycle control genes [26]. Moreover, the list of targets directly regulated by PPARγ includes many genes which lack PPRE [27]. Most likely, this is due to either the binding of activated PPARγ to other proteins that, in turn, serve as transcription factors (TFs) or the action of PPRE-containing genes providing delayed transcriptional response to PPARγ ligation [27]. Knowledge about endogenous ligands for PPARγ is limited. The list of these compounds includes polyunsaturated fatty acids (PUFAs) and eicosanoids, particularly lipoxygenase

(LOX), and cyclooxygenase (COX) products [28]. An anti-inflammatory prostaglandin, 15-deoxy-D12,14-PGJ2 (15d-PGJ2), which is formed from PGD2 in vivo, is probably the most potent endogenous PPARγ ligand [28]. Another powerful physiological stimulator of PPARγ is oxidized phosphatidylcholine [29]. It should be mentioned that synthetic ligands of PPARγ (TZDs) display stronger binding affinity to this nuclear receptor than its endogenous ligands, thus raising the question whether the list of natural PPARγ ligands is complete.

2.1. Effects of the chronic exposure of the breast epithelium to PPARγ agonists

PPARγ is expressed in normal breast tissue and in many primary breast carcinoma specimens [30, 31]. Comparative studies of PPARγ expression in breast carcinoma patients so far have produced contradictory results [32–34]. Described associations between PPARG polymorphisms and breast carcinoma are also discrepant: some researchers see a marginally significant increase in the risk of breast cancer among women homozygous for the Ala allele of PPARγ (Pro12Ala), causing a reduction in the transcriptional activity of PPARγ2 [35], while others stress that carriers of the same variant allele are at lower risk [36]. Since complete loss of PPARγ signaling in clinical breast tumors seems to be a rare event [37], it is likely that patients undergoing chronic treatment with chemical ligands for PPARγ will experience alteration in the behavior of both breast carcinoma cells and their normal counterparts. Patients with ER-positive tumors might benefit from TZD exposure more than those with ER-negative tumors, as the level of PPARγ expression is significantly associated with the ER status of carcinoma cells [38].

Chemically synthesized ligands for PPARγ (thiazolidine-diones, or TZDs) have actively been used as insulin sensitizers since the late 90s [18]. In addition to their insulin resistance-alleviating effects, TZDs may influence an incidence or a progression of breast carcinoma lesions as they have been shown to suppress the proliferation rates of many types of cancer cells and induce either their differentiation or apoptosis in vitro [20, 39, 40]. Responsiveness to TZDs has been demonstrated for both normal human mammary epithelial cells [30] and breast cancer cells [41–43], although it was not uniformly seen in all experimental conditions [44]. TZDs suppress the cell cycle by repressing cyclins D1 and D3 [45], by stimulating expression of the tumor suppressor p53 and its effector p21 (WAF1/Cip1) [46], and by inhibiting the Akt/PTEN pathway [47]. Additionally, TZDs induce marked cellular acidosis in breast carcinoma cell lines, leading to a decrease in the number of viable cells [48]. Some effects of TZDs are independent of the transcriptional activities of PPARγ [48]; these effects may be mediated through interactions of these compounds with other cellular targets.

The growth-suppressive properties of TZDs are complemented by their ability to induce apoptosis. Many breast tumors are naturally resistant to the apoptotic action of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and other similar agents. TZDs sensitize these cells to TRAIL [45], to anti-Fas IgM (CH11), and to tumor

necrosis factor (TNF)-α [49]. It is tempting to speculate that TZDs might prevent the spread of microscopic breast tumors by sensitizing malignant cells to these endogenous apoptotic signals. Interestingly, TZDs also synergize with all-trans-retinoic acid (ATRA) to induce apoptosis in MCF-7 and primary breast carcinoma cells, but not in the normal breast epithelium [43]. Some TZDs also stimulate expression of apoptosis related genes, such as growth arrest and DNA damage-inducible gene 45 (*GADD45*) [50], *BRCA1* [51] and proline oxidase encoding gene *POX* [52]. In addition to intrinsic apoptotic pathways, TZDs are also capable of the direct stimulation of the *FASL* gene encoding Fas ligand that induces an apoptosis by cross-linking with the Fas receptor located on the membranes of the adjacent cells [53].

Additionally, TZDs block the invasion of tumor cells through upregulation of the tissue inhibitor of MMP-1/TIMP-1 and a subsequent decrease in MMP-9 gelatinolytic activities [54]. These observations have been supported by experiments with the murine mammary tumor cell line LMM3, which produces less metastatic nodules in lungs of animals treated by oral rosiglitazone [55]. It should be mentioned that the pronounced antitumor effects described above occur only at suprapharmacological doses of TZDs. It remains to be seen whether chronic exposure to TZDs could have therapeutic effects in patients with established breast tumors.

The effects described above are relevant only to some TZD users, namely, patients currently with breast tumors and those diagnosed with such tumors in the past. It is still unclear whether action of PPARγ ligands is different within normal and tumor cells, and what would be effects of TXD exposure in cancer free individuals. There are some indications that PPARγ ligands may influence the initial stages of breast carcinoma development, in particular, immortalization of the breast epithelia. One recent study demonstrated that exposure to low nontoxic doses of rosiglitazone (10 nM) reduces the frequency of spontaneous immortalization of Li-Fraumeni syndrome (LFS)-derived (p53 +/−, telomerase silent) breast epithelial cells by almost four times [56]. In these experimental settings, the antimutagenic properties of this widely prescribed TZD were superior to those of well-known chemopreventive agents such as sulindac sulfide and celecoxib [56]. It will be interesting to see whether exposure to TZD is capable of lowering the incidence of malignant foci in the breast epithelia genetically predisposed to breast carcinoma development, particularly that of carriers of mutations in *BRCA, BRCA2,* or *ATM*.

Some effects outlined above result from the interference of PPARγ signaling with other pathways involved in breast carcinogenesis, particularly with estrogen receptor (ER)α and NF-κB cascades. Agonists of PPARγ may suppress NF-κB dependent transcription either through an increase in physical interaction between PPARγ and p65 [57] or through SUMOylation-dependent targeting of PPARγ to NCoR/histone deacetylase-3 (HDAC3) corepressor complexes which prevent NCoR/HDAC3 clearance from NF-κB target gene promoters [58]. The interplay between ER and PPARγ signaling seems to be more complex. Many PPARγ ligands, particularly troglitazone and ciglitazone, inhibit ERα

signaling by stimulating proteasomal degradation of ERα [59].

On the other hand, one recent study's findings are disturbing: in the breast cancer cell line MCF-7, commonly used as a model for ER-positive breast carcinoma, TZD rosiglitazone has been shown to induce both estrogen receptor response element activity and cell proliferation [44]. Even more disturbing is the fact that in dose-response assays higher concentrations of rosiglitazone inhibited proliferation, while lower concentrations of the same compound induced proliferation. Rosiglitazone-induced proliferation and ERE reporter activation were mediated by ERα and the extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) pathway [44]. The concentration-dependent nature of rosiglitazone's effects may have tremendous clinical importance for the chronic users of TZDs. Moreover, these findings point at the possibility that the effects of the rosiglitazone might vary between individuals, as the bioavailability of rosiglitazone depends on the activity of the CYP2C9 and CYP2C8 enzymes [60], which are substantially polymorphic in human populations.

2.2. Chronic exposure to PPARγ agonists influences nonepithelial cells participating in breast carcinoma development

In addition to the effects of PPARγ ligands on premalignant and malignant breast epithelia, these compounds also produce profound changes in noncancerous cells. Some of these changes may be relevant to breast carcinoma outcomes. For example, PPARγ ligands demonstrate antiangiogenic effects (reviewed in [40]), including direct suppression of the vascular endothelial growth factor (VEGF) and the angiopoietin-1 (Ang-1) gene transcription [61, 62]. On the other hand, in some noncancerous settings, PPARγ ligands stimulate angiogenesis [63, 64], thus pointing to their involvement in remodeling tumor vessels rather than in suppressing angiogenesis per se.

In vitro experiments suggest that PPARγ ligands act as differentiating agents in nonmalignant stromal cells. Malignant epithelialcells of breast tumors secrete growth factors and cytokines to prevent the differentiation of peri- and intratumoral stromal fibroblasts into mature adipocytes by downregulation of adipogenic factors such as the C/EBPα and PPARγ [65]. In turn, underdifferentiated fibroblasts provide structural and secretory growth promoting support to tumor tissue [66]. Prolonged treatment with TZDs stimulates the differentiation of fibroblasts into adipocytes instead of myofibroblasts and interferes with transforming growth factor beta (TGFβ) fibrogenic pathway, particularly, through attenuation of TGFβ-driven type I collagen protein production [67]. Taken together, these effects of TZDs may to some degree counteract desmoplastic proliferative response promoted by tumor proximity and delay the formation of the scirrhous component of the breast tumors and the subsequent spread of tumor cells.

It must be taken into account that an interference of TZDs with TGFβ signaling is a double-edged sword, since TGFβ serves as both a tumor suppressor and a tumor

promoter depending on tumor developmental stages and cellular context [19]. During the initial phase of breast tumorigenesis, the TGFβ signal inhibits primary tumor development and growth by constraining cell division and possibly inducing apoptosis [68, 69]. In the later stages of breast carcinoma development, tumors lose their sensitivity to TGFβ, but continue overproduction of the hormone. Excess TGFβ acts upon stromal components of the tumor promoting the metastatic process through desmoplastic reaction, inhibiting host immune surveillance, and stimulating invasion and angiogenesis [70]. The outcome of the crosstalk between TGFβ and PPARγ in breast carcinoma patients should be dependent on stage of the particular breast lesion.

Last but not least, TZD therapy has been shown to produce an average weight gain of 4-5 kg, which cannot be explained by fluid retention [71]. The magnitude of weight gain correlates in part with improved metabolic control, that is, better responders are more prone to increases in body weight [72]. In turn, weight gain is associated with a significant increase in postmenopausal ER-positive/PR-positive breast cancer [73, 74]. It remains to be seen whether TZD-associated increases in adiposity contribute to breast carcinoma risks similarly to nonspecific weight gain.

2.3. Effects of TZDs on breast carcinogenesis in vivo

The PPARγ agonist GW7845 delays the development of mammary tumors in immunocompetent mice treated with medroxyprogesterone acetate followed by DMBA administration by an average of 2 months [75]. In the classic rat model of mammary tumorigenesis employing nitrosomethylurea as a carcinogen, GW7845 also significantly reduces both tumor incidence and tumor weight [76]. Similarly, troglitazone, alone or in combination with RXR ligands, prevents the induction of preneoplastic lesions in a mouse mammary gland organ culture model treated by DMBA [77]. TZD treatment alone or in combination with ATRA suppresses tumor growth from breast carcinoma cells MCF-7 [43]. On the other hand, attempted rosiglitazone chemoprevention of breast carcinogenesis in the MMTV-HER-2/neu transgenic mouse model produced no encouraging data [78]. It is important to note that the mechanisms underlying various routes of the tumorigenesis in rodent breast differ substantially [79]; therefore, it is entirely possible that TZDs may modify outcomes only in some of the models studied. It is also possible that these effects might be either compound or dose-specific.

Recently, a few epidemiological studies have explored the association of TZD-based diabetes therapy and breast carcinoma incidence. The largest profiled cohort was the one covered by the Integrated Healthcare Information Services (IHCISs), Mass, USA, managed care database [80]. The relevant part of IHCIS allowed analysis of pharmacy and doctor's office claim data related to 126 971 nonelderly USA diabetics with a mean followup time of 16.6 months. Importantly, each individual case of breast carcinoma ($N = 513$) was matched to up to five diabetes controls (cumulative $N = 2557$) using matched nested case-control design. The adjusted odds ratios and 95% CI for breast cancer from any exposure to TZD (mono- or combination therapy) compared to all non-TZD antidiabetic agents were 0.89 (0.68–1.15) [80]. Thus, neither a beneficial nor a deleterious effect of TZDs on the likelihood of breast carcinoma development was found. It should be mentioned that the median duration of followup in the studied cohort was rather short for the development of breast tumors. Studies following patients for longer periods of time are warranted.

Another group of researchers analyzed 1003 adult diabetic patients participating in a Vermont Diabetes Information System (VDIS) study and revealed a significant association between any cancer and the use of any TZD (OR = 1.59, 95% CI (1.03–2.44), $P = .04$) [79]. When TZDs were analyzed by compound, a significant association was found for rosiglitazone (OR = 1.89, 95% CI (1.11–3.19), $P = .02$), but not for pioglitazone. Stratification by gender showed a highly significant association between cancer prevalence and TZD use for women (OR = 2.07, 95% CI (1.18–3.63), $P = .01$) [81], but not for men. It is important to note that the number of the patients enrolled in this study is not allowed assessment of the risks for individual cancers. Nevertheless, the increase of tumor incidence in TZD using women points at the possible vulnerability of the breast epithelia.

Slightly more encouraging results were produced in the recently completed PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events). This study reviewed longitudinal data of 5238 diabetic patients treated with pioglitazone or with a placebo [82]. The incidence of breast carcinoma was nonsignificantly reduced in the pioglitazone-treated group (3 versus 11 cases in the equally sized pioglitazone and placebo arms of the study, resp.).

Several attempts to use TZDs as a means of therapy for breast carcinoma have been made so far. One trial of TZD as a monotherapy ended 5 months after it started, because troglitazone was withdrawn from the marker. This trial—performed in the cohort of patients with advanced breast cancer refractory to at least one chemotherapy regimen—resulted in no objective responses [83]. Another attempt at TZD monotherapy enrolled 38 women with early-stage lymph node negative breast carcinomas. This intervention was even shorter as rosiglitazone treatment (8 mg/d) was given between the time of diagnostic biopsy and definitive surgery. No significant effects on breast tumor cell proliferation were observed using Ki67 expression as an endpoint. Interestingly, rosiglitazone treatment leads to down-regulation of nuclear PPARγ expression, as demonstrated by immunohistochemistry. Additionally, rosiglitazone intervention resulted in an increase of serum adiponectin concentrations ($P < .001$). Serum adiponectin negatively regulates breast cancer growth [84] and inhibits angiogenesis by suppression of endothelial cell proliferation and migration [85]. The potential therapeutic implications of rosiglitazone modulation of adiponectin levels require further study.

3. PPARα LIGANDS

The nuclear receptor PPARα regulates lipid metabolism in general and β-oxidation of fatty acids in particular.

Its gene, *PPARA*, expresses mainly in tissues with high energy requirements, particularly in the skeletal muscle, the heart, and the liver [86]. PPARα is activated by a number of natural ligands, including various derivatives of fatty acids and leukotriene B4, and by common lipid-lowering drugs, particularly fenofibrate and gemfibrozil. Activated PPARα exerts beneficial effects on lipid metabolism, raising cardioprotective high-density lipoprotein (HDL) cholesterol and lowering cardiovascular mortality [87]. In addition, activation of PPARα may limit inflammation, both in the vessel endothelium and in other tissues as well as inhibit the fibrotic response. The apparent uniformly beneficial action of PPARα agonists prompted the development of a number of these compounds. Among them, some exert dual affinity to PPARα and PPARγ. Dual agonists hold considerable promise in the management of insulin resistance, serving as major confounders for cardiovascular diseases and other comorbidities associated with metabolic syndrome.

Experimental data describing the effects of PPARα agonists on tumor initiation and progression are limited. Long-term administration of PPARα ligands clofibrate and WY-14643 in the rodent model induces hepatocellular neoplasms including adenomas and carcinomas [88]. PPARα suppresses apoptosis in liver tissue in response to various peroxisome proliferator carcinogens, especially in the presence of TNFα [89]. As levels of TNFα are substantially elevated in obesity and in metabolic syndrome, it could be hypothesized that hepatocarcinogenesis may be an issue for long-term fibrate medicated patients. So far, epidemiological observations in fibrate treated populations have not produced any evidence that fibrates are associated with elevated risk of liver cancer or any other neoplasms in humans. As PPARα-humanized mice are resistant to hepatocarcinogenic effects of fibrates, it seems that the response described in mouse models is species specific [90].

Studies of the nonhepatic tumorigenesis models indicate that in other tissues PPARα agonists exert antiproliferative effects [91]. In the mouse model of skin carcinogenesis, an animal topically treated with PPARα ligands exhibited an approximately 30% lower skin tumor yield compared with mice treated with vehicle, thus indicating that the activation of PPARα may suppress the earliest stages of tumor development [92]. Additionally, PPARα ligands possess strong antiangiogenic properties, as they suppress endothelial cell proliferation and VEGF production, upregulate TSP-1 and endostatin, and inhibit neovascularization [93, 94].

Studies concerning PPARα activation in breast carcinomas are scarce. It is known that PPARα is expressed and dynamically regulated in both ER-positive (MCF-7) and ER-negative (MDA-MB-231) human breast cancer cells. PPARα activation significantly increases proliferation of both cell lines, and this increase is proportional to the endogenous level of PPARα [95]. On the other hand, one recent study pointed at PPARα as a possible contributor to the growth inhibitory effect of n-6 PUFA arachidonic acid exerted in the same pair of breast carcinomas cell lines [96].

PPARα also reduces the sensitivity of MCF-7cells to histone deacetylase inhibitors [97]. Interestingly, there is an inverse relationship between mean PPARα and ERα mRNA levels in ER-positive breast cancer cells [97]. These observations point to the possible involvement of PPARα activation in mammary gland tumorigenesis and vouch for a longitudinal study of breast carcinoma incidence and progression in patients using fibrate therapy.

4. PPARδ LIGANDS

The nuclear receptor PPARδ, also known as PPARβ, is expressed ubiquitously. It controls a number of physiological functions, particularly cell proliferation and differentiation as well as inflammation and energy homeostasis [22]. Interestingly, PPARδ is the only PPAR isoform that maintains repressor activity when bound to DNA. When unligated, PPARδ can act as an intrinsic transcription repressor and inhibit the trans-activation activity of other PPARs [98]. It was suggested that PPARδ serves as a gateway receptor capable of modulating PPARα and PPARγ activity [98]. The ligand binding pocket domain of PPARδ is larger than that of other PPARs and is believed to accommodate the binding of various fatty acids and their derivatives [99]. A number of synthetic agonists are being developed for the same purpose with nanomolar affinities [100, 101], although none is currently marketed for clinical use in humans yet.

The physiological effects of activated PPARδ have been studied extensively [22, 102]. The results of these studies suggest that sooner or later high-affinity PPARδ synthetic drugs which uniquely target multiple components of the metabolic syndrome, including obesity, insulin resistance, hyperglycemia, dyslipidemia, and atherosclerosis will enter the market. Some of these compounds are already being subjected to phase I/II clinical trials. In light of this fact, it is important to establish experimental systems allowing rapid evaluation of the potential carcinogenic or chemopreventive effects of the synthetic PPARδ ligands. Given that the prevalence of the metabolic syndrome and comorbidities associated with the disease is on the rise in both developed and developing countries, it is extremely important to watch for possible effects of anticipated chronic exposure to PPARδ ligands upon common types of cancer, particularly upon breast carcinoma.

Alarmingly, PPARδ selective agonists stimulate the growth of the hormone-dependent breast carcinoma cell lines T47D and MCF-7. In T47D cells, activation of PPARδ stimulates expression of the proliferation marker Cdk2. In addition, an increase in the production of both VEGF and its receptor, FLT-1 has been noted, suggesting that PPARδ may initiate an autocrine loop for cellular proliferation and possibly angiogenesis. Similar pro-proliferative effects of activated PPARδ have been observed in endothelial cell cultures [103]. Further studies of angiogenic and growth-inducing properties of PPARδ agonists in breast epithelia are warranted.

5. GENERAL REMARKS

It should be mentioned that breast carcinoma is not a single disease entity, but rather an extremely polymorphic spectrum of neoplastic pathologies which are fairly diverse in
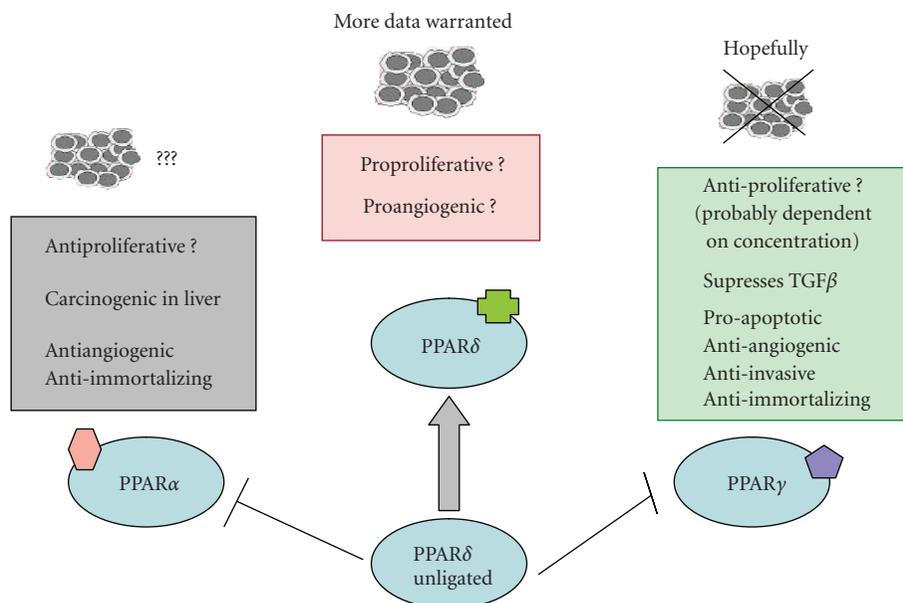
FIGURE 1: A summary of influence of PPAR ligands on the process of breast carcinogenesis.

their molecular portraits. It is likely that both chemoprevention and treatment by PPAR ligands as well as their possible tumorigenic side effects will be selective to particular molecular subtypes of tumor, or will be relevant to certain stages of carcinoma progression (Figure 1). Therefore, much larger cohorts of patients followed for longer periods of time will have to be studied in order to reveal statistically significant modifications of the disease's outcome. Chemoprevention studies of this type are prohibitively expensive, for example, the recently completed National Surgical Adjuvant Breast and Bowel Project Study of Tamoxifen and Raloxifene (STAR) trial with an endpoint of cancer incidence required the enrollment of 19 747 subjects from near 200 clinical centers throughout North America took 8 years before initial data analysis, and cost approximately $200 million [104, 105]. Before initiating large-scale efforts, a comparative study of the molecular portraits of breast carcinomas developed in chronic TZD users and in the general population needs to be completed. This kind of study could be performed using microarrays as a primary profiling means which should be complemented by validation efforts through the methods of immunohistochemistry, in situ hybridization of mRNA, and phosphoproteomics. The design of this study could be a challenge due to the difficulties with proper matching of groups compared and with eliminating common confounders. One of the possible ways to overcome this problem is to profile both malignant and normal breast epithelia samples of current TZD users to that of recently diagnosed diabetics never exposed to TZDs. Confirmed differences between the molecular portraits of tumors which initiated or progressed despite an exposure to PPAR ligand and subtype-matched tumors that arose on TZD free background may give some important clues to the design of a clinical trial aimed at chemoprevention-related endpoints.

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Review Article

To Live or to Die: Prosurvival Activity of PPARγ in Cancers

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The role of PPARγ in tumorigenesis is controversial. In this article, we review and analyze literature from the past decade that highlights the potential proneoplastic activity of PPARγ. We discuss the following five aspects of the nuclear hormone receptor and its agonists: (1) relative expression of PPARγ in human tumor *versus* normal tissues; (2) receptor-dependent proneoplastic effects; (3) impact of PPARγ and its agonists on tumors in animal models; (4) clinical trials of thiazolidinediones (TZDs) in human malignancies; (5) TZDs as chemopreventive agents in epidemiology studies. The focus is placed on the most relevant *in vivo* animal models and human data. *In vitro* cell line studies are included only when the effects are shown to be dependent on the PPARγ receptor.

1. INTRODUCTION

PPARγ is a nuclear hormone receptor that requires ligand binding for activation. In 1995, it was discovered that PPARγ is the molecular target of thiazolidinediones (TZDs, [1]), a class of synthetic compounds that are effective for the treatment of type 2 diabetes. This discovery spurred great interest in these agents, as well as in the receptor. Besides its function as an insulin sensitizer in diabetes, PPARγ was found to have a variety of roles in immunoregulation, atherosclerosis, angiogenesis, and tumorigenesis.

With regards to carcinogenesis, debate continues as to whether PPARγ is pro- or antineoplastic, despite very active research over the past few years. At the cellular level, PPARγ was found to be involved in cancer cell survival/apoptosis, proliferation, and differentiation. While the apoptotic functions of PPARγ and its agonists are addressed by others in this special issue, we will conduct a critical review of the literature that suggests that PPARγ has a prosurvival activity. The review is mainly focused on data derived from *in vivo* models and/or human studies. *In vitro* cell line-based studies are included only when the effects are shown to be dependent on the PPARγ receptor.

One important lesson learned from the past several years of research is that effects observed with agonists of PPARγ are not necessarily intrinsic effects of the nuclear hormone receptor. In tumor cell survival, the proapoptotic activities of PPARγ agonists in various tumors act through both receptor-dependent and receptor-independent mechanisms. When reviewing the literature, we advise that the readers carefully consider the following to distinguish drugs or TZDs *versus* receptor effects: (1) are high or low doses used in the studies? High or low doses should be defined with respect to EC_{50} of glitazones in the PPARγ transactivation assays (Table 1) or plasma concentrations that can be reached in humans (Table 2). Effects observed with high concentrations may not be relevant due to toxicities of certain TZDs, such as hepatotoxicity of troglitazone and potential cardiotoxicity of rosiglitazone (see below). (2) Are multiple pharmacological agents used? If a pharmacological approach is the only one used, claims of a receptor-dependent effect require demonstration with agonists of different chemical structures, such as TZDs, tyrosine analogues, 15-Deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$), and so forth. Beware that 15d-PGJ$_2$ possesses many PPARγ-independent activities, including inhibition of the NFκB pathway, that are known to have prosurvival and anti-inflammatory properties, as well as other effects [2–4]. (3) Are any antagonists included in the study? Do antagonists GW9662 or T0070907 block or reverse the observed effects? (4) Are there any experiments in the study utilizing a genetic

approach to confirm the pharmacological findings? Does the study involve cell lines or primary cells that contain or lack PPARγ, preferably in the same genetic background? For those cell lines with endogenous PPARγ, is the siRNA, shRNA or dominant negative form of PPARγ used to reduce the levels of the receptor? Are specific effects of the receptor diminished by such reduction? For readers' convenience, these questions are summarized in Table 3.

2. EXPRESSION OF PPARγ IN HUMAN TUMOR VERSUS NORMAL TISSUES

It is generally believed that expression of a gene in a particular tissue suggests that the activity of the encoded protein is required for certain cellular functions of that tissue. In so far as cancers are concerned, the general rule is that oncogenes are overexpressed due to dysregulation, and tumor suppressor genes are underexpressed or absent due to mutations or deletions. In order to clarify the roles of the PPARγ receptor, it would be informative to review the expression levels of PPARγ in tumors with respect to their normal tissue counterparts. In this article, expression data from tumor cell lines are not included.

A review of the current literature on human cancers showed that expression levels of PPARγ mRNA and protein are generally higher in neoplastic tissues than their normal counterparts (summarized in Table 4). The most convincing data came from a large study of prostate cancer that included 156 patients with prostate cancer (PC), 15 with less aggressive prostatic intraepithelial neoplasia (PIN), 20 with benign prostatic hyperplasia, and 12 normal prostate tissues. In this study, a high level of PPARγ expression, by immunohisto-chemistry, is observed in PC and PIN cases in comparison to low or no expression in the benign hyperplasia and normal tissues. The results were confirmed at the mRNA level with RT-PCR on a few cases from each category of the malignant and benign conditions [13]. A large study of 126 renal cell carcinomas also showed significantly more extensive and intensive PPARγ staining in tumor epithelium compared to the average staining levels seen in 20 normal tissues [14]. Similarly, in 22 patients with nonsmall cell lung carcinoma, higher levels of PPARγ are expressed in tumor cells than in the surrounding normal tissue, as determined by immunohistochemical staining. In addition, higher expression levels in tumor cells are confirmed by Western blotting hybridization, using homogenized tissue samples [15]. In hepatocellular carcinoma, immunostaining also demonstrates that PPARγ is overexpressed in all of 20 carcinoma tissues but not in normal hepatocytes [16]. For squamous cell carcinoma, 20 cases of primary tumor and six cases of lymph node metastasis were demonstrated to have increased PPARγ protein expression compared to normal tongue tissue [17]. Infiltrating adenocarcinoma of the breast also expresses higher nuclear staining of PPARγ compared to normal ductal epithelial cells by immunohistochemical analysis. However, only one of the three cases was shown [18]. For papillary thyroid carcinoma, six patients were studied to determine PPARγ mRNA expression using reverse transcription PCR. The message was found in three of six

tumor tissues while the corresponding normal tissues do not express PPARγ [19].

Follicular thyroid carcinoma, a less common histological subtype of thyroid cancer, is characterized by a chromosomal translocation t(2;3) that results in a fusion between paired box gene 8 on chromosome 2 and PPARγ on chromosome 3 (PAX8-PPARγ). The fusion protein was initially thought to function as a dominant-negative inhibitor of the wild-type PPARγ protein [28]. However, a recent microarray study revealed that (1) PPARγ transcript levels in all seven cases of PAX8-PPARγ-containing follicular carcinomas are more than 10-fold higher than normal thyroid tissues, as determined by both microarray and quantitative RT-PCR analyses; (2) the expression profile of the fusion-positive follicular carcinomas shows induction of genes that are involved in fatty acid, amino acid, and glucose metabolic pathways. Interestingly, many of the upregulated genes are known transcriptional targets of the wild-type receptor, suggesting that the PAX8-PPARγ fusion protein functions similarly to wild-type PPARγ, rather than antagonizing its activity. (3) Using cell lines transfected with PPARγ or the fusion protein, it is shown that expression of some genes, including angiogenic factors PGF and ANGPTL4, is specifically upregulated by the fusion protein, particularly in the absence of ligand, indicating that the fusion protein is constitutively active. Taken together, these experimental data suggest that the translocation enhances the function of PPARγ in a way that contributes to the development or progression of follicular carcinoma of the thyroid [29].

Upregulation of PPARγ has been demonstrated during tumor progression. Mueller et al. have found significant PPARγ staining in six cases of metastatic breast adeno-carcinoma. In cell lines established from the primary and metastatic tumors of one of these patients, significantly higher amounts of PPARγ transcript are shown in the cell line derived from the metastatic tumor [20]. In ovarian cancer, intensity and location of PPARγ immunostaining were examined in 28 carcinoma cases along with 28 normal, benign or borderline cases. Twenty six of 28 carcinomas showed strongly positive PPARγ staining compared to 2 weak-staining cases in the control group. Moreover, it is noted that PPARγ staining was predominantly nuclear in grade 2 or 3 tumors, as compared to a predominantly cytoplasmic staining pattern in grade 1 tumors [21]. Similar findings were made in transitional cell carcinoma of urinary bladder. Whereas no significant PPARγ immunoreactivity was observed in 20 normal tissues, elevated PPARγ was found in 168 tumors. Furthermore, the intensity of staining increased as the histological grade increased from G1 to G3 and the tumor stage increased from early (pT1 or lower) to advanced (stage 2 or higher) [22].

A recent large study of 129 cases of pancreatic ductal ade-nocarcinoma convincingly showed by array-based gene pro-filing that expression of PPARγ in the tumor cells is ~7 fold higher than that in the normal ductal epithelia. This finding was confirmed with immunohistochemical analysis of the tissue sections. Normal ductal epithelia showed insignificant staining for PPARγ. An early lesion, intraepithelial neoplasia showed occasional PPARγ expression whereas more than

TABLE 1: EC$_{50}$ of common PPARγ agonists in transactivation assays.

Agonists	Constructs used for transactivation	EC$_{50}$ (μM)	References
Ciglitazone	mPPARγ1 LBD[a]-GAL4 DBD[b]	3	[5]
Pioglitazone	Wild-type mPPARγ1	0.4	[1]
	Wild-type mPPARγ2	0.4	
	mPPARγ1[c] LBD-GAL4 DBD	0.55	[6]
	hPPARγ1[d] LBD-GAL4 DBD	0.58	
Rosiglitazone	Wild-type mPPARγ1	0.03	[1]
	Wild-type mPPARγ2	0.1	
	mPPARγ1 LBD-GAL4 DBD	0.076	[6]
	hPPARγ1 LBD-GAL4 DBD	0.043	
Troglitazone	mPPARγ1 LBD-GAL4 DBD	0.78	[6]
	hPPARγ1 LBD-GAL4 DBD	0.55	
15d-PGJ$_2$	Wild-type mPPARγ1	2	[7]
	mPPARγ1 LBD-GAL4 DBD		

(a) LBD, ligand binding domain.
(b) DBD, DNA binding domain.
(c) mPPARγ1, mouse PPARγ1.
(d) hPPARγ1, human PPARγ1.

TABLE 2: Peak plasma concentrations of PPARγ agonists.

Agonists	C$_{max}$[a] (μM)	References
Ciglitazone	15~30[b]	[8]
Pioglitazone	0.2~2.5	[9]
Rosiglitazone	0.2~1.7	*Avandia* Prescribing Information[c]
Troglitazone	0.7~8.8	[10]
15d-PGJ$_2$	Low nanomolar to picomolar range[d]	[11] [12]

(a) C_{max}, the maximum or peak plasma concentration in human unless otherwise indicated.
(b) That in dog plasma.
(c) From http://us.gsk.com/products/assets/us_avandia.pdf.
(d) Physiological concentrations in cerebrospinal fluid, urine, and the interior of adipocytes.

TABLE 3: Points to be considered to discern drugs/TZDs versus receptor effects.

(1) Are high or low doses of drugs used in the studies with respect to their K_d values for PPARγ, or plasma concentrations?
(2) Are multiple pharmacological agents of different chemical classes used?
(3) Are any antagonists included in the study?
(4) Are any genetic approaches used to confirm the pharmacological findings?

70% of invasive pancreatic carcinoma demonstrated weak to strong expression. Statistical analysis indeed revealed that expression of PPARγ correlates with high tumor stage and higher tumor histological grade. More strikingly, expression of PPARγ in pancreatic cancer is shown, by multivariant survival analysis, to be a significant prognostic indicator for shortened patient survival [23].

In parallel to the above literature, levels of PPARγ mRNA found in several well- or poorly-differentiated colorectal adenocarcinomas, were similar to normal tissues [24]. Another group also found that the PPARγ immunostaining in well-, moderately-, or poorly-differentiated gastric adenocarcinomas is comparable to that in noncancerous tissue adjacent to the tumor [25]. In liposarcomas, PPARγ transcript levels are similar to that of the adipose tissue [26]. In adrenal glands, there is, again, no significant difference in mRNA expression among cases of carcinoma, adenoma, and normal tissues [27]. Notably, at the time of composition of this manuscript, we have not yet found any reports stating that PPARγ expression is downregulated or absent in human tumor *versus* normal tissues (Table 4).

The next question is whether or not the PPARγ expressed in tumor tissues is functional. Are ligands of PPARγ present in the tumor tissues? A thorough and up to date literature search yielded few results. The English abstract of a study published in a foreign language stated that there was no significant difference in 15d-PGJ$_2$ concentration between gastric cancer tissues and controls [30]. An earlier study showed that 15d-PGJ$_2$ promotes the proliferation of HCA-7, a cyclooxygenase 2 (COX-2)-containing colon cancer cell line at nanomolar concentrations. Further characterization by HPLC and mass spectrometry identified PGJ$_2$, a chemical precursor of 15d-PGJ$_2$ in the culture medium of HCA-7 cells [31]. COX-2 is a key enzyme in the biochemical pathway that leads to the formation of cyclopentenone prostaglandins including 15d-PGJ$_2$. Overexpression of COX-2 has been documented in many cancer types and contributes to tumor growth [32]. Overall, these few and somewhat circumstantial evidences suggest that 15d-PGJ$_2$ might be present in the tumor tissues.

TABLE 4: PPARγ expression in human tumor versus normal tissues.

Tumor versus normal tissue	No. of cases	References
Overexpression		
Prostate cancer/prostatic intraepithelial neoplasia	156/15	[13]
Renal cell carcinoma	126	[14]
Nonsmall-cell lung carcinoma	22	[15]
Hepatocellular carcinoma/lymph node metastasis	20/6	[16]
Squamous cell carcinoma	20	[17]
Metastatic breast adenocarcinoma	6	[20]
Infiltrating ductal breast adenocarcinoma	3	[18]
Papillary thyroid carcinoma	6[a]	[19]
Increased expression during tumor progression		
Breast adenocarcinoma	1[b]	[20]
Ovarian carcinoma	28 versus 28[c]	[21]
Urinary bladder carcinoma	100 versus 70[d]	[22]
Pancreatic ductal adenocarcinoma	45 versus 84[e]	[23]
Similar expression		
Colorectal adenocarcinoma	11	[24]
Gastric adenocarcinoma	12	[25]
Liposarcoma	13	[26]
Adrenocortical tumors	32	[27]

(a) Of the six papillary carcinoma tissues, three expressed PPARγ mRNA.
(b) The primary and metastatic breast cancer cell lines were derived from a single patient.
(c) Normal, benign, or borderline versus malignant tumors (grades 1, 2, and 3).
(d) Lower ($\leq$pT1) versus higher ($\geq$pT2) tumor stages.
(e) Lower (pT1 & pT2) versus higher (pT3 & pT4) tumor stages.

Does PPARγ lose or gain abnormal functions through mutations other than PAX8-PPARγ translocation? A large survey of human tumor samples and cancer cell lines does not support such a notion. The exon 3 and 5 mutations, once reported in sporadic colon cancers [33], were not present in nearly 400 cell lines and primary tumor samples including lung, breast, prostate, colon cancers, and leukemias [34].

Taken together, several lines of evidence regarding PPARγ expression suggest a positive contributive role of the receptor in the development, maintenance, or progression of human malignancies: (1) PPARγ is overexpressed in the vast majority of cancers. (2) In several types of cancer, PPARγ expression is further increased during tumor progression. (3) The oncogenic fusion PAX8-PPARγ results in PPARγ overexpression and upregulation of a similar profile of transcriptional targets as the wild-type protein. (4) Expression of PPARγ in pancreatic cancer is associated with shorter survival.

3. RECEPTOR-DEPENDENT PRONEOPLASTIC EFFECTS OF PPARγ

Is there also cellular-level evidence suggesting that PPARγ promotes tumors? Most studies, especially those employing high doses of TZDs, suggest that PPARγ agonists have anti-tumor activities through inhibition of cell proliferation or induction of apoptosis or differentiation. However, receptor-independent pathways are involved in most of the cases

(reviewed elsewhere in this special issue). Then what does the receptor by itself do in tumors?

Schaefer et al. showed that inhibition of PPARγ induces apoptosis of hepatocellular carcinoma cells (HCCs) by preventing their adhesion to the extracellular matrix, suggesting that the activity of PPARγ is required for HCC cells to adhere and survive [16]. In that study, those particular effects were shown to be receptor-dependent. Loss of cell adhesion requires almost complete loss of PPARγ activity achieved by either PPARγ-targeting siRNA or PPARγ inhibitor T0070907. In addition, T0070907 causes cell death at concentrations far lower than those needed for PPARγ agonists rosiglitazone and troglitazone. Together, the data suggest that PPARγ functions to promote tumor cell adhesion and survival in HCC cells. In line with this notion, the promoter region of hepatocyte growth factor contains a functional PPAR response element (PPRE) that mediates its transcriptional upregulation by PPARγ. The growth factor plays an essential role in liver growth during embryonic development, as well as in maintenance and renewal of cells in various organs including liver, lung, and kidney, in adulthood [35].

Our laboratory studied human anaplastic large T-cell lymphomas, a common form of large cell lymphoma in the pediatric population. We first demonstrated with immunohistochemical staining that PPARγ is expressed in the malignant cells of the lymphoma tissues [36]. We then tested the effect of PPARγ activation in cell lines established from patients with this lymphoma. A pair of cell lines,
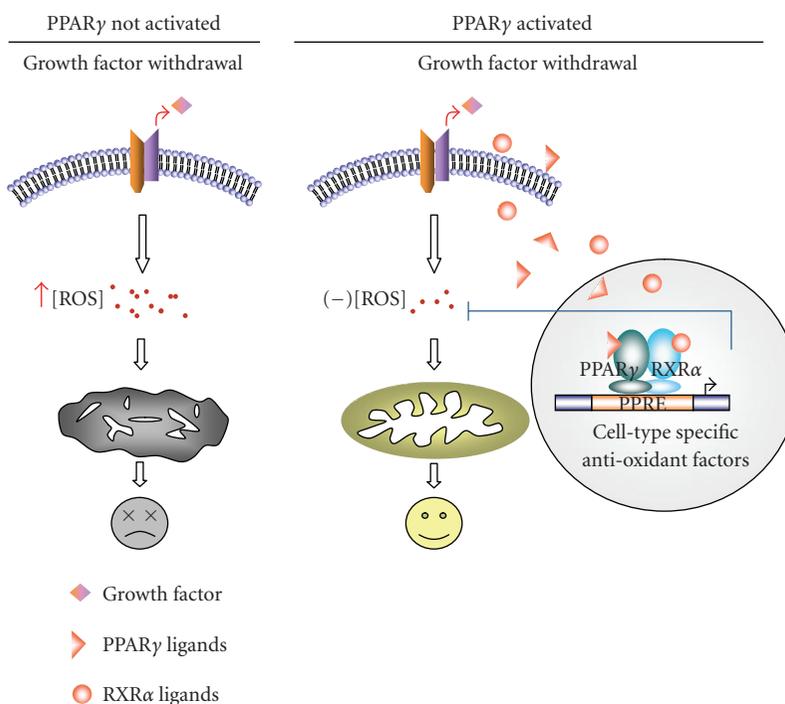
FIGURE 1: *Schematic diagram showing how PPARγ increases cell survival in growth factor/nutrient-deprived cells.* Growth factor/nutrient withdrawal induces ROS production. In the absence of PPARγ activation, increased levels of ROS inhibit mitochondrial electron transport, leading to mitochondrial depolarization, caspase activation, and cell death. When PPARγ is activated, the increase in ROS is attenuated by the receptor through transcriptional upregulation of cell type specific antioxidant factors, such as catalase, Cu/Zn-SOD (SOD1), Mn-SOD (SOD2), or UCP2. The transcriptional upregulation of these genes by PPARγ may or may not be direct (shown to be direct in the diagram for simplicity).

Karpas 299 and SUP-M2 that, respectively, contain and lack endogenous PPARγ were selected to address the receptor-dependency issue. Additionally, only low ligand concentrations were used, following initial dose titration, to minimize any off-target effects. Using this system, we have found that low doses of PPARγ agonists do not affect cell survival under normal conditions. When cell death was induced by nutrient deprivation through serum withdrawal, activation of the receptor with low doses of rosiglitazone ($0.5–2\,\mu M$) attenuated cell death, as compared to drug vehicle-treated cells. This result was reproducible with low doses of GW7845 ($0.5–2\,\mu M$) and 15d-PGJ$_2$ ($0.5–1\,\mu M$). The effect occurred only in PPARγ-containing Karpas 299 cells but not in PPARγ-lacking SUP-M2 cells. Moreover, reducing PPARγ in Karpas 299 cells with siRNA diminished the prosurvival effect of the receptor. Furthermore, we showed that the prosurvival effect is mediated through PPARγ-dependent cellular metabolic changes, including increased cellular ATP levels, stabilized mitochondrial membrane potential, and reduced reactive oxygen species (ROS) production that each favor cell survival. PPARγ does so through coordinated regulation of the expression of ROS metabolic enzymes, including the p67 subunit of NADPH oxidase, uncoupling protein 2 (UCP2), and manganese superoxide dismutase (Mn-SOD) at both mRNA and protein levels that lead to ROS limitation. Lastly, we showed that stable transfection of PPARγ into SUP-M2 cells not only improved cell survival,

but also suppressed ROS accumulation during serum starvation. These genetic manipulations have provided definitive evidence that PPARγ promotes lymphoma cell survival under conditions of nutrient deprivation.

Our group has also made similar findings in a murine cellular model [37, 38]. FL5.12 is a murine lymphocytic cell line that requires interleukin-3 (IL-3) for survival and proliferation. This cell line has been extensively used to characterize tumor cell metabolism [39]. FL5.12 cells express little PPARγ, but are killed by high concentrations of PPARγ agonists, 15d-PGJ$_2$ ($\geq 10\,\mu M$) and ciglitazone ($\geq 80\,\mu M$). In an FL5.12 cell line stably-transfected with PPARγ, low doses of PPARγ agonist do not affect cell viability under normal conditions. However, when cells are induced to die by IL-3 withdrawal, low doses of ciglitazone ($10\,\mu M$) and rosiglitazone ($0.05–2\,\mu M$) improved survival in only PPARγ-containing cells. Improved cell survival is also accompanied by stabilized mitochondria and reduced ROS. Moreover, ATP production is required for PPARγ to exert its prosurvival effect. In this system, expression of a different panel of ROS metabolic enzymes including catalase, and Cu/Zn-SOD are involved in reduction of the cellular levels of ROS. Functional PPRE sequences were shown to be present in the promoter regions of these two genes, suggesting that the upregulation of their expression could be directly regulated by PPARγ [40–42]. Taken together, data from both human and murine cell line studies suggest that PPARγ promotes

tumor cell survival under conditions of nutrient/growth factor deprivation, and that the effect is not limited to a particular system. The mechanism by which PPARγ increases cell survival is diagrammed in Figure 1 (Also see below).

In support of the prosurvival activity of PPARγ in T-cell malignancies, Ferreira-Silva et al. very recently showed that RNAi-mediated silencing of PPARγ in Jurkat T-cells caused increased DNA fragmentation and apoptosis as well as G2/M cell cycle arrest, arguing that the receptor, proper, promotes the viability of the tumor cells [43].

In parallel to these findings in tumors, the prosurvival activity of PPARγ has been well documented in certain nonneoplastic pathological conditions, especially ischemia-reperfusion injury in nutrient-sensitive tissues such as brain, heart and kidney [44–51]. Irreversible damage that results from prolonged ischemia causes stroke, and myocardial and kidney infarction. At the cellular level, cell death occurs as a result of nutrient deprivation and inflammatory responses that involve the actions of proinflammatory cytokines, chemokines and transcriptional factors. In addition, increased production of ROS plays an important role in causing damage to macromolecules and eventual cell death [52]. A recent study using a rat model of cerebral focal ischemia has shown that expression of PPARγ mRNA and protein is upregulated in the areas adjacent to infarct caused by middle cerebral artery occlusion [46]. Administration of glitazones prior to, at the time of, or shortly after ischemia induction causes an increase in DNA binding of the receptor. This is accompanied by a decrease in the expression of a number of inflammatory genes, along with an increase in the expression of antioxidant enzymes including catalase and Cu/Zn-SOD [44–47]. Consequently, these changes lead to limited cell demise, which eventually results in significantly reduced infarct size. This process apparently works through a PPARγ-dependent mechanism, as GW9662 can block these effects of TZDs in animals [47]. Another PPARγ antagonist, T0070907, even increases the infarction size, both in the presence and absence of PPARγ ligands [46].

In light of both these findings and the overexpression of PPARγ in many cancers, it is reasonable to hypothesize that the function of PPARγ in cancer is to confer a survival advantage upon the malignant cells, allowing them to survive in an adverse environment. As a result of fast growth, the center of a three dimensional tumor mass is often deprived of oxygen, growth factors, glucose, and other nutrients due to excessive demand and insufficient vascularization. However, cancer cells possess remarkable tolerance and are able to survive despite the adverse conditions [53, 54]. Besides increasing angiogenesis, increasing PPARγ might be another mechanism that allows tumor cells to enhance their survival under these unfavorable conditions (Figure 1).

4. IMPACT OF PPARγ AND ITS AGONISTS ON ANIMAL TUMOR MODELS

Animal models were employed to examine the role of PPARγ in tumors. These systems can be categorized by how the tumor models are generated and by how the dose/activity of PPARγ is altered. With respect to the former, tumors can be generated with xenografts, carcinogens, or genetic manipulations. Watch for spontaneous tumor formation in certain PPARγ genetic backgrounds has also been conducted. With respect to the dose/activity of PPARγ, it can be altered using PPARγ agonists including TZDs or GW7845, or genetic manipulations including hemizygosity or tissue-specific overexpression or deletion of PPARγ. Results differ drastically between different model systems, even for the same types of cancer (Tables 5 and 6). This review focuses on models that are more relevant to human cancers. As such, animal studies involving TZD treatment of xenografted tumors are not discussed here.

4.1. Colon cancer

$Apc^{+/Min}$ mice possess a nonsense mutation in one copy of the adenomatous polyposis coli *(APC)* gene which truncates the protein at amino acid 850. Loss-of-function mutations in the *APC* gene are common in human familial adenomatous polyposis and can be found in sporadic colon cancers as well. Using this model, which is highly relevant to human colon cancers, one study showed an increase in tumor number and size, as well as worse histological grade in mice treated with troglitazone or rosiglitazone. This is associated with a rosiglitazone-induced increase in the β-catenin protein level in the colon tissues [55]. Another study [56], which also used $Apc^{+/Min}$ mice, reported an increase in the number of colon polyps in troglitazone-treated mice, but reported no significant difference in tumor size or histology, which may be related to the shorter TZD treatments used in this study (5 weeks as compared to 8 weeks in the first study). Similar findings were made in $Apc^{+/1638N}$: $Mlh1^{+/−}$ double mutant mice. In these mice, one copy of the APC gene is truncated at amino acid position 1638 and one of the two alleles of the DNA repair enzyme *Mlh1* is absent. In the double mutant mice, troglitazone treatment significantly increased the number of mice that developed large intestine tumors [58]. In contrast to these reports, another study used $Apc^{+/1638N}$ mice crossed with hemizygous PPARγ mice. Because homozygous deletion of PPARγ is embryonic-lethal, studies examining the dose effect of the gene employed either a hemizygous $Ppar\gamma^{+/−}$ mouse strain or a conditional knock-out strategy. No differences in survival, number of colonic tumors or β-catenin expression levels were observed between mice of $Apc^{+/1638N}$: $Ppar\gamma^{+/−}$ and $Apc^{+/1638N}$: $Ppar\gamma^{+/+}$ littermates [57]. Therefore, in colon cancer induced by *APC* mutations, it appears that activation of PPARγ by TZDs promotes tumor formation, while reduction of PPARγ gene dosage has little effect on tumor formation.

In stark contrast to the *APC* genetic tumor models, carcinogen-generated colon cancer models seem to yield opposite results. In the study that evaluated PPARγ haploinsufficiency in an $Apc^{+/1638N}$ background, the investigators also determined the effect of $Ppar\gamma^{+/−}$ in azoxymethane-mediated colon cancer. Compared to the $Ppar\gamma^{+/+}$ mice, a greater number of haploinsufficient mice developed tumors in the colon. The tumor-bearing $Ppar\gamma^{+/−}$ mice also had a greater number of tumors in them that led to significantly decreased survival. In another study, mice with

TABLE 5: PPARγ and agonists in animal models (differentially shaded according to methods of tumor induction).

Cancer type	Tumor induction	PPARγ activation ($\uparrow$)/reduction ($\downarrow$)	Tumor response	PPARγ's effect	References
Colon	Apc$^{+/Min}$	$\uparrow$ Troglitazone, Rosiglitazone	Increased incidence and size of tumor	Promoting	[55, 56]
Colon	Apc$^{+/1683N}$	$\downarrow$ Ppar$\gamma^{+/-}$	No response	No effect	[57]
Colon	Apc$^{+/1683N}$: Mlh1$^{+/-}$	$\uparrow$ Troglitazone	Increased tumor incidence	Promoting	[58]
Colon	Azoxymethane	$\downarrow$ Ppar$\gamma^{+/-}$	Increased tumor incidence, number, shortened survival	Suppressing	[57]
Colon	Azoxymethane	$\uparrow$ Troglitazone, pioglitazone, or rosiglitazone	Decreased tumor incidence, number, and size	Suppressing	[59]
Colon	Spontaneous	$\uparrow$ Troglitazone (5 weeks)	No response	No effect	[56]
Colon	Spontaneous	$\uparrow$ Troglitazone (6 months)	Increased tumor incidence	Promoting	[58]
Mammary glands	Polyoma virus middle T antigen	$\uparrow$ Tissue specific constitutive activation of PPARγ	Promoted tumor development	Promoting	[60]
Mammary glands	Polyoma virus middle T antigen	$\downarrow$ Ppar$\gamma^{+/-}$	No response	No effect	[60]
Mammary glands	MNU[a]	$\uparrow$ GW7845	Decreased tumor incidence, number, and total weight	Suppressing	[61]
Mammary, ovarian, skin	DMBA[b]	$\downarrow$ Ppar$\gamma^{+/-}$	Increased tumor incidence and number, worse survival	Suppressing	[62]
Mammary glands	Spontaneous	$\downarrow$ Tissue-specific PPARγ deletion	No response	No effect	[63]
Mammary glands	Spontaneous	$\downarrow$ Ppar$\gamma^{+/-}$	No response	No effect	[62]
Prostate	SV40 T antigen	$\downarrow$ Ppar$\gamma^{+/-}$	No response	No effect	[64]
Thyroid	DN-TRβ[c]	$\downarrow$ Ppar$\gamma^{+/-}$	Increased metastases, shortened survival	Suppressing	[65]
Thyroid	DN-TRβ	$\uparrow$ Rosiglitazone	Reduced tumor growth, delayed progression	Suppressing	[65]
Gastric	MNU	$\downarrow$ Ppar$\gamma^{+/-}$	Increased tumor incidence, shortened survival	Suppressing	[66]
Gastric	MNU	$\uparrow$ Troglitazone	Decreased tumor incidence	Suppressing	[66]
Lung	Urethan	$\uparrow$ Tissue-specific PPARγ overexpression	Decreased tumor incidence	Suppressing	[67]

(a) MNU, *N*-methyl-*N*-nitrosourea.
(b) DMBA, 7,12-dimethylbenzanthracene.
(c) DN-TRβ, dominant-negative mutant of thyroid hormone receptor β.

Un-shaded: Genetic tumor models
Light grey-shaded: Carcinogen-induced tumor models
Dark grey-shaded: Spontaneous tumor formation

azoxymethane-mediated colon cancer were treated with troglitazone, pioglitazone, or rosiglitazone. This resulted in reduced incidence, number, and size of colorectal tumor [59]. Taken together, these data suggest that PPARγ suppress azoxymethane-induced colon carcinogensis.

What would happen in normal mice? Spontaneous colon tumor development was evaluated in normal mice administered with troglitazone [58]. All nine mice fed with troglitazone developed tumors in the large intestine, in contrast to none of the 10 mice in the control group. An earlier study did not find any tumors in 17 troglitazone-fed normal mice, possibly due to the short duration of feeding (5 weeks in [56] versus 6 months in [58]).

4.2. Mammary gland tumors

The mammary gland tumor is another relatively well-studied tumor in animals. Similar to colon carcinogenesis, data on PPARγ's role in mammary gland carcinogensis suggest a wide range of effect depending on the tumor models (Tables 5 and 6). Some studies indicate no effect, while others suggest that it has a tumor promoting role, while others yet

Table 6: PPARγ and agonists in animal models (differentially shaded according to methods of PPARγ manipulation).

Cancer type	Tumor induction	PPARγ activation (↑)/reduction (↓)	Tumor response	PPARγ's effect	References
Colon	APC$^{Min/+}$	↑ Troglitazone, Rosiglitazone	Increased incidence and size of tumor	Promoting	[55, 56]
Colon	Apc$^{+/1638N}$	↓ Ppar$\gamma^{+/-}$	No response	No effect	[57]
Colon	Apc$^{+/1638N}$: Mlh1$^{+/-}$	↑ Troglitazone	Increased tumor incidence	Promoting	[58]
Colon	Azoxymethane	↓ Ppar$\gamma^{+/-}$	Increased tumor incidence, number, shortened survival	Suppressing	[57]
Colon	Azoxymethane	↑ Troglitazone, pioglitazone, or rosiglitazone	Decreased tumor incidence, number, and size	Suppressing	[59]
Colon	Spontaneous	↑ Troglitazone (5 weeks)	No response	No effect	[56]
Colon	Spontaneous	↑ Troglitazone (6 months)	Increased tumor incidence	Promoting	[58]
Mammary glands	Polyoma virus middle T antigen	↑ Tissue specific constitutive activation of PPARγ	Promoted tumor development	Promoting	[60]
Mammary glands	Polyoma virus middle T antigen	↓ Ppar$\gamma^{+/-}$	No response	No effect	[60]
Mammary glands	MNU$^{(a)}$	↑ GW7845	Decreased tumor incidence, number, and total weight	Suppressing	[61]
Mammary, ovarian, skin	DMBA$^{(b)}$	↓ Ppar$\gamma^{+/-}$	Increased tumor incidence and number, worse survival	Suppressing	[62]
Mammary glands	Spontaneous	↓ Tissue-specific PPARγ deletion	No response	No effect	[63]
Mammary glands	Spontaneous	↓ Ppar$\gamma^{+/-}$	No response	No effect	[62]
Prostate	SV40 T antigen	↓ Ppar$\gamma^{+/-}$	No response	No effect	[64]
Thyroid	DN-TRβ$^{(c)}$	↓ Ppar$\gamma^{+/-}$	Increased metastases, shortened survival	Suppressing	[65]
Thyroid	DN-TRβ	↑ Rosiglitazone	Reduced tumor growth, delayed progression	Suppressing	[65]
Gastric	MNU	↓ Ppar$\gamma^{+/-}$	Increased tumor incidence, shortened survival	Suppressing	[66]
Gastric	MNU	↑ Troglitazone	Decreased tumor incidence	Suppressing	[66]
Lung	Urethan	↑ Tissue-specific PPARγ1 overexpression	Decreased tumor incidence	Suppressing	[67]

(a)MNU, N-methyl-N-nitrosourea.
(b)DMBA, 7,12-dimethylbenzanthracene.
(c)DN-TRβ, dominant-negative mutant of thyroid hormone receptor β.

Un-shaded: Activation of PPARγ by pharmacological agonists
Light grey-shaded: Reduction of PPARγ gene dosage
Dark grey-shaded: Tissue specific PPARγ overexpression

suggest a tumor suppressing role. A murine genetic model supports a tumor-promoting role [60]. In this model, the mammary gland tumor is induced by mammary gland-specific expression of polyoma middle T antigen *(MMTV-PyV)*. Mammary gland specific constitutive expression of PPARγ *(MMTV-VpPPARγ)* did not yield tumor development. However, when crossed with the *MMTV-PyV* mice, the double mutant progeny developed more mammary gland tumors sooner than *MMTV-PyV* mice. The increased tumor burden eventually led to shorter survival. Interestingly, hemizygosity of *PPARγ* in the *MMTV-PyV* background

did not change the time course of tumor development. Exacerbation of tumor formation by PPARγ was ascribed to increased Wnt-β catenin signaling as demonstrated by zebrafish developmental models.

In contrast to this genetic model, chemically induced mammary gland tumors were inhibited by PPARγ agonists. Both TZDs and GW7845, a tyrosine analog, have been shown to exhibit antitumor effects. An early study using nitro-somethylurea (MNU) to induce mammary carcinogenesis showed that GW7845 reduced the incidence, number of tumors *per* animal, and average weight of tumor at autopsy

following a two-month administration of the drug to rats [61]. In 7,12-dimethylbenzanthracene (DMBA)-mediated mouse carcinogenesis model, the animals develop multiple types of tumor, including mammary ductal papilloma and adenocarcinoma. Incidence of mammary gland tumor was significantly higher in $Ppar\gamma^{+/-}$ mice than in $Ppar\gamma^{+/+}$ mice. The hemizygous mice also had increased number of tumors and a lower survival rate [62].

Spontaneous tumor formation was also examined in $Ppar\gamma^{+/-}$ mice. Dose reduction of PPARγ does not make animals prone to increased carcinogenesis [62]. In concordance with this finding, the specific deletion of PPARγ in mouse mammary epithelia failed to induce mammary tumors in 20 mice observed for 12 months [63].

4.3. Other cancers

In a murine prostate cancer model, generated using tissue-specific SV40 T antigen, reduced $Ppar\gamma^{+/-}$ had no effects on tumor incidence, latency, size, histopathology, or disease progression [64]. However, in a murine follicular thyroid cancer model containing a dominant-negative mutant form of thyroid hormone receptor β ($TR\beta^{PV/PV}$), loss of one PPARγ allele led to increased weight of tumor-bearing thyroid gland, increased lung metastasis, and shortened survival. In addition, rosiglitazone treatment of $TR\beta^{PV/PV}$ mice reduced thyroid weight, and tumor progression [65], suggesting a tumor-suppressing role for PPARγ. Lastly, in gastric carcinoma, induced with MNU, PPARγ haploinsufficient mice had increased tumor incidence and shorter survival. Troglitazone treatment significantly reduced tumor incidence in mice with wild-type PPARγ background [66].

In summary, results from animal studies regarding the role of PPARγ are conflicting and difficult to assess. For the purpose of clarification, we attempted to analyze the published data according to the cancer types, tumor induction models, PPARγ activation/reduction methods, and tumor characteristics (Tables 5 and 6). Our extensive analysis revealed no clear pattern. However, some trends have been noted: (1) in multiple types of carcinogen-induced tumor (Table 5, light grey shaded rows), PPARγ seems to have a tumor-suppressing function. This appears to be independent of how PPARγ is activated or reduced, whereas in genetic tumor models (Table 5, un-shaded rows), the receptor exhibited all possible different effects. As to spontaneous tumors (Table 5, dark grey shaded rows), long-term use of troglitazone increased tumor formation, whereas PPARγ reduction had no effect; (2) a reduction of PPARγ dose by itself (Table 6, light grey shaded rows) is insufficient to induce spontaneous tumor formation, but in existing tumors, it either exacerbates tumor formation or have no effect at all; (3) TZDs (Table 6, un-shaded rows), in most cases, inhibits tumor formation with a rare exception of $Apc^{+/Min}$ mice.

The activity of the Wnt/β-catenin signaling pathway might account for these seemingly discrepant results, as tumor models generated by APC mutation or polyoma middle T antigen all involve overly active Wnt/β-catenin signaling. TZDs are shown to induce β-catenin in colon

[55]. Paradoxically, reduction of PPARγ ($Ppar\gamma^{+/-}$) also increases β-catenin expression in colon [57]. The appropriate activation of PPARγ signaling might also be important. Ligand-independent constitutive activation of PPARγ is involved in the development of mammary gland tumors [60] as well as in the action of PAX8-PPARγ in follicular thyroid carcinoma [29].

5. CLINICAL TRIALS OF TZDs IN HUMAN MALIGNANCIES

As discussed above, TZDs have been shown in many preclinical studies to possess antitumor effects that have prompted several early-phase clinical studies to evaluate their efficacies in various types of cancers. In this review, we analyze these studies both in terms of clinical responses and biological responses, focusing on recently published studies that include more than 10 patients (Table 7).

A phase II clinical trial of rosiglitazone in 12 patients with liposarcoma was recently conducted. Eight of 12 patients were fully evaluated for up to 16 months. As to clinical response, all patients progressed while on treatment with a mean time-to-progression of 5.5 months. Histological appearance of repeated biopsy materials did not show any signs of tumor differentiation. In one of the 8 patients, PPARγ and fatty acid binding protein (FABP) were induced after 12-week rosiglitazone therapy, but disease in this patient progressed similarly to the others [68]. Ten patients with thyroid cancers were treated with rosiglitazone. Among them, 4 had partial response, 2 had stable disease, and the remaining 4 progressed. No correlation was found between the clinical response and levels of PPARγ mRNA and protein in these patients. PAX8-PPARγ status was not assessed [69]. An early study evaluated efficacy of troglitazone in 25 patients with metastatic colorectal carcinoma. All 25 patients progressed with a median time-to-progression of 1.6 months and a median survival time of 3.9 months [70].

In breast cancer, data from two human trials have been published. An early trial on 22 women with refractory breast cancer showed no objective response to troglitazone in 18 of the 21 evaluable patients at 8 weeks after treatment. The therapy was terminated in 16 patients due to progression of their tumors. At 8 weeks, only three patients had stable disease. All patients were evaluated for serum tumor markers, CEA and CA27.29, which showed increased levels within 8 weeks of treatment. Expression of PPARγ was not determined in the study [71]. A short-term pilot trial of rosiglitazone in 38 women with early stage breast cancer was conducted. Clinical response was not assessed in this short-term (<6 week) study. Biological response, as assessed by Ki-67 staining on biopsy tissues before and after treatment, was not detected in treated patients, either. Decreased insulin levels and increased insulin sensitivity were noted in these patients, suggesting that the rosiglitazone did affect metabolism as expected [72].

An early phase II trial of troglitazone in 41 patients with metastatic prostate cancer showed a decrease in levels of prostate-specific antigen (PSA) in 20% of patients enrolled

TABLE 7: Clinical trials of TZDs in cancer patients.

Cancer type	Phase	TZDs	No. of pts	Tumor response	References
Liposarcoma	II	Rosiglitazone	12	All patients progressed, no sign of differentiation by histology	[68]
Thyroid cancer	I, II	Rosiglitazone	10	4 pts with partial response, 2 with stable disease, and 4 with progressed disease	[69]
Metastatic colorectal cancer	I, II	Troglitazone	25	All patients progressed	[70]
Refractory breast cancer	II	Troglitazone	22	Most patients progressed with increased serum tumor markers	[71]
Early-stage breast cancer	II	Rosiglitazone	38	No reduction in Ki-67 staining on tissue biopsies	[72]
Metastatic prostate cancer	II	Troglitazone	41	Decrease or stabilization of PSA	[73]
Recurrent prostate cancer	III	Rosiglitazone	106	Similar to placebo in both PSADT and time-to-disease-progression	[74]

in the study. Prolonged stabilization of PSA was seen in 39% of patients [73]. However, these encouraging results were not reproduced in a large double-blind, randomized, placebo-controlled trial of rosiglitazone in 106 patients with recurrent prostate cancer [74]. The time-to-disease-progression was not significantly different between the rosiglitazone and placebo groups. Moreover, the PSA doubling time, a predictor of clinical recurrence, was also not prolonged by the treatment.

Taken together, TZDs appear to show little benefit, both in terms of clinical response and biological response, in treating various types of human cancers despite promising results from preclinical animal studies. It is worth noting that most of the studies use low doses of TZDs which are sufficient to activate PPARγ and control diabetes. It remains possible that higher doses, even via receptor-independent pathways, would be beneficial for cancer patients. However, one should keep in mind that TZDs are not a class of drugs without dose-limiting toxicities. Troglitazone was withdrawn from the market by the FDA in 2002 due to liver toxicity. Most recently, increased cardiovascular risk has been associated with rosiglitazone in the diabetic patient population [75, 76] which has prompted the FDA to issue label warnings.

6. TZDs AS CHEMOPREVENTIVE AGENTS IN EPIDEMIOLOGY STUDIES

The clinical trials discussed above suggest that TZDs have questionable efficacy as chemotherapeutic agents in patients who already have cancers. Do they have the potential to act as chemopreventive agents? Recently, a large epidemiologic study, involving a population of 87,678 veteran men with diabetes, attempted to answer that question [77]. In this retrospective study, incidence of lung, prostate, and colon cancer in TZD users was compared to incidence in non-TZD users and risk of cancer development was analyzed. Only patients who obtained a cancer diagnosis after the date of TZD initiation were included. TZD usage significantly reduced risk of lung cancer by 33%. It also reduced risk of colon and prostate cancer, though without statistical significance. Interestingly, although the risk of prostate cancer is not significantly influenced by TZDs in the entire population, when examining distinct populations, TZDs are associated with an increased incidence of prostate cancer in both Caucasians and African Americans. These data suggest that the overall reduced risk is accounted for by the non-Caucasian, non-African Americans populations in the study. These data suggest that TZDs may be beneficial for reducing certain cancers in certain populations. Specific molecular abnormalities in specific cancers and the genetic background of different populations may account for these apparently different results.

Although this study was quite strong, we suggest the following for future investigations: (1) separate TZD-users into those using rosiglitazone and those using pioglitazone. In the cardiovascular risk studies, it was shown that rosiglitazone increases the risk while pioglitazone decreases the risk [78]. (2) Evaluate the impact of the duration of TZD exposure on risk of cancer development. (3) Determine the influence of TZDs on the behavior of existing cancers.

7. CONCLUSIONS

In this article, we reviewed literature on the roles of PPARγ in cancer with an emphasis on those that suggest a proneoplastic function for the receptor. PPARγ, unlike MYC, RAS, or p53, is neither a strong tumor promoter nor a tumor suppressor. However, it may function as a "conditional tumor promoter" or a "conditional tumor suppressor" that modulates the tumorigenic process depending upon cellular conditions, tumor types, or genetic background of an animal strain or human individuals. TZDs, as a class of pharmacological agent, may have receptor-independent antineoplastic effects, especially at doses higher than diabetic doses or after long-term use and accumulation. It remains possible that their antitumor activities would be enhanced when in combination with other drugs. Further investigation is needed to address that possibility. To help clarify the roles of PPARγ in cancer, future large epidemiological studies of diabetic populations with concurrent cancers would be helpful. In addition, investigations relating PPARγ activities to the clinical outcomes of cancer patients would also be informative.

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Review Article

Peroxisome Proliferator-Activated Receptors and Progression of Colorectal Cancer

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The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily. These receptors are also ligand-dependent transcription factors responsible for the regulation of cellular events that range from glucose and lipid homeostases to cell differentiation and apoptosis. The importance of these receptors in lipid homeostasis and energy balance is well established. In addition to these metabolic and anti-inflammatory properties, emerging evidence indicates that PPARs can function as either tumor suppressors or accelerators, suggesting that these receptors are potential candidates as drug targets for cancer prevention and treatment. However, conflicting results have emerged regarding the role of PPARs on colon carcinogenesis. Therefore, further investigation is warranted prior to considering modulation of PPARs as an efficacious therapy for colorectal cancer chemoprevention and treatment.

1. INTRODUCTION

Understanding the biology of intestinal epithelial cells may reveal the molecular pathogenesis of a number of digestive diseases. One such disease, colorectal cancer (CRC), leads to significant cancer-related morbidity and mortality in most industrialized countries. Initiation and progression of CRC are a complex process that results from the loss of the normal regulatory pathways that govern a balance between epithelial cell proliferation and death. For example, alterations in multiple pathways such as Wnt/APC, COX-2, and Ras are known to play major roles in CRC progression. The standard treatment for advanced malignancies has improved greatly over the past decade but is still not satisfactory. Therefore, significant effort has been exerted to identify novel drug targets for both the prevention and treatment of this disease. One group of compounds found to decrease the risk of colorectal cancer includes nonsteroidal anti-inflammatory drugs (NSAIDs), which target the cyclooxygenase enzymes (COX-1 and COX-2). However, prolonged use of high doses of these inhibitors (except for aspirin) is associated with unacceptable cardiovascular side effects [1–3]. Thus, it is now crucial to develop more effective chemopreventive agents with minimal toxicity and maximum benefit.

Dietary fat intake is an environmental factor that is associated with some human diseases such as diabetes, obesity, and dyslipidemias. Some nuclear hormone receptors play a central role in regulating nutrient metabolism and energy homeostasis. These nuclear receptors are activated by natural ligands, including fatty acids and cholesterol metabolites. Among these receptors, special attention has been focused on the members of the peroxisome proliferator-activated receptors (PPARs) family, which were initially identified as mediators of the peroxisome proliferators in the early 1990s [4]. PPARs play a central role in regulating the storage and catabolism of dietary fats via complex metabolic pathways, including fatty acid oxidation and lipogenesis [5]. To date, three mammalian PPARs have been identified and are referred to as PPARα (NR1C1), PPARδ/β (NR1C2), and PPARγ (NR1C3). Each PPAR isotype displays a tissue-selective expression pattern. PPARα and PPARγ are predominantly present in the liver and adipose tissue, respectively, while PPARδ expresses in diverse tissues [6]. In common with other members of the type II

steroid hormone receptor superfamily, PPARs are ligand-dependent transcription factors and form heterodimers with another obligate nuclear receptors, such as retinoid X receptors (RXRs) [4, 7, 8]. Each PPAR-RXR heterodimer binds to the peroxisome proliferator responsive element (PPRE) located in the promoter region of responsive genes.

It is well established that modulation of PPAR activity maintains cellular and whole-body glucose and lipid homeostases. Hence, great efforts have been made to develop drugs targeting these receptors. For example, PPARγ synthetic agonists, rosiglitazone and pioglitazone, are antidiabetic agents which suppress insulin resistance in adipose tissue. The antiatherosclerotic and hypolipidemic agents including fenofibrate and gemfibrozil are PPARα synthetic agonists that induce hepatic lipid uptake and catabolism. Genetic and pharmacological studies have also revealed important roles of PPARδ in regulating lipid metabolism and energy homeostasis. Genetic studies indicate that overexpression of constitutively active PPARδ in mouse adipose tissue reduced hyperlipidemia, steatosis, and obesity induced by either genetics or a high-fat diet. In contrast, PPARδ null mice treated in similar fashion exhibited an obese phenotype [9]. Pharmacologic studies demonstrate that the PPARδ selective-agonist (GW501516) attenuated weight gain and insulin resistance in mice fed with high-fat diets [10] and increased HDL-C while lowering tryglyceride levels and insulin in obese rhesus monkeys [11]. Furthermore, preclinical studies revealed that PPARδ agonists diminished metabolic derangements and obesity through increasing lipid combustion in skeletal muscle [12]. These results suggest that PPARδ agonists are potential drugs for use in the treatment of dyslipidemias, obesity, and insulin resistance. Therefore, the PPARδ agonist (GW501516) is currently in phase III clinical trials to evaluate its use for treatment of patients with hyperlipidemias and obesity. However, recent studies showing that some agonists of PPARs promote carcinogenesis in animal models have raised concerns about using these agonists for the treatment of metabolic diseases. For example, long-term administration of a PPARα agonist induces the development of hepatocarcinomas in mice but not in PPARα null animals, conclusively demonstrating that PPARα mediates these effects in promoting liver cancer [13]. Furthermore, the PPARδ agonist (GW501516) accelerates intestinal polyp growth in Apc$^{Min/+}$ mice [14, 15]. These results raise concerns for developing this class of agents for human use and support the rationale for developing PPARδ antagonists as chemopreventive agents.

2. PPARs AND COLORECTAL CANCER

Significant effort has been concentrated on deducing the role of PPARs in CRC and other cancers. A large body of evidence indicates that PPARγ serves as a tumor suppressor. Contradictory evidences suggest that PPARδ can act as either a tumor suppressor or tumor promoter. A few evidences support a role of PPARα in CRC.

2.1. PPARα

Although the tumor-promoting effects of PPARα in hepatocarcinomas are clear, less is known about the role of PPARα in human tumors. Generally, activation of PPARα by exogenous agonists causes inhibition of tumor cell growth in cell lines derived from CRC, melanoma, and glial brain tumors [16–18]. There is no evidence showing that PPARα expression is elevated in human cancers.

2.2. PPARγ

The prominent role of PPARγ in regulating cellular differentiation prompted a great effort to investigate the function of PPARγ in cancer field. While PPARγ is elevated in CRC [19], suggesting that this receptor may contribute to tumor biology, studies of PPARγ mutation in CRC from humans, animals, and cultured cells produced controversial results. One study showed that 8% of primary human colorectal tumors had a loss of function mutation in one allele of the PPARγ gene [20]. Recent data revealed that a Pro12Ala (P12A) polymorphism in the PPARγ gene is associated with increased risk of CRC [21, 22]. These results suggest a putative role for this receptor as a tumor suppressor. In contrast, another study showed that mutant PPARγ gene has not been detected in human colon tumor samples and CRC cell lines, suggesting that PPARγ mutations in human CRC is a rare event [23].

In vitro studies show that activation of PPARγ results in growth arrest of colon carcinoma cells through induction of cell-cycle arrest or/and apoptosis. Several potential downstream targets of PPARγ for mediating antitumor effects of PPARγ have been identified in various cancer cell types. Activation of PPARγ negatively regulatescell cycle progression by modulating a number of cell cycle regulators: (1) inhibiting E2F activity in transformed adipogenic cells [24], (2) Rb hyperphosphorylation in vascular smooth muscle cells and pituitary adenoma cells [25, 26], (3) cyclin D1 expression in Ras-transformed intestinal epithelial cells, pancreatic, or breast cancer cells [27–29], and (4) inducing CDK inhibitor expression such as p18, p21, and p27 in hepatoma cells [30]. Activation of PPARγ has also been reported to inhibit tumor cell growth by upregulation of the transcriptional repressor TSC22 in colon cancer cells [31] and GADD153 in nonsmall-cell lung carcinoma cells [32]. PPARγ agonists induce apoptosis by induction of PTEN expression in pancreatic, breast, and colon cancer cells [33] and inhibition of NFκB and Bcl-2 expression in colon cancer cells [34]. Moreover, PPARγ exhibits antiangiogenic effects by inhibiting VEGF expression in tumor cells and VEGF receptors in endothelial cells [35, 36]. It has also been reported that PPARγ agonists suppress tumor cell invasion in colon and breast cancer cells by downregulation of matrix metalloproteinase-7 (MMP-7) and induction of MMP inhibitors [37, 38]. In addition, the ability of PPARγ to suppress tumor growth is also through inhibiting APC/β-catenin and COX-2/PGE$_2$ signaling pathways, which are pivotally involved in colon carcinogenesis [39–42].

However, the role of PPARγ in colorectal cancer progression is controversial because there are conflicting results in mouse models of colon cancer. Although PPARγ agonists inhibit colorectal carcinogenesis in xenograft models and in the azoxymethane (AOM)-induced colon cancer model [43, 44], these drugs are reported to have both tumor-promoting and tumor-inhibiting effects in a mouse model for familial adenomatous polyposis, the Apc$^{Min/+}$ mouse. It has been reported that administration of PPARγ agonists significantly increases the number of colon adenomas in the Apc$^{Min/+}$ mice [45–47] and even in wild-type C57BL/6 mice [48]. However, other studies show that treatment of 2 different Apc-mutant models (Apc$^{Min/+}$ and Apc$^{\Delta 1309}$) with the PPARγ agonist pioglitazone resulted in reduction in the number of both small and large intestinal polyps in a dose-dependent manner [49, 50]. These paradoxical observations appear to have been resolved by genetic studies showing that the heterozygous disruption of PPARγ is sufficient to increase tumor number in AOM-treated mice and that intestinal-specific PPARγ knockout promotes tumor growth in Apc$^{Min/+}$ mice [39, 51]. These genetic evidences support the hypothesis that PPARγ serves as tumor suppressor in colorectal cancer. One possible explanation for the differences in phenotype caused by pharmaceutical versus genetic manipulation of PPARγ in mouse models may be due to the PPARγ-independent effect of the agonist drugs, drug doses used, and animal models employed. This controversial extends beyond CRC. For example, data are conflicting from different animal models of breast cancer as well. PPARγ agonist suppresses NMU-induced mammary carcinomas [52]. However, overexpression of a constitutively active form of PPARγ accelerates mammary gland tumor development in MMTV-PyV transgenic mice [53].

2.3. PPARδ

PPARδ has been shown to play an important role in embryo implantation [54], atherogenic inflammation [55], regulating cell survival in the kidney following hypertonic stress [56], and skin following wound injury [57, 58]. The role of PPARδ in colorectal carcinogenesis is more controversial than that of PPARγ. The first evidence linking the PPARδ to carcinogenesis actually emerged from studies on gastrointestinal cancer. PPARδ is elevated in most human colorectal cancers and in tumors arising in the Apc$^{Min/+}$ mice, and AOM-treated rats [59, 60]. Importantly, the PPARδ proteins are accumulated only in human CRC cells with highly malignant morphology [61]. Downregulation of PPARδ is correlated with antitumor effects of dietary fish oil/pectin in rats treated with radiation and AOM [62]. PPARδ was identified as a direct transcriptional target of APC/β-catenin/Tcf pathway and as a repression target of NSAIDs [59, 63]. A case-control study in a large population showed that the protective effect of NSAIDs against colorectal adenomas was reported to be modulated by a polymorphism in the PPARδ gene [64]. PPARδ expression and activity are also induced by oncogenic K-ras [65]. In addition, COX-2-derived PGI$_2$ directly transactivates PPARδ [60], and COX-2-derived PGE$_2$ indirectly induces

PPARδ activation in CRC, hepatocellular carcinoma, and cholangiocarcinoma cells [66–68]. These studies indicate that PPARδ is a focal point of cross-talk between these signaling pathways.

In a murine xenograft cancer model, the disruption of both PPARδ alleles in human HCT-116 colon carcinoma cells decreased tumorigenicity, suggesting that activation of PPARδ promotes tumor growth [69]. However, PPARδ has been reported to have both tumor-promoting and tumor-inhibiting effects based on conflicting data obtained from mouse models of colon cancer. For example, activation of PPARδ by a selective synthetic PPARδ agonist (GW501516) or a PPARδ endogenous activator (PGE$_2$) accelerates intestinal adenoma growth in Apc$^{Min/+}$ mice by promoting tumor cell survival [14, 66]. A subsequent genetic study showed that deletion of PPARδ attenuates both small and large intestinal adenoma growth, and PPARδ is required for the tumor-promoting effects of PPARδ ligand (GW501516) and PGE$_2$ in Apc$^{Min/+}$ mice [15, 66]. Another study showed that loss of PPARδ in Apc$^{Min/+}$ mice significantly reduced growth of tumors larger than a diameter of 2 mm, even though PPARδ deficiency did not affect overall tumor incidence [70]. In contrast to these reports suggesting that PPARδ serves as tumor accelerator, recent conflicting reports show that PPARδ deficiency enhances polyp growth in Apc$^{Min/+}$ and AOM-treated mice in the absence of exogenous PPARδ stimulation [71, 72]. Moreover, a PPARδ ligand (GW0742) inhibits colon carcinogenesis in AOM-treated mice but promotes small intestinal polyp growth in Apc$^{Min/+}$ mice [73].

One explanation for these disparate results may be due to differences in the genetic background of Apc$^{Min/+}$ mice, animal breeding, or possibly to differences in the specific targeting strategy employed to delete PPARδ. For example, the average number of polyps in 13-week old Apc$^{Min/+}$ mice on a C57BL/6 genetic background is about 50, while the polyp number in Apc$^{Min/+}$ mice on a mixed-genetic-background (C57BL/6 × 129/SV) is about 120. Our results also show that the breeding strategy affects the number and size of polyps in mice even on the same genetic background. Mice generated by breeding female PPARδ$^{-/-}$/Apc$^{Min/+}$ with male PPARδ$^{-/-}$/Apc$^{+/+}$ exhibit increased adenoma number with a larger average size than those obtained by breeding female PPARδ$^{-/-}$/Apc$^{+/+}$ with male PPARδ$^{-/-}$/Apc$^{Min/+}$. Finally, the PPARδ null mice we studied were obtained from Beatrice Desvergne in Switzerland. These mice were generated by deleting exons 4 and 5 encoding the DNA binding domain [74], while Peters group generated the PPARδ knockout mice by inserting a neomycin resistance cassette into the last exon (exon 8) [75]. It has been suggested that the strategy employed to disrupt PPARδ by the Peters group might have led to a hypomorphic allele, which retains some aporeceptor function, thus making it difficult to correctly interpret their results. Indeed, conflicting results in the context of embryonic lethality have also been observed from these two PPARδ mutant mouse strains [74, 75]. To further clarify the role of PPARδ in colorectal tumorigenesis, it is important to investigate the role of PPARδ in animal models that are dependent on activation of other oncogenes
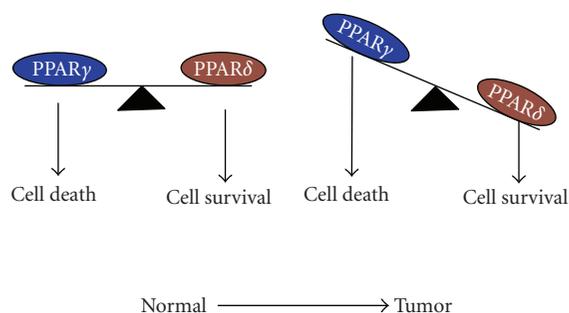
Figure 1: A potential model for PPARs regulating colorectal tumor growth.

or disruption of other tumor suppressors to verify our conclusions that activation of PPARδ is proneoplastic.

Studies in other types of cancer also support the hypothesis that PPARδ serves as a tumor accelerator. A selective PPARδ agonist (GW501516) has been shown to stimulate proliferation of human breast, prostate, and hepatocellular carcinoma cells [68, 76, 77]. In a xenograft model, blocking PPARδ activation reduced ovarian tumor growth [78]. PPARδ knockout mice exhibited significant impaired angiogenesis and tumor growth after these mice were injected s.c. with mouse Lewis lung carcinoma and melanoma cells [79]. In a mouse mammary tumor model, treatment with the PPARδ agonist (GW501516) accelerated tumor formation, while a PPARγ agonist (GW7845) delayed tumor growth [80]. Taken together, the role of PPARδ in cancer biology remains unclear.

3. SUMMARY

Despite extensive research on both PPARγ and PPARδ in CRC, the role of these receptors remains highly controversial in this disease. Emerging evidence demonstrates that cooperative interactions between Wnt, COX-2, and PPARs signaling pathways can initiate cellular transformation and promote progression of colorectal cancer. These studies provide support for evaluating the efficacy of PPARδ antagonists for cancer prevention and/or treatment. We propose a potential working model as a useful starting point for future studies (see Figure 1).

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Review Article

PPARα- and DEHP-Induced Cancers

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Di(2-ethylhexyl)phthalate (DEHP) is a widely used plasticizer and a potentially nongenotoxic carcinogen. Its mechanism had been earlier proposed based on peroxisome proliferator-activated receptor α (PPARα) because metabolites of DEHP are agonists. However, recent evidence also suggests the involvement of non-PPARα multiple pathway in DEHP-induced carcinogenesis. Since there are differences in the function and constitutive expression of PPARα among rodents and humans, species differences are also thought to exist in the carcinogenesis. However, species differences were also seen in the lipase activity involved in the first step of the DEHP metabolism, which should be considered in DEHP-induced carcinogenesis. Taken together, it is very difficult to extrapolate the results from rodents to humans in the case of DEHP carcinogenicity. However, PPARα-null mice or mice with human PPARα gene have been developed, which may lend support to make such a difficult extrapolation. Overall, further mechanical study on DEHP-induced carcinogenicity is warranted using these mice.

1. INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) a plasticizer around the world, suggesting that many people come across this chemical every day. Animal studies showed that this chemical is a nongenotoxic carcinogen. Metabolites of DEHP, mono- and dicarboxylic acids, transactivate peroxizome proliferator-activated receptor α (PPARα), which has been thought to result in nongenotoxic carcinogenesis [1, 2]. However, the latest studies also showed the involvement of non-PPARα pathways; multiple pathways might be involved in the pathway of DEHP-induced carcinogenicity [3]. There are species differences in the functional activation or constitutive expression of rodent and human PPARα, and that in humans is thought to be less active and expressive than those of rodents. Recently, inflammation-related carcinogenesis has drawn attention [4, 5]. PPARα is involved not only in the induction of target genes such as β-oxidation enzymes of fatty acids but also in anti-inflammation signaling [6, 7], suggesting that PPARα also may protect against carcinogenesis. Species differences in lipase activity (DEHP-metabolizing enzyme) among mice, rats, and marmosets have been also reported recently [8], suggesting that this kinetic difference should be considered in the species differences in DEHP-

induced carcinogenesis. In this review, we focused on DEHP-induced hepatic carcinogenesis in relation to PPARα-dependent and PPARα-independent pathways, and discussed the science policy.

2. PPARs

PPARs are involved in a member of the nuclear hormone receptor superfamily, and consist of three subunits: PPARα, PPARβ/δ, and PPARγ [9]. These three isoforms have been identified at the organ-specific level. In the respective organ, PPARs function as transcription factors through the classic ligand-dependent nuclear hormone receptor mechanism. Upon binding to their ligands, PPARs undergo conformational changes that allow corepressor release [10]. The PPAR-ligand complex binds to direct repeat 1 elements or peroxisome proliferator response elements (PPREs), usually located upstream of the target genes, which results in the induction of fatty acid transport and metabolism, glucose metabolism, and also elicitation of anti-inflammatory effects [6, 11].

As one of the three isoforms, PPARα is mainly expressed in organs that are critical in fatty acid catabolism, such as liver, heart, and kidney [7]. Thus, this nuclear receptor is

primarily involved in the regulation of fatty acid metabolism. In addition to this function, PPARα also has various functions including the promotion of gluconeogenesis, lipogenesis, ketogenesis, and anti-inflammatory effects [6].

3. PPARα LIGANDS

The ligands of PPARα represent a diverse group of chemicals including not only endogenous ligands but also exogenous synthetic ligands with a high likelihood of clinical, occupational, and environmental exposure of humans to chemicals [1, 12]. The primary endogenous ligands are fatty acids, mainly the 18–20 carbon polyunsaturated fatty acids and eicosanoids [7, 13–17]. As exogenous ligands, fibrates and thiazolidinediones are involved. Additionally, the general population is exposed to environmental chemicals such as plasticizers (e.g., phthalates), solvents (e.g., tetrachloroethylene and trichloroethylene), perfluorooctanoic acid and herbicides (e.g., 2, 4-dichlorophenoxyacetic acid, diclofopmethyl, haloxyfop, lactofen, and oxidiazon).

Of these ligands, the toxicity of DEHP is well established in relation to PPARα. This chemical is used as a plasticizer to improve the plasticity and elasticity of polyvinyl chloride products that have become ubiquitous in our daily living. These products are widely used in building materials, wallpaper and flooring, wire covering, vinyl sheeting for agriculture, food packages, and medical devices such as intravenous and hemodialysis tubing and blood bags. The recent production of DEHP in Japan has approached 14 000 tons per year, which accounts for about 54% of all plasticizers used [11]. It is noted that mono- and dicarboxylic acid metabolites of DEHP, not DEHP itself, act as ligands for PPARα [18] and have potentially adverse effects on liver, kidney, heart, and reproductive organs though monocarboxylic acid, mono(2-ethylhexyl) phthalate (MEHP), also binds to PPARγ [18].

4. SPECIES DIFFERENCES IN PPARα

Since there are species differences in the toxicity of PPARα agonists, the expression levels or functions of the receptor are thought to be different among species. Several explanations for the species differences in response to the ligands have been suggested [19, 20]. One of the major factors was considered to be due to differences in the levels of PPARα expression [21, 22] although other possibilities include differences in ligand affinity between rodent and human PPARα, differences in cellular context of PPARα expression, and those in PPRE sequences found upstream of critical target genes [23, 24]. Indeed, PPARα expression in humans is about 1/10 times less than that in rodents [25]. In addition, micro-RNA expression regulated by PPARα has been recently reported to be changed in wild-type mice, but not in mice with human PPARα gene [26]; Wy-14,643 inhibited a micro-RNA let-7C which is involved in suppression of tumorigenesis in wild-type mice, but neither in PPARα-null mice nor in mice with human PPARα gene. Mice with human PPARα gene are resistant to hepatocellular proliferation though they respond to Wy-14,643 in β-oxidation and serum triglycerides [27]. These results suggest that the function of the PPARα signaling in liver proliferation and tumorigenesis by the chemical exposure is not always similar in mice and humans.

In regard to the species differences in the PPREs, the lack of acyl CoA oxidase (ACO) induction in studies on liver biopsies from humans treated with hypolipidemic drugs or primary human hepatocytes treated with Wy-14,643 may be attributable to an inactive functional PPRE since the sequence of a PPRE for the ACO gene from a small number of human liver biopsy samples was found to be different from that of the rats [28]. However, Reddy remarked at a panel discussion that, although the sequence of ACO gene promoter in the mouse was also different from that in the rat, both rodents are responsive to some peroxisome proliferators in ACO induction [20]. In addition, differences in the ability of rodents and human PPAR to recognize and bind PPRE are unlikely since the DNA binding domains of the human and rodent PPARα are 100% homologous [29, 30]. Though characterized from only a limited number of individuals, the prevalence in the population of defective PPAR alleles cannot be determined at this point [31]. The species difference in the sequence of PPRE may not be involved in the difference in response to ligands between rodents and humans.

In addition to the lower expression levels of PPARα in human, there was a truncated, inactive form of PPARα in human liver, suggesting that the expression of full-length functional PPARα was very low. These inactive forms of PPARα may be insufficient to bind PPRE because PPREs may be occupied in vivo by other nuclear receptors that bind to similar sequences, thus affecting responsiveness to ligands [25].

5. SPECIES DIFFERENCES IN DEHP METABOLISM

In addition to the species differences in PPARα functions or expression levels, we should also be mindful of the importance of those in the metabolism of DEHP between rodents and humans. DEHP absorbed in the body is first metabolized by the catalytic action of lipase to produce MEHP and 2-ethylhexanol (2-EH) [32]. Some MEHP is then conjugated with UDP-glucuronide by UDP-glucuronosyltransferase (UGT) and excreted in the urine. The remaining MEHP is excreted directly in the urine or is oxidized by cytochrome P450 4A, then further oxidized by alcohol dehydrogenase (ADH) or aldehyde dehydrogenase (ALDH) to dicarboxylic acid or ketones. 2-EH is metabolized mainly to carboxylic acid (mainly 2-ethylhexanoic acid (2HEA)) via 2-ethylhexanal by catalytic action of ADH and ALDH. Thus, lipase may be an essential enzyme to regulate the DEHP metabolism; knowing the species difference in the lipase activity may be an important tool to clarify the species difference in metabolism.

Recently, the activities of lipase, UGT, ADH, and ALDH for DEHP metabolism in several organs were measured and compared among mice, rats, and marmosets [8]. Marmosets were used as a reference to human. Clear-cut species differences were seen in the activities of the four enzymes involved in the DEHP metabolism among mice, rats, and
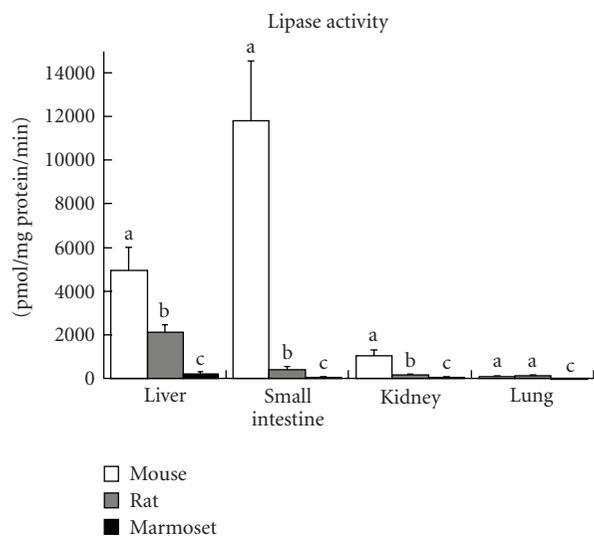
FIGURE 1: Species differences in lipase activities (pmol/mg protein in microsomal fragment/min) using hepatic microsomes in liver, small intestine, kidney, and lung from mice, rats, and marmosets. Lipase activity was measured by GC/MS. Substrate concentration (DEHP) used was 1 mM. Each white bar (6 mice), grey bar (5 rats), or black bar (5 marmosets) represents the mean ± standard deviations. Lipase activity was not detected in marmoset lung (under 1 pmol/mg protein/min). Comparisons were made using analysis of variance and the Tukey-Kramer HSD post hoc test. A logarithmic transformation was applied to lipase activities in microsome samples from the small intestine and kidneys before Tukey-Kramer analysis. Different letters (a, b, c) on the top of each bar in each organ indicate that they are significantly different from each other ($P < .05$).

marmosets. The most prominent difference was observed in the lipase activity with an almost 148- to 357-fold difference between the highest activity in mice and the lowest in marmosets (Figure 1). These differences were comparable to those in the kinetic parameter, *Vmax.* These results suggest that the constitutive levels of lipase were greater in the mice and rats than in marmosets. Indeed, lipase-mRNA levels in livers from mice or rats were much higher than those in marmoset (Figure 2). Thus, concentrations of MEHPs (ligands to PPARα) in the body were higher in mice or rats than in marmosets when the same dose of DEHP was administered [33].

Besides species differences in the constitutive levels of lipase, *Km* values of DEHP for lipase of marmosets were much higher than in rats or mice, suggesting the species differences in the DEHP affinity for lipase; the affinity of DEHP for lipase in the marmosets may be lower than that of mice or rats. The affinity in human may be even lower than that in primates; cumulative ^{14}C excretion in urine of African green monkey following bolus injection of ^{14}C-DEHP leached into autologous plasma occurred earlier than in human [34].

6. MECHANISM OF DEHP-INDUCED CANCER

DEHP causes tumors, especially in liver when chronically administered to rats and mice [35–39], similar to the other
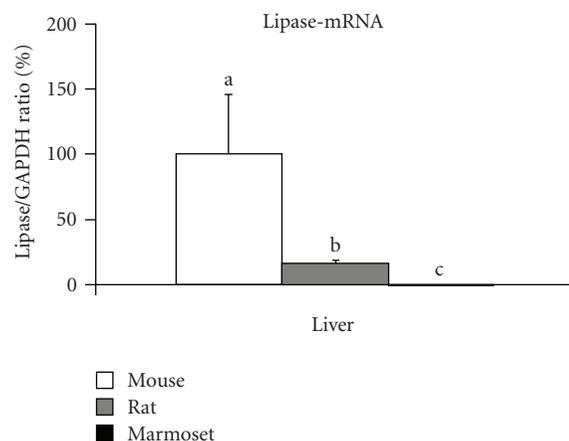
FIGURE 2: Lipase-mRNA levels in mice, rats, and marmosets. Each mRNA level was measured by real-time quantitative PCR and normalized to the GAPDH-mRNA level in the same preparation. Mouse liver mean was assigned a value of 100. Figures represent mean ± SD from 6 from mice and 5 from rats and marmosets. Comparisons were made using analysis of variance and the Tukey-Kramer HSD post hoc test. Different letters (a, b, c) on the top of each bar in each organ indicate that they are significantly different from each other ($P < .05$).

peroxisome proliferators such as Wy-14643. Table 1 shows that DEHP induces hepatic tumors in mice and rats. From the viewpoint of percentage in feed, the lowest-observed effect-level (LOEL) of DEHP carcinogenicity in the rat was 0.6%, and the no-observed effect-level (NOEL) was 0.1% [2]. In the mouse, the corresponding values may be 0.05% for LOEL and 0.01% for NOEL because the study in which male mice were exposed to 0.05% DEHP for 78 weeks exhibited a significant increase in the hepatic tumor incidence rate compared with controls, but not when exposed to 0.01% DEHP [40].

DEHP also has potential for carcinogenesis in other organs; pancreatic acinar cell adenoma and mononuclear cell leukemia incidences were significantly increased in male F344 rat but not in F344 female rat and B6C3F1 mouse of both sexes after DEHP exposure [35, 36, 44]. The reason why these cancers are not observed in female rat has not been identified.

Chronic treatment with PPARα agonist results in an increased incidence of liver tumors which were thought to have occurred through a PPARα-mediated mechanism as revealed by the resistance of PPARα-null mice to liver cancer induced by Wy-14,643 exposure for 11 months [46]. All the wild-type mice fed with 0.1% Wy-14643 diet for 11 months had multiple hepatocellular neoplasms, including adenomas and carcinomas, while thePPARα-null mice fed with the 0.1% Wy-14643 diet for the same duration were unaffected. Ward et al. [47] reported that exposure for only six months to 12 000 ppm DEHP caused induction of peroxisomal enzymes, liver enlargement, and histopathological increases in eosinophil counts and peroxisomes in the cytoplasm of wild-type mice, while there were no such toxic findings in the liver of PPARα-null mice. Thus, DEHP-derived

TABLE 1: Primary studies on DEHP-induced carcinogenesis in mice and rats (modifying the paper reported by Huber et al. [2]).

Author Species, strain	Sex	Route	Duration	Dosage	Type of tumor	Tumor frequency (%)
[39] Rat F344	M	Feed	103 w	0.00%	Hepatic tumors	6
				0.60%		12
				1.20%		24
	F	Feed	103 w	0.00%	Hepatic tumors	0
				0.60%		12
				1.20%		26
[41] Rat F344	F	Feed	2 y	0.00%	Hepatic tumors	0
				0.03%		6
				0.10%		5
				1.20%		30
[42] Rat F344	M	Oral	24 m	0 (water)	Liver carcinoma	4
				0 (vehicle)		12
				2EH 50 mg/kg		6
				2EH 150		6
				2EH 500		2
	M	Oral	24 m	0 (water)	Liver adenoma	0
				0 (vehicle)		0
				2EH 50 mg/kg		0
				2EH 150		2
				2EH 500		0
	F	Oral	24 m	0 (water)	Liver carcinoma	0
				0 (vehicle)		2
				2EH 50 mg/kg		2
				2EH 150		4
				2EH 500		0
[42] Mouse B6C3F1	M	Oral	18 m	0 (water)	Liver carcinoma	8
				0 (vehicle)		12
				2EH 50 mg/kg		12
				2EH 200		14
				2EH 750		18
	M	Oral	18 m	0 (water)	Liver adenoma	0
				0 (vehicle)		0
				2EH 50 mg/kg		0
				2EH 200		0
				2EH 750		2
	F	Oral	18 m	0 (water)	Liver carcinoma	2
				0 (vehicle)		0
				2EH 50 mg/kg		2
				2EH 200		6
				2EH 750		10
[43] Rat F344	M	Feed	2 y	0 ppm	Hepatocellular carcinoma	2
				6000 ppm		2
				12000 ppm		10
	M	Feed	2 y	0 ppm	Hepatocellular neoplastic nodule	4
				6000 ppm		10
				12000 ppm		14

TABLE 1: Continued.

Author Species, strain	Sex	Route	Duration	Dosage	Type of tumor	Tumor frequency (%)
	F	Feed	2 y	0 ppm	Hepatocellular carcinoma	0
				6000 ppm		4
				12000 ppm		16
				0 ppm	Hepatocellular neoplastic nodule	0
				6000 ppm		8
				12000 ppm		10
[43] Mouse B6C3F1	M	Feed	2 y	0 ppm	Hepatocellular carcinoma	18
				3000 ppm		29
				6000 ppm		38
				0 ppm	Hepatocellular adenoma	10
				3000 ppm		23
				6000 ppm		20
	F	Feed	2 y	0 ppm	Hepatocellular carcinoma	0
				3000 ppm		14
				6000 ppm		34
				0 ppm	Hepatocellular adenoma	2
				3000 ppm		10
				6000 ppm		2
[40] Rat F344	M	Diet	79 w	0 ppm	Hepatocellular carcinoma	10
				2500 ppm		0
				12500 ppm		40
				0 ppm	Hepatocellular adenoma	10
				2500 ppm		10
				12500 ppm		10
	F	Diet	79 w	0 ppm	Hepatocellular carcinoma	0
				2500 ppm		0
				12500 ppm		20
				0 ppm	Hepatocellular adenoma	0
				2500 ppm		0
				12500 ppm		10
	M	Diet	105 w	0 ppm	Hepatocellular carcinoma	1
				100 ppm		0
				500 ppm		2
				2500 ppm		5
				12500 ppm		30
				Recovery		13
				0 ppm	Hepatocellular adenoma	5
				100 ppm		10
				500 ppm		5
				2500 ppm		12
				12500 ppm		26
				Recovery		22
				0 ppm	Hepatocellular carcinoma	0
				100 ppm		2
				500 ppm		0
				2500 ppm		2
				12500 ppm		18
				Recovery		7

Table 1: Continued.

Author Species, strain	Sex	Route	Duration	Dosage	Type of tumor	Tumor frequency (%)
	F	Diet	105 w	0 ppm	Hepatocellular adenoma	0
				100 ppm		6
				500 ppm		2
				2500 ppm		3
				12500 ppm		10
[40] Mouse B6C3F1	M	Diet	79 w	0 ppm	Hepatocellular carcinoma	0
				100 ppm		0
				500 ppm		10
				1500 ppm		0
				6000 ppm		7
				0 ppm	Hepatocellular adenoma	7
				100 ppm		10
				500 ppm		20
				1500 ppm		10
				6000 ppm		7
	F	Diet	79 w	0 ppm	Hepatocellular carcinoma	0
				100 ppm		0
				500 ppm		0
				1500 ppm		0
				6000 ppm		13
				0 ppm	Hepatocellular adenoma	0
				100 ppm		10
				500 ppm		10
				1500 ppm		10
				6000 ppm		27
	M	Diet	105 w	0 ppm	Hepatocellular carcinoma	6
				100 ppm		8
				500 ppm		14
				1500 ppm		22
				6000 ppm		31
				Recovery		22
				0 ppm	Hepatocellular adenoma	6
				100 ppm		17
				500 ppm		20
				1500 ppm		22
				6000 ppm		27
				Recovery		5
	F	Diet	105 w	0 ppm	Hepatocellular carcinoma	4
				100 ppm		3
				500 ppm		5
				1500 ppm		15
				6000 ppm		23
				Recovery		42
				0 ppm	Hepatocellular adenoma	0
				100 ppm		3
				500 ppm		6
				1500 ppm		14
				6000 ppm		49
				Recovery		24

TABLE 1: Continued.

Author Species, strain	Sex	Route	Duration	Dosage	Type of tumor	Tumor frequency (%)
[43] Rat F344	M	Feed	2 y	0, 6000, 12000 ppm	Pituitary adenoma or carcinoma	Decrease in highest dose
	F	Feed	2 y	0, 6000, 12000 ppm	Pituitary adenoma or carcinoma	Decrease in lower dose
	M	Feed	2 y	0, 6000, 12000 ppm	Thyroid C-cell adenoma or carcinoma	Decrease in highest dose (unclear)
	M	Feed	2 y	0, 6000, 12000 ppm	Testis interstitial cells tumor	Decrease in highest dose
	F	Feed	2 y	0, 6000, 12000 ppm	Mammary gland	Decrease in highest dose
[36] Rat F344	M	Diet	78 w	0 ppm	Interstitial cells tumor or testes	90
				2500 ppm		100
				12500 ppm		30
	M	Diet	104 w	0 ppm	Interstitial cells tumor or testes	92
				100 ppm		90
				500 ppm		91
				2500 ppm		92
				12500 ppm		31
				0 ppm	Mononuclear cell leukemia	23
				100 ppm		26
				500 ppm		29
				2500 ppm		49
				12500 ppm		42
				0 ppm	Pancreatic acinar cell adenoma	0
				100 ppm		0
				500 ppm		0
				2500 ppm		0
				12500 ppm		8
	F	Diet	104 w	0 ppm	Mononuclear cell leukemia	22
				100 ppm		34
				500 ppm		20
				2500 ppm		25
				12500 ppm		26
				0 ppm	Pancreatic acinar cell adenoma	0
				100 ppm		0
				500 ppm		0
				2500 ppm		0
				12500 ppm		3
[44] Rat F344	M	Diet	79 w	0 ppm	Interstitial cells tumor or testes	90
				12500 ppm		30
				0 ppm	Mononuclear cell leukemia	0
				12500 ppm		10

Table 1: Continued.

Author Species, strain	Sex	Route	Duration	Dosage	Type of tumor	Tumor frequency (%)	
	M	Diet	105 w	0 ppm	Interstitial cells tumor or testes	92	
				12500 ppm		31	
				Recovery		32	
				0 ppm	Mononuclear cell leukemia	23	
				12500 ppm		42	
				Recovery		53	
[35] Mouse B6C3F1	M,	Diet	78 w,	0, 100, 500, 1500, 6000 ppm	No data about tumors		
[44] Rat F344	F	Diet	79 w	0 ppm, 12500 ppm	No data about tumors		
[44] Mouse B6C3F1	M,	Diet	79 w	0 ppm, 6000 ppm,	No data about tumors		
	M,	Diet	105 w	0 ppm, 6000 ppm,	No data about tumors		
[45] Mouse 129/Sv, PPARα-null	M	Diet	21 m		Liver tumors (hepatocellular adenoma, hepatocellular carcinoma, cholangiocellular carcinoma)	Wild-type	PPARα-null
				0%		0	4
				0.01%		9	4
				0.05%		10	25.8

carcinogenicity was thought to be mediated by PPARα, similar to Wy-14,643, and DEHP was considered to cause primarily PPARα-dependent carcinogenicity in rodents, but it is considered to be relatively safe in humans, similar to other ligands [2]. However, Ward et al. [47] could not directly observe DEHP-derived tumors in the wild-type mice, because exposure to DEHP for 6 months may not be sufficient to induce hepatic tumors, as suggested by Marsman et al. [48]; they reported that DEHP tumorigenesis required longer exposure periods than Wy-14,643. It is doubtful whether DEHP definitively induces hepatic tumors via PPARα.

As mentioned above, the following simple mechanism has been proposed for the DEHP-induced hepatocarcino-genesis; when DEHP was administered to rats and mice, the chemical caused an increase in cell proliferation and peroxisome proliferation [49]. The latter is accompanied by an increase in both peroxisomal and mitochondrial fatty acid metabolizing enzymes such as ACO. As a byproduct of fatty acid oxidation, enzymes involved with β-oxidation generate H_2O_2, resulting in elevated oxidative stress. DEHP also causes an increase in proinflammatory cytokines and inhibition of apoptosis [2, 24].

DEHP-induced liver carcinogenesis in rodents, however, appears to involve more complex pathways as described in the following events whereby various combinations of the molecular signals and multiple pathways may be involved [3]. DEHP is metabolized to bioactive metabolites which are absorbed and distributed throughout the body; they might induce PPARα-independent activation of macrophages and production of oxidants, and also activate PPARα and sustained induction of target genes. The inductions lead to enlargement of hepatocellular organelles, an increase

in cell proliferation, a decrease in apoptosis, sustained hepatomegaly, chronic low-level oxidative stress and accumulation of DNA damage, and selective clonal expansion of the initiated cells. Finally, preneoplastic nodules might be induced and might result in adenomas and carcinoma.

Peraza et al. [10] also suggest that PPARα is the only receptor in PPARs that is known to mediate carcinogenesis, while the prevailing evidence suggests that PPARβ, PPARγ, and their ligands appear to be tumor modifiers that inhibit carcinogenesis, albeit there is still controversy in the field. Melnick [50] also addressed non-PPARα mechanisms for DEHP-induced carcinogenicity as follows. (1) Peroxisome proliferator-induced tumorigenesis is related to the genes involved in cellular proliferations of, for example, p38 mitogen-activated protein kinase, which is not involved in peroxisome proliferations [51]. (2) DEHP and other peroxisome proliferators stimulated growth regulatory pathways such as immediate early genes for carcinogenesis (c-jun, c-fos, junB, egr-1), mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphorylation of p38, which were dissociated from PPARα activation in rat primary cultures [52–54]. These findings also support the view that peroxisome proliferators, including DEHP, may have the potential for tumorigenesis via non-PPARα signal pathways.

In recent years, an inflammation-associated model of cancers has been given attention [4, 5]. PPARα exerts anti-inflammation effects by repressing nuclear factor kappa B (NFκB) [55], which inhibits inflammation signaling and subsequent cancer [4].

Ito et al. [45] proposed possibility of DEHP tumorigenesis via a non-PPARα pathway using PPARα-null mice. They compared DEHP-induced tumorigenesis in wild-type and

PPARα-null mice treated for 22 months with diets containing 0, 0.01, or 0.05% DEHP. Surprisingly, the incidence of liver tumors was higher in PPARα-null mice exposed to 0.05% DEHP (25.8%) than in similarly exposed wild-type mice (10%), while the incidence was 0% in wild-type miceand 4% in PPARα-null mice without DEHP exposure. The levels of 8-hydroxydeoxyguanosine increased dose-dependently in mice of both genotypes, but the degree of increase was higher in PPARα-null mice than in wild-type mice. NFκB levels also significantly increased in a dose-dependent manner in PPARα-null mice. The proto-oncogene c-jun-mRNA was induced, while c-fos-mRNA tended to be induced only in PPARα-null mice fed with 0.05% DEHP-containing diet. These results suggest that chronic low-level oxidative stress induced by DEHP exposure may lead to the induction of inflammation and/or the expression of proto-oncogenes, resulting in a high incidence of tumorigenesis in PPARα-null mice. Moderate activated PPARα might protect from p65/p50 NFκB inflammatory pathway caused by chronic DEHP exposure in wild-type mice. Although cross-talk of PPARγ, but not PPARα, with cyclooxygenase 2 (Cox-2), which also was related with inflammation-induced hepatocellular carcinoma, has been suggested [56], there was neither induction of Cox-2 nor PPARγ in both genotyped mice of that study (data not shown).

Additionally, we compared the mechanisms of tumorigenesis between wild-type mice and PPARα-null mice using hepatocellular adenoma tissues of both genotyped mice [57]. The microarray profiles showed that the up- or downregulated genes were quite different between hepatocellular adenoma tissues of wild-type mice and PPARα-null mice exposed to DEHP, suggesting that their tumorigenesis mechanisms might be different. Interestingly, the gene expressions of apoptotic peptidase activating factor 1 and DNA-damage-inducible 45α (Gadd45α) were increased in the hepatocellular adenoma tissues of wild-type mice exposed to DEHP, whereas they were unchanged in corresponding tissues of PPARα-null mice. On the other hand, the expressions of cyclin B2 and myeloid cell leukemia sequence 1 were increased only in the hepatocellular adenoma tissues of PPARα-null mice. Taken together, DEHP may induce hepatocellular adenomas, partly via suppression of G2/M arrest regulated by Gadd45α and caspase 3-dependent apoptosis in PPARα-null mice. However, these genes may not be involved in tumorigenesis in wild-type mice. In contrast, the expression level of Met was notably increased in the liver adenoma tissue of wild-type mice, which may suggest the involvement of Met in DEHP-induced tumorigenesis in wild-type mice. However, we could not determine whether DEHP promoted the spontaneous liver tumor in PPARα-null mice because spontaneous hepatocellular tumors are known to occur in these mice at 24 months of age [58], while we observed DEHP-induced tumorigenesis at 22 months of age. To clarify this, gene expression profiles of liver tumors in the control group must be analyzed.

Taken together, the mechanisms of DEHP-induced carcinogenesis do not consist of only a simple pathway such as PPARα-mediated peroxisome proliferation as mentioned by Rusyn et al. [3]. PPARα-independent pathways may also exist and, by contrast, activated PPARα may protect against DEHP-induced carcinogenesis. The valance of the production of oxidative stress via the transactivation of PPARα and subsequent DNA damages versus the effective exertion of anti-inflammation by activating the receptor may determine the incidence of DEHP-induced tumors.

7. FUTURE INVESTIGATIONS

To determine the mechanism of species difference in response to peroxisome proliferators, a mouse line with human PPARα was produced and designated hPPARα^{TetOff} [27]. This mouse line expresses the human receptor in liver in a PPARα-null background by placing the hPPARα cDNA under control of the Tet-Off system of doxycycline control with the liver-specific LAP1 (C/EBPβ) promoter. Interestingly, the hPPARα^{TetOff} mice express the human PPARα protein at levels comparable to those expressed in wild-type mice; so we should not need to consider the species differences in the expression of PPARα between mice and humans. Treatment of this mouse line with Wy-14,643 revealed induction of genes' encoding peroxisomal lipid-metabolizing enzymes, including ACO, bifunctional enzyme and peroxisomal thiolase, and the fatty acid transporter CD36 at a level comparable to that in wild-type mice, expressing native mouse PPARα. This suggested that human PPARα is functionally active. Upon treatment with Wy-14,643, hPPARα^{TetOff} mice also had lower levels of fasting serum total triglycerides similar to wild-type mice. However, hPPARα^{TetOff} mice did not show any significant hepatocellular proliferation, nor did they have an induction of cell cycle control genes, in contrast to Wy-14,643-treated wild-type mice where a significant increase in mRNAs encoding PCNA, cMYC, cJUN, CDK1, CDK4, and several cyclins was found after treatment with Wy-14,643. hPPARα^{TetOff} mice were also found to be resistant to Wy-14,643-induced hepatocarcinogenesis after 11 months of Wy-14,643 feeding in contrast to a 100% incidence in the wild-type mouse group [59].

Another transgenic mouse line with human PPARα was generatedthat has the complete human PPARα gene on a P1 phageartificial chromosome (PAC) genomic clone, introduced onto the mouse PPARα-null background [60]. This new line, designated hPPARα^{PAC}, expresses human PPARα not only in liver but also in kidney and heart. hPPARα^{PAC} mice exhibited responses similar to wild-type mice when treated with fenofibrate lowering of serum triglycerides and induction of PPARα target genes' encoding enzymes involved in fatty acid metabolism. Treatment of hPPARα^{PAC} mice with fenofibrate did not cause significant hepatomegaly and hepatocyte proliferation similar to hPPARα^{TetOff} mice, suggesting that the resistance to the hepatocellular proliferation found in the hPPARα^{TetOff} mice is not due to lack of expression of the receptor in tissues other than liver.

Until now, there are no reports concerning the interaction between DEHP and hPPARα^{TetOff} or hPPARα^{PAC}. Recently, we have compared the transactivation of mouse and human PPARα by DEHP treatments using wild-type and hPPARα^{TetOff} mice (unpublished observation). A relatively

high dose of DEHP (5 mmol/kg for 2 weeks) clearly activated PPARα in liver of both genotyped mice, but the activation was very little in hPPARα^{TetOff} mice from the standpoint of the target gene expression as well as triglyceride levels in plasma and liver. Human PPARα response to DEHP may be weak when sufficient human PPARα is expressed in the human liver. Thus, the use of the hPPARα^{TetOff} mouse model is a very valuable means to solve the species differences in the toxicity of peroxisome proliferators. The results from the typical peroxisome proliferator (Wy-14643) may not always be similar to those of DEHP; a study of each case is needed using hPPARα^{TetOff} mouse model.

8. PROPOSED SCIENCE POLICY STATEMENTS

The International Agency for Research on Cancer downgraded the level of potential health risks of DEHP from 2b (possibly carcinogenic to humans) to 3 (not classifiable as to carcinogenicity to humans) in 2000 [61]. In this report, DEHP carcinogenesis via PPARα was considered not to be relevant to humans because peroxisome proliferation had not been documented either in human hepatocyte cultures exposed to DEHP or in the liver of nonhuman primates. This decision has been variously argued by several scientists in the literature [50, 62, 63]. In contrast, the Japan Society for Occupational Health has maintained the 2B class of DEHP carcinogenicity because of the obvious rodent carcinogenicity [64].

Although the US Environmental Protection Agency (EPA) had classified the risk for DEHP carcinogenicity as B2 (probable human carcinogen) in 1993, recently, the expert panel of EPA report has provided the current scientific understanding of the mode(s) of action of PPARα agonist-induced tumors observed in rodent bioassays that are associated with PPARα agonisms: liver tumors in rats and mice as well as Leydig cell and pancreatic acinar cell tumors in rats—all of which represent limited evidence [65]. Since the key events for the mode of action, which have been causally related to liver tumor formation, include the activation of PPARα, perturbation of cell proliferation and apoptosis, selective clonal expansion, and the PPARα-related key events included in the expression of peroxisomal genes (e.g., palmitoyl CoA oxidase and acyl CoA oxidase) and peroxisome proliferation (i.e., an increase in the number and size of peroxisomes) are reliable markers. Additionally, the evidence obtained from the findings that PPARa agonists did not activate the receptor in human cell culture or biopsy samples, and from epidemiological studies, shows that humans are apparently refractory to the effects of a PPARα agonist. However, the EPA maintained the DEHP carcinogenicity criterion.

In 2004, with regard to preclinical and clinical safety assessments for PPAR agonists, the Food and Drug Administration recommended that, due to the prevalence of positive tumor findings of PPAR agonists, two-year carcinogenicity studies on mice and rats are required [66].

Although IARC changed the criterion for DEHP carcinogenicity, other agencies did not because DEHP is a potential rodent carcinogen of liver and the precise mechanism has not been yet understood, though DEHP is a potentially hepatic carcinogen in rodents.

9. CONCLUSIONS

As mentioned above, some studies suggest the possibility of DEHP tumorigenesis via a non-PPARα pathway although DEHP also exerts adverse effects via PPARα-dependent pathway. Since there are species differences regarding expression levels, cellular context, and function of PPARα as well as metabolism enzyme activity of DEHP, it is difficult to extrapolate the results from rodents to humans in terms of risk. Recently, hPPARα mice have been developed, which may help to solve these differences. Re-evaluation of the risk of DEHP carcinogenicity may well be warranted if the previous decisions were based on only PPARα-dependent mechanisms.

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Review Article

PPARγ and MEK Interactions in Cancer

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Peroxisome proliferator-activated receptor-gamma (PPARγ) exerts multiple functions in determination of cell fate, tissue metabolism, and host immunity. Two synthetic PPARγ ligands (rosiglitazone and pioglitazone) were approved for the therapy of type-2 diabetes mellitus and are expected to serve as novel cures for inflammatory diseases and cancer. However, PPARγ and its ligands exhibit a janus-face behaviour as tumor modulators in various systems, resulting in either tumor suppression or tumor promotion. This may be in part due to signaling crosstalk to the mitogen-activated protein kinase (MAPK) cascades. The genomic activity of PPARγ is modulated, in addition to ligand binding, by phosphorylation of a serine residue by MAPKs, such as extracellular signal-regulated protein kinases-1/2 (ERK-1/2), or by nucleocytoplasmic compartmentalization through the ERK activators MAPK kinases-1/2 (MEK-1/2). PPARγ ligands themselves activate the ERK cascade through nongenomic and often PPARγ-independent signaling. In the current review, we discuss the molecular mechanisms and physiological implications of the crosstalk of PPARγ with MEK-ERK signaling and its potential as a novel drug target for cancer therapy in patients.

1. INTRODUCTION

1.1. The janus-face of PPARγ: tumor suppressor versus tumor promoter actions

The metabolic and cell fate regulatory functions of PPARγ place this nuclear receptor (NR) [1, 2] at the cross-road of life style and diabetic comorbidity risks, which are assumed to result from the diet and/or chronic inflammation-induced sequence of preneoplastic lesions towards manifested cancer [3]. Since decades, the association of aberrant insulin signaling in diabetics and increased cancer risk has been stated, and recently validated in patient studies with respect to colon, pancreas, breast, endometrium, prostate, liver, and bladder (see, e.g., [4–7]). Although PPARγ plays an important part in the transmission of insulin responses and physiological diet, little direct evidence exists relating these factors to PPARγ activation and the risks of the development of cancer [6–8]. One of the reasons for the lack of knowledge on the role of PPARγ is that a *bona fide* high-affinity natural ligand(s) for PPARγ has not been identified yet [2].

PPARγ can be activated by low-affinity ligands such as unsaturated long-chain fatty acids derived from nutrient uptake (e.g., linoleic acid) and/or inflammatory reactions (e.g., 15-deoxy-Δ(12,14)-prostaglandin J2) [9, 10]. However, those do not induce the full activity of PPARγ in most systems examined [2]. As of today, modulation of PPARγ activity is mediated by synthetics drugs, and among them the thiazolidinediones (TZDs) rosi- and pioglitazone are considered to be potent and selective PPARγ agonists [2]. These drugs were approved as insulin sensitizers for the treatment of type-2 diabetes mellitus [11] and have been proven helpful in vascular and atherogenic complications [12, 13]. However, TZD drugs can also exert protumorigenic actions in certain rodent models [14, 15]. In addition, the safety of the TZDs has been recently evaluated in clinical studies aimed to examine cancer prevalence in diabetic patients under TZD use [16–18]. One study stated a significant association of cancer risk in women under any TZD treatment (1003 patients) [17], while the other two stated no significant associations (126,971 patients [16]; 87,678 patients [18]). On the other hand, patients with long-term intake of nonsteroidal anti-inflammatory drugs (NSAIDs), cyclooxygenase (COX) inhibitors that prevent endogenous eicosanoid production and may act also as low-affinity PPARγ ligands, were reported to profit from a reduced risk for colon cancer formation [19].

These paradoxical effects resulting from PPARγ activation are derived from a complex balance of anti-*versus* protumor functions of PPARγ protein and its ligands in a given system. The latter are also related to the interaction of PPARγ with other oncomodulating proteins (such as MEK1 and βcatenin). In the current review, we will discuss this janus-faced role of PPARγ and its ligands in cancer with a major focus on its crosstalk with the ERK signaling cascade, which is a central signaling pathway deregulated in a majority of tumor types in humans.

1.2. The ERK cascade and cancer

The MAPK cascades are central signaling pathways that mediate the response of essentially all cellular processes stimulated by extracellular ligand, including proliferation, survival, differentiation, apoptosis, stress response, and even oncogenic transformation. Four main cascades have been identified to date, of which the Ras-Raf-MEK1/MEK2-ERK1/ERK2 cascade (ERK cascade) is the most prominent one in human cancers [20, 21]. Its multilevel organisation of kinases guarantees signal amplification and coherence, and its scaffold proteins [22] organize the pathway into a 3D module that enables crosstalk and direct interactions with other central signaling pathways such as the PPARγs.

Within the MAPK family, the ERK cascade constitutes a major signaling pathway, regulating cell proliferation and survival, as well as cell adhesion and motility, differentiation, embryonal development, and neuronal regulation [21, 23]. Its deregulation, mainly due to constitutive upregulation by receptor kinase "gain of function" mutations, contributes to cancer initiation and progression [24–26]. The majority of human carcinomas harbour increased expression or activating point mutations for the upstream components of the ERK cascade (e.g., epidermal growth factor receptor (EGFR/Her1), Her2/Neu/ErbB2, K-Ras, B-Raf) that culminate in a higher ERK activity in a large majority of human tumors. The ERK cascade currently represents the main targeted cascade (next to the angiogenic vascular endothelial growth factor/receptor (VEGF/R) system) by second-generation low molecular weight (LMW) kinase inhibitors (e.g., gefitinib, erlotinib) and monoclonal (humanized) mAbs directed against members of the EGFR family (e.g., herceptin), which are in clinical use against cancer (as reviewed in [25, 27, 28]). Therefore, inhibitors of the ERK cascade are likely to be beneficial in combating most types of cancer.

2. MECHANISMS OF CROSSTALK BETWEEN PPARγ AND THE ERK CASCADE

The mechanism of action and the regulation of PPARγ have attracted considerable attention over the years. Although this protein was initially shown to act as a transcription factor, studies using synthetic ligands suggested that it may exert its function via activation of signaling as well [1, 2]. According to the current knowledge, PPARγ signaling is mediated by several distinct mechanisms (Figure 1). The best known one is exerted by PPARγ protein itself, which is activated by ligand binding, heterodimerizes with the retinoic X receptor (RXR) and requires NR coregulator recruitment, events that lead to binding and transcriptional activation of PPAR-responsive elements (PPREs) in the DNA [29] (Figure 1(a)). Simultaneous activation of the ERK cascade (e.g., by mitogens) therein contributes to inhibition of this classical genomic action through serine phosphorylation of PPARγ (Figure 1(a)). Another mechanism is that PPARγ interacts with other transcription factors at the DNA level, which leads to PPRE-independent genomic actions of PPARγ protein and its ligands [9, 10] (Figure 1(b)). Activation of the ERK cascade participates in this mechanism by phosphorylation of the latter transcription factors that interact with PPARγ (Figure 1(b)). A third possibility is that nuclear export and cytoplasmic retention of PPARγ by MEK1 [30] results in "off-DNA"-interaction of PPARγ with distinct protein partners (e.g., cytoskeleton, lipid droplets, kinases), leading to alternative cytoplasmic signaling (Figure 1(c)). Finally, PPARγ ligands can function via activation of intracellular signalling (e.g., the ERK cascade) by a PPARγ-independent mechanism, which is derived from exogenous application of ligands that bind to plasma membrane-bound receptors [31] (Figure 1(d)). The latter mode of action can be "nongenomic," that is, involving cytosolic signaling cascades, or "genomic," that is, converging on the DNA by activation of alternative (non-PPAR) transcription factors (Figure 1(d)).

As apparent from the above description, interaction with the ERK cascade plays an important role in the regulation and signal transmission of PPARγ and its ligands. Overall, three main mechanisms of signaling crosstalk between the ERK cascade and PPARγ were described so far as follows: (1) phosphorylation of PPARγ (and its cofactors) by ERKs and other MAPKs (p38, JNK); (2) nongenomic activation of the ERK cascade by PPARγ ligands; and (3) compartmentalization of PPARγ by the ERK cascade component MEK1. Those are described in details in this section.

2.1. The functions of the PPARγ protein and its regulation by ERK phosphorylation

Genetic and pharmacologic studies in cells, rodent models, and human patients corroborated that the PPARγ protein serves as a master regulator of adipocyte and macrophage function in normal and pathophysiological conditions (inflammation, type-2 diabetes, obesity, atherosclerosis) [1]. Its expression in mesenchymal stem cells also associated this receptor with bone, skin, and muscle differentiation [2]. This 50-kDa protein consists of (from N- to C-terminal) the following: a transactivation function-1 (AF1) harbouring an MAPK-phosphorylation motif PXSP, a zinc-finger-type DNA-binding domain (DBD), a hinge region, the ligand-binding domain (LBD), and a flexible AF2 helix. Ligand-binding triggers the formation of the "charge clamp" between the AF2 and the core LBD, an event that enables the release of NR corepressors (NCoRs), heterodimerization with RXR, DNA-binding, NR coactivator (NCoA) recruitment, and transactivation of promoters [29] (Figure 1(a)). The LBD/AF2 interface also constitutes an important docking interface with unusual coregulators such as kinases and cell-cycle regulators (reviewed in [32]).
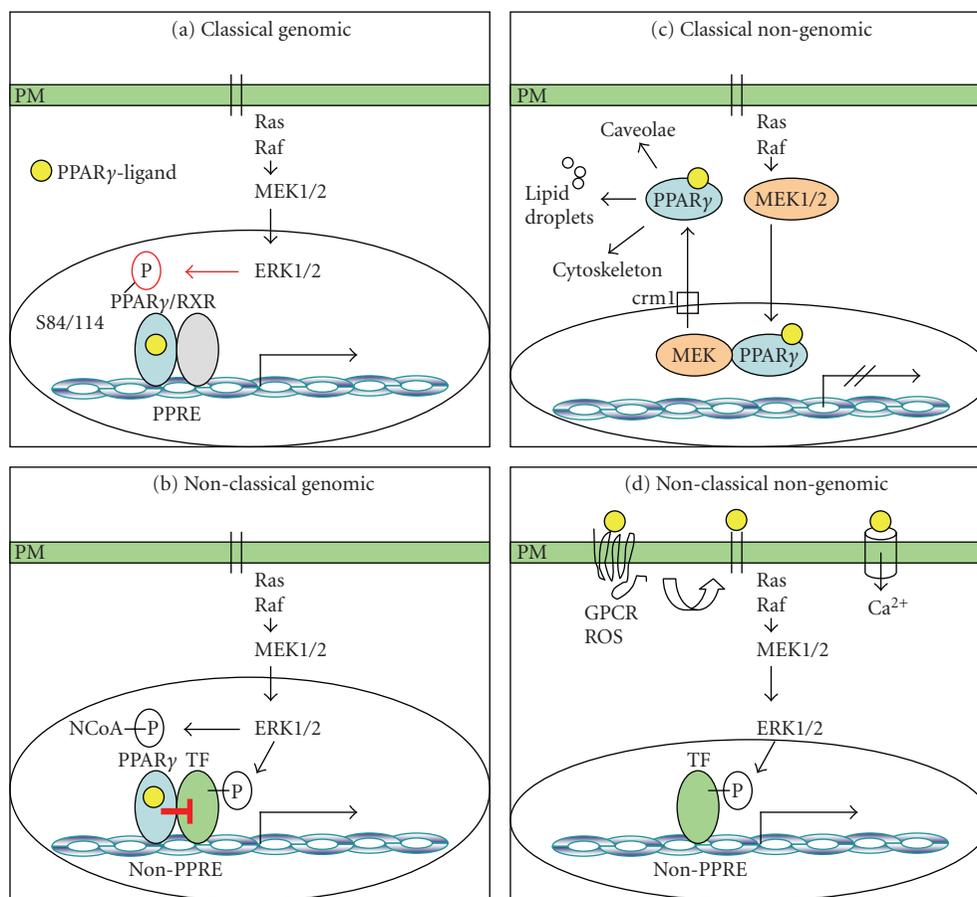
FIGURE 1: *Mechanisms of PPARγ-ERK signaling crosstalk*: (a) serine phosphorylation of PPARγ by the ERK cascade suppresses the classical genomic action of RXR/PPARγ heterodimers on PPREs in the DNA; (b) ERK cascade phosphorylation of promitotic and proinflammatory transcription factors (TF) and NR coactivators (NCoA) modulates their interaction with PPARγ "On-DNA"; (c) nuclear export of PPARγ by MEK1 may result in "Off-DNA" interactions of PPARγ with alternative protein partners in the cytoplasm; (d) PPARγ-independent ERK cascade activation by PPARγ ligands through plasma membrane GPCRs, transactivation of the EGFR (black bars), or calcium signaling.

PPARγ positively regulates the expression of a vast spectrum of target genes involved in immunity and inflammation, differentiation, proliferation, apoptosis, cell survival, and metabolism [10]. However, PPARγ can also repress transcription by negatively interacting with several proinflammatory [9] and promitotic transcription factors [33] such as ETS, STAT, AP1, and NFκB (Figure 1(b)). Thereby, this factor promotes terminal differentiation of various normal and transformed cells of epithelial and mesenchymal origin. PPARγ(−/+) knockout mice exhibit enhanced susceptibility to chemically induced tumorigenesis [34, 35], and this enhanced susceptibility is observed also upon breeding with other strains deficient in tumor suppressors (such as APC) [36]. In patients, PPARγ protein is expressed (in varying levels) in leukemias, lipo- and osteosarcomas and in many carcinomas. Gene polymorphisms within the human population result in several "loss-of-function" PPARγ variants that are associated with metabolic diseases (insulin resistance, lipodystrophy) [37] and cancers (e.g., colon, stomach) [4, 5, 38, 39]. These data initially corroborated PPARγ as a protective transcription factor.

In line with the latter findings, ERK- (and other MAPK-) mediated phosphorylation of PPARγ reduces its genomic activity. A panel of extracellular/environmental promitotic, stress and inflammatory stimuli (growth factors, hormones, cytokines, lipid mediators/eicosanoids, UV-radiation, anisomycin, acetaldehyde, etc.) trigger the activation of the MAPK-family members: ERK, JNK, and p38 (Figure 1(a)). These MAPKs phosphorylate (in humans) Ser 84 in the PPARγ1 and Ser 114 in PPARγ2 isoform, which correspond to Ser 82/112 in mouse and are both located in the AF1 region of the molecules. This phosphorylation results in suppression of the PPARγ's ability to transactivate target gene promoters and thereby its physiological functions (reviewed by [40, 41]). In addition, phosphorylated PPARγ is assumed to be more prone to other posttranslational modifications (sumoylation, ubiquitination) and subsequent degradation by the proteasome, an event that promotes its further down-regulation upon MAPK-activation [42, 43]. But these effects are not fully characterized yet. In any event, the inhibition of PPARγ activity by MAPK phosphorylation is in accordance with the anti-inflammatory and prodifferentiation action

of PPARγ and has been verified for normal (fibroblasts, adipocytes, macrophages, hepatic stellate cells) as well as cancer cell lines, various stimulating agents (as reviewed in [31, 44]) and also in vivo [45, 46]. An additional level of crosstalk is constituted by the fact that PPARγ cofactors, such as steroid receptor coactivator (SRC) family members (e.g., AIB/SRC3 in breast cancer), are phosphorylated by MAPKs and thereby are altered in their ability to coactivate transcription [47] (Figure 1(b)).

The effect of PPARγ phosphorylation by MAPKs was also supported by several in vivo studies. For example, a "knock in" of an unphosphorylable allele S112A in mice preserved their insulin sensitivity in absence of lipogenesis (weight gain) in a setting of diet-induced obesity [45]. In addition, a recent study revealed "downstream of tyrosine kinases-1" (Dok1) as an adapter protein in the insulin-signaling pathway that inhibits S112 phosphorylation of PPARγ2 in vivo [46]. Dok1 knockout mice on high fat remain lean and insulin-sensitive, and Dok1 knockout mouse embryonal fibroblasts (MEFs) show defective adipogenic differentiation, increased ERK activation and phosphorylation of PPARγ2 on S112. Mutation of S112 of PPARγ2 blocked the lean phenotype in Dok1 knockout mice, indicating that Dok1 promotes adipocyte growth and differentiation by counteracting the inhibitory effect of ERK on PPARγ. Another current intriguing example is the identification of parvinβ, a focal adhesion protein (lost in breast cancer patients), that increases the expression, S84 phosphorylation, and activity of PPARγ1 through cyclin-dependent kinase 9 (CDK) and suppressed breast cancer growth in vivo [48]. These data indicate that MAPK-mediated S84/S114 phosphorylation alters the activity of PPARγ1/2 in vitro and in vivo.

In sum, these studies initially corroborated the role of PPARγ as a tumor suppressor [2, 14], which may be shut down by MAPK-phosphorylation [44]. However, more recent evidence was collected, that PPARγ is a context-specific tumor modulator, whose effector profile is complemented and modified by PPARγ-independent effects of its ligands (e.g., TZDs and eicosanoids) and by reciprocal regulation of PPARγ through members of the ERK cascade as follows [31, 40].

2.2. PPARγ ligands influence cellular processes via a nongenomic activation of the ERK cascade

A second mechanism of crosstalk between PPARγ and the ERK cascade comprises the direct activation of ERKs by PPARγ ligands. In the past, ample data was collected on the effects of chemically distinct classes of PPARγ ligands on cells. Different ligands induce either cell growth and proliferation or growth arrest and apoptosis in various human and mouse cancer cell lines and xenografts (as extensively reviewed in [14, 31]), and also modulate angiogenesis in vitro and in vivo [49]. These effects are dose-, time-, and cell type-dependent, and manifest either in a PPARγ receptor-dependent ("genomic") or non-PPARγ receptor-mediated ("nongenomic") manner or in a combination of both. The mechanisms that underlie these context-dependent responses are largely unknown. One concept is

based on the claim that nongenomic PPARγ ligand effects manifest at higher micromolar concentrations ($>10\,\mu$M) well above the low EC50's necessary for classical genomic actions on PPARγ/RXR heterodimers at characterized PPREs in target genes (e.g., 80 nM for rosiglitazone) [50, 51]. This assumption translated into the idea that, low doses of PPARγ ligands, for example, that correspond to the pharmacological doses prescribed for diabetic patients, exert overtly beneficial efficacy, while supra-pharmacological high doses evoke adverse effects. For example, troglitazone was retracted from the market due to hepatotoxicity, which was not a TZD-class effect but due to a drug-specific (possibly "nongenomic") adverse action [2]. However, the literature provides examples for both pro- *and* antitumor actions of PPARγ ligands at similar dose ranges in similar cellular systems. Thus, an underlying principle for the separation of genomic from nongenomic PPARγ ligand effects is currently not available.

The PPARγ ligand effects are likely to be mediated either (i) through so far unknown plasma membrane-bound receptors (Figure 1(d)) or (ii) through cytoplasmatic localized PPARγ protein (Figure 1(c)). Novel G-protein coupled receptors, such as GPR30 for estradiol [52], TGR5 for bile acids [53], and GPR40 for free fatty acids [54], were identified to function as alternative signal transducers for NR-ligands. GPR40, a candidate PPAR ligand receptor, is highly expressed in the pancreas but also in monocytes and in the lower GI tract (e.g., ileum, colon) [55, 56]. Oleate, a natural PPAR ligand, increases proliferation of MCF7 human breast adenocarcinoma through binding and signaling via endogenous GPR40 [57]. TZDs were postulated as bona fide ligands for ectopic GPR40 in CHO cells and to signal via Gαi/q proteins, cAMP, calcium, and ERK activation [58]. However, in vivo proof is lacking. In addition to GPCRs, also plasma membrane-bound classical NRs interact with specific adapter or scaffold proteins in the cytoplasm and trigger the initiation of proproliferative and survival signaling [59]. For example, the estrogen receptor docks to modulator of nongenomic action of estrogen receptor (MNAR) that recruits Src and leads to activation of the p85 subunit of PI3K [60] and the ERK cascade [61]. If this situation is also relevant for PPARγ molecules remains to be shown. Many TZD effects actually target cytoplasmic proteins such as at mitochondria, the proteasome, or the translational machinery. Thus, it is possible that cytoplasmic PPARγ molecules are also involved in the transduction of "nongenomic" TZDs signals.

Downstream of the initial ligand triggering event, nongenomic responses to PPARγ ligands include transient alterations in mitochondrial functions and activation of stress (production of reactive oxygen species (ROS)) as well as kinase signaling pathways promoting proliferation and survival such as PI3K-PKB/AKT, ERK, p38, and JNK [50, 51]. Rapid signaling initiated by ligands can be mediated by membrane proximal events such as cleavage of transmembrane proteinases (ADAMs), activation of GPCRs, EGFR transactivation, calcium influx, and activation of protein tyrosine kinases (Pyk2, Src). Further downstream effects include PPARγ-independent induction of "early response genes" such as c-Fos and Egr-1. In this context, it was shown that PPARγ ligands enhance proliferation,

survival and drug resistance in cancer cells, for example, by induction of the prosurvival and promitotic hormone gastrin [62]. We showed that TZDs enhance drug resistance in human colon adenocarcinoma HT29 cells in a PPRE-independent but EGFR-dependent manner, involving Src/MAPK-signaling [63]. In colon carcinoma cells, TZDs induce matrix metalloproteinase 2 (MMP2) and membrane type 1-MMP (MT1-MMP) activation and concomitantly increase tumor cell invasion through generation of ROS and activation of the ERK cascade [64]. On the other hand, ERK cascade activation by TZDs may also translate into growth inhibition and/or apoptosis [65–69]. It is currently unknown which mechanism governs the decision for pro- versus antiproliferative responses upon TZD application.

In addition to TZD drugs, also the physiological eicosanoid-type ligands for PPARγ exert tumor-modulating effects through their ability to trigger ERK cascade activation [70]. Eicosanoids are generated by cytoplasmic phospholipase A2 and cyclooxygenases (COX1/2). Some of these arachidonic acid metabolites act as endogenous PPARγ ligands ((e.g., 15-deoxy-Δ(12,14)-PGJ2) [71]), while others, like the prostaglandins of the E and D series, activate the ERK cascade through prostanoid GPCRs at the cell membrane [72]. 15-deoxy-Δ(12,14)-PGJ2 directly inhibits inhibitor-κB kinase (IKK) in an intracellular fashion and exerts various effects on inflammation, cell growth, and apoptosis independent of a prostanoid GPCR [71]. For example, in human breast MCF7 adenocarcinoma cells, 15-deoxy-Δ(12,14)-PGJ2 upregulates VEGF synthesis through induction of heme oxygenase-1, an enzyme that stimulates proliferation and angiogenesis, and triggers ERK phosphorylation in an PPARγ-independent fashion [73]. In sum, these data point out to the important role for protumor effects of PPARγ ligands of the TZD- and eicosanoid-class in the activation of ERK cascade-related proliferation and survival pathways, which stand in sharp contrast to the otherwise reported tumor suppressive effects of the latter in similar cellular systems [65–67].

In vivo preclinical and clinical data of TZDs support the concept of an overlapping profile of PPARγ receptor-dependent and independent ligand signaling. In contrast to the lessons from PPARγ(+/−) knockout mice [34, 35] and the antineoplastic action of PPARγ receptor activation in vitro [33], ample in vivo data asserted that many potent and selective PPARγ ligands actually promote tumorigenesis. Thus, PPARγ ligands induce tumor growth in rodent xenograft models [14] and enhance in vivo angiogenesis [49]. In addition, TZDs act as procancerogenic agents in wild-type and APC-deficient mouse models of colon carcinogenesis [74–77]. Importantly, clinical studies in humans failed to show a clear benefit of TZD monotherapy in cancer patients [14, 78, 79]. PPARγ ligands are procarcinogenic in human bladder, as evaluated by the PROactive study [12], and in the rodent bladder [80, 81]. As a reaction towards the safety-toxicological data collected in preclinical studies and clinical trials regarding TZD use, the US Food and Drug Administration (FDA) (http://www.fda.gov/cder/present/DIA2004/15) issued a warning of tumor-related adverse effects of novel

potent PPARγ ligands that are currently in clinical trials as novel antidiabetics or obesity cures (reviewed in [82]) [83, 84]. The FDA classified all PPARγ ligands as multispecies and multiorgan carcinogens requiring strict dose finding for therapeutical use in humans. However, the full molecular mechanism of this interplay between tumor promoting versus suppressing action of PPARγ ligands is so far unknown.

2.3. Towards solving the tumor initiation/suppression paradox of PPARγ: interaction of PPARγ with the ERK cascade in cancer

Unlike the impression that is left by many articles to date, PPARγ protein does not always act as a tumor suppressor, and the PPARγ ligands are not always procancerogenic independently of the receptor. Notably, the PPARγ itself seems to be important for exacerbating mammary gland tumor formation in bitransgenic mice expressing a constitutive active PPARγ form independently of application of an exogenous ligand [85]. An interesting in vitro study corroborated the functional cooperation of the PPARγ receptor and the ERK cascade in the promotion of epithelial-mesenchymal transition (EMT) in the mouse small intestine and rat intestinal epithelial cells, which was dependent on an intact DNA-binding activity of the PPARγ receptor protein [86]. In this system, PPARγ induced ERK1/2 phosphorylation by activating PI3K, Cdc42, and p21-activated kinase (PAK), which in turn phosphorylated S298 of MEK1 that supports its activity [23]. Ectopic expression of dominant negative MEK1 blocked EMT induced by PPARγ, while constitutively active MEK1 overexpression promoted a mesenchymal morphology. However, as evident in the latter intriguing example, the exact molecular mechanisms and physiological relevance of the cooperative interactions between posttranslational regulation of NRs by kinases and rapid nongenomic kinase activation by NR-ligands are so far unknown.

Ample data supports the notion that mutual physical/allosterical associations between kinases and NRs exist that translate into reciprocal regulation of their activities [87, 88]. For example, 3-phosphoinositide-dependent protein kinase-1 (PDK1), that is the upstream activator of AKT/PKB, binds to and activates PPARγ during adipogenic differentiation [89]. Complexes of cyclins and CDKs are cofactors for and phosphorylate PPARγ in adipocytes [90, 91]. PPARγ also interacts with and is activated by ERK5 [92, 93] in order to inhibit (in conjunction with WNT signaling factors) the proliferation of lung cancer (NSCLC) cells and inflammation in endothelial cells upon flow (shear stress), indicative of a protective function of ERK5-PPARγ cooperation. These unusual NR cofactors [32], that also include retinoblastoma protein and transcriptional elongation factors, directly interact with regulatory domains in NRs and considerably add to the pleiotropic effector profile of a given NR. Several interaction partners for PPARγ protein have been identified including prominent oncogenic modulators such as βcatenin [94, 95] and MEK1 [30]. Therefore, it is likely that PPARγ interacts with or cooperates with several signaling pathways and particularly the ERK cascade in order to induce or

prevent oncogenic transformation dependent on the cell type and environment.

2.3.1. Spatial regulation of PPARγ activity: MEKs export PPARγ to the cytoplasm

Next to Ser84/114 phosphorylation and the nongenomic ERK activation by PPARγ ligands, the direct interaction of PPARγ with the ERK cascade component MEK1 constitutes a third mechanism of crosstalk between PPARγ and the ERK cascade. Subcellular compartmentalization is a major mechanism in regulating cellular signaling. Interestingly, PPARγ itself can regulate the membrane translocation of other proteins such as NFκB in gut intestinal epithelial cells [96] and PKC in macrophages [97]. Several reports have demonstrated a signal-mediated translocation of PPARγ between the nucleus and the cytoplasm in vitro (as reviewed in [98]). In addition, it was shown that PPARγ is expressed predominantly in the nucleus of nonneoplastic tissues, whereas it is present in both the nucleus and the cytoplasm of tumorous tissues in squamous cell carcinoma (SCC) of the lung, indicative of a correlation of malignancy with differential PPARγ compartmentalization [99]. Moreover, a dominant negative PPARγ splice variant was described in lung SCC patients, an event that leads to the loss of apoptosis sensitivity in response to oxidative stress and cisplatin [99]. Differential compartmentalization of PPARγ was also described in gastric cancer patients [100]. The ratio of cytoplasmic/nuclear PPARγ expression decreased in the progression of intestinal metaplasia to undifferentiated cancers [100]. In salivary duct carcinoma, an aggressive tumor type, PPARγ is highly expressed (80%) and topographically located in the cytoplasm [101], indicative of an inactivation of its genomic activities in the nucleus. Cytoplasmic PPARγ was also detected in the cytoplasm (58%) of infiltrating breast carcinoma samples and was proposed as an independent prognostic factor for patients with ductal carcinoma [102]. However, the function of this subcellular distribution of PPARγ molecules are yet unknown.

The mechanism that may induce the changes in localization of PPARγ upon stimulation, or upon neoplastic transformation was only recently elucidated by us [30]. We showed that PPARγ is exported from the nucleus to the cytoplasm by MEK1/2. This is induced by a reversible interaction of PPARγ with MEK1 through association of the AF2 of the first with the N-terminal docking domain of MEK1. This export to the cytoplasm (Figure 1(c)) leads to reduction in its genomic function in the nucleus [30]. We also elucidated the molecular mechanisms of the export and the physiological implications, but the question remained is whether cytoplasmatically located PPARγ is subjected to degradation or shunted to alternative signaling compartments such as lipid droplets, ER/Golgi, cytoskeleton, or the plasma membrane. To this regard, we tend to speculate that alternative locations of PPARγ in the cell may determine the balance between tumor-suppressive and tumor-promoting functions.

2.3.2. Tumor-suppressive functions of PPARγ related to ERKs and MEKs interaction

Due to the coexpression of the ubiquitous proteins PPARγ and MEK1/2 in different organs of the body, it was interesting to identify their coregulation in various physiological and pathological processes, as described below.

Differentiation

Due to the lethality of MEK1 knockout mice [103] and absence of phenotypes in MEK2 knockout mice [104], the major focus of interest was directed towards the role of MEK1 overexpression in vivo. Constitutively active MEK1 (S218E/S222E) has been conditionally overexpressed (among other tissues) in the skin and bone of mice [105]. All transgenic mice exhibited increased cell numbers (hyperplasia) and cell size and a defect in terminal differentiation. Interestingly, both in skin and in bone of mice, PPARγ was shown to be an important player promoting differentiation [2]. In addition, the constitutively active MEK1 overexpressing mice show dwarfism and reduced bone size due to defective ossification and impaired chondrocyte differentiation. In other systems, it was shown that osteoclast-specific PPARγ knockout mice are characterized by increased bone mass due to impaired osteoclast differentiation [106], suggesting antagonistic effects of PPARγ and MEK1 on different bone cell types: with PPARγ promoting osteoclast differentiation, and MEK1 inhibiting chondrocyte differentiation.

Skin-restricted MEK1 transgenic mice exhibit hyperproliferation, hyperkeratosis and of age papillomas at sites of wounding [105, 107]. Vice versa, epidermis-specific knockout of MEK1/2 in mice [108] resulted in hypoproliferation, apoptosis, skin barrier defects, and death, indicative of a positive role of MEK1 in skin proliferation and tissue homeostasis. PPARγ knockout mice are characterized by an increased sensitivity to experimentally-induced skin tumors [35], emphasizing the tumor suppressor and differentiation promoting activity of PPARγ in the skin. These "mirror-images" phenotypes in the organs where MEK1/2 and PPARγ are normally coexpressed may give some indication for the antagonistic regulation of the two proteins, MEK promoting proliferation and dedifferentiation, PPARγ promoting terminal differentiation. In line with this idea, it was shown that the kinase activity of MEK1 was actually dispensable for the hyperproliferative and integrin-inducing effects of the MEK1 in mouse skin [109]. Instead, a kinase-dead mutant of MEK1 elicited the same phenotype, indicative of an involvement of other MEK1-functions such as scaffolding inhibition of differentiation-promoting cellular factors.

In adipogenic differentiation systems originating from (mesenchymal) stem cells, synergistic cooperations between the MEK-ERK cascade and PPARγ have been described. In fibroblasts, differentiating towards the adipogenic lineage, a positive cooperation between PPARγ and MEK1 exists that facilitates the adipogenic program by MEK1-dependent induction of the C/EBPα gene [110]. In bone marrow-derived mesenchymal stem cells isolated from normal and streptozotocin (STZ)-induced diabetic FVB/N mice, high

glucose enhanced adipogenesis, lipid accumulation, and PPARγ expression via PI3K/AKT and ERK cascade signaling, events that were all inhibited by the MEK-inhibitor PD98059 [111]. In differentiated C2C12 myocytes, the free fatty acid palmitate reduces the mRNA levels of PPARγ-coactivator-1α (PGC1α) and activated MEK, while the MEK inhibitors PD98059 and U0126 prevented such downregulation of PGC1α, indicative of a MEK-mediated inhibition of an important NR coactivator protein for PPARγ in muscle cells [112]. These findings corroborated that the MEK-ERK cascade and PPARγ signaling pathways can syn- or antagonistically cooperate to control the balance of proliferation and differentiation in an organ/cell type-specific manner.

Cell cycle

The ERK cascade participates in the regulation of cell cycle at (i) G0/G1 and G1/S transitions in response to mitogenic stimulation (as reviewed in [24]) and (ii) in the process of Golgi fragmentation [113–115] during mitosis. This is mediated in part by the nuclear translocation of ERK upon cellular stimulation that promotes expression of "immediate early" genes such as members of the AP1 family that activate the promoters of the G1 cyclins D and E. However, the subcellular compartmentalization of ERK signaling by scaffold proteins (KSR, MP1/p14, Sef) (reviewed in [22]) indicates a novel mode of spatial separation of substrate specifities and signal translation. For example, MP1 via the adapter protein p14 tethers MEK1 to endosomes [116] and focal adhesions [117]. Sef translocates MEK1 to the Golgi apparatus, prevents nuclear translocation of ERKs, and, thereby, favours phosphorylation of cytoplasmic ERK substrates instead of nuclear ones [118]. The latter subcellular localization-determining systems may thus be as well exploited by the PPARγ-MEK1 nuclear export shuttle to regulate the cell cycle.

The PPARγ receptor has been involved in the inhibition of the G0/G1-transition by up-regulation of genes coding for the CDK-inhibitors p18(INK4C) [119] and p21(WAF1/CIP1) [120, 121], and in the inhibition of G1/S transition through upregulation of the p27(KIP1) gene [122, 123]. Upregulation of other genes implicated in cell cycle control such as PTEN or members of the BCL-gene family contributes to the growth-arresting and/or apoptosis-inducing action of PPARγ ligands [15]. The cell cycle modulatory actions of PPARγ are usually not mediated through classical PPRE binding at the DNA but rather through PPRE-independent "off-DNA" crosstalk to other transcription factors [15] and through nongenomic effects in the cytoplasm, such as inhibition of translation initiation [124, 125] and modulation of the proteasomal machinery [126–128]. The latter processes may be mediated by ligand-activated cytoplasmic PPARγ molecules or cytoplasmic alternative signal-transducers for PPARγ ligands. We therefore hypothesize that, by nuclear export and cytoplasmic retention of PPARγ-MEK1 complexes to other MEK1-scaffolding locations (e.g., at the Golgi, endosomes, focal adhesions), the genomic PPARγ functions may as well be redirected in favour of cytoplasmic signaling events. In sum, the cell cycle

modulating effects of PPARγ protein and its ligands may be caused by its differential subcellular compartmentalization by MEK1.

2.3.3. Tumor-promoting functions of PPARγ, related to crosstalk with the ERK cascade

Metastasis

In contrast to the initial assumption of PPARγ mainly acting as a tumor suppressor whose activity and/or expression is lost in cancers, PPARγ expression and activity can also be a negative predictor of cancer aggressiveness; and positive cooperation between PPARγ and components of the ERK cascade in malignant phenotypes takes place. For example, strong nuclear PPARγ expression was detected in thyroid carcinomas compared to normal tissue, and patient samples of thyroid carcinoma-associated lymph node metastasis also showed a higher percentage of PPARγ-positive staining than other case categories [129]. PPARγ expression was also elevated in human prostate cancer compared to normal prostate [130]. In patients with invasive breast carcinoma, cytoplasmic MT1-MMP and MMP9 expression positively correlated with PPARγ levels [131]. These data corroborated a positive relationship between PPARγ expression and malignancy state in certain tumor entities, a fact that was shown to be therapeutically exploitable by the use of PPARγ antagonists or siRNA. This was described in primary esophageal tumor specimen and in esophageal cancer cell lines [132], in human primary squamous cell carcinoma (SCC) and lymph node metastases [133] and in hepatocellular carcinoma (HCC) samples [134], where PPARγ expression is elevated compared to matched normal tissue. In all three cell systems, PPARγ antagonists (T0070907,GW9662) and RNAi-mediated knock-down of PPARγ levels reduced the invasiveness and adherence of cells to the extracellular matrix, triggered anoikis, or inhibited proliferation by decreasing the phosphorylation status of focal adhesion kinase (FAK), MEK, and ERK. Therefore, in tumors where elevated PPARγ and activated ERK and MEK levels contribute to the malignant phenotype, inhibition of PPARγ may be beneficial as a therapeutic strategy (see also Section 3).

Angiogenesis

The overall vascular protective and antiatherogenic effects of PPARγ ligands provide essential add-ons for the clinical application as insulin sensitizers (reviewed in [13]). However, the proangiogenic effects of PPARγ ligands via modulation of the VEGF/VEGF-receptor system (that signals via the ERK cascade) have gained recognition (reviewed by [49]), which may be beneficial for therapy of vascular diseases (e.g., infarction) [135, 136] but detrimental in cancer tissue. For example, in rat myofibroblasts, rosiglitazone and 15-deoxy-Δ(12,14)-PGJ2 induce expression of VEGF and its receptors (Flt1 and KDR, that signal via the ERK cascade), and augment tubule formation on a matrigel, indicative of a promoting function of PPARγ and

ERKs in angiogenesis [137]. In osteoblast-like MC3T3E1 cells, pioglitazone and ciglitazone augmented FGF2-induced VEGF release in a PPARγ-dependent manner and enhanced the phosphorylation of JNK [138]. In human RT4 bladder cancer cells, VEGF mRNA and protein are upregulated by PPARγ via activation of the VEGF promoter. Interestingly, the MEK inhibitor PD98059 reduced PPARγ ligand-induced expression of VEGF [139], indicative of a positive cooperation of PPARγ-ERK pathways in angiogenesis. These positive effects on angiogenesis were examined also in two clinical studies with rosiglitazone [140] and pioglitazone [136], in which it was demonstrated that chronic addition of the TZDs increased endothelial cell precursor counts and migration in diabetic patients, raising concern on the proangiogenic potential of TZDs.

Taken together, the data which revealed an antagonistic cooperation of PPARγ and ERK signaling in several cell or tissue-specific differentiation systems (skin, bone, muscle, fat) is now challenged by the findings of positive cooperation of the same components in tumor progression (metastasis, angiogenesis). Thus, the role of PPARγ as a MEK/ERK-regulated tumor suppressor seems to be of importance in normal tissue or in prevention of tumor initiation, while in advanced stages of certain tumors a synergistic cooperation between PPARγ and the ERK cascade may contribute to the malignancy of the disease. Future studies have to clarify whether PPARγ agonists, PPARγ antagonists, or PPARγ modulators/partial agonists (SPPARMs) with a selective effector profile [141] may be of interest for the therapy of certain tumor entities.

3. CLINICAL USE OF PPARγ INTERACTION WITH THE ERK CASCADE AS A DRUG TARGET

Reactivation ("differentiation") therapy targeting functional PPARγ protein in cancer cells/tissues by exogenous application of TZD-class PPARγ ligands was lately expected to represent a novel approach to fight cancer [142]. However, differentiation-inducing monotherapy with TZDs did not show the expected clinical benefit [11]. Instead, evidence accumulated that alternative ("nongenomic") PPARγ signaling pathways, crosstalk with the ERK cascade and elevated PPARγ expression levels in certain tumor types (where PPARγ is postulated to act as a prosurvival factor, e.g., in hepatocellular carcinoma, squamous cell carcinoma), are the cause for the observed tumor promoting effects of PPARγ ligands, and may explain the absence of clear therapeutical benefit of TZDs in cancer patients [78, 79, 143]. Therefore combination therapy of PPARγ ligands with kinase inhibitors may represent a novel strategy to circumvent the crosstalk of PPARγ and ERK cascade signaling and limit PPARγ protein activation to its classical differentiation-inducing feature (Figure 2). This dual approach is expected to avoid (a) ERK cascade-mediated downregulation of PPARγ, (b) MEK-driven nuclear export and cytoplasmic retention of PPARγ and (c) nongenomic amplification loops of PPARγ ligands towards the ERK cascade, but to promote (d) the growth-arresting and proapoptotic genomic functions of PPARγ and its ligands, and (e) the negative crosstalk of PPARγ

with promitotic and proinflammatory transcription factors in the nucleus. This concept may not be suitable for tumor types with elevated "malignant" PPARγ expression/activities. However, due to the lack of clinically approved PPARγ antagonists, no statement can be currently made on the potential therapeutical benefit of PPARγ and kinase coinhibition.

3.1. In vitro studies

The combination of PPARγ ligands and inhibitors against receptor tyrosine kinases of the EGFR-family or cytoplasmic tyrosine kinases (e.g., Abl) revealed some promising results in leukemia and carcinoma cells. Gefitinib, an inhibitor of the EGFR/Her1 kinase, exhibits antitumor activity in only a fraction of 10–20% of patients with nonsmall cell lung cancer (NSCLC) [144]. The mechanisms underlying this resistance to gefitinib are not known. However, application of rosiglitazone reduced the growth of the NSCLC A549 cells and potentiated the antiproliferative effects of gefitinib and increased PPARγ and PTEN expression in these cells, indicative of a potential benefit of this drug combination also in cancer patients. MCF7 breast cancer cells stably transfected with ErbB2/Her2 displayed reduced differentiation and enhanced resistance to TZD-driven inhibition of anchorage-independent growth [145]. Herceptin, a monoclonal antibody against Her2 kinase, sensitized cells for the differentiation-promoting and growth-inhibitory effects of troglitazone. This concept also held true for chronic myeloid leukemia (CML) cell lines, where TZD18 (a dual PPARα/γ ligand) enhanced CDK-inhibitor p27(KIP1) expression and inhibited cyclin E, cyclin D2 and CDK2 [122]. TZD18 synergistically enhanced the antiproliferative and proapoptotic effect of imatinib, a clinically used kinase inhibitor of the Bcr-Abl fusion protein. Collectively, this work demonstrated that the targeting of receptor tyrosine kinase signaling with LMW inhibitors or monoclonal antibodies can improve the sensitivity of cancer cells to PPARγ ligand-mediated growth inhibition.

3.2. In vivo rodent and clinical studies

The clinical outcome of selective MEK inhibitors in patients studies was disappointing (CI-1040, PD0325901, AZD-6244) (reviewed in [146, 147]). On the other hand, a Raf inhibitor, sorafenib, was recently approved for clinical use; and novel selective Raf inhibitors are under development [148]. So far no clinical studies were performed using MEK or Raf inhibitors in combination with PPARγ ligands. However, successful treatment data in mouse models or patients are available for combinations of PPARγ ligands and three other types of inhibitory drugs: classical chemotherapeutics, COX-inhibitors (NSAIDs), and established tyrosine kinase inhibitors (imatinib, gefitinib, herceptin).

NSAID/COX-inhibitors have been shown to reduce the risk for colon carcinoma formation, however at the expense of gastric ulcer and cardiovascular complications [19]. Several NSAIDs are also low-affinity PPARγ ligands, a fact that led to the speculation that a part of the clinical profile of these compounds is related to low-level
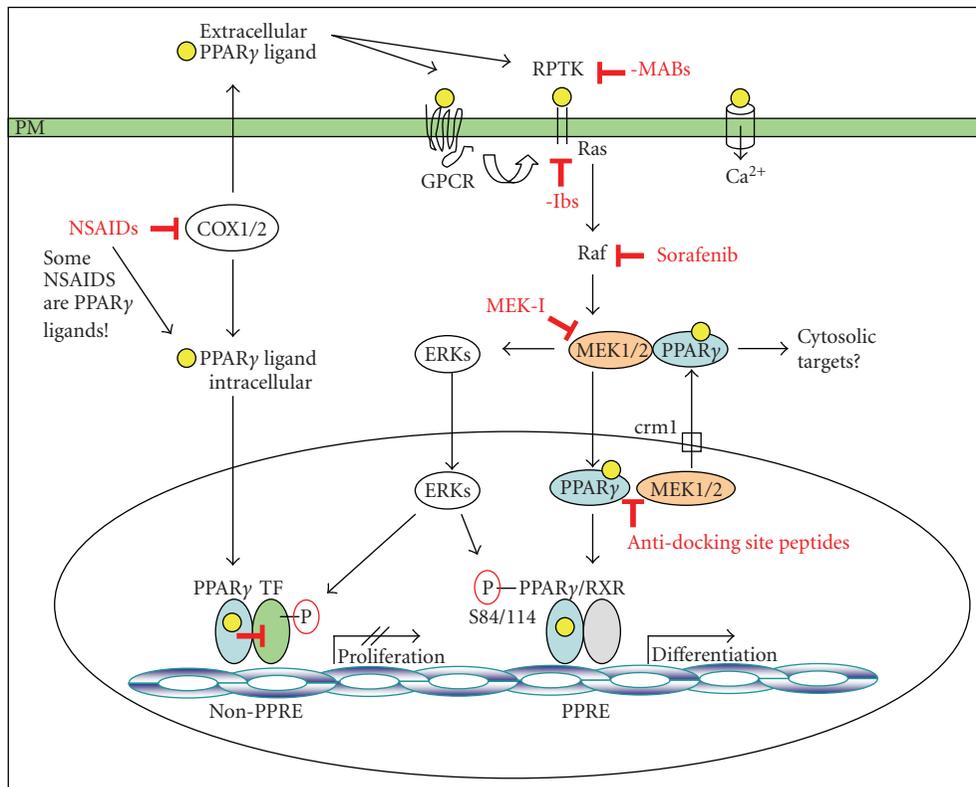
FIGURE 2: *Model of the combination therapy using PPARγ ligand and ERK cascade inhibitors.* The simultaneous inhibition of EGF receptor-initiated ERK cascade activation by specific kinase inhibitors (-ibs) or antibodies (-MABs) and supply of PPARγ ligands (in tumors that have a need for restored PPARγ activity) will avoid: (a) ERK-mediated downregulation of PPARγ through Ser84/114 phosphorylation, (b) MEK1-driven nuclear export and cytoplasmic retention of PPARγ, (c) activation of prosurvival and proproliferative ERK cascade signaling by exogenous PPARγ ligands (e.g., by TZD drugs) or endogenous eicosanoid type of PPARγ ligands (e.g., generated by COX1/2), but is expected to (d) restore the differentiation-inducing and proapoptotic functions of PPARγ and its ligands, and (e) promote the transrepressive activity of PPARγ on other promitotic and proinflammatory transcription factors (e.g., AP1, ETS, STAT, NFκB). *Legend*: Yellow circles = PPARγ-ligand; TF = transcription factors; ROS = reactive oxygen species; GPCR = G protein coupled receptor; RPTK = receptor protein tyrosine kinase; crm1 = exportin1; NSAID = nonsteroidal anti-inflammatory drug; COX = cyclooxygenase; -Ibs = LMW tyrosine kinase inhibitors; MABs = monoclonal tyrosine kinase antibodies.

activation of PPARγ [19]. Therefore, clinical trials with combination therapies were initiated to exploit PPARγ activation and simultaneous blockage of the promitotic and proinflammatory COX1/2-mediated eicosanoid production, which contributes to nongenomic signaling in cancer tissues (**Figure 2**). Pilot clinical studies with an angiostatic triple combination of pioglitazone, rofecoxib (a selective COX2 inhibitor), and trofosfamide showed benefit in patients with angiosarcoma and hemangioendothelioma [151, 152] and advanced sarcoma [153]. A phase-II trial with the same triple combination in patients with metastatic melanoma or soft-tissue sarcoma evinced disease stabilization [152], indicative of a beneficial effect of COX2 inhibition (whose eicosanoid metabolites activate the ERK cascade) and simultaneous PPARγ activation in sensitization of tumor cells to differentiation and/or apoptosis. A recently published outcome of a phase-II trial in high-grade glioma patients (glioblastoma or anaplastic glioma) under pioglitazone and rofecoxib combined with chemotherapy (capecitabine or

temozolomide) also stated some disease stabilization [157]. However, due to the severe side effects of selective COX2-inhibitors this therapeutic regimen may raise concerns.

Preclinical studies in rodents provided evidence for a therapeutic potential of combination therapy with other inhibitory agents. In mice xenografted with NSCLC A549 cells, the PI3K inhibitor PX-866 potentiated the antitumor activity of gefitinib [149]. The glucose intolerance related to PX-866 in mice was reversed by insulin and pioglitazone. PX-866 in combination with insulin sensitizers may thus be useful in facilitating the response to EGFR inhibition. The antitumoral action of rosiglitazone on experimentally induced mammary tumors induced by N-nitroso-N-methylurea (NMU) in Sprague-Dawley rats was potentiated by the selective estrogen-receptor modulator (SERM) tamoxifen with respect to the extent of tumor cell apoptosis and necrosis [150]. The PPARγ ligand RS5444 in combination with paclitaxel had additive antiproliferative effect in vitro and minimized tumor growth in nude mice xenografts

TABLE 1: Combination therapy with PPARγ ligands.

Cancer type	PPARγ ligand	Combination	Inhibitor type	Reference
In vitro				
CML	TZD18	Imatinib	Abl, other RPTKs	[122]
NSCLC A549	Rosiglitazone	Gefitinib	EGFR/Her1	[144]
Breast MCF7	Troglitazone	Herceptin	Mab-Her2/ErbB2	[145]
In vivo (human xenografts or chemically-induced tumors in rodents)				
NSCLC A549	Pioglitazone	PX-866 Gefitinib	PI3K-p110α Her1/EGFR	[149]
Breast (by NMU)	Rosiglitazone	Tamoxifen	SERM	[150]
Thyroid ATC	RS5444	Paclitaxel	Chemotherapeutic	[120]
Clinical studies				
Melanoma Sarcoma	Pioglitazone	Rofecoxib Trofosfamide	COX2 Chemotherapeutic	[151–153]
Advanced Solid tumors	LY293111	Irinotecan Gemcitabine	Chemotherapeutic Chemotherapeutic	[154–156]
Glioblastoma Anaplastic Glioma	Pioglitazone	Rofecoxib Capecitabine Temozolomide	COX2 Chemotherapeutic Chemotherapeutic	[157]

of anaplastic thyroid carcinoma (ATC) cells [120]. These preclinical studies underline that the combination of PPARγ ligands and established anticancer drugs may be of clinical benefit also in cancer patients.

Interestingly, two studies provided already first-line evidence for the potential of an in vivo reactivation of PPARγ protein function by simultaneous inhibition of the COX pathway-mediated activation of the ERK cascade: LY293111, an oral PPARγ ligand, leukotriene B4 receptor antagonist and 5-lipoxygenase inhibitor, was validated for its antineoplastic efficacy in combination with chemotherapy (irinotecan, gemcitabine) in preclinical models [154] and evoked disease stabilization in patients with advanced solid tumors [155, 156]. The NSAID R-etodolac inhibits growth of prostate cancer (CWRSA6, LuCaP35) xenografts in mice by downregulation cyclin D1. However, the combination of R-etodolac with herceptin elicited an additive antitumor effect, reduced ERK phosphorylation and stabilized PPARγ protein levels [158]. These therapeutic regimens inhibited the eicosanoid-mediated activation of the ERK cascade, and in conjunction with PPARγ activation, may provide a basis for differentiation-inducing therapy in combination with classical chemotherapeutics or biologicals.

So far no clinical evidence was published on the combined use of ERK cascade inhibition and PPARγ activation (in tumors with low PPARγ expression/activity) or PPARγ inhibition (in tumors with high PPARγ expression/activity). In the future, the combination of PPARγ ligands with kinase inhibition selectively targeted by MABs against the EGFR tyrosine receptor kinase family or LMW selective inhibitors

of the downstream ERK cascade, such as Raf and MEK, may constitute a possible new approach to treat cancer.

4. CONCLUSION AND PERSPECTIVES

In conclusion, PPARγ emerges as a tumor-type and tumor-stage-specific modulator that is regulated by at least three mechanisms through the ERK cascade. Downregulation is carried out through (1) MAPK-mediated Ser84/114 phosphorylation, (2) ERK cascade activation through PPARγ ligands, and (3) cooperation of PPARγ with tumor modulating proteins (such as MEK1). The overlay of these 3 mechanisms of crosstalk is likely to determine the physiological outcome of PPARγ effector functions. Consequently, interference with these interactions by LMW inhibitors, antibodies, or peptidomimetic drugs against protein docking interfaces may constitute a novel approach to redirect PPARγ effector functions from a protumorigenic towards an antitumorigenic profile. Simultaneous inhibition of ERK cascade-mediated signaling is expected to prevent adverse promitotic and prosurvival pathways triggered by PPARγ and its ligands. This therapeutic approach is assumed to be reasonable in tumors where the tumor-suppressor activities of PPARγ are lost/reduced/dysfunctional and should be restored. However, it may not be applicable for tumors where high PPARγ expression/activity levels positively correlate with the state of malignancy. Since no PPARγ antagonist or PPARγ modulator is in clinical use so far, future studies have to evaluate whether (depending on the tumor type and stage) the combination of the latter drugs with kinase inhibitors

may be of therapeutic benefit in tumor entities with high PPARγ expression.

ABBREVIATIONS

AF:	Activation function
DBD:	DNA binding domain
COX:	Cyclooxygenase
ERK:	Extracellular signal-regulated kinase
LBD:	Ligand binding domain
MAPK:	Mitogen-activated protein kinase
MEK:	MAPK/ERK kinase
NSAID:	Nonsteroidal anti-inflammatory drug
NCoA:	NR coactivator
NCoR:	NR corepressor
NR:	Nuclear receptor
PPAR:	Peroxisome proliferator-activated receptor
PPRE:	PPAR responsive element
RXR:	Retinoid X receptor
ERK cascade:	Ras-Raf-MEK1/2-ERK1/2 cascade.

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Review Article

NO-Donating NSAIDs, PPARδ, and Cancer: Does PPARδ Contribute to Colon Carcinogenesis?

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The chemopreventive NO-donating NSAIDs (NO-NSAIDs; NSAIDs with an NO-releasing moiety) modulate PPARδ and offer the opportunity to revisit the controversial role of PPARδ in carcinogenesis (several papers report that PPARδ either promotes or inhibits cancer). This review summarizes the pharmacology of NO-NSAIDs, PPARδ cancer biology, and the relationship between the two. In particular, a study of the chemopreventive effect of two isomers of NO-aspirin on intestinal neoplasia in *Min* mice showed that, compared to wild-type controls, PPARδ is overexpressed in the intestinal mucosa of *Min* mice; PPARδ responds to *m*- and *p*-NO-ASA proportionally to their antitumor effect (p- > m-). This effect is accompanied by the induction of epithelial cell death, which correlates with the antineoplastic effect of NO-aspirin; and NO-aspirin's effect on PPARδ is specific (no changes in PPARα or PPARγ). Although these data support the notion that PPARδ promotes intestinal carcinogenesis and its inhibition could be therapeutically useful, more work is needed before a firm conclusion is reached.

1. INTRODUCTION

Cancer represents a major health challenge of our time. In the last decade, biomedical science has pursued with unusual vigor the molecular understanding of cancer. Cell signaling cascades have, in particular, been examined or even recognized in the context of cancer research. The implicit assumption (as well as the expectation) has been that understanding the mechanisms of carcinogenesis will facilitate the development of rational, mechanism-driven interventions for either the treatment or even better the prevention of cancer. The ultimate "deliverable" of such systematic efforts will be successful cancer therapeutic or preventive agents.

As is, however, sometimes the case in science, mechanistic progress can also be made while trying to understand the mode of action of agents already developed. Such appears to be the case with the opportunity that presented itself while we were exploring the mode of action of a novel chemopreventive agent, nitric oxide-donating aspirin (NO-ASA), and its relationship to peroxisome proliferator-activated receptor δ (PPARδ). Here, we discuss our findings, and to provide an appropriate perspective, we summarize relevant aspects of the pharmacology of nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs), PPARδ cancer biology, and the relationship between the two.

2. NO-NSAIDs AND CANCER

NO-ASA, initially intended for rheumatologic and cardiovascular applications [1, 2], is a member of a large family of pharmacologically active compounds known as NO-donating NSAIDs (NO-NSAIDs). NO-NSAIDs consist of a conventional NSAID to which the NO-releasing moiety–ONO$_2$ has been attached via a chemical linker [3, Figure 1]. In the case of NO-ASA, the spacer can vary in its chemical structure, generating a great number of derivatives. There are three positional isomers of the NO-ASA molecule (*ortho*, *meta*, and *para*), generated by varying the position of the –CH$_2$ONO$_2$ group with respect to the ester bond linking the two benzenes [4]. NO-ASA is the best studied NO-NSAID to date.
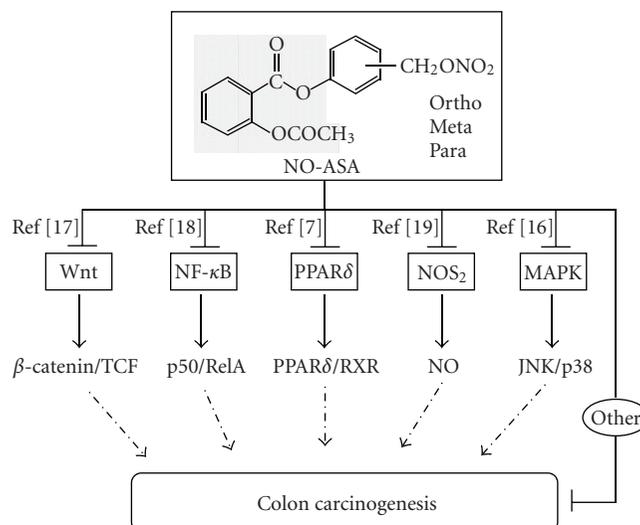
FIGURE 1: Effect of NO-ASA on PPARδ and other signaling pathways. NO-ASA consists of a traditional ASA molecule (shaded), the spacer, and –ONO$_2$, which releases NO, with the molecule being considered responsible for much of its pharmacological properties. There are three positional isomers of NO-ASA (*ortho*, *meta*, and *para*), depending on the position of –ONO$_2$ in the benzene ring with respect to the ester bond linking the ASA and spacer moieties. NO-ASA affects several cell signaling pathways, all relevant to carcinogenesis. The modulation of these often cross-talking pathways culminates in a net inhibitory effect on cell growth, one of the crucial determinants of the fate of a tumor. It is likely that such mechanistic pleiotropism by NO-ASA is central to its efficacy against cancer.

The impetus for the development of NO-NSAIDs for cancer prevention has been provided by extensive epidemiological data and interventional studies which over fifteen years have established conclusively conventional NSAIDs as chemopreventive agents against colon and other cancers [5, 6]. The limited efficacy (less than 50%) and side effects that accompany NSAIDs have prompted the search for better performing agents. NO-NSAIDs, especially NO-ASA, promise to be such an alternative, and their anticancer properties are now under intense study by our group and others. Even though significant progress has been made, the mechanism by which NO-ASA exerts its chemopreventive effect against colon cancer is still not completely understood [2]. Our data indicate that NO-ASA could exert its colon chemopreventive effect, at least in part, by modulating PPARδ function [7].

Extensive preclinical results have established that NO-ASA, which is now FDA approved for clinical trials, displays properties consistent with a chemopreventive effect [8]. These findings can be grouped into those documenting a favorablein vitro cytokinetic effect and those demonstrating chemopreventive efficacy in animal models of cancer. Compared to their corresponding parent compounds, several NO-NSAIDs (including NO-ASA, NO-sulindac and NO-ibuprofen, NO-salicylic acid, NO-indomethacin, and NO-flurbiprofen) have greater potency in inhibiting the growth of cancer cell lines, for example, colon, prostate, lung, pancreas, tonsil, breast cancer, and leukemia [9–12]. For example, in the case of colon cancer cell lines, the IC$_{50}$ values of NO-NSAIDs were enhanced between 1.7- and 1083-fold. The growth-inhibitory effect of NO-NSAIDs is due to a profound cytokinetic effect, consisting of reduced cell proliferation, enhanced cell death, and inhibition of cell-

cycle-phase transitions. Beyond classical apoptosis, NO-ASA induced another form of cell death, termed as atypical cell death [4]. Likely, a form of cell necrosis, atypical cell death, was initially described in vitro, but may actually occur in vivo [7].

The in vivo studies used orthotopic animal models of cancer as well as xenotransplants of human cancer cell lines in appropriate murine hosts. For colon cancer, the results from the various models are congruent and demonstrate a clear-cut chemopreventive effect. In *Min* mice, 3 weeks of treatment with NO-ASA decreased the number of tumors by 55% [13]. In F344 rats treated with the carcinogen azoxymethane, NO-indomethacin and *meta* NO-ASA significantly suppressed both tumor incidence and multiplicity (NO-indomethacin was more effective than NO-ASA). Of the two NO-ASA isomers, the *para* was more efficacious than the *meta* in *Min* mice [4]. When combined with 5-fluorouracil or oxaliplatin, *para* NO-ASA showed additive effects [14]. Sequential NO-ASA and oxaliplatin treatment reduced tumor growth more effectively than single-drug treatments, perhaps by sensitizing colon cancer cells to the effect of antitumor drugs. Studies using a hamster model of pancreatic cancer generated impressive results [15]. Compared with the control group, NO-ASA reduced the incidence and multiplicity of pancreatic cancer by 88.9% and 94%, respectively, whereas conventional ASA had no significant effect.

An exciting aspect of NO-ASA is its extraordinarily enhanced potency. We and others have attempted to understand this through studies assessing their effects on potentially informative pathways (summarized in [8]). It appears that NO-ASA has a pleiotropic effect involving several pathways, as depicted in Figure 1. PPARδ is a significant

component of this array of signaling molecules [7, 16–19]. Below, after an overview of the role of PPARδ in cancer, we discuss the relationship between NO-ASA and PPARδ.

3. PPARδ AND CANCER

PPARs, having their first member cloned in 1990 [20], are ligand-activated transcription factors belonging to the super-family of nuclear receptors. They facilitate the response of cells to extracellular stimuli by transcriptionally regulating gene expression [21, 22]. Three distinct PPAR subclasses have been identified: PPARα, PPARδ (also referred to as PPARβ/δ), and PPARγ. These isoforms are encoded by separate genes and differ in their tissue distribution and function. PPARδ is the more ubiquitously expressed isoform. Each of the PPAR isoforms heterodimerizes with the 9-cis-retinoic receptor, their obligate partner. PPARs regulate diverse physiological processes ranging from lipogenesis to inflammation, and have been implicated in several disorders including the metabolic syndrome, diabetes, and atherosclerosis, as well as cancer. More recently, PPARγ was shown to play a significant role in cell growth, inflammation, apoptosis, and angiogenesis [23–27].

The study of PPARδ lags behind our fairly advanced understanding of PPARα and PPARγ; the development of high-affinity PPARδ agonists has recently expedited progress [28, 29]. PGI$_2$ and cPGI are naturally occurring PPARδ agonists [30]. PPARδ is involved in a wide range of phenomena affecting several functions, and some of them are critical to the life of an organism. PPARδ stimulates fatty acid oxidation in heart and skeletal muscle [31, 32], and plays a role in cell differentiation [33–35], placental development [36], cancer, wound repair [37], and atherosclerosis [38–41]. PPARδ-null mouse models revealed that PPARδ deficiency is associated with multiple developmental and metabolic abnormalities, including frequent embryonic lethality [36].

4. THE PROS AND CONS FOR A ROLE OF PPARδ IN CANCER

There have been both significant work on and significant excitement about a potential role of PPARδ in cancer. As with any evolving field, some controversy is almost inevitable. This controversy arises mainly from the varying results from animal studies (summarized in [42]). Available data can be divided into two: those which support the notion that PPARδ plays a crucial role in carcinogenesis, and those which indicate that PPARδ is devoid of any such role. Below, we present the main points supporting each one of these antithetic conclusions (Table 1).

4.1. Pros

PPARδ was ascribed as an oncogenic function after being identified as a direct transcriptional target of β-catenin, and as a repression target of the NSAID sulindac, a potent suppressor of colorectal tumors [44]. A close association between PPARδ and colon carcinogenesis was suggested by immunohistochemical analyses showing that the expression of PPARδ increases progressively as the colonic epithelium advances from normal to malignant [45].

A series of observations in *Min* mice support a procarcinogenic role of PPARδ. When *Min* mice were treated with azoxymethane, PPARδ levels were increased in flat dysplastic aberrant crypt foci [65], although the same authors indicate that PPARδ expression in adenomas from *Min* mice does not differ compared to normal epithelium [65]. Deletion of PPARδ decreased intestinal adenoma growth and inhibited the tumor-promoting effects of a PPARδ agonist [51]. Interestingly, the same group also showed that prostaglandin E$_2$ (PGE$_2$), the predominant prostanoid found in most colorectal cancers, indirectly transactivates PPARδ promoting cell survival and intestinal adenoma formation [53]. PGE$_2$ treatment did not increase intestinal adenoma burden in *Min* mice lacking PPARδ, concluding that PPARδ is a focal point of cross-talk between the prostaglandin and Wnt signaling pathways, which results in a shift from cell death to cell survival, leading to increased tumor growth [53]. Treatment of *Min* mice with a synthetic agonist of PPARδ increased significantly the number and size of intestinal polyps. The most prominent effect was on polyp size; the PPARδ activator increased the number of polyps by >2 mm five-fold [47]. The same group also showed that compared with control $Apc^{\text{Min}/+}$ mice ($Ppard^{+/+}/Apc^{\text{Min}/+}$), small intestinal polyps in PPARδ-deficient $Apc^{\text{Min}/+}$ mice ($Ppard^{-/-}/Apc^{\text{Min}/+}$) were reduced three-fold; the number of large polyps (>1 mm) was reduced about ten-fold. Heterozygous deletion of PPARδ ($Ppard^{+/-}/Apc^{\text{Min}/+}$) did not significantly reduce the total number of small and large intestinal polyps in malemice, but this disruption significantly diminished the number of small intestinal polyps that were >1 mm [51].

In cultured colon cancer cells, PPARδ inhibited differentiation, conferred apoptotic resistance, and promoted cell migration [43], whereas prostacyclin, a metabolic product of COX-2 which modulates intestinal tumorigenesis [66], increased PPARδ activity [43]. PPARδ expression was elevated in colon cancer cells and was repressed by *apc* via the β-catenin/TCF-4 response elements in its promoter [44]. Genetic disruption of *Ppard* decreased the tumorigenicity of human coloncancer cells [46]. HCT116 $Ppard^{-/-}$ cells, inoculated as xenografts onto nude mice, exhibited decreased ability to form tumors compared to $Ppard^{+/-}$ and wild-type controls [46]. Dietary fish oil/pectin protected rats against radiation-enhanced colon cancer by upregulating apoptosis in colonic mucosa, in part, by suppressing PPARδ [48].

Data from noncolonic cell lines and tissues also support a role for PPARδ in cancer. Activation of PPARδ results in increased growth in sex hormone-responsive breast (T47D, MCF7) and prostate (LNCaP, PNT1A) cell lines [52]. Epithelial ovarian cancer cells express high levels of PPARδ, and inhibition of PPARδ reduced tumor growth [50]. In epithelial ovarian cancer cells, aspirin suppressed PPARδ function and cell growth by inhibiting ERK1/2 [50]. Activation of PPARδ by its pharmacologic ligand GW501516 enhanced the growth of human hepatoma cell lines, whereas PPARδ knockdown by siRNA prevented cell

TABLE 1: The *pros* and *cons* for a role of PPARδ in cancer.

Evidence	Reference
Pros	
PPARδ expression is enhanced in colon cancer cells	[43]
PPARδ expression is repressed by the APC gene	[44]
PPARδ expression increases as tumor progresses	[45]
PPARδ genetic disruption decreases tumorigenicity of colorectal cancer cells	[46]
PPARδ activation accelerates intestinal adenoma growth in *Min* mice	[47]
$Ppard^{-/-}$ HCT116 cells exhibit decreased ability to form xenograft tumors	[46]
Dietary fish oil/pectin protects against radiation-enhanced colon cancer by upregulating apoptosis, in part, through PPARδ suppression	[48]
PPARδ expression levels are correlated with advanced pathological tumor stage in tumor patients	[49]
PPARδ-targeted removal of a hub node of the angiogenic network markedly impairs angiogenesis and tumor growth in mice	[49]
Inhibition of PPARδ function reduces growth of epithelial ovarian cancer	[50]
Activation of PPARδ upregulates VEGF in colon cancer cells	[51]
PPARδ activation stimulates the proliferation of human breast and prostate cancer cell lines	[52]
PGE$_2$ indirectly transactivates PPARδ promoting cell survival and intestinal adenoma formation	[53]
Cons	
PPARδ-null *Min* mice exhibit increased predisposition to intestinal tumorigenesis	[54]
PPARδ-deficient mice show higher polyp formation	[55]
PPARδ agonists do not increase cell growth in human cancer cell lines	[56]
PPARδ is dispensable for polyp formation in the intestine and colon of *Min* mice	[36]
RNA interference against *Ppard* promotes proliferation of HCT116 cells	[57]
Lung tumorigenesis is attenuated in mice with disrupted *Ppard*	[58]
PPARδ does not modify impaired mismatch repair-induced neoplasia	[59]
PPARδ promotes differentiation, inhibiting cell proliferation in keratinocytes	[60]
PPARδ ligands inhibit TNFα-induced expression of the vascular cell adhesion molecule-1 and E-selectin in HUVEC, preventing inflammation	[61]
Inhibition of colon carcinogenesis by a PPARδ agonist in an azoxymethane mouse model	[62]
PPARδ activators inhibit TNFα-induced endothelial inflammation, in part by interfering with the NF-κB signaling pathway	[63]
PPARδ activation by a PPARδ agonist produces no change in colon cancer cell growth	[52]
PPARδ activation by GW0742 inhibits colon polyp multiplicity in $Ppard^{+/+}$ mice, but not in $Ppard^{-/-}$ mice	[64]

growth [67]. In murine knockout experiments, targeted removal of a hub node (PPARδ) of the angiogenic network markedly impaired angiogenesis and tumor growth [49]. In human cholangiocarcinoma, a positive feedback loop between PPARδ and PGE$_2$ was recognized; this interaction plays an important role in cell growth [68]. In patients with pancreatic cancer, PPARδ levels were correlated with advanced pathological tumor stage, increasing the risk of tumor recurrence and distant metastases [49].

4.2. Cons

The strongest evidence that PPARδ plays no appreciable role in carcinogenesis comes from a series of animal studies, cell culture data, and from studies evaluating the role of PPARδ

in inflammation, the latter being considered as a contributor to carcinogenesis.

Barak et al. evaluated the hypothesis that if PPARδ is a critical transducer of the tumorigenic signal, then its loss should substantially reduce, ifnot eliminate, intestinal polyps in *Min* mice [36]. *Min* mice that were *Ppard*-null harbored intestinal and colonic polyps. Histologically, all of the 12 intestinal polyps from $Ppard^{+/+}$ mice and the 9 from $Ppard^{-/-}$ mice were low-grade tubular adenomas. The number of intestinal polyps was not significantly different between $Ppard^{+/+}$, $Ppard^{+/-}$, and $Ppard^{-/-}$ *Min* mice. Loss of PPARδ did not significantly change the median size of intestinal polyps, although polyps > 1 mm were decreased upon PPARδ dosage reduction, which was further

pronounced for polyps > 2 mm. The number of polyps < 1 mm was essentially identical in all PPARδ genotype groups. Their conclusion was that PPARδ is qualitatively dispensable for the tumorigenic process, although they could not rule out the possibility that it influences the pace of polyp growth. In agreement with these findings, Marin et al. showed that PPARδ activation by GW0742 inhibits colon polyp multiplicity in $Ppard^{+/+}$ but not in $Ppard^{-/-}$ mice, suggesting that ligand activation of PPARδ attenuates azoxymethane-induced colon carcinogenesis [64].

The most striking resultis provided by a study demonstrating that in *Min* mice differing in their *Ppard* genotype ($Ppard^{-/-}$, which did not express PPARδ protein; $Ppard^{+/-}$; and $Ppard^{+/+}$), the incidence of polyp formation was not significantly different between groups [54]. In fact, $Ppard^{-/-}$ *Min* mice had about 3–6 times as many colon polyps as those of $Ppard^{+/+}$ or $Ppard^{+/-}$ mice. No significant differences in polyp size were found between any of the genotypes. Congruent results were obtained when they examined colon carcinogenesis with a more colon-specific, azoxymethane-induced model. The data from these two different colorectal cancer models suggest that PPARδ attenuates colon carcinogenesis.

Finally, Reed et al. reported that PPARδ-null *Min* mice exhibited increased predisposition to intestinal tumorigenesis [55]. Another report from the same group, evaluating the incidence and severity of intestinal neoplasia in mice deficient in both PPARδ and the mismatch repair gene *Mlh1*, showed that deficiency of PPARδ in mice with compromised mismatch DNA repair failed to affect intestinal neoplasia [59], with the implication being that PPARδ is not required for intestinal adenoma formation.

Similar results have been obtained for noncolonic tumors. For example, mice lacking one or both alleles of *Ppard* had enhanced growth of lung tumors [58]. In another example, the onset of tumor formation, tumor size, and tumor multiplicity of the skin was significantly enhanced in PPARδ-null mice compared with wild-type mice [69].

There are also data from cell culture models contradicting the notion that PPARδ plays a role in carcinogenesis. For example, in several human cancer cell lines, two PPARδ ligands failed to increase cell growth, Akt phosphorylation, or the expression of VEGF or COX-2 [56]. PPARδ activation by a PPARδ agonist does not induce cell growth in HT29, SW480, and HCA-7 colon cancer cells [64]. Furthermore, Raf oncogenes can contribute to tumorigenesis by augmenting the secretion of tumor growth promoting prostaglandins, such as PGI_2. However, using several cell lines, Fauti et al. showed that the increase in PGI_2 synthesis did not induce the transcriptional activity of PPARδ, suggesting that the oncogenic effect of PGE_2 does not involve PPARδ [70]. Another PPARδ function is the modulation of cell cycle. Knockdown of the PPARδ gene by siRNA promoted proliferation of HCT116 cells, suggesting that PPARδ may, in fact, inhibit their proliferation by arresting them in the G_1 phase of the cell cycle [57].

The chemopreventive action of PPARδ is also suggested by studies showing that in many cell types PPARδ promotes differentiation and inhibits proliferation [33, 60, 69, 71]. For example, Hollingshead et al. examined in azoxymethane-treated PPARδ-null mice whether PPARδ activation and COX2 inhibition attenuate colon cancer independently. Inhibition of COX2 by nimesulide attenuated colon cancer, and activation of PPARδ by GW0742 had inhibitory effects. The effects of these compounds occurred through independent mechanisms as increased levels of differentiation markers resulting from ligand activation of PPARδ were not found with COX-2 inhibition, and reduced PGE_2 levels resulting from COX-2 inhibition were not observed in response to ligand activation of PPARδ [62]. In another study by the same group, wild-type ($Ppard^{+/+}$) and $Ppard^{-/-}$ mice were treated with azoxymethane, together with GW0742, a specific PPARδ ligand, to test if $Ppard^{-/-}$ mice exhibit increased colon polyp multiplicity [64]. Ligand activation of PPARδ in $Ppard^{+/+}$ mice increased the expression of mRNA encoding the adipocyte differentiation-related protein, fatty acid-binding protein, and cathepsin E, all being indicative of colonocyte differentiation [64]. Thus, the induction of differentiation and the inhibition of proliferation in response to PPARδ activation support the hypothesis that PPARδ attenuates colon carcinogenesis [62].

Another contrarian point of view concerns the role of PPARδ in inflammation, with studies suggesting that activation of PPARδ has anti-inflammatory effects. In hepatocytes, the PPARδ agonist suppressed IL-6-mediated acute phase reaction, prompting the speculation that PPARδ agonists may be used to suppress systemic inflammatory reactions in which IL-6 plays a central role [72]. Two synthetic PPARδ ligands inhibited TNFα-induced expression of the vascular cell adhesion molecule-1 and E-selectin in human umbilical vein endothelial cells, suggesting that PPARδ activation has a potent anti-inflammatory effect [61].

Relevant to cancer is the presumed role of PPARδ in inflammation and NF-κB regulation [63, 73, 74]. Such a role is exemplified by studies on the skin, where activation of PPARδ by IFN-γ and TNFα accelerated keratinocyte differentiation [75]. Studies with PPARδ agonists have shown anti-inflammatory properties of PPARδ attributed to inhibition of NF-κB DNA-binding activity [74, 76]. Inflammation induced by TPA (*O*-tetradecanoylphorbol-13-acetate) in the skin was lower in wild-type mice fed sulindac than in similarly treated PPARδ-null mice [77]. In human endothelial cells, PPARδ activators inhibited TNFα-induced endothelial inflammation (VCAM-1 expression, monocyte adhesion, and MCP-1 secretion), in part by interfering with the NF-κB signaling pathway [63]. Lipopolysaccharide-induced TNFα production in cultured cardiomyocytes through NF-κB activation was inhibited by overexpression of PPARδ or the PPARδ synthetic ligand GW0742 [73].

The foregoing arguments and counterarguments make it clear that this controversy remains unresolved. This is the reason why we attempted to obtain an insight into the role of PPARδ in carcinogenesis by exploiting the unique opportunity offered by studying the effect of NO-ASA on PPARδ. Our work is presented in the following section.

5. NO-NSAIDs AND PPARδ

Our limited understanding of the mechanism by which NO-ASA exerts its colon chemopreventive effect combined with the possibility that PPARδ plays a role in colon carcinogenesis prompted us to assess the expression of PPARδ during intestinal carcinogenesis, and also whether NO-ASA modulates it [7].

We studied *Min* mice and their congenic (wild-type) mice, C57BL/6J$^{+/+}$. Three groups of each type of mice were treated for 21 days with vehicle or *m*-NO-ASA or *p*-NO-ASA, each at 100 mg/kg/day. As expected from their relative in vitro potency, after 21 days *m*-NO-ASA suppressed the number of intestinal tumors in *Min* mice (wt mice had no tumors) by 38%, and *p*-NO-ASA by 59%.

Most of the PPARδ positive cells (staining being always nuclear) were in the intestinal villi, with only few in the crypts. PPARδ was minimally expressed among the three groups of wild-typemice. In contrast, the expression of PPARδ in *Min* mice, similar in tumors and histologically normal mucosa, was more than ten-fold increased compared to wild-type mice. The two NO-ASA positional isomers inhibited the expression of PPARδ in both normal and neoplastic cells of *Min* mice. *m*-NO-ASA suppressed PPARδ expression in histologically normal mucosa by 23% and in neoplastic tissue by 41%; *p*-NO-ASA suppressed PPARδ expression in histologically normal mucosa by 27% and by 55% in neoplastic tissue. The reduction in the number of tumors by each NO-ASA isomer and the respective suppression of PPARδ expression in neoplastic cells are strikingly similar; the *meta* isomer reduced tumor incidence by 38% and PPARδ expressionby 42%, whereas the corresponding reduction for the *para* isomer was 59% and 55%. Of note, the expression of PPARα and PPARγ was sparse, and treatment with NO-ASA had no appreciable effect on either of them.

The changes in PPARδ expression induced by NO-ASA seemed to have a significant impact on the cell kinetics of the intestinal mucosa, rendering such an effect mechanistically important. The induction of apoptosis by NO-ASA, more prominent in neoplastic epithelial cells, followed closely the pattern of PPARδ reduction. Thus, in the neoplastic tissues, *m*-NO-ASA increased apoptosis by 22% and *p*-NO-ASA by 70%. The percentage of changes in PPARδ expression and apoptosis is significantly correlated ($P < .03$), suggesting a potential etiological association between the two events.

We have previously reported that NO-ASA induces two types of cell death, classical apoptosis as well as atypical cell death, which based on a variety of criteria appears to be a variant of necrosis [4]. Documentation of atypical cell death in vivo had been elusive. This study, however, provided a glimpse into this phenomenon in vivo. As shown in Figure 2, we were able to record the evolution of necrotic areas in NO-ASA-treated intestinal tumors. Initially, TUNEL positive cells coalesce and, as the necrotic area develops, they populate its margins (being extremely rare in the surrounding tissue). As the necrotic area increases in size, the TUNEL positive cells persist at the margins. We have identified multiple TUNEL positive spots within the necrotic areas, suggesting their cellular origin. We believe that these

TUNEL positive cells are necrotic cells [78]. The relationship of PPARδ and cell death induced by NO-ASA was ascertained by studying successive sections of intestinal tumors from both treated and untreated animals (Figure 2). Untreated tumors show strong PPARδ expression and few apoptotic cells. After treatment with *meta* or *para* NO-ASA, tumors show decreased PPARδ expression and increased apoptosis. If the apoptosis index of tumors from NO-ASA-treated mice is plotted against the expression of PPARδ, the association between the two is statistically significant; Figure 2 makes this correlation obvious. It should, however, be pointed out that these data have two methodological limitations. First, the specificity of the antibody is not considered by experts in the field ideal for immunohistochemistry, as nonspecific binding is possible. Second, no corroborating methodology was employed such as determination of PPARδ protein levels in these tissues by immunoblotting.

Other NSAIDs such as aspirin (of which NO-ASA is a derivative) have been reported to have PPARδ as one of their molecular targets. In epithelial ovarian cancer cells, aspirin suppressed PPARδ function and cell growth by inhibiting ERK1/2 [50]. Sulindac sulfide and indomethacin inhibit both 14-3-3 proteins and PPARδ levels in HT29 cells, suggesting that this could be the mechanism by which NSAIDs induce apoptosis in colorectal cancer [79]. Furthermore, in SW480 cells, sulindac sulfone significantly decreased PPARδ expression more potently than the sulfide metabolite [80]. A case-control study in a large population showed that a polymorphism in the promoter of PPARδ modified the protective effect of NSAIDs on colorectal adenomas [81]. However, the opposite was observed by another group, which found that regular NSAIDs use reduced the risk of colorectal cancer, but none of the polymorphic genes studied, including PPARδ, modified their protective effect [82].

Several reports present evidence that NSAIDs induce apoptosis independently of PPARδ. For example, sulindac significantly inhibited chemically induced skin carcinogenesis in both wild-type and PPARδ-null mice [83]. In addition, aspirin-induced apoptosis in Jurkat cells was not mediated by PPARδ [84]. Aspirin at a concentration which induces apoptosis did not affect the DNA binding of PPARδ, whereas neither addition of a specific PPARδ ligand nor transient transfection of PPARδ expression vectors protected Jurkat cells from aspirin-induced apoptosis. Finally, as the work of Hollingshead et al. presented above suggested, COX-2 inhibition by the NSAID nimesulide and PPARδ activation during colon carcinogenesis occurred through independent mechanisms [62].

6. CONCLUSIONS AND FUTURE DIRECTIONS

The contrast in data that were reviewed here on the potential role of PPARδ in cancer, with colon cancer being most extensively evaluated, could not be starker. Excellent studies from fine laboratories led "conclusively" to diametrically opposite results. As no grey zone seems to exist, the reader is left in bewilderment.

Our data indicate that, compared to wild-type mice, the nuclear receptor PPARδ is overexpressed in the intestinal
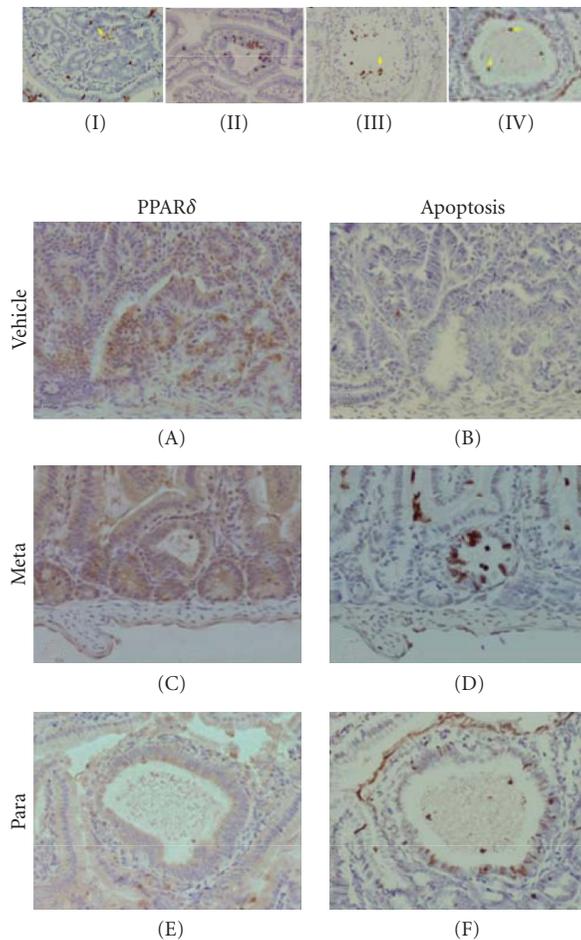
FIGURE 2: Effect of NO-ASA on PPARδ and apoptosis in colon tissue of Min mice. Upper panel: the evolution of necrotic areas in intestinal tumors treated with *p*-NO-ASA; sections are stained by the TUNEL method. (I) Coalescence of TUNEL positive cells (arrow), representing the earliest stage; (II) abundant apoptotic cells at the margins of the developing area with contrast to their rarity in the surrounding area; (III) and (IV) the necrotic area is increasing in size, but TUNEL positive cells persist at its margins; TUNEL positive areas within the necrotic areas (arrows) suggest their cellular origin. *Lower panel*: the relationship of PPARδ and apoptosis in NO-ASA treated intestinal tumors. Successive sections of intestinal tumors were stained for PPARδ expression and apoptosis. The untreated tumor shows strong PPARδ expression (A) and rare apoptotic cells (B). After treatment with *meta* or *para* NO-ASA, tumors show decreased PPARδ expression (C) and (E) and increased apoptosis (D) and (F). Magnification is x400, adapted from Ouyang et al. [7].

mucosa of *Min* mice, and that two isomers of NO-ASA, which suppress their intestinal neoplasia, inhibit to a commensurate degree the expression of PPARδ as well. This effect is accompanied by the induction of epithelial cell death, which correlates well with the antineoplastic effect of NO-ASA. As discussed earlier, these findings are, however, limited by the fact that PPARδ was detected using an antibody whose specificity may not be perfect and also by the lack of any corroborating methodology (e.g., immunoblot detection of PPARδ levels).

One could, nevertheless, consider that these findings support the notion that PPARδ promotes colon carcinogenesis. The key elements of support come from three findings. First, PPARδ is overexpressed in the intestinal mucosa of the *Min* mice but not in the wild-type control mice; being the same

in histologically normal and neoplastic mucosas further suggests that it has a role in early events of carcinogenesis. There is also specificity in the induction of PPARδ, as neither PPARα nor PPARγ was induced. Second, PPARδ responds to two NO-ASA molecules that are structurally identical except for their positional isomerism, proportionally to their antitumor effect. And, third, changes in tumor response, PPARδ, and cytokinetic parameters (apoptosis and necrosis) are closely correlated and mechanistically congruent.

Clarifying the role of PPARδ in colon carcinogenesis and the response to medications is of substantial interest. The mechanistic significance of this question is apparent. The implications for the rational design of therapeutic and/or preventive approaches are also clear. Finally, the fact that PPARδ agonists may be used for other indications raises the

concern of unintended consequences of such modulation of PPARδ, which may have a direct effect on the patient's risk of colon and perhaps other cancers.

At this stage, the jury should be considered out on the role of PPARδ in cancer. As with any evolving field, the mundane but accurate conclusion is that more work is needed to clarify such an important question.

ABBREVIATIONS

COX: Cyclooxygenase
MAPK: Mitogen-activated protein kinase
NSAIDs: Nonsteroidal anti-inflammatory drugs
NO-ASA: NO-donating aspirin
NO-NSAIDs: Nitric oxide-donating nonsteroidal
               anti-inflammatory drugs
PPAR: Peroxisome proliferator-activated
               receptor
TPA: O-tetradecanoylphorbol-13-acetate.

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Review Article

Potential of Peroxisome Proliferator-Activated Receptor Gamma Antagonist Compounds as Therapeutic Agents for a Wide Range of Cancer Types

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PPARγ is a therapeutic target that has been exploited for treatment of type II diabetes mellitus (T2DM) with agonist drugs. Since PPARγ is expressed by many hematopoietic, mesodermal and epithelial cancers, agonist drugs were tested and shown to have both preclinical and clinical anticancer activity. While preclinical activity has been observed in many cancer types, clinical activity has been observed only in pilot and phase II trials in liposarcoma and prostate cancer. Most studies address agonist compounds, with substantially fewer reports on anticancer effects of PPARγ antagonists. In cancer model systems, some effects of PPARγ agonists were not inhibited by PPARγ antagonists, suggesting noncanonical or PPARγ-independent mechanisms. In addition, PPARγ antagonists, such as T0070907 and GW9662, have exhibited antiproliferative effects on a broad range of hematopoietic and epithelial cell lines, usually with greater potency than agonists. Also, additive antiproliferative effects of combinations of agonist plus antagonist drugs were observed. Finally, there are preclinical in vivo data showing that antagonist compounds can be administered safely, with favorable metabolic effects as well as antitumor effects. Since PPARγ antagonists represent a new drug class that holds promise as a broadly applicable therapeutic approach for cancer treatment, it is the subject of this review.

1. INTRODUCTION

PPARγ is one of the three known peroxisome proliferator-activated receptors and is a member of the nuclear receptor (NR) superfamily. Since it has a predominantly nuclear location, regardless of whether cognate ligands are present, it is classified as a type II NR. It functions as a transcription factor by heterodimerizing with the retinoid X receptor (RXR), after which this complex binds to specific DNA sequence elements called peroxisome proliferator response elements (PPREs) [1]. In order to become fully active as a transcription factor, PPARγ must be bound by ligand. RXR can be affected by binding its own cognate ligands, usually resulting in incremental increases in transcriptional activity. After the PPARγ/RXR heterodimer binds to PPREs in promoter regions of target genes, coactivator proteins, such as p300 (CBP), SRC-1, and Drip205 (or TRAP220) family members, are recruited to this complex to modulate gene transcription [2–4]. Different PPARγ ligands appear to

be able to recruit different coactivators, which may explain differences in the biological activity between ligands [5].

The cardinal biologic activity of PPARγ is the induction of differentiation of adipocytes, the cell type that expresses the highest levels of PPARγ amongst normal tissues. Lower levels of PPARγ are, however, found in other normal tissues and cell types such as skeletal muscle, liver, breast, prostate, colon, type 2 alveolar pneumocytes, some endothelial cells as well as monocytes, and B-lymphocytes. There are three *PPARγ* mRNA isoforms (γ1, γ2, and γ3) and two major protein species (γ1 and γ2). The mRNA isoforms are generated by alternate promoter usage, resulting in an additional 28 amino acids at the N-terminus of PPARγ2 compared with PPARγ1. Most tissues express PPARγ1, whereas the PPARγ2 isoform is expressed mostly by adipocytes. The longer N-terminal domain of PPARγ2 may affect function, since this isoform was shown to confer a higher level of ligand-independent transcriptional activity, which was further increased by physiologic concentrations of insulin [6]. High

levels of PPARγ expression by fat and its role in adipogenesis led to the recognition that agonistic PPARγ ligands have antidiabetic effects. The chemical class of PPARγ agonists known as thiazolidinediones (TZDs) demonstrated high-affinity binding to PPARγ [7] as well as favorable therapeutic properties, and such drugs were eventually registered for the treatment of type II diabetes mellitus (T2DM). Three TZD drugs have been registered in the U.S.: rosiglitazone (Avandia), pioglitazone (Actos), and troglitazone(Rezulin). Subsequent to its marketing and widespread use, troglitazone was associated with idiosyncratic and, in rare cases, fatal hepatic toxicity, and, thus, was withdrawn from the market. The former two drugs, however, have remained as safe and effective therapeutic options for the management of T2DM.

Not long after reports of the cloning of PPARγ and its expression in normal tissues [8, 9], PPARγ expression was observed in an array of primary cancers and derivative cell lines. Its expression was reported initially in liposarcoma [10], and soon thereafter in colon, breast, and prostate carcinomas and additional cancer types [11–14]. In addition to the in vitro and preclinical in vivo anticancer effects of TZDs, pilot clinical studies using troglitazone showed antitumor activity in patients with liposarcoma and prostate cancer [15, 16]. Compounds from other chemical classes were also shown to bind PPARγ and to have antiproliferative effects in cancer models, such as the naturally occurring eicosanoid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$(15-d-PGJ$_2$), the N-aryl tyrosine derivative, GW1929 [17], and the triterpenoid, 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid, CDDO [18]. While compounds that exhibit PPARγ agonist activity, such as TZDs, have PPARγ-dependent antiproliferative effects, they have also been shown to have antiproliferative effects in cell types that are genetically PPARγ-null [19]. Also, uncertainty about mechanisms of anticancer effects of PPARγ ligands has resulted from variability in the classification of some compounds (e.g., bisphenol A diglycidyl ether [BADGE], which has been shown to have both agonist and antagonist activities) [20, 21].

2. EFFECTS OF PPARγ ANTAGONIST COMPOUNDS IN EPITHELIAL CANCER MODEL SYSTEMS: CELL GROWTH AND APOPTOSIS

The initial report of Fehlberg et al. [22] showed an inhibitory effect of this class of agents on a colon cancer and a lymphoma cell line using the compound, BADGE, which as noted has been classified as both an agonist and antagonist. This initial study did not examine effects on proliferation, but showed that apoptotic effects, such as increases in annexin-V binding and reductions in DNA content as assessed by propidium iodide staining, required 50–100 μM concentrations of BADGE, which would tend to increase off-target effects. Subsequently, Seargent et al. [23] showed that a higher affinity, selective PPARγ antagonist, GW9662, had direct antiproliferative effects on three breast cancer cell lines of differing phenotypes (ER+, ER−, and p53-null). This antagonist compound was somewhat more potent in its effects than an agonist (rosiglitazone). In this report, the role of PPARγ in mediating growth inhibition

was addressed, but not fully elucidated. All three cell lines expressed it and the predicted, canonical PPARγ-related transactivation effects were demonstrated, with the agonist inducing transactivation and the antagonist suppressing it, thus excluding PPARγ-mediated transactivation as the mechanism of this effect. There are data, however, that suggest that antagonist-type compounds may also act via other PPARγ-dependent pathways. Lea et al. reported similar results using a range of agonist and antagonist compounds on both murine and human cell lines [24]. Schaefer et al. showed that the antiproliferative effect of the PPARγ antagonist, T0070907, on hepatocellular carcinoma cell lines was attenuated by knockdown of PPARγ by siRNA [25]. These data are consistent with a PPARγ-mediated transrepression mechanism, which has been demonstrated with respect to anti-inflammatory effects of PPARγ ligands mediated by the NF-κB signaling pathway. Pascual et al. showed similar effects of a pure agonist (rosiglitazone) and a mixed agonist/antagonist (GW0072) on the repression of a NF-κB-regulated gene, iNOS, suggesting that pure antagonists may also be capable of mediating this effect [26].

There are also data that PPARγ ligands (both agonist and antagonist) exert PPARγ-independent effects suggesting other cellular targets of these compounds. This was demonstrated clearly by Palakurthi et al., who demonstrated in vitro and in vivo growth inhibition of two agonist compounds, troglitazone and ciglitazone, in experiments utilizing PPARγ$^{-/-}$ and PPARγ$^{+/+}$ embryonic stem cell lines (ES), both of which exhibited very similar sensitivity to these compounds [19]. This effect was shown to be mediated in part by the inhibition of the initiation of protein translation, since these TZD compounds increased the phosphorylation and consequent inactivation of elongation-initiation factor 2 (eIF2) both in cells that expressed and were null for PPARγ. The effect of antagonist compounds on this pathway has not been reported. As noted, BADGE had similar proapoptotic effects in a colon cancer line expressing PPARγ and a T-lymphoma line that showed no detectable expression of it (by immunoblotting and RT-PCR) of this target [22]. But, given the variable classification of this compound as both an antagonist and agonist, the mechanism underlying this effect and its attribution are unclear.

3. OTHER EFFECTS OF PPARγ ANTAGONIST COMPOUNDS

PPARγ antagonist compounds have also been shown to affect cell shape, adhesion, and invasiveness of cancer cell lines. Masuda et al. evaluated the effects of the PPARγ antagonists, BADGE, GW9662 and T0070907, on four squamous carcinoma cell lines derived from tumors of the oral cavity. Antiproliferative effects were shown for the three antagonists, but not for the agonist, pioglitazone [27]. Effects of these agents on adhesion and anoikis were also evaluated. Antagonists were found to inhibit adhesion and induce cell death related to loss of adhesion (known as anoikis) under normal tissue culture conditions on untreated plastic dishes. T0070907 induced similar inhibition of adhesion to fibronectin-coated plates, and this was significantly reversed

by coincubation of cells with this antagonist and the agonist, pioglitazone, suggesting a PPARγ-dependent effect. Since adhesion and detachment are related to cytoskeletal structure and function, this was assessed by fluorescent staining of F-actin. Using confocal microscopy, T0070907 was shown to cause dose-dependent disruption of F-actin, associated with rounding of the cells. Additional experiments showed inhibition of FAK and MEK-ERK signaling pathways, as well as decreased expression of integrin $\alpha5$ and CD151, both of which are adhesion proteins that have been implicated in cancer cell invasion and metastasis. Schaefer et al. showed similar effects of PPARγ antagonists on hepatocellular carcinoma cell lines including inhibition of adhesion, induction of anoikis, and inhibition of phosphorylation and activation of FAK [25]. These effects were shown to be dependent on the degree of PPARγ inhibition, and could be mediated by the antagonist or knockdown of PPARγ via specific, cognate siRNA. T0070907 was also shown to have substantially greater growth inhibitory effects on the HepG2 line compared with the agonist drugs, troglitazone, and rosiglitazone. Takahashi et al. demonstrated anti-invasive and growth inhibitory effects of the antagonists, GW9662 and T0070907, on esophageal cancer cell lines. The anti-invasive effects were observed at levels substantially lower than those required for growth inhibition [28]. In summary, all of these studies addressing anticancer effects of PPARγ antagonist compounds have show effects on cell growth, adhesion, and invasion in multiple epithelial cancer models.

Some of these effects are PPARγ-dependent, but the potential role of other targets is suggested by the similar effects of BADGE on a PPARγ+ colon cancer line and a PPARγ-negative T-lymphoma line. Also, the substantially different concentrations of PPARγ antagonists needed to induce anti-invasive effects versus growth inhibition in esophageal cancer lines suggest different mechanisms with differing degrees of PPARγ dependence or lack of involvement of the PPARγ-signaling pathway for some effects. A PPARγ-independent effect of antagonists on colorectal cancer cell lines and in an in vivo tumor xenograft derived from one of the lines was shown in a more recent report by Schaefer et al. [29]. A decrease in tubulin levels was observed that was independent of PPARγ, PPARδ, and proteasome function. This downregulation of tubulins α and β may explain the antimigratory, anti-invasive, and antimetastatic effects that were observed. Thus, in summary, PPARγ antagonist compounds with varying chemical structures (though GW9662 and T0070907 are similar) have several significant anticancer effects in vitro and in vivo in epithelial cancer model systems including breast, colon, aerodigestive squamous cell, and hepatocellular.

4. EFFECTS OF PPARγ ANTAGONISTS IN HEMATOPOIETIC CANCER MODEL SYSTEMS

Studies were conducted in our lab to assess the effects of PPARγ antagonists on hematopoietic cell lines. Initial screening showed that several myeloma (MM) cell lines had the greatest sensitivity to the antiproliferative effects of the antagonists, GW9662 and T0070907. Thus multiple MM

lines were tested, including one that is IL-6-dependent, for sensitivity to these compounds as well as to the agonist, pioglitazone. MM lines as well as non-Hodgkin lymphoma (NHL) lines showed significantly greater sensitivity to the growth inhibitory effects of the two antagonist drugs compared with the agonist [30]. As a group, the MM lines were more sensitive than the other groups of cancer cell lines to the antiproliferative effects of the antagonists, particularly T0070907. Other goals were to directly compare the sensitivity of previously tested epithelial cancer types (breast and colon) to hematopoietic lines (MM and NHL) as well as to evaluate a chemoresistant epithelial cancer type (renal cell). These experiments showed that in all the epithelial and hematopoietic cell lines tested, the antagonists were significantly more potent in their growth inhibitory effects compared with the agonist drug.

The IC_{50} values for the panel of 16 cell lines tested in these studies are shown in Table 1. For each of the cell lines in the panel, significant differences in the IC_{50} values of the antagonist compounds and the agonist drug, pioglitazone, were observed (P values ranging from <.04 to <.001, with 12 of 16 lines at <.001). While the MM lines showed the greatest sensitivity to the antagonists, similar degrees of sensitivity to the antagonists were also seen in the subset of breast cancer lines, which included two lines that are estrogen receptor-negative. Though not quite as sensitive as a subset, significant differences between the antagonists and the agonist were also observed in the renal cell lines, which are among the most chemoresistant epithelial lines. The differential sensitivities within and across cell lines did not appear to be related to the levels of PPARγ expression. Also, neither the agonist nor the antagonist induced significant upregulation of PPARγ as has been reported in some studies with PPARγ ligands. Consistent with prior reports, combinations of the agonist and with each of the antagonists did not result in attenuation of growth inhibitory effects. In fact, schedule-dependent increases in growth inhibition were observed, particularly when the antagonists were added to cells 24 hours prior to the agonist. Aspects of the mechanisms of cytotoxicity of the antagonists and agonists were also compared. It was shown that both classes of PPARγ ligand-induced apoptotic effects, but this effect was found to be caspase-independent for the agonist, pioglitazone [30].

Another question that was addressed was the impact of IL-6 on the responses of the MM lines to PPARγ antagonists, since this is a cytokine that plays a central role in the pathogenesis and progression of MM, as well as other cancer types. For these studies, 4 of the 5 MM lines that were utilized were IL-6-independent in order to follow up on a previous report of Wang et al. that analyzed the responses of three MM lines to the PPARγ agonists, 15-d-PGJ$_2$ and troglitazone. This report showed that growth inhibition and certain downstream signaling events were PPARγ-dependent, and also that two IL-6-dependent MM lines expressed PPARγ while an IL-6-independent line did not [31]. Also, GW9662 was reported to block the effects of the agonists, and had no antiproliferative activity on its own. We utilized five different MM lines, of which four are IL-6 independent (CAG, KMS12-BM, KMS12-PE, and OPM-6)

Table 1: Mean IC_{50} values (μM) for the PPARγ ligands.

Cell lines	Pioglitazone	T0070907	GW9662
Colon			
Moser[#]	26.5 ± 2.6	15.9 ± 1.0	20.1 ± 0.3
HT29[§]	53.0 ± 4.7	11.2 ± 0.0	14.1 ± 0.5
LS174T[#]	38.7 ± 7.4	7.8 ± 1.9	9.5 ± 0.5
HCT-15[§]	53.1 ± 2.5	13.0 ± 0.5	19.0 ± 0.8
RCC			
A498[#]	38.9 ± 4.9	24.3 ± 0.7	29.1 ± 0.3
ClearCa-2[§]	56.4 ± 3.1	20.8 ± 1.9	21.5 ± 0.7
Breast			
ZR75-30[§]	77.9 ± 7.0	3.9 ± 0.3	10.6 ± 0.9
MCF7[§]	54.8 ± 3.9	10.2 ± 1.9	16.6 ± 2.4
MDA-MB-231[§]	78.7 ± 3.5	20.1 ± 1.1	26.8 ± 1.0
MM			
CAG[*]	62.4 ± 9.9	12.2 ± 1.2	13.8 ± 0.1
KMS12-BM[§]	33.2 ± 5.1	3.2 ± 0.6	11.8 ± 1.6
KMS12-PE[§]	56.4 ± 1.5	4.3 ± 0.3	9.5 ± 0.9
OPM6[§]	48.9 ± 1.8	4.1 ± 0.3	11.5 ± 0.1
U266B1[§]	56.6 ± 1.3	9.9 ± 0.2	29.7 ± 1.5
NHL			
Ramos[§]	66.5 ± 7.4	12.7 ± 0.7	15.1 ± 0.1
SU-DHL6[§]	53.1 ± 1.4	11.8 ± 0.4	14.8 ± 0.3

Mean IC_{50} values from replicate experiments with this panel of cells for each of the three PPARγ ligands are shown above, expressed in μM $\pm$ SEM. Cell lines are grouped according to cancer type. IC_{50} values from each cell line were compared by single factor ANOVA analysis, with all lines showing significant differences as indicated: [§] $P < .0001$; [*] $P < .005$; [#] $P < .04$.

as well as a fifth that is dependent on an IL-6 autocrine loop (U266B1). In contrast to the prior report cited above, of the lines analyzed, CAG expressed PPARγ, while the autocrine IL-6-dependent line, U266B1, did not express PPARγ by immunoblotting. Also, three of the four of IL-6-independent MM lines were more sensitive to the growth inhibitory effects of both of the two PPARγ antagonist compounds compared with the IL-6-dependent line, U266B1 (see Table 1).

In MM cell lines, which are more often IL-6 dependent compared with other B cell lines, the strict dependence on exogenous IL-6 is indicative of ongoing requirement for this signaling pathway, which in pathophysiologic states, such as MM, usually depends on production of this cytokine by stromal cells. In MM, clinically more aggressive or treatment-resistant disease is associated with production of IL-6 by the myeloma cell themselves as opposed to the bone marrow stroma [32]. MM lines show a spectrum of IL-6 dependence, with some being dependent on exogenous IL-6, others being dependent on its autocrine production, and yet others being IL-6-independent for their growth. Even those MM lines that are not strictly dependent on IL-6 for their growth (exogenous or autocrine) can still be affected by the addition of exogenous IL-6 [33] (also shown in one of the lines tested, OPM-6, [34]). Addition of IL-6 to such MM lines has been shown to induce either incremental stimulation of proliferation or induction of resistance to various agents such

as dexamethasone, standard chemotherapy drugs such as melphalan and other agents. Thus the interaction of IL-6 and PPARγ antagonist compounds were examined in two MM lines (KMS12-PE and OPM-6). MTT assays were performed in the presence and absence of exogenous IL-6 (5 ng/mL). For both of these MM lines, addition of IL-6 did not induce resistance, but instead appeared to increase the sensitivity of these lines to T0070907, with a similar trend observed with GW9662 [30].

5. DOSE-RESPONSE EFFECTS OF PPARγ ANTAGONIST COMPOUNDS AND INTERACTION WITH OTHER AGENTS

The PPARγ antagonist compounds, GW9662 and T0070907, differ in their antiproliferative dose-response effects compared with the agonist as well as other agents. Not only are the corresponding IC_{50} values for the antagonists significantly lower than the agonist, pioglitazone, but a greater degree of growth inhibition (85–97% versus 50–80%) was observed with the former compounds. Also, of note was that the maximal effects of these agents were seen at concentrations that were only 2- to 3-fold greater than the IC_{50} across the entire panel of cell lines tested that included cell lines with relative and very high levels of chemoresistance (colon and renal cell, resp.). The dose-response curves were much steeper with the antagonist compounds compared to the agonist, pioglitazone, and also much steeper than what is observed with most other agents, including standard chemotherapy drugs and other agents (see Figure 2). This dose-response relationship suggests either a positive cooperative effect, potentially via increased, cooperative recruitment of corepresssors, thereby increasing transrepression. The alternate possibility is that different targets are being engaged with gradually increasing concentrations, which together exhibit additive or supra-additive interactions.

Since MM lines as a group were the most sensitive of the cell lines we tested, interaction with other novel agents for therapy of MM were evaluated. One such agent is anti-CD74 monoclonal antibody (mAb). CD74 was shown to be strongly expressed by the malignant plasma cells in the vast majority of clinical MM specimens as well as the majority of MM lines [35]. It was also shown that this mAb in unlabeled (cold) form exhibited in vitro growth inhibitory effects on both NHL and MM lines [36]. The anti-CD74 mAb used in these studies, LL1, also showed significant therapeutic effects in two preclinical murine NHL xenograft models. In preliminary in vitro studies, the humanized anti-CD74 mAb was combined with T0070907 in two MM lines. These studies also evaluated a sixth MM line (KMS11), which is IL-6 independent, expresses CD74 and is useful as a murine MM xenograft model. This line showed similar sensitivity to T0070907 as the other IL-6-independent lines, with an (unpublished observations, J Burton). Another IL-6-independent MM line that was used in initial studies, KMS12-PE, was also used to evaluate interactions between T0070907 and the hLL1 mAb. While KMS11 line showed moderate sensitivity to hLL1 (maximum growth inhibition
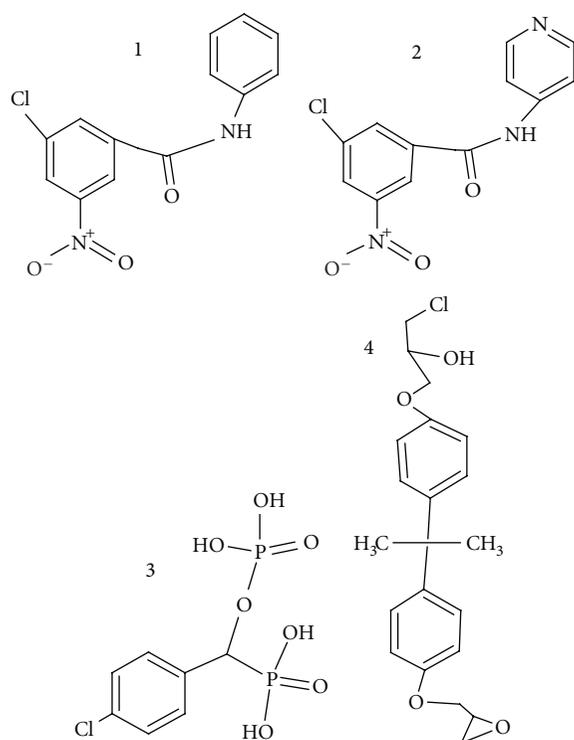
FIGURE 1: The chemical structures of four PPARγ antagonists: (1) GW9662, (2) T0070907, (3) SR-202, and (4) BADGE.

FIGURE 2: Dose-response curves for the MM line, KMS12-PE, to T0070907, both in the presence and absence of the hLL1 mAb. Square symbols represent the dose-response curve in the presence of hLL1, and diamond symbols represent the curve in the absence of LL1. The ordinate shows percent growth inhibition values and the abscissa the concentration of T0070907 in micromolar.

of 50–70%), the KMS12-PE line was resistant to single-agent hLL1 (<10% inhibition). However, in combination with T0070907, there was a sizable shift to the left of the dose-response curve, as is shown in one representative experiment in Figure 2. Current data indicate that the IC_{50} value decreases by from a mean value of ∼4.1 μM for T0070907 alone versus ∼3.0 μM with T0070907 in combination with hLL1, suggestive of a supra-additive effect (25–30% observed versus <8% expected based effect of hLL1 alone). This is a promising initial preclinical lead given that hLL1 is now being evaluated in several phase I/II clinical trials in B-cell cancers such as NHL and MM, and appears to be safe and well tolerated.

6. OVERVIEW OF MECHANISMS OF ACTION OF PPARγ AGONIST AND ANTAGONIST COMPOUNDS

The studies reviewed above have shown that the effects of PPARγ ligands are mediated by various mechanisms. Some studies show or suggest canonical PPARγ-mediated effects (i.e., via transactivation), as exemplified by early in vitro studies with agonist compounds that showed fat accumulation, a major PPARγ-mediated effect, in both breast cancer and liposarcoma cell lines [10, 12]. This was also demonstrated in liposarcoma patients in whom increased fat content within tumors was demonstrated by serial CT scanning before and after treatment with an agonist drug [10]. The studies of Wang et al. showed that the growth-inhibitory effects of PPARγ agonist compounds on

MM lines was seen only in lines expressing PPARγ and that these effects were reversed by cotreatment with an antagonist compound [31]. In contrast, completely PPARγ-independent effects were demonstrated for both agonist and antagonist compounds in reports from Palakurthi et al. [19] and Schaefer et al. [29]. This was clearly shown for the agonist compounds, troglitazone and ciglitazone, which showed similar antiproliferative effects in PPARγ-wild type and PPARγ-null (knockout) embryonic stem cell lines, both in vitro and in vivo [19]. PPARγ-independent growth inhibitory and antimetastatic effects of several antagonist compounds were shown in both in vitro and in vivo studies using three colon carcinoma cell lines. These effects were associated with reductions in tubulin levels and were also shown to be independent of PPARδ and proteasome function. The PPARγ-independent effect of agonist compounds was shown to be associated with inhibitory effects on the protein translation pathway. The mechanism of PPARγ-independent effects of antagonist compounds on tubulin levels has not been elucidated.

The mechanism of PPARγ-mediated transrepression may explain some of the effects of antagonist compounds. This was suggested by the attenuation of the effects of antagonist compounds by PPARγ knockdown by siRNA in hepatocellular carcinoma cell lines [25]. Also, the observation that combinations of PPARγ agonist and antagonist compounds result in additive antiproliferative effects in various cancer cell lines [24, 30] is consistent with this mechanism. This mechanism is plausible, as it has been shown to inhibit the NF-κB signaling pathway, which is central to inflammation and to the proliferation and survival of multiple cancer types including hepatocellular and colon carcinomas as well as multiple myeloma. The potential role of this and other mechanisms remain to be determined.

7. SUMMARY OF PRECLINICAL STUDIES OF PPARγ ANTAGONIST COMPOUNDS AND THEIR CLINICAL POTENTIAL

The studies reviewed above have shown that PPARγ antagonists have in vitro and preclinical in vivo anticancer effects that are as broad and potent as agonist compounds. These effects have been demonstrated in a wide range of epithelial cancer cell lines as well as hematopoietic cancer cell lines. Exploration of the underlying mechanisms of action for antagonist compounds has shown either involvement of PPARγ or a PPARγ-independent effect. One study suggested the involvement of the canonical transactivation mechanism in that antagonist effects were antagonized by coincubation with an agonist compound, pioglitazone [27]. In another study, where knockdown of PPARγ affected responses to antagonist compounds, the effect was not consistent with the canonical transactivation mechanism, but may be consistent with a transrepressive mechanism [25]. Another study showed that anticancer effects were associated with reductions in tubulin levels (a validated cancer-related target), but this was not mediated by PPARγ, PPARδ, or the proteasome [29].

While there have been numerous preclinical in vivo studies in cancer models with PPARγ agonists, there have been relatively few with antagonist compounds. Also agonists have been tested clinically. Some studies with antagonists have been conducted in noncancer models at low doses (≤ 1 mg/kg), which were not toxic and biologically active [37, 38]. A chemically distinct, but selective PPARγ antagonist, SR-202, has been synthesized and evaluated in preclinical models (Figure 1). It was given at a dose of 400 mg/kg for periods of up to 10 weeks with favorable metabolic effects such protection against diet-induced hyperinsulinemia and reduction in hyperinsulinemia and hyperglycemia in genetically predisposed (ob/ob) mice [39]. In pilot studies, we have administered moderate doses of GW9662 (15 mg/kg) and T0070907 (7.5 mg/kg) daily for 3 weeks by the intraperitoneal route to immunodeficient mice. These doses and schedules were well tolerated and resulted in no signs of toxicity (unpublished observations). These data indicate that the doses of these antagonists that may be sufficient for anticancer therapy are well tolerated, paving the way for further development of these agents for treatment of cancer.

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Review Article

PPARγ Inhibitors as Novel Tubulin-Targeting Agents

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The microtubule-targeting agents (MTAs) are a very successful class of cancer drugs with therapeutic benefits in both hematopoietic and solid tumors. However, resistance to these drugs is a significant problem. Current MTAs bind to microtubules, and/or to their constituent tubulin heterodimers, and affect microtubule polymerization and dynamics. The PPARγ inhibitor T0070907 can reduce tubulin levels in colorectal cancer cell lines and suppress tumor growth in a murine xenograft model. T0070907 does not alter microtubule polymerization in vitro, and does not appear to work by triggering modulation of tubulin RNA levels subsequent to decreased polymerization. This observation suggests the possible development of antimicrotubule drugs that work by a novel mechanism, and implies the presence of cancer therapeutic targets that have not yet been exploited. This review summarizes what is known about PPARγ inhibitors and cancer cell death, with emphasis on the tubulin phenotype and PPAR-dependence, and identifies potential mechanisms of action.

1. INTRODUCTION

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear hormone receptors that act as transcriptional modulators. They have important roles in control of metabolism, inflammation, and cell growth and differentiation. There are three PPAR isoforms (γ, β/δ, and α) with overlapping but distinct tissue expression patterns and cellular functions [1]. Much evidence suggests that PPARγ activity can modulate tumor development, implicating PPARγ as an important therapeutic cancer target [2].

PPARγ (NR1C3) is able to both activate and repress transcription, depending on the promoter that is involved [3]. In the classical pathway, PPARγ binds to promoters containing PPAR-response elements (PPREs) in combination with its heterodimer partner, the retinoid X receptor. Activator ligand binding to PPARγ causes a structural shift that increases its ability to recruit transcriptional coactivators while decreasing its basal ability to bind to corepressors [4]. PPARγ also exhibits transrepressive functions at promoters lacking a PPRE [5], by binding in a ligand-dependent manner to transcription factors, cofactors, or repressor com-plexes. In these cases, PPARγ binding inhibits transcription, either by binding/sequestering the transcription factors or by preventing clearance of repressor complexes. In at least one case of transrepression, the specific PPARγ conformational shift required is different from that required for cofactor recruitment at PPREs [6]. It is worth noting here that since PPARγ has basal ligand-independent repression [5] and activation functions [3], the effects of PPARγ inhibitor binding and PPARγ knockdown may not be the same.

PPARγ can be activated pharmacologically by thiazoli-denedione (TZD) compounds, including the antidiabetic drugs pioglitazone and rosiglitazone. There are multiple studies showing that high doses of TZDs can inhibit tumor growth in cell lines and mouse models. Clinical trials are currently underway testing TZDs as chemopreventive and therapeutic agents in human cancers [11]. While TZDs act to stimulate PPARγ activity, they also have multiple PPARγ-independent effects, and the specific role of PPARγ activation itself in the therapeutic effects of TZDs is still an active area of research. These topics are reviewed, from the point of view of cancer therapeutic effects, in several recent reviews [11–18] and elsewhere in this special issue of *PPAR research.*

TABLE 1: Effects of PPARγ inhibitors on PPARγ, PPARα, and PPARδ activity IC50 (nM) for ability to compete with a PPAR agonist.

	Binding	Direct binding assay			Activation of GAL4 chimera			References
		PPARγ	PPARα	PPARδ	PPARγ	PPARα	PPARδ	
GW9662	Irreversible				4	188	471	[7]
		5	39	1200	8	630[1]	4100	[8]
T0070907	Irreversible	1	850	1800	1 μM completely inhibits γ with no effect on α or δ[2]			[9]
BADGE	Reversible	100 000			100 μM $\sim$70% inhibits γ with little or no effect on α or δ[2]			[10]

[1] GW9662 is also a partial activator of PPARα with an EC50 of 22 nM [8], leading to the apparently higher concentrations of GW9662 required to inhibit PPARα than would be predicted by the direct binding assay.
[2] Dose curves were not performed, but the indicated concentrations suppressed the GAL4 chimera as indicated.

Several studies indicate that PPARγ inhibitor compounds are also able to reduce tumor growth in preclinical models [9, 19–29]. As with the TZDs, the precise role of the loss of PPARγ activity in cell death is an active research area, and may depend on the specific cell type. Our recent observation that PPARγ inhibitors can cause rapid dissolution of the microtubule network in colon cancer cells [26] suggests that these compounds might act as microtubule-targeting agents (MTAs), similar to the taxanes or *Vinca* alkaloids that are in current clinical use. However, unlike MTAs [30], they markedly reduce concentrations of α and β tubulin proteins long before a commitment to apoptosis, and do not strongly affect microtubule polymerization in vitro. This review will focus on the strong possibility that PPARγ inhibitor compounds represent a new class of tubulin-targeting agents [31].

2. BINDING ACTIVITY OF PPARγ ACTIVATORS AND INHIBITORS

The PPARγ ligand-binding pocket can accommodate a variety of lipophilic molecules [32]. Many cellular fatty acids activate PPARγ, including oxidized low-density lipoproteins, unsaturated fatty acids, 15-hydroxyeicosatetraenoic acid, and 9- and 13-hydroxyoctadecadienoic acids. In addition, the putative endogenous ligand prostaglandin 15-deoxy delta-(12,14)-prostaglandin J2, as well as the TZD anti-diabetic drugs [32], are able to activate PPARγ. The anti-inflammatory drug 5-aminosalicylic acid binds to PPARγ at therapeutic doses [33], as do other nonsteroidal anti-inflammatory drugs [34], although both classes of medications are lower affinity ligands than the TZDs. Ligand binding introduces PPARγ conformational shifts that favor recruitment of transcriptional coactivators over corepressors or that promote specific posttranslational modifications, and it is these changes that dictate the transcriptional activity of PPARγ. All of these ligands also have multiple effects that are independent of PPARγ, especially at high doses [13, 32]. In addition, the identity and regulation of true endogenous ligands is poorly understood at the present time.

PPARγ also binds to a number of compounds that are able to inhibit TZD-mediated PPARγ activation (see [35] for chemical structures). These include halofenate [36] and its enantiomer metaglidasen [37], SR-202 [38], G3335 and its derivatives [35, 39], T0070907 [9], GW9662 [8],

and bisphenol-A-diglycidyl-ether (BADGE) [10]. PPARγ inhibitors probably suppress PPARγ activation both by preventing binding by endogenous or exogenously added ligands, and by inducing specific conformational shifts that actively promote repression [9]. However, the details of these conformational changes are less well understood than for the activators. Of the known PPARγ inhibitors, only T0070907, GW9662, and BADGE have been tested for their effects on cancer cell death; all three can cause cell death in multiple cancer cell types at high-micromolar concentrations.

Interpreting the effects of the cancer-targeting PPARγ inhibitors is difficult, since they can act as activators or inhibitors, depending on the concentration used. They also bind to multiple members of the PPAR family (and quite possibly to other molecules) at high doses. At low micromolar doses, T0070907 and GW9662 also bind to and inhibit PPARα and PPARδ (Table 1). In addition, at low nanomolar doses, GW9662 is a partial activator of PPARα. While the ability of T0070907 to activate PPARα has not been checked, it is possible that this compound may behave in the same manner. Similarly, there are reports that BADGE can act as a PPARγ activator at lower doses (10–30 μM) than those needed for inhibitory effects [28, 40].

3. STANDARD MICROTUBULE TARGETING AGENTS ACT BY INTERFERING WITH MICROTUBULE DYNAMICS

Microtubules are long, tube-shaped polymers, formed by ordered arrays of α/β tubulin heterodimers (Figure 1), that make up one of the major components of the cellular cytoskeleton. Precise regulation of microtubule function is essential for maintenance of cell shape and polarity, migration, regulation of cell signaling cascades, intracellular transport, and cell division [41]. Microtubule function is governed by a variety of active changes in microtubule structure collectively termed *microtubule dynamics*. Microtubules normally alternate between phases of growth and rapid shrinking (dynamic instability), and also move tubulin heterodimers from one end of the polymer to the other (treadmilling) [42]. These processes are regulated in the cell by a host of microtubule-associated proteins with varied functions [43–46]. Both dynamic instability and treadmilling are required for mitosis, and are almost certainly necessary for the other functions of microtubules [44].
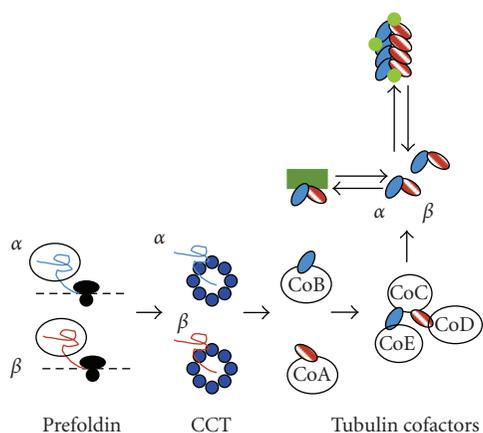
FIGURE 1: Microtubule formation depends both on chaperone-mediated production and assembly of α/β heterodimers and on microtubule-associated proteins. Production of α and β tubulin proteins requires assistance from chaperone proteins. The chaperone prefoldin associates with nascent tubulin polypeptide chains and delivers them to the CCT chaperone. CCT folds them into stable forms, which are delivered to the tubulin cofactors A and B [47]. CoA and CoB both transfer tubulin monomers to the CoC/D/E complex, which assembles the monomers into α/β heterodimers ready for introduction into microtubules. Tubulin reservoirs are held by the microtubule-associated protein stathmin (green box), which, depending on phosphorylation, binds to free tubulin and also destabilizes microtubule polymers. A host of micotubule-associated proteins (green circles) associate with the microtubule and regulate addition and removal of heterodimers from both ends of the microtubule; in some cases, they have been shown to regulate tubulin levels. While MTA therapies like the taxanes and *Vinca* alkaloids target the equilibrium between α/β tubulin heterodimer and the microtubule polymer, PPARγ inhibitors could be affecting any of the chaperone proteins or one of the microtubule-associated proteins that is involved in control of tubulin levels.

Given the importance of rapid cell proliferation and migration to tumor development, it is not surprising that the microtubule-targeting agents (MTAs) are one of the most successful classes of cancer therapeutics, with clinical applications in hematological cancers and solid tumors of the head/neck, breast, ovaries, testes, lung, gastric tissue, and prostate [48]. MTAs are a chemically and structurally diverse sets of small molecules that bind to microtubules, tubulin, or both, and interfere with microtubule function [44]. Despite this diversity, all known MTAs bind at or near to one of three domains: the taxane domain, the *Vinca* domain, or the colchicine domain. Historically, MTAs have been divided into microtubule-stabilizing and microtubule-destabilizing agents, based on their effects at high doses on polymer mass in in vitro polymerization assays. However, while the classification remains in use, and these effects clearly occur in vivo at high doses, it is becoming generally accepted that MTAs at clinically relevant concentrations primarily act by disrupting microtubule dynamics, rather than by affecting bulk polymerization [30, 49]. The microtubule-disrupting effect leads to cell cycle arrest. In addition, MTAs may also

cause apoptosis by mechanisms that ultimately prove to be at least partially independent of the effect on the mitotic spindle [50, 51].

Discovery and development of new microtubule-binding compounds is an area of active research. In contrast, less effort has been spent on considering whether reducing tubulin levels directly, and thereby altering microtubule function, could be used to impair cancer cells. Our recent results, showing that PPARγ inhibitors reduce tubulin levels in HT-29 colon cancer cells, before a commitment to apoptosis [26], suggest that targeting tubulin itself may be a viable strategy.

4. PPARγ INHIBITORS CAUSE APOPTOSIS IN MULTIPLE CANCER CELL TYPES AND CAUSE RAPID LOSS OF TUBULIN PROTEINS IN COLON CANCER CELLS

Experiments with many different cancer cell lines show that high doses of PPARγ inhibitors can cause cell death. T0070907 and/or GW9662 exhibited antiproliferative effects in both hematopoietic cell lines from non-Hodgkin's lymphoma and multiple myeloma [27] and epithelial cancer cell lines, including carcinoma cell lines from renal [27], breast [21, 22, 27], liver [23, 52], oral squamous [29], esophageal [24], prostate [22], and colon tissue [22, 26]. IC50 concentrations for inhibition of growth in the epithelial lines ranged from 3–50 μM for T0070907 and 10–50 μM for GW9662. While the reasons for this reduction in cell number have not been explored in all cases, T0070907 and GW9662 clearly caused apoptosis in several epithelial cell lines. BADGE also exhibited cytotoxic effects against colon cancer cell lines [20, 22, 25, 31] and a T lymphoma cell line [19, 20], at doses in the 100–200 μM range.

In HT-29 colon cancer cells, treatment with 50 μM T0070907 led to dissolution of the microtubule network within 12 hours [26]. At this timepoint, the effects of T0070907 were reversible. However, after longer exposure, the cells became committed to caspase-dependent apoptosis. Cells treated with T0070907 also assumed a rounded shape that occurred prior to commitment to apoptosis, and that was not affected by caspase inhibitors. Similar effects and timing were seen with the PPARγ inhibitor GW9662 ([26] and KLS, unpublished data).

Loss of the microtubule network is associated with the microtubule-depolymerizing class of MTAs that includes vinblastine, vincristine, vinorelbine, and nocodazole [30, 53]. Thus, it was especially striking that, unlike nocodazole, T0070907 and GW9662 did not affect microtubule polymerization in in vitro assays [26]. Instead, α and β tubulin protein levels in the cells dropped rapidly after treatment with these compounds, suggesting that the microtubule network disappeared because tubulin protein levels were below critical thresholds needed for polymerization. BADGE also caused tubulin loss, although the timing of this loss relative to commitment to apoptosis was not determined. It is not currently known whether PPARγ inhibitors cause loss of tubulin in other cell lines. While many of the other experiments with inhibitors documented altered cytoskeletal

structure [23–25, 29], it was not clear in these papers whether the altered shape was the result or the cause of apoptosis, and tubulin levels were not measured directly.

5. IS TUBULIN LOSS REQUIRED FOR CELL DEATH INDUCED BY T0070907 AND GW9662?

The effects of T0070907 and GW9662 on tubulin are striking, especially as there have not been any reports of cancer cell-targeting small molecules that affect tubulin levels without dramatic effects on microtubule polymerization. However, it is not clear that the ultimate cause of cell death is loss of tubulin. These compounds may independently target a combination of signaling pathways that ultimately trigger the apoptotic response as well as modulating tubulin levels. Given the fact that the PPARγ inhibitors also trigger loss of γ and δ tubulin isoforms (KLS, unpublished data), it will be difficult to do genetic replacement experiments to address this issue in the absence of other information about the reasons for tubulin loss. Regardless of whether or not microtubule disruption is the first trigger for apoptosis, the reduction in tubulin should serve as a barrier that is impossible for the tumor cells to surmount. This effect could have profound advantages, in that simply modulating the levels of anti-apoptotic proteins in response to chemotherapy would not be sufficient to allow tumor escape.

Intriguingly, commitment to apoptosis in HT-29 cells occurred at about the same time that tubulin levels dropped below a threshold level for normal function observed in yeast. After 12 hours of T0070907 treatment, when the tumor cells had lost $\sim$50% of their tubulin, the effects of the drug were still reversible. By the time, 50% of the cells had committed to apoptosis ($\sim$20 h), the average tubulin level was less than 10% of control ([26] and data not shown), suggesting that apoptosis may be triggered by tubulin levels below 50% of normal. These numbers parallel observations in the yeast *Saccharomyes cerevisiae*, which was able to tolerate a 50% reduction in either α or β tubulin, as long as there were not excess unpaired β tubulin molecules [54, 55], but which began to show defects in mitosis when levels dropped to $\sim$20% of normal.

6. WHAT COULD BE CAUSING THE LOSS OF TUBULIN INDUCED BY T0070907?

The reasons for T0070907-mediated tubulin loss remain to be elucidated, and may well be the result of multiple coordinated changes taking place in the context of alterations in PPAR function. This point is of critical interest, as identification of the mechanism(s) of tubulin loss will serve as an important step in identifying the therapeutic targets that are exploited by T0070907, and in design of better ways to target them. In HT-29 cells, α/β tubulin RNA levels were unaffected, suggesting a post-transcriptional mechanism. Several aspects of tubulin production could be involved, including degradation, translation initiation, chaperone-mediated folding/assembly of tubulin heterodimers (Figure 1), and disruption of microtubule-associated protein interactions with tubulin.

Because the protein half-life of tubulin is believed to be long ($\sim$50 hours) [56, 57], detectable loss of tubulin within 6 hours suggests a mechanism involving increased decay. Tubulin can be targeted for proteasomal degradation by the tubulin cofactor-like protein El [58] and probably by other regulatory factors. However, proteasome inhibitors did not prevent T0070907-induced tubulin loss [26], suggesting that ubiquitin-mediated proteasomal degradation is not a major factor. Other degradation pathways must be investigated, especially the aggresome pathway, which can replace proteasomal degradation, resulting in autophagic clearance and lysosomal degradation [59, 60]. It is also worth noting that the estimate of long tubulin half-lives is based on measurement in only two cell types, and may not apply to HT-29 cells.

T0070907-induced tubulin loss is unlikely to be the result of acute increases in tubulin monomer protein. Many eukaryotic cells respond to a sudden increase in unpolymerized tubulin by reducing synthesis of tubulin [61–65] in a process termed *autoregulatory control*. This mechanism is associated with large reductions in tubulin mRNA; later work showed that polysomal mRNA in the process of being translated was specifically susceptible to an increased mRNA decay [66, 67]. T0070907 induces little difference in tubulin mRNA concentrations as measured by real-time PCR [26]. In addition, at least in in vitro polymerization assays, T0070907 did not inhibit polymerization or cause depolymerization, although it is important to note that depolymerization might occur in the cell as a result of alterations in microtubule-associated protein function. However, nocodazole, which at high (10 μM) doses increases the amount of soluble tubulin by inhibiting bulk microtubule polymerization [53], did not affect the tubulin protein levels in HT-29 cells. This result strongly suggests that tubulin in these particular cells is not strongly subject to autoregulatory control. However, it is formally possible that T0070907 might increase the soluble tubulin pool far more strongly than nocodazole. Direct measurement of the amount of tubulin in the polymerized and free pools after T0070907 treatment should resolve these questions.

There is also the potential for T0070907 to control translation initiation or other aspects of protein synthesis. The TZD PPARγ activators have been shown to suppress translation initiation in a PPARγ-independent manner through a mechanism involving intracellular Ca^{2+} store depletion and subsequent inhibition of the eIF2 translation inititation factor [68]. It is possible that T0070907 also affects some aspect(s) of the translation machinery.

The loss of tubulin and cell death phenotypes induced by T0070907 can be mimicked by knockdown of chaperone proteins involved in folding and assembly of tubulin, suggesting that PPARγ inhibitors may interfere with this pathway. Tubulin production and assembly into α/β heterodimers require the presence of multiple chaperone proteins, including the multisubunit chaperones prefoldin [69] and CCT [70], and tubulin cofactor proteins A–E [71] (Figure 1). Additional chaperone modulatory proteins, including PhLP3 [72] and E-like (El) [58], also modulate the function of the tubulin chaperone system. Knockdown of CCT subunits

causes reduced tubulin levels [73], as does knockdown of tubulin cofactor A [74]. It is possible that PPARγ inhibitors bind directly to and inhibit some of the chaperone proteins. Alternatively, they may change the expression or function of any of the chaperone subunits or cofactors.

Knockdown of microtubule-associated proteins (MAPs) can also cause loss of tubulin, and PPARγ inhibitors could interfere with MAPs or dysregulate MAP expression. MAPs are a functional class of proteins that physically interact with microtubules or microtubule precursors and regulate microtubule functions. Some MAPs directly control the rate of association or dissociation of α/β tubulin heterodimers with the ends of the microtubule, as well as the levels of soluble tubulin in the cell, and thus affect microtubule dynamics (Figure 1). Others link microtubules to signaling complexes and other cytoskeletal components [75]. Mutations in stathmin, a multifunctional MAP that both destabilizes microtubules and sequesters α/β tubulin heterodimers so that they are not part of the freely polymerizing pool, led to reduced α tubulin levels (β tubulin was not checked) and fewer microtubules in Drosophila oocytes [76]. In the same system, stathmin overexpression increased tubulin levels. Knockdown of MAP4, generally thought to be a microtubule-stabilizing MAP, also caused reduced tubulin levels [77]. In both cases, it is possible, but has not been shown directly, that these effects were subsequent to autoregulatory control.

7. ARE THE CELL DEATH AND TUBULIN EFFECTS OF T0070907 OR GW9662 DEPENDENT UPON PPARγ?

It is important to establish whether the effects of T0070907 and GW9662 on cell death can be separated from their ability to target PPARγ. Although results in two cell lines [23, 29] have shown that PPARγ knockdown causes cell death or potentiates the effects of the inhibitors, this result does not occur in all cell lines [26]. In addition, the inhibitor doses required for the cell death (3–50 μM for T0070907 and 10–50 μM for GW9662) in all cell lines tested are much higher than those needed to inhibit the transcriptional effect of PPARγ by at least 90%, given an approximate effective dissociation constant in the low nanomolar range (Table 1). This result suggests that at least some of the cell death effects are indeed independent of PPARγ. The differences in cell lines may reflect a true disparity in the role of PPARγ in maintaining cell growth and survival in different cell types, and suggests that the HT-29 system is ideal for examining the PPARγ-independent effects of T0070907.

The effect of T0070907 and GW9662 on tubulin has been examined primarily in HT-29 colon cancer cells, although the loss of adhesion, cell rounding, and cell death occur in multiple colorectal cancer cell lines ([26] and KLS, unpublished data). In HT-29 cells, the effects on tubulin are not replicated by PPARγ knockdown, or reductions in the closely related PPARδ. However, given the fact that at 50 μM T0070907, PPARγ, PPARα and PPARδ transcriptional activities are all expected to be at least partially repressed, based on the predicted dissociation constants (Table 1), it is possible that multiple PPAR molecules must be inactivated

in order to create the conditions necessary for tubulin loss. In addition, it is entirely possible that a non-PPAR-dependent event must occur in the context of knockdown of one or more of the PPAR transcription factors. This idea is somewhat contradicted by the observation that BADGE, which does not strongly affect PPARα/δ at 100 μM [10], does cause a reduction in the amount of tubulin [26]. However, in this case, it is possible that the tubulin loss was a separate phenomenon, secondary to extensive cell death. Further experiments will be needed to address this issue.

8. ADVANTAGES AND DISADVANTAGES TO USING PPARγ INHIBITORS AS TUBULIN-TARGETING THERAPIES

To our knowledge, the PPARγ inhibitors are the first described instance of a possible small molecule cancer therapeutic that reduces tubulin levels. This result suggests the exciting possibility that tubulin levels can be modulated therapeutically, in a tumor-specific manner, to kill cancer cells. While the current microtubule-targeting agents have significant antitumor activity in many cancer types, they are not effective in all cancers, and acquired resistance is a problem [78]. In addition, because these drugs differentially targeting rapidly proliferating cells, they cause leukopenia [78]. For the same reason, they are not expected to target cancer stem cells, owing to their generally slow proliferation rate [79]. Microtubule-targeting drugs also induce peripheral neuropathy [80] and may interfere with mental function [81], presumably as a result of their effects on neuronal microtubule function. T0070907 and/or GW9662, or second-generation compounds, by virtue of acting by a different mechanism, might ameliorate some of the difficulties of the standard MTAs.

Several questions must be addressed, when considering these compounds, or others like them, as cancer therapies. To date, preclinical data on the bioavailability of either T0070907 or GW9662 has not been published, although a pilot experiment with radiolabeled GW9662 indicates that these compounds are delivered to tumors [82]. It is also important to consider tumor specificity and whether the tubulin-targeting effect can be separated from the PPAR inhibitory effect.

Inherent or acquired resistance to current microtubule-targeting agents is a serious problem in microtubule-based cancer therapy. One major source of resistance is the expression of alternate tubulin isoforms with inherently different microtubule dynamics [83]. These differences antagonize the effects of the drug, and allow the cell to continue proliferating. It appears that T0070907 causes concurrent loss of multiple tubulin isoforms (KLS, unpublished data), presumably by some regulatory mechanism common to all isoforms. It is therefore reasonable to suspect that T0070907 would suppress levels of the tumor-specific alternate isoforms as well. The microtubule targeting drugs are also generally good substrates for drug efflux pumps that prevent accumulation of therapeutic levels of drug [78]. For this reason, it would be useful to test whether T0070907 and GW9662 are substrates for the common drug efflux pumps.

As tubulin is a constituent of all cells, the effects of T0070907 or similar compounds on normal cells is a serious consideration. To date, these compounds have only been tested in one mouse model of cancer. At oral doses that reduced tumor growth (1–5 mg/kg/d), the compounds did not cause weight loss or malaise in mice [26]. In addition, recent unpublished results from our laboratory showed that 10 mg/kg/d orally, maintained daily for three weeks, did not cause any alterations in values from a standard complete blood count with differential. The reasons for this apparent specificity will need to be examined in more detail, as well as whether tubulin levels are reduced in normal and tumor tissues in vivo. The fact that radioactively labeled GW9662 preferentially accumulated in tumor cells as compared to many normal tissues in mice [82] suggests that part of the specificity may simply reflect where the compound is accumulating. Another possibility is that these compounds do act through the tubulin chaperone system. Components of this system are upregulated in some tumor cells [84–86], and tumor cells may require higher levels of tubulin chaperone function, as they do with the HSP90 chaperone [87, 88]. If this were true, this might explain why tumor cells are preferentially susceptible to the PPARγ inhibitors.

A major question is whether suppression of PPARγ (or PPARα/δ) function is required for the tubulin-targeting effects of T0070907 and/or GW9662. If suppression of PPARγ cannot be separated from the tubulin targeting effects, it will be necessary to carefully balance the therapeutic effects of PPARγ inhibitors on tubulin with the possible deleterious effects of PPARγ inhibition on physiologic processes that affect tumor growth. In addition to the question of whether PPARγ is a tumor suppressor or has tumor-promoting activity in each cancer cell type, the effects of PPARγ on angiogenesis [14, 16] and the immune system [32] must also be considered.

The role of PPARγ itself in angiogenesis, in contrast to the effects of TZD PPARγ activators, is still relatively unclear [16]. TZDs can reduce the production of proangiogenic FGF and VEGF factors, interfere with endothelial cell migration, and inhibit vascular tube formation, as well as reduce production of inflammatory mediators that stimulate angiogenesis. However, to date, only CD36 upregulation [89] and decreased iNOS production [6] are known to be PPARγ-dependent. It is also noteworthy that standard microtubule-targeting agents in clinical use disrupt tumor-specific vasculature [90, 91]. It will be interesting to determine whether the PPARγ inhibitors also suppress tumor angiogenesis, and whether the effects are linked to PPARγ and/or tubulin.

In all probability, the net effect of PPARγ inhibition on the immune system will depend upon the individual characteristics of the tumor, including the site of the tumor, the role of PPARγ in tumor-intrinsic biology, and the presence and type of immune infiltration. A variety of immune cells infiltrate tumors, including macrophage lineage cells, T lymphocytes, mast cells, and natural killer (NK) cells [92]. All of these cells have the potential to promote or hinder tumor growth, depending on the cytokines they secrete and the cell-mediated cytotoxic effects they are able to promote. Both macrophage and regulatory T cell (T_{reg}) functions are modulated by PPARγ. Monocytes can differentiate into M1 or M2 macrophages in response to different stimuli [93, 94]. In general, M1 macrophages secrete large quantities of inflammatory cytokines, have cytotoxic activity toward tumor cells, elicit the adaptive immune response, and are associated with a better tumor prognosis. In contrast, M2 macrophages secrete immunosuppressive cytokines, have poor antigen-presenting capacity, and promote angiogenesis and tissue remodeling; these macrophages are generally associated with a poorer prognosis [93]. Since PPARγ activation of human monocytes promotes M2 polarization [95], PPARγ inhibitors might be expected to favor production of M1 tumor-suppressing inflammatory macrophages. T_{reg} cells lacking PPARγ are unable to suppress colitis in a regulatory T cell-dependent model of inflammatory bowel disease [96], arguing that PPARγ is required for suppressive regulatory function. As T_{reg} activity can impair tumor rejection [97], PPARγ inhibitors should suppress T_{reg} thereby aiding tumor rejection.

9. CONCLUSIONS

The microtubule-targeting agents are one of the most successful classes of cancer therapeutics, but ongoing issues with resistance make the development of additional strategies for targeting microtubules extremely desirable. The recent discovery that the small molecule PPARγ inhibitor compounds reduce tubulin protein levels, without affecting in vitro polymerization rates, suggests the exciting possibility that targeting tubulin levels directly, rather than microtubule dynamics, might be an additional way to manipulate microtubule biology to kill cancer cells. Several questions, including whether inhibition of PPAR function is required for the tubulin effect, the nature of the tumor specificity, the ultimate targets of these compounds, and whether better compounds with similar tubulin targeting effects can be designed, must be answered before this strategy can be fully realized.

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Review Article

The Role of PPARs in the Endothelium: Implications for Cancer Therapy

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The growth and metastasis of cancers intimately involve the vasculature and in particular the endothelial cell layer. Tumours require new blood vessel formation via angiogenesis to support growth. In addition, inflammation, coagulation, and platelet activation are common signals in the growth and metastasis of tumour cells. The endothelium plays a central role in the homeostatic control of inflammatory cell recruitment, regulating platelet activation and coagulation pathways. PPARα, -β/δ, and -γ are all expressed in endothelial cells. This review will discuss the roles of PPARs in endothelial cells in relation to angiogenesis, inflammation, coagulation, and platelet control pathways. In particular, we will discuss the recent evidence that supports the hypothesis that PPARα and PPARγ are antiangiogenic receptors, while PPARβ/δ is proangiogenic.

1. IMPORTANCE OF THE ENDOTHELIAL CELL IN CANCER

Endothelial cells play critical roles in vascular biology, being both the protective inner lining of vessels and the local site for delivery of oxygen to all tissues. It has become clear, particularly from the seminal work of Professor Judah Folkman, whom this issue is dedicated to, that the endothelium plays a critical role in the growth and spread of cancer [1–4]. The growth of tumours, or indeed any tissue growth requires new blood vessel formation to sustain it. This process of angiogenesis as a target for modulating cancer growth has been a major research theme. The critical initial stimulus for angiogenesis appears to be hypoxia in the growing tumour. The hypoxia leads to upregulation of hypoxia-induced transcription factors, for example, hypoxia inducible factor (HIF)-1α and HIF-2α [5–8], which stimulate the expressions of genes involved in oxygen homeostasis, and secretion of proangiogenic mediators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [4, 9, 10]. Although these are key growth factors for endothelial cell growth and morphogenesis, it is clear that there are an increasing number of endogenous proangiogenic factors

(PGDF, IL-8, angiopoietin-1, leptin, matrix metalloproteinases, thrombin, plasminogen activators) and antiangiogenic factors (endostatin, angiostatin, thrombospondin-1, angiopoietin-2, IL-4, IL-12, IL-18, tissue inhibitor of MMPs, TGF-β, IFNα, -β, and -γ) [1, 4, 10, 11]. When the cumulative actions of the proangiogenic mediators outweigh their antiangiogenic counterparts an "angiogenic switch" occurs [12]. In particular, VEGF (VEGF-A; VEGF$_{165}$) is a central mediator of endothelial cell growth and angiogenesis [13]. Two endothelial VEGF tyrosine kinase receptors have been identified: VEGFR-1/Flt-1, and VEGFR-2/KDR/Flk1, with the latter being the most important in VEGF-induced mitogenesis and permeability [13]. The lymphatic system and in particular lymphangiogenesis also contributes significantly to tumour metastasis. Unlike angiogenesis, where VEGF-(A) and VEGFR1/2 are key regulators, lymphangiogenesis is regulated by VEGFR-3 and VEGF-C/D isoforms (along with PROX1, podoplanin, LYVE-1, ephrinB2, and FOXC2) [14, 15]. Once stimulated by VEGF, the receptors initiate a signal transduction cascade, activating kinases such as ERK1/2 and Akt, which phosphorylate and activate further mediators of endothelial cell proliferation, apoptosis, and angiogenesis, such as eNOS [16].

Anti-angiogenic

Endostatin
Angiostatin
Thrombospondin-1
Angiopoietin-2,
IL-4, IL-12, IL-18
TIMPs,
TGF-β,
INFα, -β, and -γ

Anti-inflammatory

Prostacyclin
NO
IL-4, IL-12, IL-18
TIMPs,
TGF-b,
INFα, -β, and -γ

Anti-coagulant

Prostacyclin
NO
uPA, tPA

Endothelial cell

Pro-angiogenic

VEGF
bFGF
PGDF
IL-8
Angiopoietin-1
Leptin
Matrix metalloproteinases
Thrombin
Plasminogen activators

Pro-inflammatory

PGE$_2$
NO
Adhesion molecules
Chemokines
MMPs
Thrombin

Pro-coagulant

PGE$_2$
Tissue factor
PAI-1
Thrombin

Figure 1: The endothelial cell is the interface between the circulation and underlying tissue, and as such plays an important homeostatic role both producing and responding to a variety of pro- and antiangiogenic, inflammatory, and coagulation factors. The balance between these opposing pathways is critical in the growth, development, spread, and metastasis of tumours.

The endothelium local to the tumour itself also contributes to tumour growth and metastasis via mechanisms independent of angiogenesis. Of increasing importance is the role of chronic inflammation in tumour progression. Chronic inflammation, in particular the presence of neutrophils, macrophages, and mast cells, correlates with poor prognosis and the angiogenic state of the tumour [17, 18]. The activation of the endothelium and its subsequent expression of adhesion molecules and chemokines is the interface for local inflammatory cell recruitment and extravasation. Central to these processes are proinflammatory transcription factors such as NFκB. NFκB regulates many inflammatory processes including inducible cytokine/chemokine and adhesion molecule expressions that are central to inflammatory cell recruitment, as well acting as a potent prosurvival signal within the cell [19].

In addition to angiogenesis and inflammation, cancer progression and metastasis is also facilitated by circulating cells and mediators regulated by the endothelium. The endothelium provides an antithrombotic surface and produces powerful antiplatelet and anticoagulant mediators such as prostacyclin, nitric oxide, and tissue- and urokinase-plasminogen activators [20]. Under physiological conditions, the endothelial surface is antithrombotic. Activated endothelial cells, however, are able to release prothrombotic/procoagulation mediators such as prostaglandin PGE$_2$ [21, 22], plasminogen activator inhibitor (PAI)-1 [23], and tissue factor [23]. In cancer, thrombocytosis is common [24], suggesting that the physiological protective system usually provided by endothelial cells may be dysfunctional or overpowered by prothrombotic pathways. Driving this thrombosis may be tumour-derived thrombopoietin, and tumour- and platelet-derived growth factors and micropar-

ticles [24]. The consequence of activation of the coagulation cascade in cancer progression can be seen using thrombin as an example. Thrombin activates tumour cell adhesion to platelets and endothelial cells, and induces tumour cell growth, metastasis, and angiogenesis [25].

The movement of tumour cells into and out of the circulation (or the lymphatics) involves interaction with, and crossing of, the endothelial barrier. Although tumour endothelial cells are generally highly permeable (induced by factors such as VEGF), it is still unlikely that tumour cell movement is a passive process [26]. Within the circulation, transit of tumour cells is facilitated by their interactions with activated platelets [26]. The platelets are believed to act as a shield, protecting tumour cells from both physical forces and immune-mediated killing [26].

In summary, along with angiogenesis and lymphangiogenesis, endothelial cells regulate tumour progression not only by directly interacting with tumour cells, but also by regulating local inflammatory cell recruitment, the coagulation cascade, and platelet activity. When discussing the actions of PPARs in endothelial cells it is, therefore, important to consider all these properties.

2. PPARs AND ENDOTHELIAL CELLS

PPARα, PPARβ/δ, and PPARγ are expressed in endothelial cells [27, 28], where they regulate cell proliferation, angiogenesis, inflammation, thrombosis, and coagulation (Figure 1). PPARα is expressed in human aortic endothelial cells, carotid artery endothelial cells, and human umbilical vein endothelial cells [27, 29–31]. PPARγ is similarly expressed in human endothelial cells both in vitro and in vivo [27, 28, 31, 32], while PPARβ is ubiquitously expressed. The role of PPARγ has been well characterised in endothelial cell inflammation and angiogenesis [33, 34]. In contrast, the functions of PPARα and PPARβ/δ in endothelial cells, especially in terms of angiogenesis, are only just beginning to be understood. Indeed, although the role of PPARγ will be discussed in this review, since there is considerable information on PPARγ in cancer [35] and an article on PPARγ regulation of the angiogenic switch in this review series [36], this manuscript will focus more on recent observations highlighting novel roles for PPARα and PPARβ/δ in endothelial cell function and in particular on the regulation of angiogenesis. The focus of this review is the endothelial cell, but it is important to note that PPARα, β/δ, and γ expression and activity have been demonstrated in a variety of cancers, inflammatory cells [34], and in platelets [37–39]. Therefore, any effects of PPAR ligands on the development of cancer may be influenced by responses in these nonendothelial cell types as well.

3. PPARα AND PPARγ: ANTICANCER TARGETS IN THE ENDOTHELIUM

3.1. PPARα and PPARγ ligands

When discussing the roles of PPARs it is important to note the types of ligands potentially used in studies. Activators

of PPARα include a variety of eicosanoids, fatty acids, and synthetic compounds including the clinically used dyslipidemic drugs, the fibrates (gemfibrozil, fenofibrate, bezafibrate, ciprofibrate) [40, 41]. Similarly, PPARγ activators also include a variety of eicosanoids, fatty acids, and synthetic compounds including the clinically used insulin sensitising thiazolidinedione drugs (rosiglitazone, pioglitizone, troglitizone (now withdrawn) [40, 41]. (See Figures 2 and 3.)

3.2. PPARα and PPARγ in cancer

One early observation regarding PPARα activation by peroxisome proliferators was the induction of hepatocarcinogenesis in rodents; an effect absent in PPARα (−/−) knockout mice [42, 43]. Although there has been a considerable amount of interest in the field, especially as the PPARα activating fibrates are in clinical use, there is no evidence that long-term activation of PPARα in nonrodent species including man is linked to hepatocarcinogenesis [42, 43].

In extrahepatic tissues, there have been fewer studies regarding PPARα and cancer. Initially, it was suggested that PPARα may prevent skin cancer [44, 45]. However, topical PPARα agonists were only moderately protective against tumour promotion in mouse skin, despite the upregulation of PPARα in tumours compared to normal epidermis [46]. Recent studies have revealed that PPARα is commonly expressed in tumour cell lines, including lung, liver, leukaemia, prostate, pancreas, bladder, colon, glioblastoma, hemangioma, melanoma, ovarian, and breast [47–49]. PPARα ligands inhibit the growth of colon, breast, endometrial, and skin cells in vitro [46, 48, 50–52] and human ovarian cancer [53], melanoma, lung carcinoma, glioblastoma, and fibrosarcoma [48]. PPARα ligands also decrease tumour development in colon carcinogenesis [52] and inhibit melanoma cell metastasis in vitro and in vivo [50, 54].

PPARγ is expressed in prostate, thyroid, colon, breast and hepatocellular carcinoma, gastric, pancreatic and lung cancer, neuroblastoma, astrocytoma, and glioma, where the receptors' ligands are antiproliferative and proapoptotic [35]. It is beyond the scope of this review to discuss all the findings of PPARγ in cancer, and there are a number of excellent reviews in the field [33, 35, 55, 56] including one on PPARγ and angiogenesis in this series [36].

The majority of the evidence points towards PPARγ ligands suppressing tumourgenesis, for example, the receptors' ligands inhibit the growth of xenografts of many of the aforementioned tumours in vivo [35]. However, in colon cancer, the beneficial role for PPARγ agonists is controversial [57]. In the APC^{min}/+ mouse, PPARγ ligands increased precancerous polyp formation and the frequency and size of tumours in the colon [58, 59]. In contrast, heterozygous loss of PPARγ increases colon cancer incidence in mice [60]. This latter study corresponds with most of the available data, suggesting that PPARγ has antineoplastic effects in colon cancer; a point further supported in colon cancer patient studies by the detection of mutations causing loss
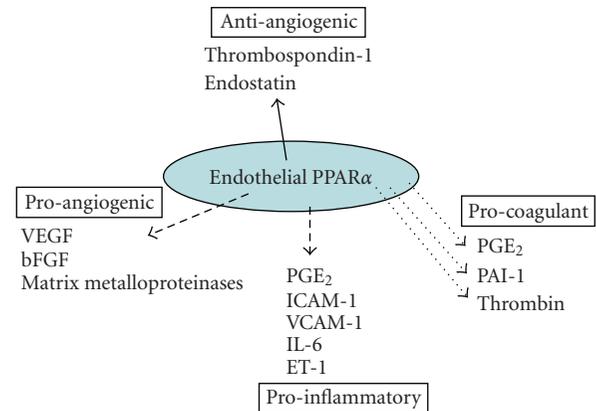
Figure 2: Endothelial PPARα has predominantly inhibitory actions on endothelial cell activation. The majority of studies so far indicate that PPARα activation induces (solid line) antiangiogenic factors, while reduces (broken line) proangiogenic factors, proinflammatory pathways, and procoagulant mediator release.
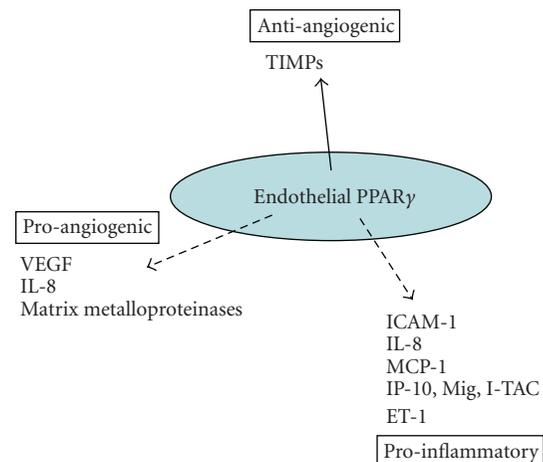
Figure 3: Endothelial PPARγ has predominantly inhibitory actions on endothelial cell activation. The majority of studies so far indicate that PPARγ activation inhibits (broken line) proangiogenic factors, proinflammatory pathways, and procoagulant mediator release, while inducing (solid line) antiangiogenic factors.

of function or impaired ligand binding of PPARγ [61] and polymorphisms of the PPARγ gene [62].

There have been positive results using PPARγ ligands to treat tumours experimentally both in vitro and in vivo, but so far this has not been successfully translated into a beneficial anticancer therapy in man. There have been a number of small scale clinical trials testing PPARγ ligands in cancer in man with varying success [63]. The most promising results were from small phase II studies treating prostate cancer [64] and liposarcoma patients [65] with troglitazone. In contrast, a phase II study treating liposarcoma patients with rosiglitazone did not significantly improve clinical outcome [66] and so far no beneficial effects of PPARγ ligands have been observed in trials for breast or colon cancer patients [35].

3.3. PPARα and PPARγ regulation of angiogenesis

Early studies showed no effect of the selective PPARα ligand WY-14643 on endothelial cell proliferation [27], however, recent studies using immortalised human dermal microvascular endothelial cells show that the PPARα ligand fenofibrate inhibits endothelial cell proliferation, migration, and tube formation (on a fibrin matrix) in vitro and angiogenesis in vivo [67]. Fenofibrate acts by disrupting the formation of the actin cytoskeleton and inhibits bFGF-induced Akt activation and cyclooxygenase 2 (COX-2) gene expression [67]. Similar results were found in a porcine model of vascular remodelling after coronary artery angioplasty where fenofibrate increased lumen size and vessel area and inhibited constrictive remodelling and inflammatory cell infiltration [68]. Importantly, adventitial angiogenesis was significantly reduced by fenofibrate in the injured vessels 3 days after angioplasty [68].

In contrast to this vascular study, the investigation of PPARα regulation of tumour angiogenesis has only just begun. In a recent report, Panigraphy et al. provide compelling evidence for PPARα inhibition of tumour growth by targeting angiogenesis [48]. Similar to previous findings, PPARα activation had direct effects on endothelial cells, inhibiting VEGF-induced endothelial cell migration in vitro and FGF2 induced corneal angiogenesis in vivo [48]. Tumour cell synthesis of VEGF and FGF2 was also suppressed by PPARα activation in conjunction with an increased expression of antiangiogenic thrombospondin-1 (TSP-1) [48]. In subcutaneously implanted human pancreatic cancer cells grown in mice, as well as in human prostate cancer, PPARα expression was detected not only in the tumour cells, but also in the new invading microvessels [48]. Systemic treatment of mice with PPARα ligands inhibited the growth of melanoma, glioblastoma, and fibrosarcoma tumours implanted in vivo, which was associated with a reduction in vessel density and inflammation [48]. To dissect the mechanism by which PPARα suppressed tumour growth (i.e., direct effects on the tumour and/or angiogenesis), embryonic fibroblasts from PPARα (−/−) knockout mice were transformed with SV40 large T antigen and H-ras oncogenes then implanted into wild-type and PPARα−/− mice. The growth of these cells into tumours could be suppressed by PPARα ligands in wild-type mice only, indicating that tumour suppression by PPARα ligands was completely dependent on the expression of PPARα in the host but not in the tumour cells [48]. Fenofibrate strongly induced the antiangiogenic factors TSP-1 and endostatin in wild-type, but not PPARα−/− mice, supporting the role of PPARα as an antiangiogenic regulator [48]. Angiogenesis and inflammation are central processes through which the tumour interacts with its surroundings to influence tumour growth. Although this study does not rule out an anti-inflammatory effect of the PPARα ligands, it is highly unlikely that the antitumour host-derived effects are due to suppression of inflammation because mice deficient in PPARα generally exhibit enhanced inflammation [64].

TSP-1 is a potent angiogenesis inhibitor that targets endothelial cells for apoptosis by initiating a signalling cascade through the CD36 receptor. PPARα directly induces TSP-1 and can enhance TSP-1 signalling indirectly by upregulating CD36 in the endothelium. PPARα activation upregulates CD36 expression in the liver [69] and in macrophages [70]. Moreover, coadministration of PPARγ ligands with exogenous TSP-1 or the TSP-1 peptide derivative ABT510 synergises to suppress angiogenesis and induce endothelial cell apoptosis [71]. The improvement of the antiangiogenic efficacy of TSP-1 was attributed to PPARγ-induced CD36 expression via a PPAR response element in the CD36 promoter [69, 71].

The vast majority of studies have indicated an antiangiogenic role for PPARα and PPARγ in a variety of models. However, it is important to note that the VEGF promoter contains a PPAR response element and PPARα and -γ ligands can induce VEGF in certain cell types [72–75]. Moreover, in contrast to the majority of findings, a recent study suggests that both PPARα and PPARγ ligands may also have proangiogenic properties in vitro in an endothelial/interstitial cell coculture assay and in a murine corneal angiogenesis model in vivo [72]. The angiogenesis induced by PPARα and PPARγ ligands was associated with the induction of VEGF, accompanied by increased activation of AKT and eNOS (by phosphorylation) [72]. How the levels of PPARα- or PPARγ-mediated angiogenesis are compared to traditional growth factor-induced angiogenesis is not known? Indeed, these results are controversial, as previous corneal angiogenesis models clearly demonstrate antiangiogenic effects of PPARα and PPARγ ligands [28, 48, 76].

Multiple mechanisms have been proposed by which PPARα and PPARγ regulate the changes in pro- and antiangiogenic factors. Here, we will focus on the central target for PPAR regulation of angiogenesis, the proangiogenic VEGF/VEGFR signalling pathway. PPARγ can downregulate VEGF either directly through a PPAR response element within the VEGF promoter [77] or by decreasing PGE_2, an endogenous stimulator of angiogenesis [78]. PPARγ can also decrease VEGF responses by suppressing transcription of its receptor VEGFR2, by interacting with and preventing Sp1 binding to DNA [79].

In colorectal cancer cell lines, PPARα also inhibits the transcription factor AP-1, impairing its binding to response elements in the VEGF and COX-2 genes and inhibiting c-jun transactivation activity, thus downregulating VEGF and COX-2 expression [80]. It is, therefore, clear that the regulation of angiogenic factors by PPARα and PPARγ may be determined by cell and cancer type and the experimental models used. Much more research is required to fully understand whether PPAR activation will be pro- or antiangiogenic in specific human cancers.

3.4. The effects of PPARα and PPARγ on endothelial progenitor cells

Endothelial progenitor cells (EPCs) present in peripheral blood promote angiogenesis and improve endothelial function. The research on the effects of PPARs on EPCs has focused on PPARγ. Despite PPARγ generally being considered antiangiogenic, the PPARγ ligands rosiglitazone and pioglitazone in diabetic patients increase endothelial

progenitor cell (EPC) number and migratory activity [81, 82]. Pioglitazone and rosiglitazone also improve the adhesive capacity of EPCs to fibronectin and collagen [82] and promote EPC colony formation, [83, 84]. In vitro, pioglitazone increased EPC proliferation, colony formation, and attenuated apoptosis [85]. Similarly, in mice pioglitazone induced the number and migratory activity of EPCs while decreasing their apoptosis, resulting in increased in vivo neoangiogenesis [86]. From these results, it has been proposed that PPARγ ligands may have a double-edged role in angiogenesis, with proangiogenic effects on EPCs at low-systemic concentrations and antiangiogenic effects at higher local concentrations [86]. Indeed, biphasic effects of pioglitazone were observed on EPCs in culture, when the number of EPC colonies and amount of adhesion were increased by $1\,\mu M$ but not $10\,\mu M$ [87]. This higher concentration of pioglitazone induced TGF-β1 and its receptor endogolin, which suppress EPC function [87]. These findings have important clinical implications suggesting that the pro-/antiangiogenic properties of PPARγ ligands may be largely dose-driven. Moreover, understanding this mechanism by which PPARγ may regulate both pro- and antiangiogenic pathways at least in EPCs may help to explain some of the contradictions in the studies examining the role of PPARγ in angiogenesis.

3.5. Effects of PPARα and PPARγ on endothelial cell inflammation

The role of PPARα in inflammation has been studied in animal models, particularly in wound healing and cardiovascular disease models (atherosclerosis and restenosis) [55, 56]. PPARα is a negative regulator of inflammation [34] in inflammatory models. Supporting this, PPARα−/− mice exhibit enhanced inflammation [88], although this may be due in part to deceased β-oxidation and accumulation of biologically active lipid mediators.

In addition to these experimental models, PPARα agonists decrease the expression of inflammatory markers both in human cells and patients treated with fibrates [89, 90]. In human endothelial cells in culture, PPARα ligands inhibit the cytokine/LPS induction of COX-2 [38, 69], ICAM-1 [91], VCAM-1 [29, 31], endothelin-1 [92], IL-6, and prostaglandin E$_2$ [32, 93]. Similarly, PPARα ligands repress thrombin-induced expression of endothelin-1 [32]. The PPARα ligand fenofibrate, but not the PPARγ ligand rosiglitazone, also reduces the induction of tissue factor in human endothelial cells [94], while PAI-1 levels remain unchanged [31]. PPARα inhibits proinflammatory mediators by interfering with the transactivation activity of NFκB and AP-1, the main transcription factors mediating inflammatory and growth factor responses. PPARα via direct protein-protein interactions can bind and inhibit the actions p65 and c-jun subunits, respectively [95, 96].

Although the weight of evidence points towards an anti-inflammatory role for PPARα, oxidised lipids that can activate PPARα have been shown to increase the release of
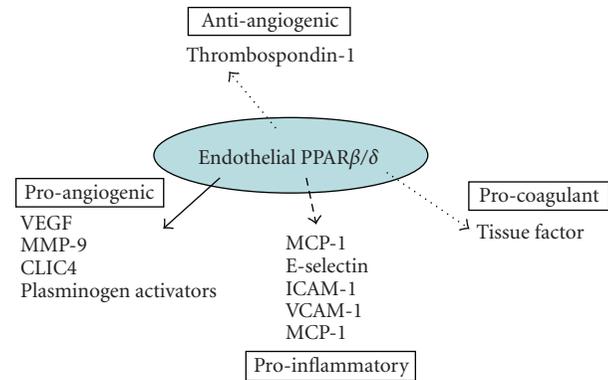
Figure 4: Endothelial PPARβ/δ has predominantly proangiogenic actions on endothelial cells. The majority of studies so far indicate that PPARβ/δ activation induces (solid line) proangiogenic factors, while reduces (broken line) antiangiogenic factors. Similar to PPARα and PPARγ, PPARβ/δ also appears to be anti-inflammatory by reducing proinflammatory pathways and potentially anticoagulant by reducing tissue factor release.

neutrophil chemoattractant IL-8 and MCP-1 from endothelial cells [30]. Similarly, PPARα ligands induce COX-2 in human breast and colon cancer cells [97, 98].

PPARγ, similarly, is a well-established negative regulator of the inflammatory response in vitro and in vivo [34]. PPARγ agonists have been shown to mediate effects on cell survival, surface-protein expression, and cytokine and chemokine production. In endothelial cells, PPARγ ligands can induce apoptosis [27] and decrease inflammatory cell recruitment by inhibiting the production of chemokines IL-8, MCP-1 [30, 99], IP-10, Mig, and I-TAC [100] and reducing ICAM-1 expression [101]. Similar to PPAR-α, PPARγ ligands repress thrombin-induced expression of endothelin-1 [32].

4. PPARβ/δ

4.1. PPARβ/δ ligands

PPARβ/δ (Figure 4) is almost ubiquitously expressed [102], although compared to PPARα and -γ, less is known regarding its role in the body. However, like PPARα and -γ, it appears able to regulate lipid metabolism, cellular proliferation, and the inflammatory response [55, 56]. Activators of PPARβ/δ include a variety of eicosanoids (the COX product prostacyclin [40, 41], COX/prostacyclin synthase-derived endocannabinoid metabolites [103]); fatty acids and synthetic compounds including GW0742X, GW501516, L-165,461, and compound F [40, 41].

4.2. PPARβ/δ and cancer

There has recently been an increasing amount of contradictory literature published regarding PPARβ/δ regulation of tumour cell growth and tumour cell release of VEGF. PPARβ/δ ligands induce VEGF in bladder cancer [104], human breast (T47D, MCF7) and prostate (LNCaP, PNT1A) cancer cell lines, along with its receptor VEGFR1 [105],

but not in colon (HT29, HCT116, LS-174T) and hepatoma (HepG2, HuH7) cell lines [106].

Much of the research into PPARβ/δ in cancer has focused on gastrointestinal cancer. PPARβ/δ expression is enhanced in human and rodent colorectal tumours, as well as preneoplastic colonic mucosa [107, 108]. PPARβ/δ is transcriptionally regulated by β-catenin/Tcf-4, which can be suppressed APC. Therefore, in colorectal cancer cells that commonly carry an APC mutation, PPARβ/δ is upregulated [108]. Interestingly, PPARβ/δ accumulation was localised to human colorectal carcinoma cells with a highly malignant morphology [109], suggesting PPARβ/δ promotes tumourogenesis. Supporting this theory, the growth of PPAR$\beta/\delta-/-$ HCT-116 human colon carcinoma cell xenografts was reduced compared to wild-type PPARβ/δ expressing cells [83].

Using animal models, a positive link has been made between PPARβ/δ and colon cancer development, especially using the intestinal polyp model, APC^{min}/+ mice. In this model, deletion of PPARβ/δ decreases intestinal adenoma growth and inhibits the tumour-promoting effects of the PPARβ/δ agonist GW501516 [85, 110]. PPARβ/δ activation induces VEGF in colon carcinoma cells, promoting cell survival by activation of Akt signalling [85]. Angiogenesis was not studied in these experiments, however, for a tumour to grow greater than 2 mm in diameter a functional vessel network is required [111]. Indeed, the most prominent effect of PPARβ/δ activation in APC^{min}/+ mice, observed by Gupta et al., was a significant increase in the number of polyps greater than 2 mm in diameter [110]. Whereas there was a significant decrease in the growth of polyps greater than 2 mm in diameter in PPAR$\beta/\delta-/-$ APC^{min}/+ mice, despite a lack of effect on overall polyp incidence [112]; indicating that PPARβ/δ promotes tumour growth via angiogenesis.

In contrast, deletion of PPARβ/δ in APC^{min}/+ mice enhanced colon polyp formation in untreated mice and in mice with chemically induced colon carcinogenesis [113, 114]. The PPARβ/δ ligand GW0742 inhibited chemically induced colon carcinogenesis in PPARβ/δ wild-type but not PPAR$\beta/\delta-/-$ mice [115]. The differences between these contrasting results have been suggested to be due to differences in genetic background, breeding, or the PPARβ/δ knockout strategy of the APC^{min}/+ mouse models [116]. However, this would not explain why in human colon and liver cancer cell lines, PPARβ/δ ligands had no effect on cell growth, Akt phosphorylation, or VEGF and COX-2 expression in vitro or on these markers in the liver, colon and colon polyps in mice treated in vivo [106]. The role of PPARβ/δ in VEGF-mediated tumourgenesis, therefore, still requires further study and clarification.

4.3. PPARβ/δ and angiogenesis

Initial reports using prostacyclin as a ligand suggested that similar to PPARα and PPARγ, PPARβ/δ promoted endothelial cell apoptosis [117], and potentially decreased angiogenesis. In contrast, with the development of highly selective synthetic ligands, there is an increasing evidence to propose

a role for PPARβ/δ in regulating endothelial cell survival, proliferation, and angiogenesis. Indeed, treating endothelial cells with the selective PPARβ/δ ligand GW501516 induces proliferation, VEGF receptor (Flt-1; VEGF R1) expression, and VEGF production [105, 118]. In addition to inducing proliferation, PPARβ/δ also protects the endothelial cell from oxidant injury via induction of the antiapoptotic and anti-inflammatory protein 14-3-3α [119].

PPARβ/δ potently induces angiogenesis by human and murine vascular endothelial cells in tumour extracellular matrix in vitro and in a murine matrigel plug model in vivo [118]. The stimulated release of VEGF from human endothelial cells was a major trigger for morphogenesis, although mRNA for the matrix metalloproteinase (MMP)-9, a protease important for cell migration, was also elevated [118]. In addition to VEGF, genomic and proteomic analysis of PPAR$\beta/\delta-/-$ endothelial cells isolated from matrigel plugs identified a number of additional candidate genes that may mediate the angiogenic actions of PPARβ/δ. Cdkn1c, which encodes the cell cycle inhibitor p57^{Kip2}, is induced by PPARβ/δ [120]. The chloride intracellular channel protein (CLIC)-4 is decreased in migrating endothelial cells from PPARβ/δ knockout mice, whereas the expression of cellular retinol binding protein CRBP1 is increased [121]. CLIC-4 plays an essential role during tubular morphogenesis [122], while CRBP1 inhibits cell survival pathways by blocking the Akt signalling pathway [123]. The combination of these studies indicates that PPARβ/δ may induce endothelial cell mitogenesis and differentiation signals, including VEGF, 14-3-3α, CLIC4, CRBP-1, and p57^{KIP2}, which may combine to bring about the functional morphogenic changes associated with the angiogenic switch.

Two recent studies in particular have addressed the regulation of angiogenesis by PPARβ/δ in matrigel plugs in PPARβ/δ wild-type and knockout mice [120, 124]. Xenograft tumours in PPAR$\beta/\delta-/-$ mice exhibited a diminished blood flow and immature hyperplastic microvascular structures when compared to wild-type mice. Moreover, the reintroduction of PPARβ/δ into the matrigel plugs was able to rescue the knockout phenotype by triggering microvessel maturation [120]. In addition, tumour angiogenesis and growth are markedly inhibited in PPAR$\beta/\delta-/-$ mouse models of subcutaneous Lewis lung carcinoma and B16 melanoma. PPARβ/δ expression correlated with advanced pathological tumour stage and increased risk for tumour recurrence and distant metastasis in pancreatic tumours from patients who had undergone the "angiogenic switch" [124]. PPARβ/δ has, therefore, been suggested as a "hub node" transcription factor, regulating the tumour angiogenic switch [124].

4.4. The effects of PPAR$\gamma\beta/\delta$ on endothelial progenitor cells

Little is known about the effects of PPARβ/δ on EPCs, but there is one study that shows that PPARβ/δ is a key regulator of EPC proangiogenic functions. Prostacyclin is a putative PPARβ/δ ligand and proangiogenic factor, produced by COX and PGI$_2$ synthase in the endothelium. EPC tube formation

and proliferation are induced by the selective PPARβ/δ ligand GW510516. EPCs treated with an inhibitor of COX or COX-1, prostacyclin synthase, or PPARβ/δ specific siRNA, exhibit decreased cell proliferation and tube formation [125]. Thus the proangiogenic effects of human EPCs appear in part dependent on the biosynthesis of prostacyclin and the subsequent activation of PPARβ/δ.

4.5. The effect of PPARβ/δ on endothelial cell inflammation

Little is known regarding the role of PPARβ/δ in endothelial cell inflammation and mediator secretion. PPARβ/δ ligands, similar to PPARα and PPARγ ligands, inhibit cytokine-stimulated upregulation of adhesion molecules ICAM-1, VCAM-1, and e-selectin and NFκB translocation [126, 127]. These anti-inflammatory effects of PPARβ/δ in endothelial cells occur when the complex between PPARβ/δ and the transcriptional repressor BCL6 is removed by ligand activation, identical to the mechanism identified in monocytes [128]. PPARβ/δ and BCL6 are then free to act on PPARβ/δ targets (including SOD and catalase) and BCL6 targets which importantly include the repression of NFκB. In addition to anti-inflammatory effects, endogenous PPARβ/δ ligands are continuously produced in endothelial cells to suppress the release of tissue factor, the primary initiator of coagulation [103].

5. PPAR THERAPY FOR CANCER

The PPARs have pleiotrophic actions on nonvascular and vascular cells. PPARα and PPARγ ligands (although there are well-detailed current concerns for rosiglitazone) are in clinical use, are considered safe, and have high tolerability with chronic use. There is considerable evidence that PPARγ and increasing evidence that PPARα are vascular protective and reduce angiogenesis. Unfortunately, as yet, there is a little clinical evidence to support these actions, apart from the promising results with the PPARγ ligand troglitazone in liposarcoma and prostate cancer previously mentioned [64, 65]. Clinically, PPARα and γ ligands do not appear to be strong antiangiogenic drugs. However, since PPARα and PPARγ ligands are in clinical use and lack severe side effects, the potential for their use to complement or augment current and new therapies to treat a variety of cancers is currently being tested in small scale trials. For example, a phase II trial combining anti-inflammatory and angiostatic therapy (PPARγ ligand pioglitazone and COX-2 inhibitor, rofecoxib) with metronomic low-dose chemotherapy (trofosamide) found that the progression-free survival rates of advanced melanoma patients were longer with the combination treatment than with metronomic chemotherapy alone [129]. This combination therapy was also successful in achieving disease stabilization or remission in patients with advanced progressive malignant vascular tumours [130] and partial remission in a single patient with endemic Kaposi sarcoma [131]. However, a similar phase II study on high-grade glioma patients, showed disease stabilisation in only 4 out of 14 patients, suggesting that

this combined therapy may only be suitable for a subset of patients [132]. The COX-2 inhibitor rofecoxib was included in the trial because COX-2 plays a role in endothelial tube formation, pericyte recruitment, and endothelial cell survival during early angiogenesis [133]. As PPARα and γ ligands have been shown to inhibit COX-2 induction in endothelial cells, it would be interesting to test the combined effects of PPARα or $-\gamma$ ligands with metronomic chemotherapy alone.

In contrast to PPARα and PPARγ, there is increasing evidence that PPARβ/δ is proangiogenic and an important transcription factor in the angiogenic switch. PPARβ/δ has an interesting activity profile in that like the other PPARs it also appears to have anti-inflammatory properties. As PPARβ/δ is considered a target to treat dyslipidaemia, its proangiogenic properties should, therefore, be considered in the long-term use of PPARβ/δ ligands to treat chronic metabolic diseases. The development of selective antagonists for PPARβ/δ offers great potential for cancer treatment. One such antagonist has recently been identified, GSK0660, which can compete with agonist in a cellular context and by itself exhibits inverse agonist activity [134]. This antagonist appears to act by promoting PPARβ/δ-mediated repression of gene expression. Unfortunately, this compound lacks in vivo bioavailability, but will be a valuable tool for elucidating the role of PPARβ/δ in cancer and angiogenesis in vitro and a basis for further development of a selective bioavailable PPARβ/δ antagonist [134]. Selective modulators of PPARβ/δ, which maintain the beneficial metabolic (and anti-inflammatory) effects while exerting no proangiogenic effects would also be beneficial. Interestingly, there is a newly developed PPAR-α agonist (R)-K-13675, which inhibits the secretion of inflammatory markers without affecting cell proliferation or endothelial tube formation [135], which suggests that selective modulators for the other PPARs may soon be available.

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Review Article

PPARγ and Agonists against Cancer: Rational Design of Complementation Treatments

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PPARγ is a member of the ligand-activated nuclear receptor superfamily: its ligands act as insulin sensitizers and some are approved for the treatment of metabolic disorders in humans. PPARγ has pleiotropic effects on survival and proliferation of multiple cell types, including cancer cells, and is now subject of intensive preclinical cancer research. Studies of the recent decade highlighted PPARγ role as a potential modulator of angiogenesis in vitro and in vivo. These observations provide an additional facet to the PPARγ image as potential anticancer drug. Currently PPARγ is regarded as an important target for the therapies against angiogenesis-dependent pathological states including cancer and vascular complications of diabetes. Some of the studies, however, identify pro-angiogenic and tumor-promoting effects of PPARγ and its ligands pointing out the need for further studies. Below, we summarize current knowledge of PPARγ regulatory mechanisms and molecular targets, and discuss ways to maximize the beneficial activity of the PPARγ agonists.

1. INTRODUCTION

PPARs are nuclear hormone receptors and targets for the compounds inducing peroxisome proliferation. The family encompasses three species, PPARα, PPARβ/δ, and PPARγ. PPARγ, the best researched of the three, is presented by the two isoforms, γ1 and γ2 whereas PPARγ2 contains 30 extra amino acids at the N-terminus due to initiation from the alternative transcription start (see Figure 1(a)). PPARγ, a key player in adipocyte differentiation and glucose metabolism, is abundantly expressed in adipose tissues [1]. On the other hand, it is expressed in all the cells of the normal and pathological vascular beds, including endothelial cells (EC), macrophages (MΦ), and vascular smooth muscle cells (VSMCs), in a variety of tumor cells, and, at lower levels, in lymphatic tissue, intestinal epithelium, retina, and skeletal muscle [2]. PPARγ is a potent modulator of the EC and VSMC function and inflammation: its effects on the tumor cells, tumor-associated MΦs (TAM), and tumor vasculature (EC and VSMCs) significantly attenuate tumor progression [3, 4], suggesting that PPARγ ligands may become new convenient therapeutic modifiers targeting simultaneously tumors and their microenvironment [5].

Unfortunately, recent studies reveal the tumor-promoting and pro-angiogenic PPARγ activities; while in most cases PPARγ agonists attenuate tumor growth and angiogenesis, troglitazone (TGZ, a now rejected PPARγ agonist) promotes hepatic carcinogenesis and liposarcomas. Moreover, some PPARγ agonists promote the differentiation of the circulating endothelial progenitor cells (EPC) [6] and elicit angiogenesis in vivo [7]. In some instances, PPARγ ligands increase the production of angiogenic stimuli, including VEGF or NO, by the EC or tumor cells [8]. Thus, the use of PPARγ modulators to manage tumor progression is more complex than it appears at a glance and requires precise knowledge of the molecular events involved in their pro- and antitumorigenic actions. Below we summarize the current knowledge of PPARγ effects and molecular mechanisms and delineate ways to augment PPARγ anti-angiogenic and antitumor effects while minimizing its pro-angiogenic and tumor-promoting capacities.

2. PPARγ AND ANGIOGENESIS

Angiogenesis is a complex process involving diverse cell types and controled by the pro- and anti-angiogenic factors
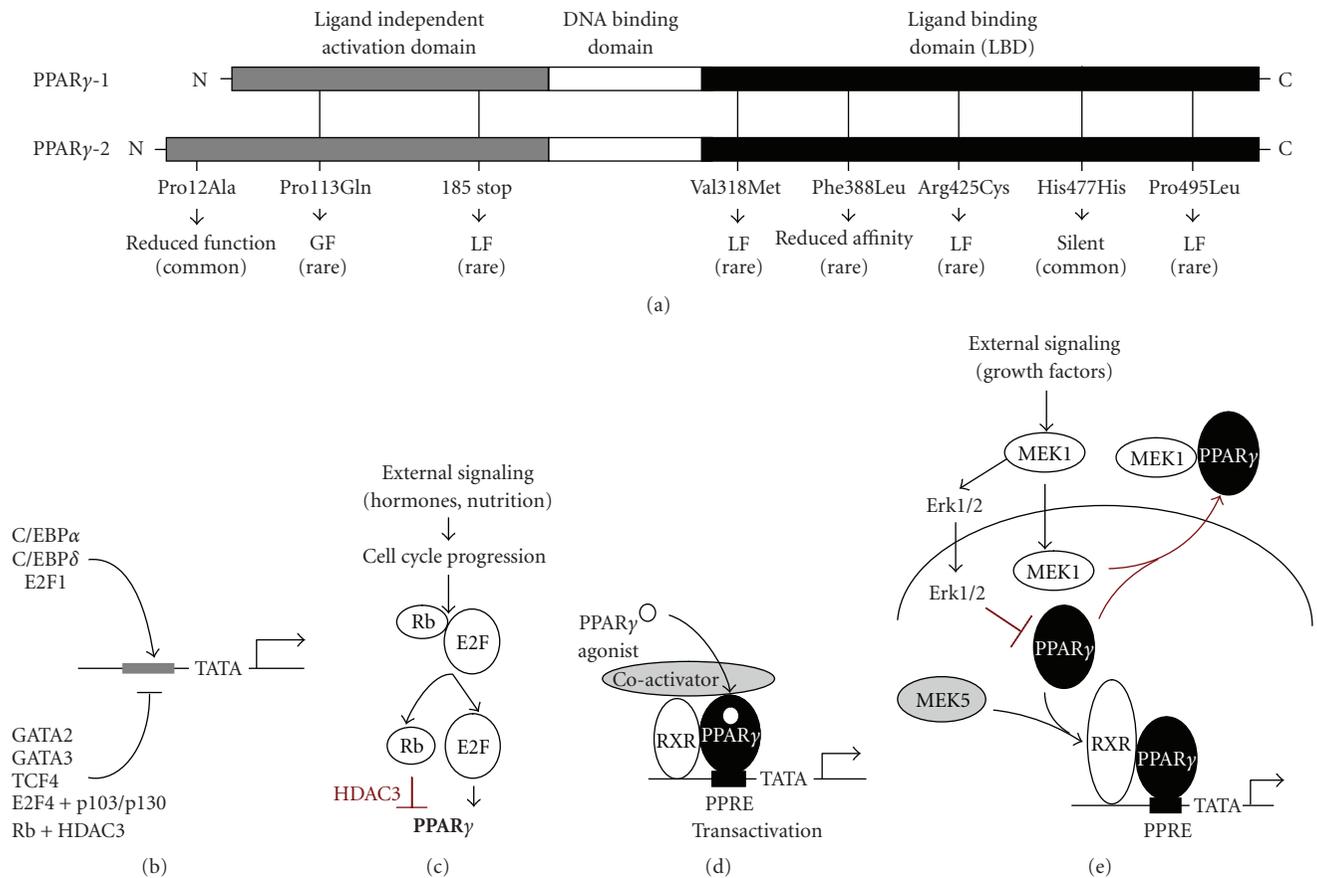
Figure 1: PPARγ structure and regulation. (a) Schematic representation of the domain structure of the PPARγ-1 and PPARγ-2. The mutations associated with metabolic syndrome are indicated. LF: loss of function; GF: gain of function. (b) Positive and negative regulators of the PPARγ gene transcription. (c) The regulation of PPARγ levels by Rb and E2F. (d) The mechanism of ligand-dependent PPARγ activation. (e) The regulation of PPARγ activity by MEK and Erk kinases: MEK1 activates Erk-1/2, which phosphorylates PPARγ and targets it to proteasomes; in addition, MEK1 binds PPARγ in the nucleus and exports it to the cytoplasm. MEK5 can serve as coactivator for the PPARγ.

produced by the ECs, VSMCs, and in vascular microenvironment by the stromal, tumor, and inflammatory cells. The balance between positive and negative angiogenesis regulators determines if the existing capillaries would expand, regress, or remain quiescent [9]. Active angiogenesis involves invasion, migration, and proliferation of the EC followed by the morphogenesis (assembly) of the neovessels. It is aided by the recruitment of the EPCs, which may constitute up to 50% of the cells in a neovessel [10]. The newly formed capillaries recruit vascular smooth muscle cells (VSMCs), which stabilize and render quiescent the newly formed capillaries: in thus stabilized mature vessels, the interactions between angiopoietin-1 (Ang-1) on the EC and Tie-2 receptor on the VSMCs generate signals that dampen EC sensitivity to the pro- and anti-angiogenic molecules [11]. Brown adipose tissue, a thermogenic organ in mammals responds to cold by increasing VEGF, thus creating permissive conditions for the fat expansion. Treatment of brown adipocytes with PPARγ ligands reduces VEGF-C mRNA pointing to their anti-angiogenic potential [12]. Moreover, chimeric mice null for PPARγ show gross defects in placental vascularization [13]. Natural and synthetic PPARγ ligands block VEGF-

driven angiogenesis in vivo, in matrigel implants, in rodent cornea, and choroid [14–16]. RGZ suppresses the growth and angiogenesis of the glioblastoma, Lewis lung carcinoma, liposarcoma, and rhabdomyosarcoma in mouse models [17], which is partly due to the PPARγ-mediated apoptosis of the tumor EC and the repression of VEGF production by the tumor cells. Below, we elucidate the PPARγ pleiotropic effects on angiogenesis and suggest optimization strategies.

3. PPARγ REGULATORY MECHANISMS

PPARγ can be regulated at expression level: PPARγ gene is repressed by the GATA-2 and 3, TCF4 [18] (see Figure 1(b)), and transactivated by CAAT enhancer binding proteins (C/EBPs), predominantly C/EBPα, ADD1/SREBP1, and E2F1 (see Figure 1(b)) [19]. E2F proteins have dual effect on PPARγ expression: during cell cycle progression, phospho-Rb releases E2F1 to activate PPARγ promoter (see Figure 1(c)), however, E2F4, if bound to the p103 or p130 Rb, represses PPARγ transcription [2, 18]. Moreover, hypo-phosphorylated Rb binds PPARγ and recruits histone
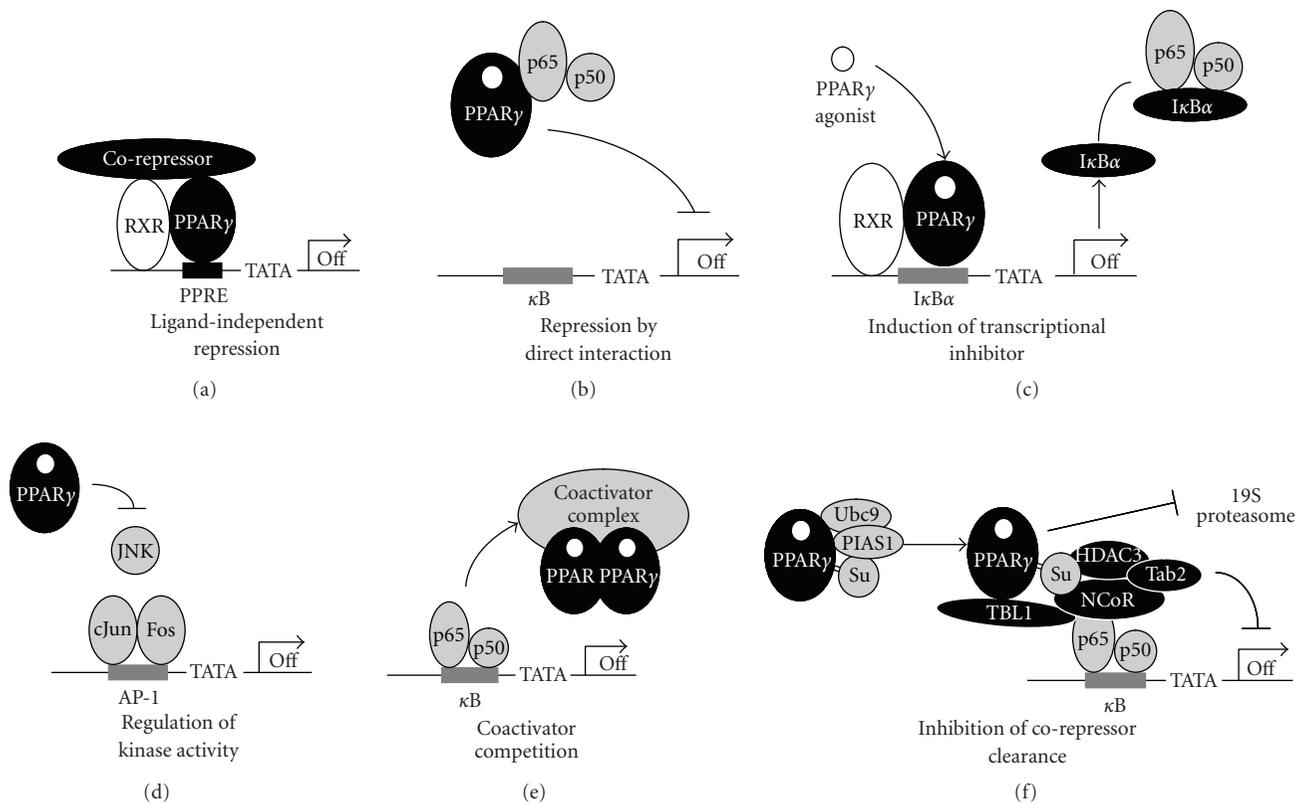
FIGURE 2: Mechanisms of transrepression by PPARγ. (a) Ligand-independent repression: preferential recruitment of corepressors in the absence of agonists. (b) Direct binding and sequestration of transcription factors on example of NFκB. (c) Activation of genes encoding inhibitors of transcription factor (e.g., NFκB inhibitor, IκBα). (d) Direct binding and inactivation of kinases, which activate transcription factors (e.g., the blockade of JNK activation of cJun). (e) Competitive binding of the coactivator complex. (f) The blockade of corepressor clearance: sumoylated PPARγ stabilizes corepressor complexes (NCoR, Tab2, and TBL1) on the promoter and facilitates the recruitment of HDAC3. In the absence of sumoylation, NCoR, Tab2, and TBL1 are subject to ubiquitination and proteasomal clearance.

deacetylase (HDAC) 3 to the complexes, causing transcriptional repression (see Figure 1(c)) [19]. Multiple growth factors including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiotensin II, tumor necrosis factor (TNF) α, interleukin (IL) 1β, and tumor-derived growth factor β(TGF-β) increase PPARγ expression by the vascular smooth muscle cells (VSMCs), via Egr-1. In contrast, AP-1 aided by Smad3/4 represses PPARγ promoter activity [20]. Mitotic, stress, and inflammatory signals cause PPARγ degradation via phosphorylation on Ser84 of the mouse PPARγ (Ser112 of the human molecule) in a consensus MAPK target motif PXSPP [21] by ERKs, JNKs, and p38, which leads to ubiquitination and proteasomal clearance [22]. Ser to Ala PPARγ mutant shows increased transcriptional activity, similar effect is caused by coexpression of a phosphoprotein phosphatase [21]. In human PPARγ, substitution of proline to glutamine at position 115 results in constitutive activation by blocking MAPK phosphorylation at position 114: patients with such mutation display extreme obesity [23]. Likewise, increased phosphorylation on Ser112 in Dok-1 null mice caused lean phenotype, which is lost in mice expressing phosphorylation-defective PPARγ [24]. The effect of PPARγ on angiogenesis remains to be determined.

The next regulatory step involves cofactor recruitment: upon ligand binding, PPARγ forms heterodimers with the retinoic acid X receptor (RXR), and occupies twin PPAR response elements AAGGTCAnAAGGTCA (PPRE); binding of the RXR ligands further increases transcriptional activity of the PPARγ/RXR dimers (see Figure 1(d)). Coactivators including SRC1, CBP/p300, pCAF/GCN, and PGC bind PPARγ/RXR complexes in a ligand-dependent manner [19]; PGC-1α has recently been linked to HIF-independent induction of vascular endothelial growth factor (VEGF) and angiogenesis [25]. PPARγ activity can be suppressed due to phosphorylation, which results in nuclear export, both executed by MEK-1 (see Figure 1(e)) [26]. In contrast, MEK-5 acts as PPARγ coactivator (see Figure 1(e)) [27].

In addition to its activator function (see Figure 1(d)), PPARγ represses transcription of select genes. PPARγ transrepression of AP-1, nuclear factor of the activated T-cells (NFAT), NFκB, and STAT-1 is well documented [19, 28]. Typical PPARγ corepressors SMRT and NCoR corecruit HDAC3, transducin beta-like protein-1 (TBL-1) and TBL-1-related protein 1 (TBLR1) [29]. The repression can be ligand-independent, with PPAR/RXR dimers forming repressor complexes in the absence of the ligands (see Figure 2(a)). Ligand-dependent repression may occur by

direct interaction with target transcription factors (see Figure 2(b)), modulation of the transcriptional regulators (see Figures 2(c) and 2(d)), by coactivator sequestration (see Figure 2(e)), or the blockade of corepressor clearance (see Figure 2(f)). The latter requires PPARγ sumoylation, which keeps HDAC3 associated with repressor complexes and prevents proteasomal clearance of their components [19]. NCoR complexes interact with a limited subset of promoters, which explains gene-specific repression by PPARγ.

4. LIGANDS

PPARγ ligands encompass wide range of structurally diverse compounds, natural and synthetic. Natural ones include long chain polyunsaturated fatty acids and derivatives (eicosanoids, prostaglandins, like 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15D-PGJ$_2$)) and nitrolinoleic acids. Synthetic ones include thiazolinediones (TZDs, or glitazones), of which rosiglitazone (RGZ) and pioglitazone (PGZ) are marketed for the treatment of type 2 diabetes and tyrosine-based derivatives (glitazars) including tesaglitazar and farglitazar, the dual agonists of PPARα and PPARγ [30]. Although their ability to alleviate insulin resistance, vascular complications, and angiogenesis is well documented, the adverse effects include hepatotoxicity, renal toxicity, weight gain, and fluid retention [30], all of which complicate the long-term use. Thus further work is required to develop PPARγ ligands into safe and efficacious treatment for diabetes, cancer, and angiogenesis-related disease. Selective PPARγ modulators (SPPARMs) represent one way to overcome this problem: they are designed to retain the desired PPARγ properties, while minimizing adverse side effects. SPPARMs can be categorized as tightly binding partial agonists (GW0072) or weakly binding full agonists of PPARγ (MCC-555/netoglitazone, NC-2100) [31].

5. ANTI-ANGIOGENIC EFFECTS OF PPARγ IN DIVERSE CELL TYPES: ENDOTHELIAL-SPECIFIC EVENTS

Human micro- and macrovascular endothelial cells (EC) express PPARγ [32]. PPARγ activation by the natural (15D-PGJ$_2$) or synthetic ligands (TGZ, RGZ, ciglitazone, and pioglitazone) potently inhibits in vitro proliferation and morphogenesis by EC of diverse tissue origin [33]. 15D-PGJ$_2$ and ciglitazone (CGZ) also induce EC apoptosis through PPARγ-dependent pathway. The PPARγ involvement is supported by (1) nuclear translocation, (2) increased transcriptional activity, (3) attenuation of the EC apoptosis by the decoy PPRE oligonucleotide, and (4) increased background apoptosis in PPARγ overexpressing EC, further enhanced by the ligand exposure [15]. PPARγ activation interferes with EC migration: TZDs block EC chemotaxis up the VEGF or leptin gradients, by blocking PI3K/Akt and Erk1/2 signaling [34–37]. In both cases, PPARγ/SREBP1 complex drives the transcription of PTEN tumor suppressor, which opposes the induction of Akt [38], see Figure 4(a).

PPARγ ligands hamper the response of the vascular EC to VEGF by lowering VEGFR1 (Flt-1) and VEGFR2 (KDR). The regulation of VEGFR2 is biphasic: in the absence of the

ligands, PPARγ enhances Sp1/Sp3 binding to the promoter and opposes it if ligands are present [39]. VEGFR2 decrease also reduces EC survival under stress or in the presence of anti-angiogenic factors, see Figure 4(a).

PPARγ induction decreases UPA and increases PAI-1 expression by the EC, thus lowering their ability to invade surrounding tissues [14, 16]. In the brain microvasculature, PPARγ stimulation dampens the activation of RhoA and Rac1 GTPases critical for the cell adhesion and migration [40], see Figure 4(a).

Proapoptotic PPARγ effects in the EC can be mediated by p53 [41–43] or by the opening of Maxi-K channel (Ca2$^+$ activated K$^+$ channel) whereas the protective Bcl-2 levels plummet and apoptotic Bax increases. In addition, increased eNos production causes elevated NO, which, in contrast with its usual protective effect contributes to EC death [44]. Downmodulation of the thioredoxin (Trx-1) by PPARγ via vitamin D3 upregulated protein (VDUP-1) also contributes to the EC killing, likely via formation of inactive PTEN/Trx-1 complexes [45]. PPARγ also ameliorates EC activation by glucose via the induction of diacylglycerol kinase (DGK), the reduction of diacylglycerol, which attenuates PKC activity and decreases angiogenesis [46]. Importantly, PPARγ activation enhances surface CD36, a lipid scavenger receptor, which transmits the anti-angiogenic signal of thrombospondin-1 (TSP1) [47] a potent endogenous inhibitor of angiogenesis, see Figure 4(a).

PPARγ produces complex effect on the endothelial progenitor cells (EPC): RGZ enhances the expression of the endothelial markers CD31 and VEGFR2 on the circulating EPCs, however VE-cadherin and CD146 remain low; increased uptake of oxidized lipids suggests elevated CD36, which increases the sensitivity to TSP1. EPCs from the diabetic patients treated with RGZ display better adherence to fibronectin than those from untreated diabetics and normal donors [6]. This is consistent with reduced oxidative stress and improved re-endothelialization by the EPCs from diabetic patients in RGZ-treated mice [48]. EPCs from the RGZ-treated diabetics migrate more vigorously than those from untreated subjects, but similarly to the EPC from untreated normal donors [6] suggesting that RGZ rather normalizes than increases the EPCs migratory potential. PGZ effect on cultured EPCs is twofold: it enhances the expression of endothelial markers at a lower dose (1 μm) and reduces it at higher (10 μm) concentration. PGZ also stimulates the expression of TGFβ and TGFβ receptor [49], and thus initiates EPC conversion to the VSMC phenotype [50]: increased VSMC presence may stabilize the neovasculature and thus reduce angiogenesis. This may explain why PPARγ agonists ameliorate glomerulonephritis in mouse model without increase in EPC homing [51].

6. IN VASCULAR SMOOTH MUSCLE CELLS

Genetic variations associated with atherosclerosis point to PPARγ role in associated metabolic and vascular events [52]. In atherosclerotic lesions, PPARγ promotes vascular repair and re-endothelialization, while suppressing neointima formation. PPARγ attenuates vasoconstrictive remodeling by

blocking NADPH oxidases [53] and inhibits VSMCs proliferative and migratory responses to multiple cytokines and growth factors including PDGF-BB, bFGF, thrombin, insulin, and angiotensin II (AngII). PPARγ interferes with VSMC proliferation and survival by blocking the downstream targets of ERK1/2 and PI3K/Akt, SHIP2 and two important regulators of mRNA translation, p70S6 kinase and 4-EBP translation initiation inhibitor [54]. In addition, PPARγ activation enhances the expression of Shp-2 phosphatase, which dephosphorylates/inactivates Vav, a guanidine exchange factor for RhoA, impairs the activation of Rho-associated kinase (ROCK), and suppresses VSMC proliferation and migration [55]. PPARγ inhibits VSMC migration but not the attachment and motility components of the migratory response: the inhibition of PDGF-BB driven VSMC migration is due to the transcriptional repression of Ets-1, which, in turn, drives MMP-9 and invasion [56], see Figure 4(b).

PPARγ activation causes VSMC growth arrest via multiple pathways: (1) by suppressing proteasomal degradation of the p27/Kip; (2) via transrepression of the E2F target, minichromosome maintenance protein, MCM7, which blocks replication [2]; (3) by blocking Ets-1 dependent transactivation of telomerase promoter [57]. PPARγ and its agonists potently induce VSMC apoptosis (1) through direct upregulation of GADD45 and p53 via an Oct-1 dependent mechanism (PPRE are identified in GADD45 and p53 promoters) [58, 59]; (2) by inducing the TFG-β/ALK/Smad pathway, subsequent Bcl-2 repression, and Smad-dependent induction of GADD45 [60]; (3) through transcriptional upregulation of the interferon regulatory factor-1 (IRF-1), a proapoptotic, antiproliferative transcription factor [61], see Figure 4(b).

All PPARγ-dependent changes in VSMC behavior can contribute to its anti-angiogenic function: decreased VSMC migration, and proliferation, plus increased apoptosis restrict VSMC incorporation in the vasculature and therefore the stability of neovessels. Moreover, ECs of the immature, VSMC-poor vessels are vulnerable to the apoptotic signals by angiogenesis inhibitors, see Figure 4(b).

7. ANTI-INFLAMMATORY EFFECTS

PPARγ affects inflammation directly, by driving CD36-dependent apoptosis in MΦs [62, 63], or indirectly, by reducing VCAM-1 expression by the ECs and thus blocking transendothelial migration (TEM) of monocytes and MΦs during chronic inflammation typical for diabetes and cancer. In contrast, E-selectin, a mediator of the acute immune response, is not altered by PPARγ [64]. Statins increase anti-inflammatory Cox-2 in MΦs, which, in turn, increases endogenous 15D-PGJ$_2$, activates PPARγ, and upregulates its downstream target, CD36 [65]. In addition, PPARγ ligands cause NFκB transrepression, thus reducing the production of inflammatory cytokines (IL-8, IL-6, MCP-1, and CX3CL1-1) by MΦs, and thus disrupting paracrine loop that attracts tumor-associated MΦs (TAM) and thus stimulates angiogenesis and tumor growth [66], see Figure 4(c).

8. IN TUMOR CELLS AND STROMA

PPARγ is expressed in human carcinomas of the breast, colon, esophagus, liver, lung, pancreas prostate, stomach, and thyroid, also in neuroblastoma, astrocytoma, and glioma: in all of these PPARγ ligands repress or delay xenograft growth in mouse models [67].

PPARγ ligands affect tumor cells in several ways: they reduce proliferation, enhance apoptosis, and modulate angiogenic phenotype of the tumor cells. PPARγ targets cyclin D1 via the inhibitors of cyclin-dependent kinases (Cdk), p18, p21, and p27, causing a decline in Rb phosphorylation [1] and arresting cells in G1 phase: PPARγ acts via p21 and p27 in pancreatic cancer and via p18 in hepatoma (see Figure 3(a)). On the other hand, glitazones repress the production of Cdk2, 4 and 6 in carcinomas of the bladder, breast, lung, and pancreas via GADD45 [67] (see Figure 3(b)). PPARγ activation also restores PTEN expression in tumor cells and thus blocks PI3K/Akt axis [38], it can also initiate a negative feedback loop, which consists of calcineurin phosphatase, nuclear factor of the activated T-cells (NFAT), and down syndrome critical region 1 (DSCR1), which inhibits calcineurin and blocks NFAT activity necessary for proliferation and survival (see Figure 3(c)) [68], see Figure 4(c).

PPARγ induction also causes tumor cell apoptosis by downmodulating prosurvival proteins cFLIP and Bcl-2, while increasing proapoptotic Bax and BAD, as occurs in glioblastoma [69] or by the interference with the PI3K/Akt signaling [38]. Conversely, PPARγ often augments the expression of TNF-related apoptosis inducing ligand (TRAIL), which selectively eliminates cancer cells [70], see Figure 4(c).

In some cases, PPARγ activation induces tumor cell differentiation (e.g., liposarcoma, breast and pancreatic cancer, neuroblastoma, glioma, bladder carcinoma, and lung carcinoma). The differentiation is evidenced by the increase of the general markers of differentiated state, such as E-cadherin, and downregulation of the specific markers of progenitor lineages, also by morphology changes consistent with differentiated state (see Figure 3(d)) [1, 67].

Finally, treatment with the PPARγ ligands frequently downregulates the expression of pro-angiogenic factors VEGF [17], IL-8 [71], Ang-1 [72], and Cox-2 [73] and thus suspends tumor angiogenesis. Moreover, mice null for PPARγ show impaired tumorgenesis, due to the dramatic increase in TSP-1 [5], see Figure 4(c).

9. PPARγ PRO-ANGIOGENIC/TUMORIGENIC EFFECTS

In contrast to the majority of findings, a recent study suggests that PPARγ ligands may have pro-angiogenic properties both in vitro [74], in an endothelial/interstitial cell coculture assay, and in a murine corneal angiogenesis model in vivo [74]. The magnitude of the angiogenic response caused by PPARγ ligands has not been compared to the angiogenesis elicited by typical stimuli (VEGF, bFGF); also, the contradiction between these results and previous studies has not yet been addressed.
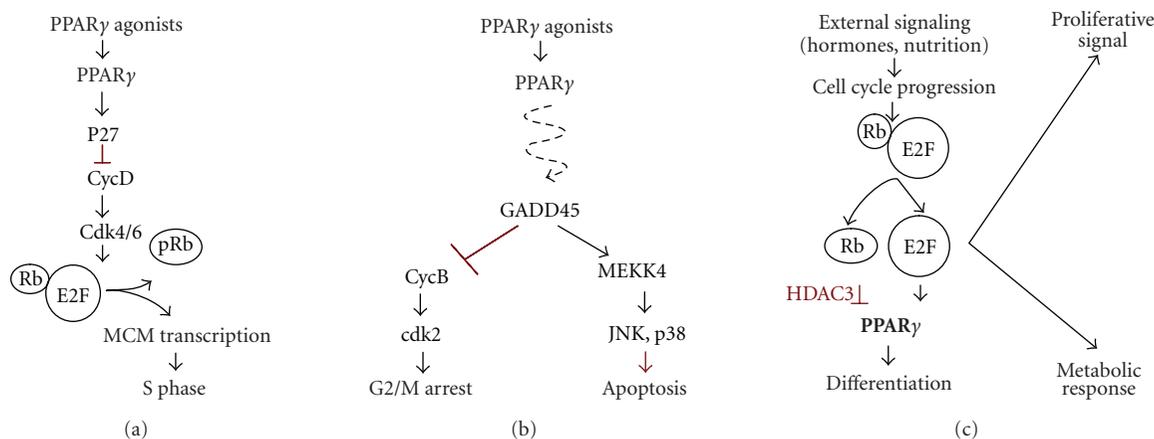
Figure 3: PPARγ effects in cancer cells. (a) The induction of Cdk inhibitor, p27 causes growth arrest due to reduced MCM7 activity and subsequent blockade of replication. (b) The induction of GADD45 impairs Cyclin B and causes G2M growth arrest. In addition, the activation of JNK and p38 kinases via MEKK4 initiates cell death by apoptosis. (c) PPARγ activation by hormones and nutrition in normal cells and by agonists in cancer cells may activate the differentiation programs.

PPARγ pro-angiogenic effects are associated with the induction of VEGF and increased phosphorylation of eNOS and AKT [7, 75], which cause elevated VEGF production in human and rodent VSMCs, MΦs and tumor cells [76–79], VEGF and VEGFR levels in the ECs and myofibroblasts [80]. Although PPARγ ligands inhibit xenografted human tumors [1, 33], in one study using mouse model of colon cancer (APC/Min) PPARγ ligands increased the number of precancerous polyps, tumor frequency and size [81]. However, in two other models, APC-deficient HT-29 xenografts and azoxymetane-induced tumors PPARγ ligands suppress tumor growth and angiogenesis [82, 83]. Of the multiple small-scale clinical trials using PPARγ ligands for cancer treatment, only two showed promising results: in an early study TGZ caused prolonged PSA stabilization in prostate cancer patients [84], while PGZ combined with low-dose chemotherapy and rofexoxib produced moderate improvement in the patients with high-grade glioma [85]. In contrast, patients with breast, colon, and thyroid cancers showed no significant response [86–88]. Thus, the use of PPARγ ligands in clinical practice obviously requires optimization, and the answers may come from the use of combination or complementation treatments.

10. PPARγ LIGANDS IN COMBINATION TREATMENTS: CAN WE AUGMENT THE BENEFICIAL EFFECTS?

The information above narrows down the list of PPARγ targets critical for its anti-angiogenic and antitumor effects (see Figure 4(a)). PPARγ reverses angiogenic functions in the ECs by blocking the expression of VEGF-A and its receptor, VEGFR2 by blocking Ets-1 transcription factor, and by dampening the prosurvival PI3K/Akt cascade, likely via PTEN induction. It also deactivates RhoA/Rac1 small GTPases which enable EC migration. NFAT deactivation lowers the levels of the apoptosis inhibitors, cFLIP and Bcl-2, and critical invasion molecules UPA and MMP 9. In addition, PPARγ promotes the following proapoptotic

events: it elevates expression of the proapoptotic CD36 and TSP1 receptor-ligand duo; increases p53 stability; opens of the Maxi-K channel to upregulate nitric oxide (NO), which, paradoxically, causes apoptosis. In addition, PPARγ suppresses Trx-1 and ROS levels by upregulating VDUP-1, a vitamin D3 target. Finally, PPARγ ligands block protein synthesis via 4-eBP and p70S6 kinase, both the targets of mTOR pathway.

In the VSMC, PPARγ represses the activation of prosurvival Erk-1 and PI3K/Akt and SHIP thus sustaining the unphosphorylated, active state of 4-EPB, a negative regulator of translation. It also enhances the activity of Shp-2 phosphatase, which blocks Vav, the trigger of RhoA/ROCK pathway necessary for survival and migration; PPARγ also interferes with VSMC Bcl-2 expression by enhancing TGFβ/Smad2 and disrupts MMP-9 production by blocking Ets-1 (see Figure 4(b)).

In MΦs and tumor cells, PPARγ through transrepression of NFκB and NFAT lowers the production of multiple growth factors and inflammatory cytokines including VEGF, Ang-1, cyclo-oxygenase (Cox) 2, IL-6, IL-8, MCP-1, and CX3CL-1. PPARg also enhances the production of thrombospondin (TSP) 1: therefore angiogenic balance tips in favor of vascular quiescence. In addition, PPARγ lowers the resistance of tumor cells and tumor-associated MΦs (TAM) to stress and apoptotic stimuli by blocking cyclin D1 via cdk inhibitors p18, p21, p27, by repressing antiapoptotic Bcl-2 and FLIP, by upregulating proapoptotic CD36 in MΦs, and Bax and BAD in tumor cells (see Figure 4).

This comprehensive list of PPARγ targets and interacting proteins can be used for intelligent design of the optimal combination therapies based on PPARγ ligands to achieve the best anti-angiogenic and anticancer activity. For example, it stands to reason to expect that EC apoptosis caused by PPARγ can be augmented by supplying CD36 ligand, TSP1 or its peptide mimics, such as ABT-510 [89]. Indeed, PPARγ ligands 15PG-E2, TGZ and RGZ, and TSP1 anti-angiogenic peptide ABT-510 synergistically block angiogenesis and
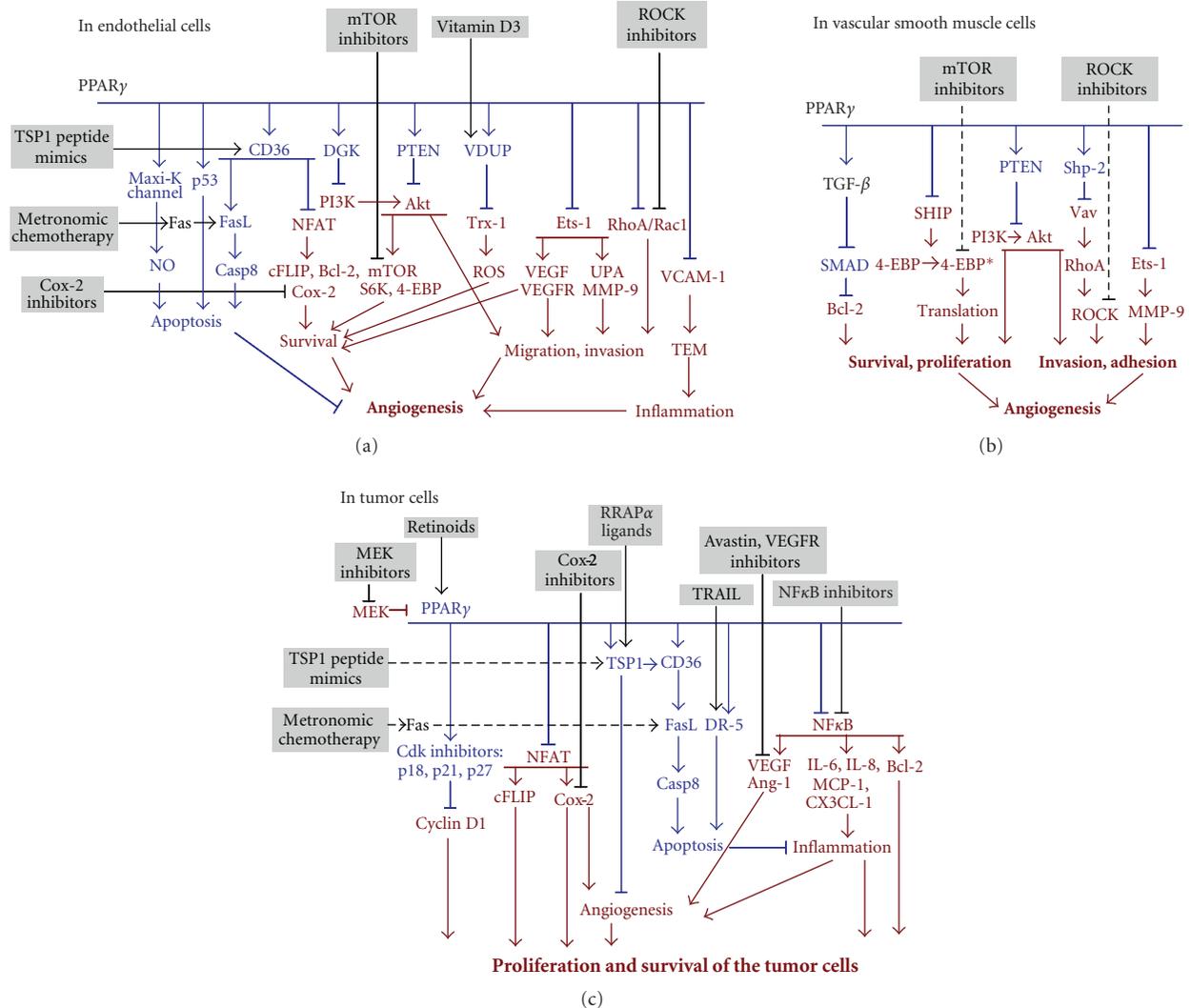
FIGURE 4: PPARγ effects on the endothelial, pericytic, tumor and immune cells in the tumor microenvironment: the consequences of angiogenesis and possible ways to augment antitumor actions. Pro-angiogenic and tumor-promoting events are shown in red. The opposing effects are in blue. The proposed drugs are shown in black. (a) Summary of the PPARγ molecular effects in the endothelial cells. TEM, transendothelial migration. (b) PPARγ molecular effects on the VSMCs. (c) The effects on macrophages and tumor cells.

curtail the growth of lung and bladder carcinoma xenografts, by initiating CD36-dependent apoptotic events in remodeling tumor endothelium [47]. Furthermore, TSP1 expression is enhanced by the low-dose metronomic chemotherapy, including cytoxan, docetaxel, and 5-fluorouracil [90–92]. Thus cytoxan, docetaxel, and 5-fluorouracil are likely to potentiate the PPARγ anti-angiogenic effects in EC and to reduce tumor-associated inflammation responses by killing TAMs. This is supported by the fact that 15D-PGE₂ enhances antitumor activity of docetaxel against lung carcinoma cell lines [93]. In addition, metronomic chemotherapy enhances the expression of Fas, a critical apoptosis mediator induced by the TSP1/CD36 interaction and thus potentiates the activity of TSP1 derivatives, such as ABT-510 [94, 95]. Hence, combined use of PPARγ ligands and metronomic regimens of chemotherapy agents is likely to be more effective than individual treatments.

PPARγ blockade of the EC and VSMC migration involves the inhibition of RhoA/ROCK signaling [40, 65], which makes ROCK inhibitors likely candidates for the use in combination with PPARγ ligands. This is doubly important, since ROCK activates Myc pathway and thus abolishes TSP1 expression by the tumor cells [96]. ROCK inhibitors show strong toxic effects at therapeutic doses, thus their clinical use is problematic. However, combined use with PPARγ ligands may allow to lower their effective concentration and therefore limit drug-induced toxicity.

Since PPARα strongly increases TSP1 production, combined use of PPARα and PPARγ agonists or the use of dual PPARα/γ ligands may present an advantage. Interestingly, TZD18, a novel PPARα/γ dual agonist induces apoptosis of glioma cells with high efficiency [97]. Unfortunately, glitazars have carcinogenic activity of their own [98].

PPARγ ligands sensitize leukemic, lung and endothelial cells to the TRAIL-induced apoptosis by enhancing DR5 expression [99, 100] pointing to possible synergy between PPARγ agonists and TRAIL therapies.

The inhibition of VEGFR2 expression by vascular endothelium, which contributes to the antiangiogenesis by the PPARγ, could be assisted by VEGF sequestering agents, such as Avastin, or by the inhibitors VEGF RTK activity, such as sunitinib, sorafenib or VEGF decoy receptor. This hypothesis is yet to be tested.

The downstream target of the PI3K/Akt pathway, which is blocked by PPARγ via PTEN activation, is tuberous sclerosis tumor suppressor complex, which, when phosphorylated by Akt, allows the activation of mammalian target of rapamycin (mTOR) kinase, protein synthesis, and cell survival [101]. On the other hand, PPARγ ligands interfere with translation by augmenting the activity of 4-EBP and blocking S6 kinase [102]. Thus PPARγ disrupts mTOR regulation of protein synthesis at two distinct steps. Moreover, the blockade of mTOR pathway is likely to suppress VEGF in all cell types in the tumor microenvironment [103]. Hence, mTOR inhibitors such as tacrolimus are likely to complement the anti-angiogenic and antitumor activity of PPARγ agonists. Cyclic AMP analogs, which block mTOR activity via AMPK1 pathway [101], may also contribute to the PPARγ beneficial effects: this is particularly important, since cAMP analogs are capable of increasing PPARγ activity (Schulze-Hoepfner and Volpert, unpublished observations). The fact that amino acid deprivation, the main off switch for the mTOR, enhances PPARγ proapoptotic effects in tumor cells [104] lends further support to this hypothesis.

PPARγ transrepression of NFκB and NFAT signaling leads to the inhibition of multiple angiogenic stimuli, including interleukins 6 and 8, MCP-1 and CX3CL-1, as well as protective Ang-1 and proinflammatory Cox-2. This PPARγ function suggests a wide range of possible treatment combinations with NFκB inhibitors, including synthetic inhibitors of IKK kinases [105] or naturally occurring plant substances, like curcumin [106]. On the other hand, the inhibition of Cox-2 with highly selective agents, like celexoxib, has direct anti-angiogenic tumor-preventing effects [107] and is quite likely to contribute to the PPARγ antitumor and anti-angiogenic activities, especially in the light of potentiating effect of celexoxib on docetaxel treatment [108] and beneficial effects of PGZ combined with rofexoxib and low-dose chemotherapy [85].

PPARγ activity is opposed by MEK kinases: thus MEK inhibitors are likely to improve the efficacy of PPARγ ligands: indeed, MEK-1 inhibitor, PD98059, improves CGZ antitumor effect in colon cancer xenografts [109]. PPARγ activity is also augmented by RXR ligands: 9-cis retinoic acid (RA) enhances PPARγ-induced differentiation and gene expression. In colon cancer, PPARγ and RXR ligands induce differentiation and apoptosis more potently than each individual compound [110, 111]. Nine-cis retinoic acid partially overcomes RXR phosphorylation, which reduces PPARγ/RXR dimerization and opposes PPARγ activity: MEK-1 inhibitors improve the combined effect of CGZ and 9-cis RA [109]. Finally, HDAC inhibitor, trichostatin A,

potentiates the effects of phenolfibrate on the differentiation and attenuation of stemness of the lung adenocarcinoma cells [112]. While combining PPARγ agonists with other drugs, particular attention should be paid to the agonist dosage: studies of PPARγ effects metabolic syndrome demonstrate that overactive and hypoactive mutants cause similar metabolic consequences and suggest the use of SPPARMs versus full agonists [113].

The list of agents with the potential to enhance the antitumor and anti-angiogenic effects of PPARγ ligands is not limited by the examples above, however we hope that it provides a convincing example of rational design of the complementation therapies, based on the knowledge of molecular mediators of a given agent. The examples, which demonstrate the improved efficacy of predicted combinations, provide an impetus for the evaluation of the combinations, which have not yet been tested.

ABBREVIATIONS

ADD1:	Adducin 1
AMPK1:	Adenosine Monophosphate Protein Kinase
Ang-1:	Angiopoietin-1
APC:	Adenomatous Polyposis Coli
Bcl:	B-cell Leukemia
bFGF:	Basic Fibroblast Growth factor
C/EBPs:	CAAT enhancer binding proteins
CBP:	CREB binding Protein
Cdk:	Cyclin-Dependent Kinases
cFLIP:	FLICE Inhibitory Protein, a caspase-8 inhibitor
CGZ:	Ciglitazone
Cox:	Cyclooxygenase
DGK:	Diacylglycerol Kinase
15D-PGJ$_2$:	15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
DSCR1:	Down Syndrome Critical Region 1
EC:	Endothelial Cells
Egr:	Early Growth Response
EPC:	Endothelial Progenitor Cells
ERKs:	Extracellular Signal-Regulated Kinase
GATA:	GATA-binding transcription factor
HDAC:	Histone deacetylase
HIF:	Hypoxia Inducible factor
IKK:	Inhibitor of Kappa Beta Kinase
IL:	Interleukin
MAPK:	Mitogen-Activated Protein Kinase
MCM7:	Minichromosome Maintenance Protein
MCP:	Monocyte Chemotactic Protein-1
MMP:	Matrix Metalloproteinase
mTOR:	Mammalian Target of Rapamycin
NCoR:	Nuclear Co-Repressor
NADPH:	Nicotinamide Dinucleotide Phosphate
NFAT:	Nuclear Factor of the Activated T-cells
NFκB:	Nuclear Factor Kappa Beta
JNKs:	Jun N-terminal Kinase
MΦ:	Macrophages
PPAR:	Peroxisome Proliferator Activated Receptor
PAI-1:	Plasminogen Activator Inhibitor
PDGF-BB:	Plateled-Derived Growth Factor
PI3K:	Phosphatidil Inositol-3 Kinase

PGZ: Pioglitazone
PPRE: PPARg Response Element
PTEN: Phosphatase and Tensin Analog
Rb: Retinobalsoma
ROCK: Rho-associated kinase
RXR: Retinoic Acid X receptor
STAT: Signal Transducers and Activators of Transcription
SMRT: Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor
SPPARMs: Selective PPARγ modulators
SREBP: Serum response Element Binding Protein
TAM: Tumor-associated Macrophages
TBL-1: Transducin Beta-Like Protein-1
TCF: T-cell factor
TNF: Tumor Necrosis Factor
TGF-β: Tumor-Derived Growth Factor β
TGZ: Troglitazone
TRAIL: TNF-related apoptosis inducing ligand
Trx: Thioredoxin
TSP1: Thrombospondin-1
TZDs: Thiazoliediones
UPA: Urokinase Plasminogen Activator
VDUP: Vitamin D3 Upregulated Protein
VEGF: Vascular Endothelial Growth Factor
VSMC: Vascular Smooth Muscle Cells
VCAM: Vascular Cell Adhesion Molecule-1.

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Review Article

Peroxisome Proliferator-Activated Receptor-γ Ligands: Potential Pharmacological Agents for Targeting the Angiogenesis Signaling Cascade in Cancer

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Peroxisome proliferator-activated receptor-γ (PPAR-γ) has currently been considered as molecular target for the treatment of human metabolic disorders. Experimental data from in vitro cultures, animal models, and clinical trials have shown that PPAR-γ ligand activation regulates differentiation and induces cell growth arrest and apoptosis in a variety of cancer types. Tumor angiogenesis constitutes a multifaceted process implicated in complex downstream signaling pathways that triggers tumor growth, invasion, and metastasis. In this aspect, accumulating in vitro and in vivo studies have provided extensive evidence that PPAR-γ ligands can function as modulators of the angiogenic signaling cascade. In the current review, the crucial role of PPAR-γ ligands and the underlying mechanisms participating in tumor angiogenesis are summarized. Targeting PPAR-γ may prove to be a potential therapeutic strategy in combined treatments with conventional chemotherapy; however, special attention should be taken as there is also substantial evidence to support that PPAR-γ ligands can enhance angiogenic phenotype in tumoral cells.

1. INTRODUCTION

Angiogenesis, the development of new capillaries from preexisting microvessels, plays a crucial role in several normal physiological processes, such as embryonic development, ovulation, wound healing, as well as tissue and organ regeneration. Angiogenesis also constitutes a crucial step in the aetiology of diverse pathological states, including cancer, diabetic retinopathy, age-related macular degeneration, psoriasis, and rheumatoid arthritis [1, 2]. In the last few years, the complicated biochemical mechanisms governing neovessel formation have been well established. These include the proliferation of endothelial cells (ECs) from preexisting capillaries, the breakdown and reassembly of the extracellular matrix (ECM) and the morphogenic process of endothelial tube formation [2, 3]. Numerous growth factors, including vascular endothelial growth factor (VEGF) family, basic fibroblast growth factors (bFGFs), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), placenta growth factor (PGF), matrix metalloproteinases (MMPs), ephrin family, angiopoetin-1 (Ang-1), interleukins (IL-2, -6, -8), as well as various endothelial surface molecules such CD31, CD34, CD36, CD144, and a_vb_3 integrins, have been found to control essential steps within angiogenesis process [1–3]. The generation and release of antiangiogenic factors, such as interferon (INF) -α, -β, -γ, platelet factor 4 (PF4), and tissue inhibitors of MMPs (TIMPs) contribute to the coordinated downregulation of the angiogenic process within physiologic angiogenesis [4].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors and include three different isotypes: PPAR-α, PPAR-β/δ, and PPAR-γ [5, 6]. PPAR-γ, the most extensively studied amongst them, functions as ligand-activated transcription factor by binding to specific DNA sequences, termed to as peroxisome proliferator response elements (PPREs), in the promoter of the target genes only as a heterodimer with the retinoid X receptor

(RXR) [7–9]. PPRE has been mainly identified in the upstream regulatory sequences of genes related to metabolic pathways [7–9]. In addition, recent studies have revealed that PPAR-γ can regulate gene expression independently of PPRE, either by suppressing growth hormone protein-1 (GHP-1), a transcription factor involved in pituitary specific gene expression, or by interfering with the function of activator protein-1 (AP-1), signal transducer and activator of transcription-1 (STAT-1) and nuclear factor-κB (NF-κB) [7, 10–12]. In this context, the identification of a sumoylation-dependent pathway by which PPAR-γ represses transcriptional activation of inflammatory response genes has recently been reported [13]. This mechanism provides a possible explanation for how ligand-bound PPAR-γ activation can be converted from an activator of transcription to a promoter-specific repressor of NF-κB target genes [13].

A wide range of natural and synthetic structurally diverse compounds has been reported as potent PPAR-γ ligands. The long chain polyunsaturated fatty acids and their derivatives, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ2), as well as nitrolinoleic acids are known natural occurring PPAR-γ ligands [14, 15]. Recently, curcumin, a well-documented anticancer phytochemical component of turmeric, has been shown to exert anti-inflammatory functions via upregulation of PPAR-γ activation [16]. Thiazolidinediones (TZDs) and tyrosine-based derivatives, such as glitazars (tesaglitazar, farglitazar), constitute the most well-known synthetic ligands [17, 18], while relatively lower binding affinity for PPAR-γ has also been reported for some nonsteroidal anti-inflammatory drugs (NSAIDs) [19]. TZDs represent a promising class of oral antidiabetic agents, some of which are already marketed drugs (pioglitazone-PGZ and rosiglitazone-RGZ) for the treatment of type II diabetes mellitus [20]. Interestingly, a wide spectrum of action for TZDs beyond the treatment of diabetes, including anti-inflammatory and antineoplastic properties, as well as targeting signaling pathways implicated in atherosclerosis and osteoporosis has been reported [21–23]. In the last decade, more than 1000 PPAR-γ ligands belonged to several distinct chemical classes have been synthesized and evaluated for their binding and transactivation to their receptor. In this aspect, screening drug-like characteristics in the chemical space of PPAR-γ ligands have currently been considered as an emerging demand in the aim to discover more potent compounds with improved absorption, distribution, metabolism, excretion/toxicity (ADME/Tox) properties, avoiding potential toxic side effects, as well as pharmacokinetic and pharmacodynamic problems [24, 25].

To date, there has been a substantial accumulation of evidence that PPAR-γ ligands exert regulatory effects on angiogenesis process related to diverse disease states, including cancer and diabetes [26–28]. It is also well documented that they directly affect tumor cells by inhibiting cell growth and inducing cell differentiation and apoptosis in various cancer types [21, 29, 30]. In view of the fact that angiogenesis is implicated in tumor development and metastasis and its inhibition could serve as potent antitumor side-therapeutic approach, the current review summarizes the latest knowledge of the role of PPAR-γ ligands in angiogenesis related to cancer, highlighting in the underlying mechanisms.

2. ANGIOGENESIS IN CANCER

Tumor angiogenesis constitutes an essential component of tumor growth, invasion, and metastasis that depends on a net balance of angiogenic and antiangiogenic mediators, which are secreted by both tumor and host infiltrating cells [31]. Currently, it is well established that this dynamic balance between angiogenic stimulators and inhibitors, controls the angiogenic signaling cascade governing the transformation of a tumor from a nonangiogenic to an angiogenic phenotype [32]. The acquisition of angiogenic phenotype has been considered as a rate-limiting step in tumor progression, which allows the tumor to transform from a small lesion to a rapidly expanding mass with metastatic potency [33]. On the other hand, human tumors arise in the absence of angiogenic activity and may exist in a microscopic dormant state for months to years without neovascularization [34]. In this context, hypoxia, developed within rapidly proliferating tissues or as a result of the occlusion of blood vessels, has been considered as a primary physiological regulator of the angiogenic switch [35]. The key mediators of this response are members of the hypoxia-inducible factor (HIF) family of proteins that function as transcriptional regulators, stimulating the expression of a multitude of genes important for oxygen homeostasis [36, 37]. In addition, HIF has been found to enhance the expression of several angiogenic mediators, including VEGF-R1, VEGF-R2, Ang-1, Ang-2, MMP-2, and MMP-9 in malignant tumors [36, 38].

In response to hypoxia, tumor cells turn on the angiogenic signaling cascade by secreting various potent angiogenic mediators, such as VEGF, PDGF, bFGFs, angiopoetins, HGF, fibronectin, and heparanase that in turn activate endothelial cells of preexisting capillaries to produce MMPs for the collapse of ECM [39]. Degradation of ECM by MMPs allows endothelial cells to migrate in response to chemotactic growth factors, including VEGF, PDGF, and bFGFs [33, 39]. Members of CXC chemokine family, such as IL-2, -6, -8, and integrins $\alpha_v\beta_3$, are also involved in the angiogenic cascade. It should be noted that in the case of high progressive tumors, the release of endogenous antiangiogenic factors are insufficient to counteract the net effect of angiogenic ones. Thus, the formation of new blood vessel is formed after attracting accessory cells, mainly pericytes and smooth muscle cells, producing a new basement membrane and a firm ECM [39, 40]. The above-mentioned angiogenic mediators have been joined by others including Notch/Delta, semaphorin, ephrin, and roundabout/slit families of proteins [40]. Besides this, blockage of NF-κB activity has been shown to reduce VEGF gene expression in highly malignant tumor cells, since a binding site for this transcription factor has been identified within the VEGF promoter [41]. Each of the sequential steps within angiogenic cascade could be considered as a potential single target for the development of new drug candidates against tumor vasculogenesis.

Currently, numerous therapeutic approaches have been designed in the aim to control tumor angiogenic cascade

Hypoxia: **HIF-1α**, HIF-2α

Angiogenic mediators

VEGFs, VEGFRs
bFGF, PDECGF, EGFR
Chemokines: CXCL8, CXCL5, CXCL1
Ang-1, MMPs, gelatinases
Leptin, CD31, tPA, PGE$_2$

Antiangiogenic mediators

TSP-1, ABT510, TIMPs
Endostatin, angiostatin
Tumstatin, 16K hPRL
TGF-α, -β, IL-1, -12
INF-α, -β, -γ, PF4
Ang-2, fibronectin
Retinoic acid

$\longleftarrow$ PPAR-γ ligands $\longrightarrow$

PEDF, PDECF, PGF, TNF-α
PGE$_1$, HGF, ephrin family
Integrins a$_v$b$_3$, PDGF, CD34, CD36
Notch/delta, heparanase

Endothelial cells
proliferation & migration

FIGURE 1: The network of the components implicated in the angiogenesis process in cancer and the impact of PPAR-γ ligands illustrated by blue color.

by targeting the above-mentioned angiogenic mediators [40, 42]. In this context, more than a few angiogenesis inhibitors have already been approved for the treatment of cancer, while several compounds are in the late stage of clinical trials. The main category of the antiangiogenic compounds exerts its action indirectly either by neutralization of tumor-derived angiogenic factors or preventing the receptors/signaling pathways of these growth factors. In this regard, VEGF isoforms and their tyrosine kinase receptors VEGFRs, as well as epidermal growth factor (EGF) and its receptor (EGFR) are currently explored in clinical trials as drug candidates against cancer [43–45].

With respect to angiogenesis inhibitors, several angiostatic compounds, such as endostatin, thrombospodin-1 (TSP-1), tumstatin, angiostatin, and 16-kDa N-terminal fragment of human prolactin (16K hPRL) have been reported to directly and selectively suppress endothelial cell migration inducing EC apoptosis and cell cycle arrest within tumor neovascularization [46, 47]. It should be mentioned that most of these angiostatic compounds are also naturally occurring molecules that compensate with angiogenic factors in order to control angiogenic cascade in normal physiologic conditions. In addition, targeting MMPs by such agents has been reported, underlining the importance of ECM remodeling during angiogenesis process. Activation of NF-κB may also be a possible mechanism of such angiostatic agents to induce EC apoptosis and to improve immune response within angiogenesis process [46, 47].

3. INHIBITION OF ANGIOGENESIS BY PPAR-γ LIGANDS

PPAR-γ ligands can regulate tumor angiogenesis via direct effects on ECs proliferation and migration and/or through indirect mode of action by affecting the counterbalance between angiogenic and antiangiogenic mediators (Figure 1, Table 1).

3.1. Direct effects on endothelium

PPAR-γ has been reported to be expressed in endothelial cells and PPAR-γ ligands are well established to exert direct effects

on them [48, 49]. PPAR-γ activation by either naturally occurring or synthetic ligands resulted in potent inhibition of growth factor-induced differentiation and proliferation in human umbilical vein endothelial cells (HUVECs) and choroidal endothelial cells (CECs) [48, 49]. In this regard, PPAR-γ dependent mode of action has been shown to stimulate caspase-mediated ECs apoptosis [50]. Importantly, RGZ levels able to inhibit ECs proliferation are readily achieved in patients undergoing standard antidiabetic RGZ treatment [51]. Moreover, both RGZ and PGZ, at relative pharmacological concentrations, resulted in a strong prevention of VEGF-induced tube formation and ECs migration [52, 53]. Mechanistically, it has been supported that angiogenesis inhibition by RGZ in HUVECs involves a proapoptotic mechanism which includes the implication of the PPAR-γ-mediated NO production and the maxi-K channel activation [54]. Maxi-K channels, essential mediators of vascular remodeling and angiogenesis, are synergically regulated by various intracellular second messengers including NO [54]. Hence, a possible proapoptotic mechanism for the PPAR-γ-mediated NO production has been suggested [55]. Recently, pigment epithelium-derived factor (PEDF), a potent antiangiogenic glycoprotein, has been shown to stimulate HUVECs apoptosis through sequential induction in the expression and transcriptional activity of PPAR-γ. PEDF upregulated p53 expression via PPAR-γ, supporting evidence that p53 may be a major target in PPAR-γ mediated ECs apoptosis [56].

PPAR-γ has also been reported to be expressed in tumor ECs, presenting a relative overexpression in tumor-induced endothelial sprouts compared to normal endothelium. In this case, endothelial and tumoral cells have been shown to display inhibition even at low TZDs doses [57]. Importantly, TZDs inhibited tumor cell invasion across blood vessel endothelium. In fact, RGZ at concentrations close to the range of its binding affinity for PPAR-γ [8] exerted inhibitory effects on tumor angiogenesis in malignant cell lines and in immunodeficient mice with transplanted tumors [57]. In this regard, it should be mentioned that RGZ concentrations of $5\,\mu$M and higher led to the phosphorylation of eIF-2α in HUVECs, supporting evidence that the inhibition of ECs proliferation could also be mediated through a PPAR-γ independent pathway. However, at even lower concentration

TABLE 1: Effects of PPAR-γ ligands on tumor angiogenesis.

PPAR-γ ligands	Type of cells/organisms	Effects	Ref.
RGZ	In vitro		
	Glioblastoma U87	VEGF↓	[57]
	Lewis lung carcinoma	VEGF↓	[57]
	Pancreatic tumor AsPC-1 cells	tPA↓	[58]
	Human breast cancer cell line MDA-MB-231	TIMP-1↑ gelatinases↓	[59]
	Transformed human endometrial cells (transiently transfected Ishikawa cells)	VEGF↓	[60]
	Human anaplastic thyroid carcinoma cells MSA, IAA, ROA, K119, KOA-2	invasive potential↓	[61]
	In vivo		
	Chick chorioallantoic membrane	Choroidal neovascularization↓	[57]
	C57/BL6 xenografted with 253J B-v bladder tumor cells	Neovascularization↓ EC apoptosis↑	[62]
TGZ	In vitro		
	Human non small cell lung cancer cells A459	ELR + CXC chemokines↓	[63]
	In vivo		
	C57/BL6 xenografted with 253J B-v bladder tumor cells	Neovascularization↓ EC apoptosis↑	[62]
CGZ	In vitro		
	Human non-small-cell lung carcinoma A427 and A549 cell	PGE$_2$, COX-2↓	[64]
	Human ovarian cancer cells OVCAR-2, DISS	VEGF, PGE$_2$↓	[65]
	In vivo		
	BALB/c nu/nu mice xenografted with OVCAR-2 or DISS	VEGF, PGE$_2$↓	[65]
PGZ	In vitro		
	Renal cell carcinoma cells SMKT-R-1, -2, -3, -4	VEGF, bFGF↓	[66]
	Human non small cell lung cancer cells A459	ELR + CXC chemokines↓	[63]
	Human anaplastic thyroid carcinoma cells MSA, IAA, ROA, K119 and KOA-2	invasive potential↓	[61]
15d-PGJ2	In vitro		
	Renal cell carcinoma SMKT-R-1, -2, -3, -4	VEGF, bFGF↓	[66]
	Human gastric cancer cells MKN45	Ang-1↓	[67]
	Human PC-3 cells	VEGF↑	[68]
	Human 5637 urinary bladder cells	VEGF↑	[68]
	Human breast MCF-7 cells	VEGF↑	[69]
	Human anaplastic thyroid carcinoma cells MSA, IAA, ROA, K119, KOA-2	invasive potential↓	[61]
	Human pancreatic cancer cells BxPC-3	MMP-2, -9↓	[70]
	Transformed human endometrial cells (transiently transfected Ishikawa cells)	VEGF↓	[60]
	In vivo		
	C57/BL6 xenografted with 253J B-v bladder tumor cells	Neovascularization↓ EC apoptosis↑	[62]

PPAR-γ ligands	Type of cells/organisms	Effects	Ref.
RS5444	In vitro Human anaplastic thyroid carcinoma cells DRO90-1, ARO81	CD31↓	[71]
	In vivo Nude mice xenografted with DRO90-1 or ARO81 tumor cells	CD31↓	[71]
RS1303	In vitro Human anaplastic thyroid carcinoma cells MSA, IAA, ROA, K119, KOA-2	Invasive potential↓	[61]
Nimesulide	In vitro Human pancreatic cancer cells BxPC-3 and MIA PaCa-2	VEGF↑	[72]

range (0.1–1 μM), at which PPAR-γ is activated, RGZ was capable of exerting even stronger antiproliferative effects on ECs in vitro [57]. In this context, the concentration range of PPAR-γ ligands should be taken into careful consideration, because over a concentration limit, which may be varied amongst the different types of cells, in vitro, as well as amongst different species, in vivo, receptor-independent actions could be elicited. Such PPAR-γ mode of action has recently been reviewed by Feinstein et al., who suggested an alternative mitochondrial target for TZDs, termed as mitoNEET [12]. To this point, it should be noted that higher doses of RGZ were less effective in inhibiting angiogenesis and hence lung metastasis than lower doses that are actually comparable to the serum levels of RGZ in diabetic patients [27, 51]. Overall, although PPAR-γ ligands can also induce EC apoptosis as mentioned in the previous paragraph, it is unlikely that they do this under physiological conditions as this may result in a severe thrombosis. Thus, it should be emphasized the fact PPAR-γ ligands may target better EC proliferation as shown by Panigrahy et al. [27] and Freed et al. [51].

Orthotopic implantation of H2122 nonsmall cell lung adenocarcinoma cells overexpressing PPAR-γ into the lungs of nude mice attenuated tumor growth and metastasis by selective inhibition of invasive metastasis, and activation of pathways that promote a more differentiated epithelial phenotype [73]. This evidence deserves special attention since both angiogenesis and invasion are crucial for the formation of metastasis and the recurrence of tumors. Moreover, reintroduction of exogenous TSP1 or its peptide derivative ABT510 can reverse the angiogenic switch, and thus blocking tumor expansion. TSP-1 is a well-known potent angiogenesis inhibitor that targets ECs for apoptosis through signaling cascade at its receptor CD36. In tumor xenografts, TGZ, RGZ, and 15d-PGJ2 coupled to ABT510 suppressed angiogenesis and induced ECs apoptosis in a CD36 dependent manner [62]. In this context, 15d-PGJ2 treatment upregulated CD36 surface expression in human monocytic cell line THP-1 by enhancement of CD36 gene transcription [74]. Thus, PPAR-γ could be considered as a critical regulator of CD36 expression, as both natural and synthetic PPAR-γ ligands are capable of increasing CD36 expression [75].

Receptor-mediated effects for PPAR-γ ligands in inhibiting angiogenesis through direct mode of action on endothelium seem to be dominated [28, 57]. In this regard, PPAR-γ knockout mice embryos died on day 10 of life because of interference with the terminal differentiation pattern of trophoblasts, as well as the loss of vascular development in the placenta [76, 77]. It has also been suggested that PPAR-binding protein (PBP), a coactivator of PPAR-γ, may function as a negative modulator of ECs proliferation [77]. Such genetic data provides additional evidence that PPAR-γ functions as modulator of angiogenesis; however, receptor-independent action should not be excluded. In this aspect, Artwohl et al. showed PPAR-γ-independent antiproliferative effects on HUVECs associated with lactate release, possibly due to inhibition of mitochondrial function [78].

3.2. Indirect effects on the net balance between angiogenic and antiangiogenic factors

Beyond the direct mode of action on the endothelium, PPAR-γ ligands have been reported to downregulate angiogenesis process via indirect mechanisms by modulating the levels of the endogenous angiogenesis mediators (Figure 1, Table 1). In this context, VEGF/VEGFR signaling pathway seems to be a key target for PPAR-γ ligands in inhibiting angiogenesis. Xin et al. provided the first evidence that 15d-PGJ2 reduced VEGFRs m-RNA levels in HUVECs [48]. It has also been supported that PPAR-γ ligands may have bifunctional properties in KDR gene expression that involve the enhancement of Sp1-DNA binding in absence of ligand by PPAR-γ itself and the suppression of Sp1-DNA-binding in presence of PPAR-γ ligands [79]. Moreover, PPAR-γ activation has been shown to downregulate leptin and tumor necrosis factor (TNF-α), two well-known angiogenesis-inducing factors [80, 81]. In fact, PPAR-γ activation by TZDs attenuated leptin gene expression both in vivo and in vitro [82, 83] and blocked leptin-induced ECs migration through inhibition of Akt and eNOS signaling [84]. This evidence suggests that endothelial phosphatase and tensin homologue

mutated on chromosome ten (PTEN), a negative regulator of PI3K → Akt signaling, may play a crucial role in the ECs antimigratory actions of TZDs [84].

Tumor-associated angiogenesis has been reported to be indirectly suppressed by blocking the expression of angiogenic stimulators in response to PPAR-γ ligand activation. In this regard, PPAR-γ activation by TGZ or PGZ diminished the production of the angiogenic ELR + CXC chemokines IL-8 (CXCL8), ENA-78 (CXCL5), and Gro-α (CXCL1) in human non-small-cell lung cancer cell line A459 [63]. This effect was ascribed to the negative modulation of NF-κB activation [63]. In addition, CGZ was found to decrease PGE$_2$ production through downregulation of cyclooxygenase-2 (COX-2) expression in human non-small-cell lung carcinoma A427 and A549 cell lines [64]. Interestingly, utilization of a dominant negative PPAR-γ construct revealed that the effect of CGZ on both COX-2 and PGE2 was mediated through PPAR-γ independent pathways [64]. Another study demonstrated that 15d-PGJ2 attenuated the expression of Ang-1 and hence the angiogenic process through the angiopoietin-Tie2 system in the gastric cancer cell line MKN45 [67]. Ang-1 is involved in the regulation of maturation and stabilization of the vascular wall, and thus it might be a potential target for inhibiting tumor angiogenesis. Moreover, in a model of human anaplastic thyroid carcinoma, RS5444, a novel high-affinity PPAR-γ agonist exerted potent antiangiogenic action, in vivo, by decreasing CD31, a specific molecular marker of blood vessels [71]. In this regard, PPAR-γ ligand treatment (TZDs, 15d-PGJ2, and RS1303) dose-dependently suppressed cell proliferation by inducing apoptosis instead of differentiation in five human anaplastic carcinoma cell lines (MSA, IAA, ROA, K119, and KOA-2) [61]. Recently, CGZ has also been shown to produce antitumor effects against ovarian cancer, in vitro and in vivo, in conjunction with reduced angiogenesis and induction of apoptosis [65]. In this case, CGZ induced antitumor effects were comparable to that of cisplatin and were ascribed to inhibition of VEGF production in relation to PGE$_2$ reduction, an endogenous stimulator of angiogenesis and invasiveness [65]. PPAR-γ ligands have also been shown to repress VEGF gene expression via a PPAR-γ-responsive element (PPRE) in the VEGF gene promoter in both primary and transformed human endometrial cell cultures [60]. This study provided substantial evidence that PPAR-γ ligands may be exploited pharmacologically to inhibit pathological vascularization in complications of pregnancy, endometriosis, and endometrial adenocarcinoma [60].

As mentioned in Section 3.1, RGZ suppressed tumor angiogenesis by direct mode of action in endothelium; however, indirect antiangiogenic effects have also been reported [57]. More to the point, RGZ, at low doses, in vitro, inhibited bovine capillary ECs and reduced VEGF production by tumor cells [57]. RGZ also suppressed angiogenesis in the chick chorioallantoic membrane, in the avascular cornea, in vivo, as well as in a variety of primary tumors, such as glioblastoma U87 and Lewis lung carcinoma cells, in vitro [57]. Likewise, both PGZ and 15d-PGJ2 have been shown to inhibit, dose- and time-dependently, VEGF and

bFGF secretion in human renal cell carcinoma cells [66]. Importantly, antiangiogenic effects were observed at the dose of 5 μM PGZ, a level that is also obtained in diabetic patients after standard PGZ treatment [66]. On the other hand, there is nonavailable data so far concerning the effect of PPAR-γ ligand treatment on the expression and/or secretion of antiangiogenic mediators. In this regard, future studies focused on the impact of PPAR-γ ligands in mediators, such as endostatin, TSP-1, tumstatin, angiostatin, and 16K hPRL are strongly recommended.

Angiogenesis constitutes a crucial step for tumor invasion and formation of metastasis. In this aspect, PPAR-γ ligand treatment attenuated the invasiveness of pancreatic tumor cells, reducing MMP-2 and -9 protein levels and activity [70]. Moreover, the secretion of the invasive factor tissue plasminogen activator (tPA) was decreased by RGZ treatment in pancreatic tumor AsPC-1 cells through receptor mediated mechanisms [58]. Treatment of the highly aggressive human breast cancer cell line MDA-MB-231 with synthetic and natural PPAR-γ ligands, at noncytotoxic concentrations, also resulted in a significant inhibition of the invasive capacity [59]. In fact, TIMP-1 was upregulated by PPAR-γ ligand treatment, while the gelatinolytic activities of gelatinases in the conditioned media were decreased [59]. Moreover, PPAR-γ ligands downregulated the invasive potential of anaplastic thyroid carcinoma cells, and this effect was prominent in 3 cell lines, which exhibited higher expression level of the PPAR-γ gene or protein [61].

Clinical evidence from a pilot study enrolled 6 patients with angiosarcoma and hemangioendothelioma, revealed that the angiostatic triple combination of PGZ, rofecoxib, and metronomic trofosfamide exhibited high efficacy in the palliative care of patients [85]. Until this study, antiangiogenic drugs such PGZ and rofecoxib had not been considered for the treatment of human angiosarcoma. In support of this view, a case report study has demonstrated that this novel antiangiogenic therapy was effective in a patient with endemic Kaposi sarcoma and led to partial remission that was stable for 18 months without significant side effects [86]. Hence, targeting PPAR-γ may prove to be a potential therapeutic strategy in combined treatments with conventional chemotherapy for patients with vascular disorders [87].

4. INDUCTION OF ANGIOGENESIS BY PPAR-γ LIGANDS

The most comprehensive data so far render PPAR-γ ligands as potent inhibitors of angiogenesis; however, there are several lines of evidence to support that PPAR-γ ligand activation can also trigger angiogenic cascade (Table 1). In fact, increased VEGF mRNA levels and induction of angiogenesis in response to PPAR-γ ligands treatment have been reported both in vitro and in vivo [88–91]. Interestingly, TZDs have been considered as potential pharmacological agents for angiogenesis induction in the treatment of ischemic artery disease [89]. Recent clinical evidence has also demonstrated that RGZ treatment improved endothelial progenitor cell (EPC) number and migratory activity in

diabetic patients [92, 93]. In addition, PGZ treatment was found to improve endothelial function by increasing the number and the migratory capacity of EPCs in animal and human studies [94, 95]. Another study has revealed that eNOS upregulation induced by RGZ may be the dominant mechanism through which RGZ enhanced angiogenesis [91]. However, Gensh et al. did not observe upregulation of vascular eNOS mRNA expression or setback of the PGZ-induced increase of EPCs in the presence of 1-NAME, a NOS inhibitor [94]. These authors suggested that TZDs may regulate EPCs by a mechanism independent of eNOS [94]; however, further studies based on pharmacologic blocking or knockout modeling of eNOS are strongly recommended in order for precise conclusion to be drawn. Importantly, taking into account the discrepancy in literature, Gensh et al. assumed that TZDs may play a double-edged role in angiogenesis signaling by promoting the number and migration of EPCs at lower tissue concentrations obtained by systematic treatment, whereas the antiangiogenic effects are elicited at higher local concentrations [94]. This major remark has also been reported in the case of breast cancer cells where low concentration of PPAR-γ ligands increase cell proliferation in contrast to the higher concentrations that suppress cell growth [96]. The urgent demand to define and monitor the dosage of PPAR-γ ligands in clinical trials for cancer therapy is thoroughly discussed by Panigrahy et al. [27]. In this aspect, special attention deserves the fact that atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, and PGZ increased myocardial 15d-PGJ2 levels in the rat myocardium and HUVECs [97]. 15d-PGJ2 was produced mainly via COX-2 and activated PPAR-γ. Interestingly, it was supported that PPAR-γ activation was exclusively mediated by 15d-PGJ2 in the case of atorvastatin, whereas PGZ activated directly PPAR-γ or indirectly via 15d-PGJ2 [97]. Thus, these recent findings raise the question whether the final effect of PPAR-γ ligands is completely ascribed to the dose of PPAR-γ ligand treatment or in addition to the induction of endogenous PPAR-γ activators, such as 15d-PGJ2. It should also be taken into account the fact that endogenous nitrated fatty acids that comprise a class of nitric oxide-derived, PPAR-γ dependent and cell signaling mediators can modulate systematic inflammatory responses within physiological concentration ranges [98].

There is also substantial evidence, which suggests that PPAR-γ ligands stimulate tumor angiogenesis. In this context, 15d-PGJ2 treatment was found to dose-dependently increase the VEGF mRNA expression in both human androgen-independent PC-3 prostate and 5637 urinary bladder carcinoma cells [68]. In addition, 15d-PGJ2 resulted in upregulation of VEGF expression through the induction of heme oxygenase (OH)-1 ERK1/2 phosphorylation in human breast cancer MCF-7 cells, thus contributing to increased angiogenesis in this type of tumor cells [69]. Nimesulide, a selective COX-2 inhibitor, although at relatively high concentrations, enhanced VEGF secretion from pancreatic cancer cells in vitro, as well as from both COX-2-positive and COX-2-negative pancreatic tumors through PPAR-γ activation [72]. Importantly, in the case of COX-2-negative

pancreatic tumors, nimesulide-stimulated VEGF production was considerably associated with enhanced angiogenesis and tumor growth [72]. Besides this, VEGF was differentially increased, according to the differentiation state of the cells, by the three PPAR isotypes, -α, -β/δ, and -γ, in two different human urinary bladder cancer cell lines, RT4 and T24, derived from grade-I and grade-III tumors, respectively [99]. The PPAR ligand-induced VEGF expression seemed to be PPAR-specific and involved an indirect mechanism requiring an intermediary regulatory protein through the MAP (ERK1/2) kinase pathway, probably by a modulation of the phosphorylation state of PPARs [99]. Immunohistochemical analysis in human bladder tumor specimens also revealed statistically significant associations between PPAR-γ and several angiogenic factors, such as VEGF, bFGF, platelet-derived endothelial cell growth factor (PDECGF), and EGFR in respect to the incidence of tumor recurrence or progression [100]. On the other hand, no statistically significant differences were observed between PPAR-γ immunoreactivity and angiogenesis parameters in skin cancer, whereas the microvessel density was significantly higher in actin keratosis and squamous cell carcinoma that expressed PPAR-β/δ [101]. These clinical data on PPAR-γ-induced signaling implicated in the expression of crucial angiogenic factors in human neoplasia may unfold the development of new therapeutic approaches in those types of cancer in which excessive angiogenesis represents a negative prognostic factor.

5. THE IMPACT OF PPAR-γ LIGANDS IN HYPOXIA-ASSOCIATED SIGNALING PATHWAYS

As hypoxia is a key regulator of the angiogenic switch, hypoxia-induced angiogenesis is gaining gradually increasing interest as a potential target for cancer therapy. In human bladder tumors and cell lines, several components of the hypoxia response pathway, including HIF-1α and HIF-2α have been considered as important cofactors of the regulation of VEGF [102]. Recent findings have revealed that PPAR-γ can modulate arterial remodeling associated with hypoxic hypertension [103]. In fact, RGZ was found to attenuate and reverse pulmonary arterial remodeling and neomuscularization in rats subjected to chronic hypoxia [104]. Decreased pulmonary arterial (PA) remodeling in RGZ-treated animals was associated with decreased smooth muscle cell proliferation, decreased collagen and elastin deposition, and increased matrix MMP-2 activity in the PA wall [104]. In this aspect, PPAR-γ mRNA levels were found significantly lower in human adhesion fibroblasts compared to normal ones in response to hypoxia [105]. Moreover, hypoxia has demonstrated to reduce the mRNA levels of PPAR-γ protein in human proximal renal tubular epithelial cells (HPTECs). However, knockout of HIF-1α with its dominant negative form did not block the hypoxia-induced reduction in PPAR-γ expression [106]. In this regard, substantial evidence has revealed that 15d-PGJ2 can modulate the activities of several transcriptional factors, such as NF-κB and AP-1, including also HIF-1 [107]. The regulation of the aforementioned redox-sensitive transcription factors by

15d-PGJ2 was not necessarily mediated via PPAR-γ activation, but rather involves covalent modification or oxidation of their critical cysteine residues acting as a redox sensor [107]. Overall, targeting hypoxia-induced angiogenesis by PPAR-γ ligands may prove to be a promising therapy for the treatment of cancer; however, the precise mechanisms involved in hypoxia-induced angiogenesis process remain to be clarified.

6. CONCLUSION

At the present, there is quite a lot of evidence to support that PPAR-γ may be considered as therapeutic target for diverse disease states in which excessive angiogenesis is implicated, including cancer. The most comprehensive data so far have revealed that PPAR-γ ligands are capable of inhibiting angiogenesis implicated in tumor malignant transformation and expansion. Targeting ECs proliferation and migration seems to be a dominant effect of PPAR-γ ligands on tumor angiogenesis. Indirect mechanisms that involve the counterbalance between a multitude of endogenous angiogenic and antiangiogenic factors further account for the inhibitory effects of PPAR-γ ligands on tumor angiogenesis. According to these data, PPAR-γ ligands may unfold new perspectives in clinical use against primary tumor growth and metastasis, since tumors that exhibit multidrug resistance are effectively targeted by antiangiogenic chemotherapy. Such perspectives could be clinically relevant, as PGZ and RGZ are orally administered FDA-approved drugs, already been used by million patients undergoing standard antidiabetic treatment.

On the other hand, there are several lines of evidence that PPAR-γ ligands can also enhance tumor angiogenesis progression under certain conditions. This controversy could be attributed to the pleiotropic action of PPAR-γ ligands, possibly via cofactors, either coactivators or corepressors. Such discrepancies may also be ascribed either to differences in time and dose of PPAR-γ ligand treatment, or to differences among the various organisms and types of cells that have been studied. It should be taken into account that angiogenesis is a multifaceted process that involves a wide range of mediators capable of inducing or suppressing angiogenesis in addition to the degree of tissue hypoxia. Consequently, the final outcome is difficult to be assessed accurately and depends significantly on experimental models and/or treatment conditions. Moreover, each type of cancer in humans presents individual and distinct vascular pattern on the microenvironment in which it is located. Thus, it should be taken into careful consideration the type of cancer being treated when deciding an appropriate therapeutic strategy.

In this aspect, the use of different cancer models, in vitro and in vivo, are strongly recommended to further define the molecular interactions amongst PPAR-γ, angiogenic/antiangiogenic factors, and tumor progression markers within the distinct cancer types. Future research effort should also be orientated to the clinical evaluation of PPAR-γ expression in aggressive tumor cancers in which various angiogenic/antiangiogenic factors exhibit high prognostic value. Such studies could delineate the potential of PPAR-γ ligands in future anticancer therapeutic strategies, either alone or combined with conventional chemotherapy.

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Review Article

PPARα Ligands as Antitumorigenic and Antiangiogenic Agents

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Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor family of ligand-activated transcription factors. This subfamily is composed of three members—PPARα, PPARδ, and PPARγ—that differ in their cell and tissue distribution as well as in their target genes. PPARα is abundantly expressed in liver, brown adipose tissue, kidney, intestine, heart, and skeletal muscle; and its ligands have been used to treat diseases such as obesity and diabetes. The recent finding that members of the PPAR family, including the PPARα, are expressed by tumor and endothelial cells together with the observation that PPAR ligands regulate cell growth, survival, migration, and invasion, suggested that PPARs also play a role in cancer. In this review, we focus on the contribution of PPARα to tumor and endothelial cell functions and provide compelling evidence that PPARα can be viewed as a new class of ligand activated tumor "suppressor" gene with antiangiogenic and antitumorigenic activities. Given that PPAR ligands are currently used in medicine as hypolipidemic drugs with excellent tolerance and limited toxicity, PPARα activation might offer a novel and potentially low-toxic approach for the treatment of tumor-associated angiogenesis and cancer.

1. THE PEROXISOMAL PROLIFERATOR-ACTIVATED RECEPTORS (PPARs)

PPARs nuclear receptors that regulate many physiological processes, including lipid and glucose homeostasis, inflammation, and wound healing [1]. Three PPAR isotypes have been identified: α, δ (or β), and γ. Upon ligand binding, PPARs form heterodimers with the retinoic acid receptor and interact with specific response elements in the promoter region of target genes [2]. Although PPARs share extensive structural homology, each isotype appears to possess distinct functions. PPARγ is expressed mainly in adipose tissue and at lower levels in intestine and immune cells [3–5]. It controls adipocyte differentiation, glucose and lipid homeostasis [5–7] and has been implicated in the pathophysiology of insulin resistance and atherosclerosis [1, 8]. PPARγ ligands include long-chain fatty acids, prostaglandins, and other eicosanoids [4]. Among the synthetic PPARγ ligands, the thiazolidinediones are currently used as insulin sensitizers in patients with type-2 diabetes [9]. PPARδ is ubiquitously expressed and it is most abundant in brain, colon, and skin [10, 11], and binds molecules such as fatty acids and prostaglandins [4].

PPARα is primarily expressed in liver, brown adipose tissue, kidney, intestine, heart, and skeletal muscle. This receptor controls fatty acid metabolism and transport, peroxisomal and mitochondrial β-oxidation [3, 4]. Moreover, this receptor has been implicated in the pathophysiology of inflammation and cardiovascular diseases [12]. Several compounds bind PPARα, including fatty and phytanic acids [4], as well as the fibric acid derivatives used in medicine for the treatment of hyperlipidemias [1].

2. PPARs AND CANCER

The observation that members of the PPAR family are expressed by tumor and endothelial cells [13, 14] together with the finding that PPAR ligands regulate cell growth, survival, migration, and invasion [15, 16] prompted investigators to determine whether these receptors play a role in the pathophysiology of tumorigenesis and angiogenesis [17, 18].

The anticancer effects of PPARγ agonists have been extensively studied because of their antiproliferative, proapoptotic, antiapoptotic, and differentiation-promoting activities [19]. In this context, activation of PPARγ has been

reported to reduce tumor cell proliferation and invasion [20] and to enhance apoptosis [21]. PPARγ ligands also regulate endothelial cell growth, migration, and angiogenesis [22–25], and influence the progression of vascular inflammation and tumorigenesis [26, 27]. Moreover, disruption of the PPARγ gene in the intestine enhances tumorigenesis in Apc$^{Min/+}$ mice [28]. Although these studies suggest that PPARγ functions as a tumor suppressor factor and its activation might be beneficial for patients with tumors, PPARγ agonists have been shown also to increase the frequency of colon tumors [29] and to promote edema [30].

In contrast to PPARγ, PPARδ has been described as protumorigenic as its ligand-mediated activation increases tumor-associated angiogenesis [31]. Moreover, treatment of Apc$^{Min/+}$ mice with PPARδ antagonists or crossing these mice with PPARδ-null mice prevents tumor growth and angiogenesis [31]. However, a recent study showed that activation of this receptor attenuates chemically-induced colon carcinogenesis, and that PPARδ-null mice exhibit increased colon polyp multiplicity, suggesting that ligand activation of this receptor can also inhibit carcinogenesis [32].

The analysis of the antitumorigenic properties of PPARα ligands has been less studied mostly due to the observation that long-term administration of certain PPARα agonists (Clofibrateand WY14643) induces hepatocarcinogenesis in rodents [33–35], despite the fact that PPARα ligands are widely used in medicine as antilipidemic drugs with excellent tolerance and little or no reported side effects. The finding that fenofibrate decreases VEGF levels in patients with hyperlipidemiaand atherosclerosis [36] provided a rationale for analyzing PPARα and its ligands as a molecular target for cancer therapy. In this review, we highlight some of the key functions attributed to PPARα in the context of endothelial and tumor cell biology.

3. PPARα TARGETS IN ANGIOGENESIS

PPARα controls the transcription of many genes involved in cell functions such as lipid metabolisms, inflammation, cell cycle progression, and angiogenesis. Among the angiogenic targets, PPARα has been shown to regulate the expression of the vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), members of the arachidonic acid P450 monooxygenases, thrombospondin and endostatin to name few (see also Figure 1 and Table 1). Biscetti et al. have recently shown that the selective PPARα agonist WY14643 promotes cornea angiogenesis in vivo and enhances endothelial tubulogenesis in vitro [37]. Interestingly, WY14643 can enhance endothelial cell tubulogenesis in vitro only when endothelial cells are cocultured with interstitial cells and this effect is accompanied by upregulation of interstitial-derived VEGF synthesis [37]. However, WY14643 does not directly promote endothelial cell migration or proliferation, and when used at 10–20 μM range it reduces both endothelial cell proliferation and migration [37]. Thus, this study indicates that while WY14643 might directly prevent endothelial cell functions, it might also promote angiogenesis by stimulating the production of nonendothelial VEGF. The observation

that activation of PPARα prevents endothelial cell proliferation/migration parallels our findings that WY14643 prevents—in a PPARα-dependent fashion—endothelial cell proliferation in vitro and tumorigenesis in vivo [38]. The antiangiogenic properties of WY14643 are associated with a PPARα-dependent downregulation of the epoxygenase branch of the cytochrome P450 arachidonic acid monooxygenases [38]. The arachidonic acid epoxygenases are expressed by endothelial cells both in vitro and in vivo [39–41] and catalyze the oxidation of arachidonic acid to four regioisomeric epoxyeicosatrienoic acids (EETs) [42, 43]. EETs have been shown to possess proangiogenic activities [39, 44–47] and we have demonstrated that WY14643-mediated PPARα activation directly prevents endothelial cell migration and proliferation by downregulating endothelial arachidonate epoxygenase expression and EET biosynthesis [38]. Most importantly, in vivo treatment with WY14643 prevents primary tumor growth and tumor-associated angiogenesis by downregulating the levels of circulating EETs [38].

Consistent with the observation that PPARα ligands might act as potent direct and/or indirect antiangiogenic factors, Panigrahy et al. have recently shown that fenofibrate suppresses VEGF-mediated endothelial cell proliferation as well as tumor cell-derived VEGF and FGF2 synthesis with concomitant stimulation of tumor-cells derived thrombospondin and endostatin [48]. Moreover, fenofibrate and WY14643 prevent VEGF-mediated endothelial cell migration by inhibiting Akt phosphorylation [24] and fenofibrate prevents endothelial cell proliferation by inhibiting cyclooxygenase-2 expression [25]. Finally, PPARα agonists were found to inhibit endothelial VEGFR2 expression by preventing Sp1-dependent promoter binding and transactivation [23]. Some of the major PPARα targets known to control endothelial cell functions and the effects of PPARα ligands on angiogenesis are summarized in Figure 1 and Table 1.

In conclusion these studies strongly suggest that by preventing endothelial cell functions PPARα ligands may protect the vasculature from pathological alterations associated with either metabolic disorders (i.e., atherosclerosis, diabetes) or cancer. Thus, PPARα can be considered as a new class of "antiangiogenic" gene, and suggest that its ligands may function as effective antiangiogenic drugs.

4. PPARα TARGETS IN CANCER

The observation that PPARα is expressed by tumor cells [59–61] started studies of the role of this nuclear receptor and its ligands on the prevention of tumor cell proliferation in vitro and in vivo. In this context it has been shown that PPARα ligands suppress the growth of several cancer lines—including colon, liver, breast, endometrial, and skin—in vitro [62–66], as we all inhibit the metastatic potential of melanoma cells in vitro and in vivo [67, 68]. Furthermore, PPARα ligands decrease colon carcinogenesis [62] and the growth of human ovarian cancer in mice [49]. Although the mechanisms whereby PPARα directly prevents tumor cell functions have not been investigated

Table 1: Effect of PPARα activation on angiogenesis and tumorigenesis.

Ligand	Cell type	Effect	Target	Reference
WY14643	Endothelial cells	Inhibition of cell proliferation and tubulogenesis in vitro Antiangiogenic activity in vivo	Downregulation of arachidonate epoxygenase synthesis	[38]
WY14643	Endothelial cells	Enhanced endothelial tube formation in vitro Proangiogenic activity in vivo	Upregulation of VEGF production	[37]
Fenofibrate WY14643 ETYA	Endothelial cells	Inhibition of VEGF- or FGF2-mediated cell proliferation in vitro Antiangiogenic activity in vivo	Downregulation of VEGF production Upregulation of thrombospondin and endostatin production	[48]
Fenofibrate WY14643	Endothelial cells	Reduced cell migration	Inhibition of Akt activation	[24]
Fenofibrate	Endothelial cells	Reduced cell proliferation	Inhibition of cyclooxygenase-2 expression	[25]
Fenofibrate	Endothelial cells	Reduced cell proliferation	Inhibition of VEGFR2 expression	[23]
Clofibrate	Ovarian cancer cells	Reduced cell proliferation in vitro Antitumorigenic activity in vivo	Reduced prostanoid and VEGF levels via upregulation of carbonyl reductase expression	[49]
Methylclofenapate	Colonic adenocarcinoma	Reduced cell proliferation	Not investigated	[50]
Methylclofenapate	$Apc^{Min/+}$ mice	Reduced number of intestinal polyps	Not investigated	[50]
Bezafibrate	APC^{1309} mice $Apc^{Min/+}$ mice	Reduced number of intestinal polyps	Reduced serum level of triglycerides and increased lipoprotein lipase synthesis	[27, 51]
WY14643	Wild-type mice	Enhanced hepatocellular proliferation and tumorigenesis in vivo	Downregulation of the miRNA let-7C with increased c-myc expression	[52]

↑ Carbonyl reductase
↓ Serum triglycerides
↑ Lipoprotein lipase synthesis
↑ Tumor suppressor p16INK4a

↑ Thrombospondin synthesis
↑ Endostatin generation
↓ VEGF synthesis

↓ P450 arachidonate epoxygenase-derived EETs
↓ Cyclooxygenase-2 expression
↓ VEGFR2 expression
↓ VEGF-mediated Akt activation
↓ VEGF- or FGF2-mediated proliferation and migration

■ Direct PPARα ligand-mediated anti-angiogenic functions
■ Direct PPARα ligand-mediated anti-tumorigenic functions
■ Indirect PPARα ligand-mediated anti-angiogenic functions

Figure 1: *Schematic representation of the antiangiogenic and antitumorigenic properties of PPARα.* PPARα ligands reduce tumor growth by direct inhibition of tumor cell functions (black pathway). In addition, they prevent tumor-associated angiogenesis via direct (red pathway) as well as indirect (green pathway) inhibition of endothelial cell functions.

TABLE 2: PPARα and tumorigenesis: lessons from the PPARα-null mice.

Ligand	Host	Challenge	Effect	Target	Reference
WY14643	PPARα-null mice		Resistant to the development of spontaneous hepatocarcinoma	Inability to downregulate the miRNA let-7C	[52]
WY14643 Fenofibrate	PPARα-humanized transgenic mouse		Resistant to the development of spontaneous hepatocarcinoma	Inability to downregulate the microRNA let-7C	[53, 54]
WY14643	PPARα-null mice	Injection of isogenic tumor cells	Resistant to the Wyeth-mediated antiangiogenic and antitumorigenic activities	Inability to downregulate arachidonate epoxygenase expression	[38]
DEHP	PPARα-null mice		Development of hepatocarcinoma	Increased PPARα-independent oxidative stress	[55]
WY14643 Fenofibrate	PPARα-null mice	Carotid arterial injury	Intimal hyperplasia	Inability to induce the expression of the tumor suppressor p16INK4a	[56]
	PPARα-null mice	Injection of isogenic tumor cells	Resistant to the development of primary and metastatic tumor growth	Increased recruitment of granulocyte responsible for thrombospondin production	[57]
	PPARα-null mice		Increased susceptibility to spontaneous adenomas and hepatocellular carcinomas	Not explored	[58]

in details, potential targets have been identified. Clofibrate, a PPARα ligand, significantly suppressed the growth of OVCAR-3 xenotransplanted tumors and inhibited ovarian tumor cell proliferation by increasing the expression of carbonyl reductase, an enzyme that promotes the conversion of protumorigenic prostaglandin E2 to inactive PGF2α [49]. Moreover, clofibrate reduced the levels of circulating VEGF in tumor-bearing mice [49], while bezafibrate, another PPARα ligand, decreased the number of intestinal polyps in Apc[Min/+] mice possibly by lowering serum level of triglycerides and upregulating lipoprotein lipase synthesis [27, 51]. Finally, PPARα activation has been shown to inhibit vascular smooth muscle cell proliferation underlying intimal hyperplasia by inducing the expression of the tumor suppressor p16INK4a [56].

Whereas these studies clearly suggest that PPARα activation might be beneficial in reducing cancer growth, studies from the Gonzales laboratory demonstrate that long-term administration of certain PPARα agonists (clofibrate and WY14643) induces liver adenoma and carcinomas in rats and mice [35, 52, 69, 70]. The ability of PPARα ligands to induce hepatocarcinoma is PPARα-dependent and mediated by the novel microRNA let-7C/c-myc axis [52]. Let-7C is a micro RNA that controls cell growth by directly down-regulating c-myc expression [52]. Upon treatment of mice

with WY14643, the hepatic expression of let-7C decreases with the concomitant induction of c-myc and the increased expression of the oncogenic mir-17-92 cluster [52]. Thus, this novel rodent specific PPARα-regulated pathway might be responsible for increased hepatocellular proliferation and tumorigenesis.

All together, these findings indicate that, with few exceptions, PPARα ligands can be viewed as antitumorigenic agents either by directly preventing tumor cell functions or by preventing tumor-derived production of proangiogenic molecules. Some of the potential PPARα targets that control tumor cell functions and the effects of PPARα ligands on tumorigenesis are summarized in Figure 1 and Table 1.

5. PPARα LIGANDS AND TUMORIGENESIS: LESSONS FROM MICE

The generation of PPARα null mice has provided an excellent tool not only to determine whether the effects exerted by PPARα ligand are indeed PPARα-dependent, but also for discerning between host versus tumor-mediated PPARα responses (see Table 2 for details). In this regard, we have shown that wild-type mice injected with isogenic PPARα expressing tumor cells respond to WY14643 treatment and develop fewer and smaller tumors than untreated wild-type mice [38]. In contrast, the growth of the same tumor cells is

not prevented in WY14643-treated PPARα null mice [38]. In agreement with our finding, absence of PPARα in the host animals abrogated the potent antitumor effect of fenofibrate [48]. Finally whereas in vivo activation of PPARα prevents vascular smooth muscle cell proliferation underlying intimal hyperplasia, PPARα deficiency leads to hyperplasia [56]. Taken together, these results strongly suggest that activation of PPARα in the host is a key element in preventing unwanted pathological cell growth.

Although rodents are the only species in which activation of PPARα promotes liver cancer, for a long time it was thought that Di(2-ethylhexyl)phthalate (DEHP), a commonly used industrial plasticizer, might cause liver tumorigenesis presumably via activation of PPARα [55, 71]. The use of PPARα null mice has disproved this idea, as this plasticizer is able to induce tumorigenesis in both wild-type and PPARα-null mice [55, 71]. These results suggest the existence of pathways for DEHP-induced hepatic tumorigenesis that are independent of PPARα, but most likely dependent on DEHP-mediated oxidative stress [55].

PPARα null mice have been also instrumental to determine the role of rodent versus human PPARα in the promotion of liver carcinogenesis. Morimura et al. have generated a PPARα-humanized mouse in which the human PPARα is expressed in liver under control of the Tet-OFF system. Interestingly, prolonged exposure to WY14643 in these mice only led to a 5% incidence of liver tumors—including hepatocellular carcinoma—compared to the 71% observed in mice expressing the mouse PPARα [53]. More recently, Yang et al. generated a PPARα-humanized transgenic mouse where the complete human PPARα gene was introduced onto a PPARα-null background [54]. These PPARα-humanized mice express the human PPARα in liver as well as other tissues and respond to fenofibrate treatment by lowering serum triglycerides and by inducing the expression of enzymes involved in fatty acid metabolism [54]. However, in contrast to wild-type mice, treatment with fenofibrate did not cause significant hepatomegaly, hepatocyte proliferation, and most importantly hepatocarcinoma [54]. Thus, this study shows that the protumorigenic let-7C/c-myc pathway is activated only by the rodent, but not the human PPARα receptor. Most importantly, this work highlights the possibility that PPARα ligands might be used as safe drugs for the treatment of cancer in humans.

Although activation of PPARα in either endothelial or tumor cells has been proven to be beneficial in inhibiting cancer growth, it has also been shown that loss of host-derived PPARα can be advantageous as it prevents tumor growth and development [57]. The host cells responsible for this protection, however, are granulocytes rather than endothelial cells. Loss of PPARα leads to an increased infiltration to the side of injury of granulocytes that suppress tumor-associated angiogenesis via excess production of the endogenous angiogenesis inhibitor thrombospondin [57]. This study clearly indicates that both activation of PPARα in specific host cells (i.e., endothelia cells) and concomitant inhibition of PPARα in immuno cells (i.e., granulocytes) might lead to the same effects, namely protection from tumor growth.

6. CONCLUSIONS

The studies summarized in this review identify PPARα as a potential host-based target for the development of new antiangiogenic approaches to inhibit and/or prevent tumor growth. As an established modulator of gene transcription, PPARα regulates the expression of genes known to be involved in energy metabolism, cellular proliferation, and angiogenesis and to have positive effects on the control of dyslipidemia, inflammation, and cardiovascular diseases. Furthermore, several fibric acid derivatives bind to and activate human PPARα with limited or no documented unwanted consequences and have proven to be safe and effective hypolipidemic drugs. In this context, gemfibrozil safely reduced the risk of death from coronary heart disease, nonfatal myocardial infarction, or stroke by raising HDL cholesterol levels and lowering levels of triglycerides [72, 73].

The effects of PPARα ligands in animal models of tumor angiogenesis should help not only to stimulate further research of their usefulness as antitumorigenic agents, but also to facilitate their evaluation as valid tools for the treatment and/or prevention of human cancers. In this context, it is our hope that these studies will serve to encourage epidemiological studies of cancer incidence in patients using hypolipidemic drugs, and help to identify their potential beneficial effects as agents for tumor prevention and/or treatment. The urgency of new approaches for cancer treatment are indicated by the fact that most current antitumorigenic therapies are oriented towards a general inhibition of tumor cell growth and, as such, they suffer from lacking target selectivity and, in most cases, causing severe side effects and overall systemic toxicity. Thus, targeting PPARα may prove to be a potential therapeutic strategy—either alone or in combination with conventional chemotherapy—to inhibit and ideally prevent cancer with excellent tolerance and limited toxicity.

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Review Article

Role of Peroxisome Proliferator-Activated Receptor Alpha in the Control of Cyclooxygenase 2 and Vascular Endothelial Growth Factor: Involvement in Tumor Growth

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A growing body of evidence indicates that PPAR (peroxisome proliferator-activated receptor) α agonists might have therapeutic usefulness in antitumoral therapy by decreasing abnormal cell growth, and reducing tumoral angiogenesis. Most of the anti-inflammatory and antineoplastic properties of PPAR ligands are due to their inhibitory effects on transcription of a variety of genes involved in inflammation, cell growth and angiogenesis. Cyclooxygenase (COX)-2 and vascular endothelial growth factor (VEGF) are crucial agents in inflammatory and angiogenic processes. They also have been significantly associated to cell proliferation, tumor growth, and metastasis, promoting tumor-associated angiogenesis. Aberrant expression of VEGF and COX-2 has been observed in a variety of tumors, pointing to these proteins as important therapeutic targets in the treatment of pathological angiogenesis and tumor growth. This review summarizes the current understanding of the role of PPARα and its ligands in the regulation of COX-2 and VEGF gene expression in the context of tumor progression.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor family of transcription factors. They modulate gene transcription in response to specific ligands by binding as heterodimers with the retinoid X receptor (RXR) to a specific peroxisome proliferator-response element (PPREs) on target genes (reviewed in [1, 2]). Three distinct isoforms of PPARs have been described PPAR α, β/δ, and γ, each encoded by a different gene and showing a distinct tissue distribution [3]. Originally found to be involved in the regulation of lipid and glucose metabolism, [4, 5] interest in these receptors has increased dramatically as a consequence of recent studies showing their involvement in tumoral cell growth and inflammation (reviewed in [6–9]). Therefore, in addition to their well-known effects in diabetes and hyperlipidemic disorders, pharmacological agents that target PPARs may have therapeutic applications in inflammatory diseases and cancer.

PPARα was the first PPAR identified [10]. It has a wide tissue distribution being expressed in liver, skeletal muscle, intestine, kidney, adipose tissue, and vascular endothelial cells, tissues in which fatty acids are predominantly catabolized [11, 12]. Several ligands have been shown to bind PPARα and to regulate transcriptional activity of target genes involved in the regulation of fatty acid metabolism as fatty acid transporters, catabolic enzymes involved in mitochondrial, and peroxisomal oxidation as well as genes necessary for the maintenance of redox balance during the oxidative catabolism of fatty acids [4, 5, 13]. PPARα-activating ligands include a number of pharmacological compounds as well as fatty acid and fatty acid-derived molecules (reviewed in [2]). Lipid-lowering fibrates as Bezafibrate, Clofibrate, Fenofibrate and Gemfibrozil as well as certain nonsteroidal anti-inflammatory drugs (NSAIDs) bind and activate PPARα [14, 15]. In addition, a variety of unsaturated and saturated fatty acids including arachidonic acid, palmitic acid, linoleic acid, linolenic acid, and oleic acid can act as natural ligands of PPARα as they can bind and activate this receptor. Some eicosanoids derived from the metabolism of arachidonic acid such as leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), and prostaglandins (PGs) can also be effective

ligand agonists for specific PPAR isoforms [16–19]. However, it is not clear whether the concentrations at which binding of these compounds occurs are physiologically relevant.

In addition to its known role in the regulation of genes involved in fatty acid metabolism, this isoform has been shown to attenuate the inflammatory response [6, 7, 9]. Most recently, an antitumoral role of PPARα and its ligands has been proposed [7, 8]. This assumption is supported by recent experimental evidence, revealing antitumoral and anti-angiogenic properties of PPARα activators both in vitro and in vivo.

2. ANTI-INFLAMMATORY ACTIONS OF PPARα

In recent years, considerable attention has focused on the involvement of PPARs in inflammatory processes. Anti-inflammatory effects of PPAR ligands, in particular those of PPARα and PPARγ, have been ascribed to inhibition of the expression of inflammatory genes and negative interference with pro inflammatory transcription factor signaling pathways in vascular and inflammatory cells (reviewed in [1, 6, 20]. The first evidence for the involvement of PPARα in the control of inflammation came from the studies of the inflammatory response elicited by LTB4 in PPARα deficient mice. In these animals, inflammatory response to LTB4 was prolonged compared to WT mice, correlating with the ability of LTB4 to activate PPARα and regulate the expression of genes involved in its own catabolism [21]. Thus, proinflammatory lipid metabolites may serve as ligands for PPARs thereby activating PPARα responsive enzymes responsible for their clearance. Therefore, some of the anti-inflammatory actions of these receptors can occur through this autoregulatory loop in lipid homeostasis. Several lipids mediators, as the polyunsaturated fatty acids (PUFAs), can function as ligands for PPARs [2]. Arachidonic acid is a precursor of several eicosanoids that have pro inflammatory properties whereas the ω-3 PUFAs are precursors of anti-inflammatory eicosanoids as EPA (eicosapentanoid acid) and docosahexaenoic acid (DHA). These ω-3 PUFAs derivatives have been reported to decrease the production of several pro inflammatory cytokines, having beneficial effects in several inflammatory diseases as rheumatoid arthritis and inflammatory bowel disease [22, 23].

Several studies have confirmed the anti-inflammatory properties of PPARs in vitro and in vivo through the regulation of genes involved in the inflammatory response. PPAR agonists decrease plasma concentrations of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in humans [24, 25]. In vascular smooth-muscle cells, PPARα ligands inhibit IL-1-induced production of IL-6, COX-2, and prostaglandins [25]. PPARα activation inhibits cytokine-induced expression of vascular cell-adhesion molecule-1 (VCAM-1) and vascular endothelial growth factor receptor-2 (VEGFR-2) in endothelial cells [26, 27]. PPARα have been also involved in the downregulation of the production of IL-2 and TNFα by T lymphocytes [28].

At the molecular level, most of the inhibitory actions of PPARα on gene transcription result from its ability to transrepress the activities of many activated transcription factors

such as nuclear factor (NF)-κB [25, 29], activator protein-1 (AP-1) [29, 30] C/EBPβ [31, 32], signal transducers and activators of transcription (STATs) [33], and transcription factor specificity protein 1 (Sp-1) [27]. Negative regulation of gene expression by PPARα can occur by several mechanisms (Figure 1). Activated PPAR-RXR complexes may compete for limited amounts of essential coactivators shared with other transcription factors. Direct physical interactions between PPARs and specific transcription factors have been also proposed to mediate transcriptional inhibition by activated PPARα, resulting in reduced binding to their cognate response elements. Agonists-activated PPARα can effectively antagonize NF-κB and AP-1-mediated signaling pathways in a bidirectional manner by physical interaction with the Rel homology domain of NFκB-p65 and with the aminoterminal of c-Jun, respectively [34]. Physical association of the C-terminal DNA binding region of c/EBPβ with PPARα mediates inhibition of alpha1-acid glycoprotein gene expression [31]. PPARα agonists can also influence transcriptional activation by modulating the expression of transcriptional repressors such as IκBα (Kleemann, 2003 #1223; Vanden Berghe, 2003 #2170}). Finally, an additional mechanism of transrepression relies on the ability of some PPARα ligands to interfere with the activation of certain members of the mitogen-activated protein kinase (MAPK) cascade as Jun kinase (JNK) and p38 MAPK [30, 35, 36].

3. INVOLVEMENT OF PPARα IN CANCER

Rapidly accumulating evidence links members of the PPAR family and their agonists to cellular growth and tumor progression. The role of the PPARγ isotype in cancer has been widely studied with a large number of reports demonstrating antitumoral properties of PPARγ agonists in a variety of different malignancies [8]. Concerning to the involvement of the PPARα form and its ligands in cancer, both tumor promotion and suppression properties have been reported [37]. Sustained PPARα activation by agonists as clofibrate and Wy-14643 induce hepatocarcinogenesis in rodents [38, 39]. However, epidemiological data on long-term administration of PPARα activators in the clinic discard the occurrence of these effects in human [40–43]. Discrepancies on the effects of PPARα ligands in rodents and human liver seem to be due to several differences between species [44]. PPARα mediated signaling is less efficient in human than in mice [37, 43, 45] and expression of this receptor is 10 to 20 times higher in rodent hepatocytes compared to human liver [46].

Emerging evidence indicates that PPARα ligands are able to suppress the growth of different types of human carcinomas. PPARα is expressed in a variety of human and murine tumor cell lines [47–49]. Expression of PPARα have been also reported in clinical samples of several types of human cancers as colorectal carcinoma [50], prostate adenocarcinoma, [47, 51], testicular cancer [52], bladder carcinoma [53], and medulloblastoma [54]. PPARα ligands are able to arrest the growth of human cancer cell lines in vitro and to slow the growth of transplanted human tumor cells in nude mice. These anticancer effects have been
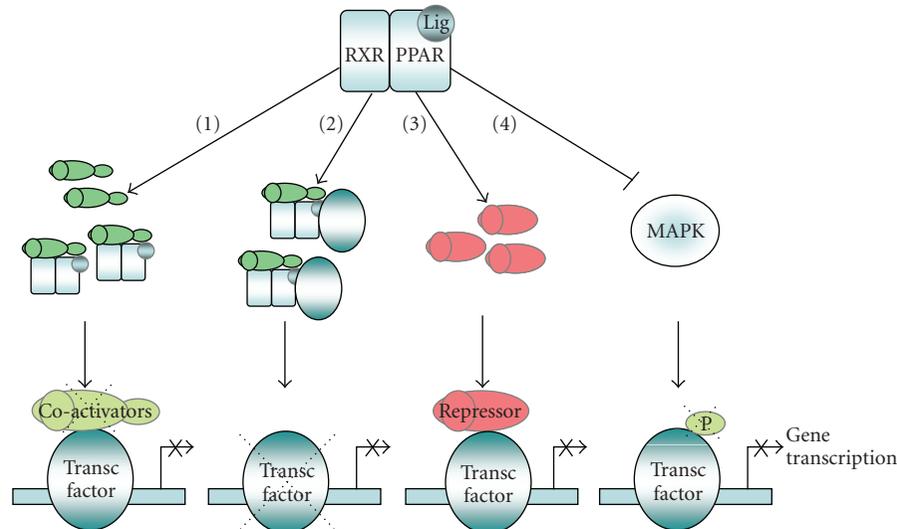
FIGURE 1: *Negative regulation of gene expression by PPARs.* Different mechanisms of transrepression through interference of activated PPARs with activation of transcription factors have been described. (1) Activated PPAR-RXR complexes may sequester essential coactivators shared with other transcription factors. (2) Physical association of PPARs with specific transcription factors results in reduced binding to their cognate response elements. (3) PPARα agonists can also influence transcriptional activation by upregulating the expression of transcriptional repressors such as IκBα. (4) PPARα-mediated interference on the activation of members of the mitogen-activated protein kinase (MAPK) cascade influences transcription factor activation.

observed in several different cancer cell types including hepatoma [55], melanoma [56], glioblastoma and fibrosarcoma [49], endometrial and ovarian cancer [57–59], as well as colon carcinoma [50, 60, 61]. Clofibric acid, a ligand for PPARα inhibits growth of ovarian cancer both in vivo and in vitro [58, 59]. Mice treated topically with the PPARα ligand Wy-14643 exhibited diminished skin tumorigenesis [62]. Moreover, Fenofibrate suppresses the metastatic potential of melanoma cells [56, 63].

Even though accumulative evidence shows data suggesting that PPARα ligands may display antitumoral properties, the precise mechanism remains unclear. A number of reports suggest that antitumor properties of PPARα activators reside on their anti-inflammatory and anti-angiogenic effects [27, 64, 65] (Figure 2). The dependence on the presence of PPARα for the antitumorigenic and anti-angiogenic role of PPAR ligands has been determined by the analysis of their effects in PPARα null mice. Wy-14643-mediated antitumoral and anti-angiogenic responses on tumor and endothelial cells are absent in PPARα KO mice [66]. Panigrahy and coworkers [49] have shown the importance of the microenvironment in tumor progression in such a way that the activation of PPARα expressed in endothelial and inflammatory cells of the host rather than in the tumoral cells is critical for anti-inflammatory, antitumor and anti-angiogenic activity of PPARα agonists. Consistent with the anti-inflammatory role of this receptor, PPARα null mice exhibit an increase of inflammatory infiltrates in tumors. Paradoxically, in spite of the enhanced inflammatory response in the absence of PPARα, tumor growth is suppressed in these animals as a consequence of an increased production of anti-angiogenic factors TSP-1 and endostatin [67]. The immune system

can have a multitude of effects on cancer development and progression, both favorable and detrimental [68, 69]. This apparent contradiction has been explained by the severity and duration of the inflammatory response associated to tumor growth. While acute inflammation is part of the defense response that may participate in the remission of preclinical cancers, chronic inflammation can promote tumor development with infiltrating innate immune cells providing proinflammatory and proangiogenic factors including cytokines, chemokines, VEGF, and prostanoids [70–72]. The association between cancer and inflammation has been also illustrated by epidemiological studies showing that the use of anti-inflammatory compounds in chronic inflammatory diseases reduces cancer risk tumor [73, 74]. In this sense, PPARα-mediated anti-inflammatory actions can be responsible for their potential chemopreventive effects in tumor progression.

An emerging area of interest is the association of anti-inflammatory actions of dietary PUFAs as potential natural agonists of PPARs with cancer risk. Increasing evidence suggests that dietary fat regulation of gene expression can play a critical role in initiation and progression of human cancer and epidemiological studies have suggested an association between dietary fat and cancer risk [75, 76]. A number of reports have shown the beneficial effects of consumption of ω-3 PUFAs, associated with anti-inflammatory effects and with protection against primary tumor development [22, 23, 75]. Although some of the effects of dietary lipids can be linked to PPARs-mediated signaling, additional research is needed to understand the potential connection between dietary fat intake and PPARs in the control of inflammation and tumor progression.
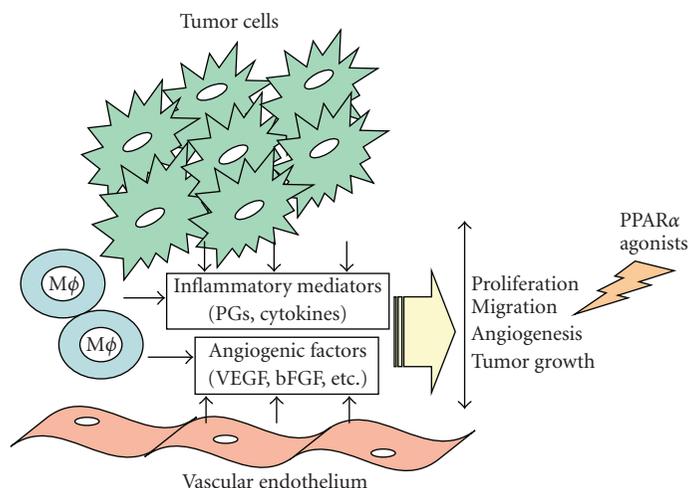
Figure 2: *Antitumoral effects of PPARα ligands*. Tumor progression depends on a cascade of cellular signals involving: (a) proinflammatory factors (cytokines, COX-2 derived prostaglandins (PGs), chemokines); (b) proangiogenic factors (VEGF, bFGF, and PGs) produced by endothelial and inflammatory cells, stromal fibroblasts, and tumor cells. These factors promote cell proliferation, migration, and induce new vessels that deliver nutrients and oxygen to the malignant cells and therefore allow tumor growth and metastasis. PPARα ligands may display antitumoral properties by their inhibitory effects on the transcription of genes involved in inflammation, cell growth, and angiogenesis thus leading to the inhibition of tumor growth.

Table 1: Effects of PPARα ligands on COX-2 signaling.

PPARα ligand	Action/effect	References
Wy-14643		
	Inhibition of IL-1-induced COX-2 expression in vascular smooth muscle cells	[25]
	Inhibition of LPS-induced COX-2 expression in THP-1 monocytes	[94]
	Inhibition of TPA-induced COX-2 expression in colon carcinoma cell lines	[30]
	Inhibition of COX-2 expression in B16-F10 melanoma tumor	[49]
	Inhibition of COX-2 up regulation by experimental steatohepatitis in liver	[95]
Fenofibrate		
	Inhibition of COX-2 expression in B16-F10 melanoma tumor	[49]
	Inhibition of b-FGF induced COX-2 expression in endothelial cells	[65]
Clofibric acid		
	Inhibition of COX-2 expression in tumor xenografts	[58]
	Inhibition of mPGES-1 expression in tumor xenografts	[59]

4. PPARα, COX-2, AND CANCER

4.1. COX-2: an essential role in inflammation and tumor growth

Cyclooxygenases (COX-1 and COX-2) convert arachidonic acid to prostaglandin $H_2(PGH_2)$, an endoperoxide intermediate that, via specific synthases, is then transformed to prostaglandins (PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2) and thromboxanes (TXA_2). Whereas COX-1 is constitutively expressed in most tissues, COX-2 is transcriptionally upregulated in response to mitogens, tumor promoters, and pro inflammatory stimuli in a discrete number of cell types (reviewed in [77–79]). These enzymes are the target of nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most widely used therapeutic for the relief of pain and inflammation (reviewed in [80–82]). The anti-inflammatory and analgesic efficacy of drugs arises essentially from inhibition of the enzymatic activity of COXs. As COX-2 is though to be the predominant isoform involved in the inflammatory response, the ability of NSAIDs to inhibit COX-2 activity may explain their therapeutic effects as anti-inflammatory drugs. Therefore, most of the new research on anti-inflammatory drugs has been aiming at targeting the COX-2 inducible production of PGs.

In addition to its essential role in inflammation, accumulating evidence links COX-2 with cancer and angiogenesis, suggesting that drugs that target COX-2 and related signaling cascades could be used as antitumoral agents [74]. Many human cancers display elevated COX-2 expression and studies in COX-2 null mice have demonstrated the role of this enzyme in tumor progression and metastasis [83–85].

TABLE 2: Effects of PPARα ligands on VEGF signaling.

PPARα ligand	Action/effect	References
Wy-14643		
	Inhibition of VEGF-mediated endothelial cell migration	[27, 49, 64]
	Inhibition of VEGF production by glioblastoma U87 cells	[49]
	Inhibition of VEGF-induced phosphorylation of Akt	[64]
	Inhibition of VEGF-mediated angiogenesis in vitro	[27]
Fenofibrate		
	Inhibition of VEGF production by glioblastoma U87 cells	[49]
	Reduction of plasma VEGF	[96]
	Inhibition of VEGF-induced phosphorylation of Akt	[64]
	Inhibition of VEGFR2 expression in endothelial cells	[27]
Clofibric acid		
	Inhibition of VEGF expression in tumor xenografts	[58]

Moreover, epidemiological studies have revealed a role of selective COX-2 inhibitors in decreasing the risk of developing colon cancer and in suppressing tumor formation and growth in animal models [73, 86–88]. COX-2 inhibitors can block both the production of angiogenic factors and the migration of vascular endothelial cells, and thus decrease tumor growth [89, 90]. Although some of the effects of these drugs on tumor regression might result from modulation of COX-2-independent pathways [91], it is likely that COX-2 is an important mediator of tumor growth.

4.2. Effects of PPARα ligands on COX-2 expression

The activity of COX-2 is closely linked to PPARs as COX-2 catalyzes the production of fatty acid derivates as prostanoids that are PPAR activators [2]. Modulation of COX-2 activity should influence the local availability of PPAR ligands; therefore, indirectly modulating PPAR activity. Moreover, some NSAIDs may act as PPARα and γ ligands, suggesting that, in addition to inhibit prostaglandin production, they might regulate gene expression as part of their anti-inflammatory and chemopreventive mechanisms [92, 93]. Downregulation of COX-2 expression by PPARα ligands may account for some of the anti-inflammatory, anti-angiogenic and antitumoral properties of these drugs in a variety of cell types [20] (Table 1). PPARα agonists as Wy-14643 inhibit macrophage differentiation and COX-2 gene expression [94]. In liver, COX-2 upregulation upon experimental nutritional steatohepatitis is suppressed by the PPARα agonist Wy-14,643 in *wt* but not in PPARα KO mice. This effect has been ascribed to the ability of activated PPARα to interfere with the NF-κB-signaling pathway [95]. Transcriptional interference of activated PPARα with NF-κB also explains the inhibition of COX-2 induction and PG production in response to IL-1 in vascular smooth muscle cells [25]. The NF-κB target genes VCAM-1 and COX-2 are also downregulated by PPARα ligands in response to cytokine activation [29]. Regarding to experimental support for anti-angiogenic and antitumoral actions of these drugs related to their effects on COX-2 expression, it has been

shown that Fenofibrate inhibits bFGF-mediated angiogenesis and COX-2 mRNA expression in endothelial cells [65]. Panigrahy and cols have observed suppression of COX-2 expression in Fenofibrate and Wy-14643 treated tumors [49]. Clofibric acid suppresses the growth of tumor xenograts of ovarian cancer cell lines with decreased microvessel density, PGE$_2$ production, and COX-2 and mPGES-1 expression [58, 59]. Diminished expression of COX-2 upon PPARα agonist treatment was parallel with reduced expression of AP-1. Similarly, in colon carcinoma cells, PPARα agonists severely diminish phorbol ester-mediated AP-1-dependent induction of COX-2 expression [30].

5. PPARα, VEGF, AND CANCER

5.1. VEGF: a therapeutic target in tumoral angiogenesis

Angiogenesis, defined as the formation of new blood vessels from preexisting vasculature, occurs under physiological conditions during embryonic development and is required for wound healing and reproduction in the adult. Indispensable for physiological processes, angiogenesis is highly regulated via fine tuning of the balance between pro- and anti-angiogenic factors [97]. Excessive angiogenesis is tightly linked to human disease, including chronic inflammatory disease, diabetic retinopathy, and cancer [98, 99]. Ample evidence shows that blockade of tumoral angiogenesis often relieves the severity of cancer [100].

Both cancer cells and cells attracted to the sites of inflammation are able to produce proangiogenic factors that cause endothelial cell recruitment and proliferation for the supply of oxygen and nutrients that favor the growth of solid tumors and facilitate metastasis. In this context, tumor-associated hypoxia plays an essential role in the regulation of angiogenesis [101]. Response to hypoxia is mediated by members of the hypoxia-inducible transcription factors (HIFs) involved in the regulation of the expression of genes participating in oxygen homeostasis [102, 103]. In addition, hypoxia has been also found to drive induction

of potent angiogenic and inflammatory factors including VEGF, VEGF-R1 and -R2, angiotensin, metalloproteinases, and COX-2 [104–106]. This response of tumors to hypoxia contributes to the malignant phenotype and to more aggressive tumor progression [107].

It has been well established that VEGF signaling pathway is one of the key regulators in angiogenesis [108, 109]. Cytokines, growth factors, tumor promoters, and hypoxia modulate the expression of VEGF [110, 111]. Activated VEGF may bind to two types of tyrosine kinases receptors: VEGFR-1 and VEGFR-2. Proangiogenic actions of VEGF as vascular endothelial cell permeability, proliferation migration, and survival are mediated mainly through binding an activation of the VEGFR-2 [112, 113]. Accumulating evidence supports a key role of VEGF in cancer, contributing to tumor neovascularization and dissemination. Increased expression of this factor has been found in most tumors, and agents neutralizing VEGF expression or activity inhibit tumor growth in vivo (reviewed in [99, 100, 114]).

5.2. Inhibition of VEGF signaling by PPARα agonists

PPARα ligands can inhibit endothelial cell proliferation and migration and induce endothelial cell apoptosis in vitro, suggesting an important role of this receptor in tumor angiogenesis. Part of these effects occurs through the ability of PPARα ligands to interfere with VEGF-mediated signaling. (Table 2). At the transcriptional level, PPAR agonists have been shown to inhibit endothelial VEGFR-2 expression by repressing transactivation and binding of Sp1 to DNA [27]. Wy-14643 downregulates TPA-mediated transcriptional induction of VEGF by interference with activation of AP-1 [30]. Interestingly, lipid-lowering therapy with Fenofibrate induces a significant reduction of VEGF levels in serum [96]. Anti-angiogenic actions of PPARα agonists may explain some of their antitumoral effects. Fenofibrate reduces adventitial angiogenesis and inflammation in a porcine model of coronary angioplasty [115]. Both Fenofibrate and Wy-14643 are able to suppress VEGF secretion in glioblastoma cells and Lewis lung carcinoma cells and to inhibit angiogenesis both in vivo and in vitro [49]. Moreover, Clofibric acid inhibits VEGF expression in tumor xenografts of ovarian cancer cell lines with a reduction in angiogenesis and decreased microvessel density in solid tumors [58, 59].

6. CONCLUSIONS

Taken together, findings on the effects of PPARα ligands in inflammation and cancer, suggest that PPARα activation may be beneficial against tumorigenesis through the inhibition of transcriptional activation of genes involved in inflammation and angiogenesis such as COX-2 and VEGF. Although COX-2 and VEGF are one of the many proinflammatory and proangiogenic factors that drive tumor growth and metastasis, targeting these proteins suffices to significantly impair tumor growth and angiogenesis. Inhibition of tumor inflammation and tumor angiogenesis by PPARα ligands might be responsible for their potential chemopreventive effects in a variety of experimental models of cancer.

However, it must be taken into account that many of the reported effects of PPARα ligands on tumor progression have been obtained in vitro and await confirmation by additional basic and clinical research to ascertain whether they can be considered of pharmacological significance in vivo.

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Review Article

Anticancer Properties of PPARα-Effects on Cellular Metabolism and Inflammation

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Peroxisome proliferator-activated receptors (PPARs) have lately attracted much attention as therapeutic targets. Previously, PPAR ligands were associated with the treatment of diabetes, hyperlipidemia and cardiovascular diseases, as they modulate the expression of genes regulating glucose and lipid metabolism. Recently, PPAR ligands have been also considered as potential anticancer agents, with relatively low systemic toxicity. The emerging evidence for antiproliferative, proapoptotic, antiinflammatory and potential antimetastatic properties of PPARα ligands prompted us to discuss possible roles of PPARα in tumor suppression. PPARα activation can target cancer cells energy balance by blocking fatty acid synthesis and by promoting fatty acid β-oxidation. In the state of limited nutrient availability, frequently presents in the tumor microenvironment, PPARα cooperates with AMP-dependent protein kinase in: (i) repressing oncogenic Akt activity, (ii) inhibiting cell proliferation, and (iii) forcing glycolysis-dependent cancer cells into "metabolic catastrophe." Other potential anticancer effects of PPARα include suppression of inflammation, and upregulation of uncoupling proteins (UCPs), which attenuates mitochondrial reactive oxygen species production and cell proliferation. In conclusion, there are strong premises that the low-toxic and well-tolerated PPAR ligands should be considered as new therapeutic agents to fight disseminating cancer, which represents the major challenge for modern medicine and basic research.

1. PPARα AND CANCER CELL ENERGY BALANCE

The concept that neoplastic transformation based on the failure of energy homeostasis is currently regaining considerable interest. This notion was associated with the hypothesis by Otto Warburg who indicated a distinctive dependence of tumor cell metabolism from glycolysis, even when there is sufficient amount of oxygen available for much more efficient oxidative phosphorylation [1, 2]. Only recently, it has been established that the inclination of tumor cells for glycolysis is mainly driven by mitochondrial dysfunction or oncogenic activity of Akt, Ras, or Myc [3, 4].

PPARα, which is a transcriptional activator of fatty acid β-oxidation machinery (e.g., acyl-CoA oxidase (ACO), acyl-CoA synthetase (ACS), carnitine palmitoyl transferase (CPT1), fatty acid binding protein (FABP), and fatty acid transporter (FAT)), can switch energy metabolism toward fatty acid degradation, and decrease glucose uptake by repressing glucose transporter GLUT4 [5, 6]. Interestingly, PPARα acts as a direct sensor for fatty acids, which are considered natural ligands for this nuclear receptor [7, 8]. According to fatty acid, glucose cycle paradigm increased rate of fatty acid and ketone bodies oxidation forces the decline in glucose utilization through the inhibition of glycolytic enzymes [9, 10]. This concept was supported by the results of animal studies, showing that during fasting-activated PPARα can divert energy metabolism from the glucose to fatty acid utilization as a primary source of energy.

Mitochondria are the main organelles that carry out fatty acid β-oxidation and produce ATP through oxidative phosphorylation [11]. Oncogenic transformation is frequently associated with mitochondrial dysfunction, however, it is still controversial if this is a result, cause, or contribution to the malignant phenotype [12]. A direct link between

aerobic respiration and carcinogenesis has been provided by the demonstration that the loss of p53, which is most commonly mutated gene in cancer, results in decrease of synthesis of cytochrome c oxidase (SCO2) gene expression [13]. SCO2 is crucial for the incorporation of mitochondrial DNA-encoded cytochrome c oxidase subunit (MTCO2) into the cytochrome c oxidase complex, and the proper assembly of this complex is essential for the mitochondrial respiration. Therefore, SCO2 downregulation in p53-deficient cells heavily inpairs oxidative phosphorylation and triggers the switch toward glycolysis [13].

Furthermore, loss of function mutations in the nuclear genes encoding the Krebs cycle enzymes (such as succinate dehydrogenase and fumarate hydratase) are frequently observed in uterine leiomyomas, renal carcinomas paragangliomas, and phaeochromocytomas [14]. The clinical data suggest that these proteins might have other functions besides energy metabolism and can be involved in the induction of apoptosis, similarly to mitochondrial apoptosis inducing factor (AIF) [15]. Nevertheless, it is likely that the glycolysis-promoting metabolism of cancer cells relieves the selection pressure and permits clonal growth of the cells with defective mitochondrial system. Such cells could be brought to the verge of metabolic catastrophe in the condition of limited glucose availability or when the oxidative metabolism is forced pharmacologically. This opens an opportunity for the use of PPARα ligands, as they should be selectively toxic for cancer cells and neutral for normal cells.

Energetic function of mitochondria is not restricted to ATP generation in the process of oxidative phosphorylation. Systemic thermal homeostasis maintained by mammals relies broadly on nonshivering thermogenesis carried on by brown adipocytes. In these cells, uncoupling protein (UCP1) is responsible for the "proton leak" of mitochondrial inner membrane, which separates respiration from ATP synthesis. The energy released through the proton flow in line with electric potential gradient is dissipated as heat.

Recently, several mammalian UCP homologues have been discovered, among which ubiquitously expressed UCP2 and muscle—specific UCP3 gained deep interest [16]. They share high degree of structural similarity with UCP1 though their primary function, which still remains elusive, is not limited to thermogenesis, but their mitochondrial uncoupling activity is connected with fatty acid anion transport. The expression of both UCP2 and UCP3 is regulated by PPARα [6, 17–19], and this notion provides an interesting link with cancer cell metabolism and behavior.

The recent report by Pecqueur and colleagues [20] has revealed that UCP2 controls proliferation through driving cellular metabolism to fatty acid oxidation and limiting glycolysis. UCP2- deficient cells proliferate significantly faster than wild-type cells and rely on glycolysis-derived pyruvate catabolism, like all rapidly normal and transformed dividing cells do. Remarkably, the higher proliferation rate in these cells is a result of cell cycle shrinkage and not the decrease in the quiescent (G0/G1) cell fraction, even though the proproliferative PI3K/Akt and MAPK signaling pathways are more activated in UCP −/− than wt cells [20]. Interestingly, UCP2 is also involved in cellular adhesion and invasive potential,

as was revealed in the studies on the THP1 monocytes with UCP2 overexpression, which showed impaired β2 integrin—mediated adhesion and transendothelial migration [21]. Taking together, these data suggest that PPARα- mediated UCP2 upregulation might have a negative impact on cancer progression.

Uncoupling proteins due to their ability to reduce ATP bisynthesis inhibit production of reactive oxygen species (ROS) during respiration. ROS and products of their activity, such as lipid peroxides, are not only toxic and mutagenic, but also stimulate inflammatory response, and therefore contribute to cancer development. PPARα regulates the expression of three proteins which govern the transport of fatty acids in and out of mitochondria. This includes CPT1 and UCP3 as well as mitochondrial thioesterase 1 (MTE-1) [17, 22]. This trio controls the mitochondrial pool of fatty acids in order to keep the danger of their peroxidation at minimal level. CPT1 supplies mitochondria with long chain fatty acid—CoA (LCFA-CoA) complexes, which undergo β-oxidation. At a high rate of β-oxidation, UCP3 in the conjunction with MTE-1 acts to prevent LCFA-CoA accumulation: MTE-1 releazes CoA-SH and enables its recycling, whereas UCP3 exports fatty acid anions outside the mitochondrial matrix, and therefore reduces the chance of their peroxidation by the superoxide generated in the complex I and III of mitochondrial electron chain [23–25]. Simultaneously, UCP2 and UCP3 due to their proton leak activity reduce the rate of ROS production, which is proportional to the protonmotive force [16, 26]. The hypothesis of protective role of PPARα in oxidative stress is supported by the results from in vivo studies showing that PPARα-deficient mice have higher level of oxidative damage in cardiac muscle, and that fenofibrate diminishes inflammatory response and oxidative stress in the neural tissue in rats subjected to traumatic brain injury [27, 28].

The above described evidence indicates that PPARα activation might metabolically target neoplastic cells through inhibition of glycolysis and promotion of fatty acid catabolism, but also might elicit chemopreventive effect through the decrease of respiratory ROS production.

Interestingly, the metabolic peculiarities of cancer cells are not restricted to aerobic glycolysis but paradoxically include also fatty acid synthesis. Some types of tumors, particularly of hormone responsive epithelial origin, are characterized by the abnormally high activity of fatty acid synthase (FAS), which is an enzyme with barely detectable levels in normal tissues. The FAS produces palmitate from the condensation of acetyl-CoA and malonyl-CoA. Interestingly, FAS overexpression correlates well with prostate cancer progression in which the highest levels of FAS activity have been observed in bone metastases [30]. For this reason, FAS has been named a "metabolic oncogene" [31]. FAS is also involved in biosynthesis of phospholipids, which are substrates for the new membrane synthesis in rapidly dividing cells, protein myristoylation, and lipid partitioning into membrane microdomains [31, 32]. FAS activity provides a significant growth advantage for transformed cells. Indeed, pharmacological inhibition of FAS induced apoptosis in cancer cell, possibly by the accumulation of
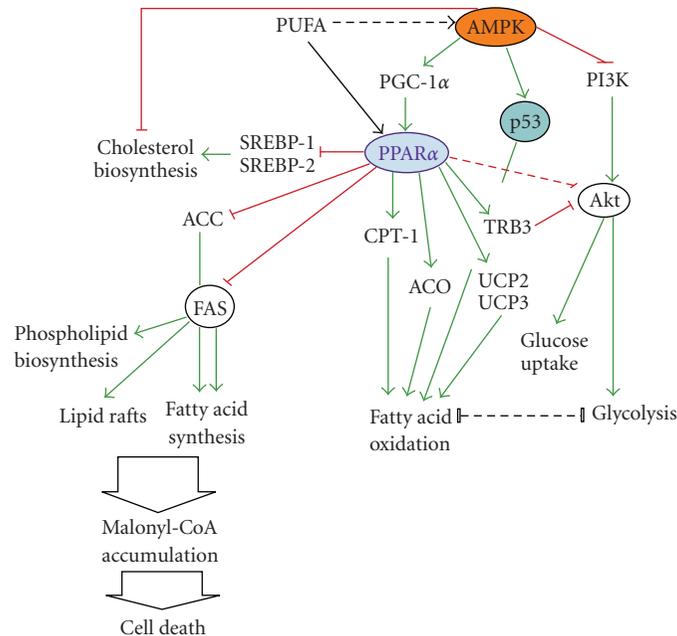
FIGURE 1: PPARα interferes with the metabolic pathways in the cancer cells. In the state of energy deprivation, AMPK activates fatty acid oxidation through PPARα- and p53-dependent pathways and blocks anabolic processes, for example, cholesterol biosynthesis. AMPK is a potent inhibitor of Akt-induced glycolysis. In response to nutrient deficiency, PGC-1α and PPARα upregulate expression of TRB3, which inactivates Akt via direct interaction [29]. PPARα promotes fatty acid β-oxidation as a transcriptional activator of fatty acid catabolic enzymes and transport proteins (e.g., ACO, CPT1, UCP2, and UCP3). Simulateneously, PPARα blocks lipid synthesis by repression of SREBP-1 and -2, ACC, and FAS. FAS inhibition in various cancer types results in toxic accumulation of malonyl-CoA and apoptosis. For more details, see the text. Arrowheads represent activation/upregulation, and blunted lines indicate inhibition/downregulation of the cellular proteins or processes. ACC—acetyl-coA carboxylase; ACO—acyl-coA oxidase; AMPK—AMP-dependent kinase; CTP-1—carnitine palmitoyltransferase-1; FAS—fatty acid synthase; PGC-1α—PPARγ coactivator 1α; PUFA—polyunsaturated fatty acids; SREBP—steroid response element binding protein; TRB3—mammalian homolog of *tribbles*; UCP2, UCP3—uncoupling proteins.

malonyl-CoA [33]. In addition, pharmacological or RNA silencing-mediated inhibition of FAS significantly reduced the expression of the oncogenic Her-2/neu (erbB-2) [34, 35], but it also induced a dramatic increase in VEGF expression by activating the Erk1/2 pathway [36].

Importantly, activation of PPARα has been shown to block FAS pathways through the transcriptional repression of genes, which are directly involved in its metabolic activity (FAS; acyl-CoA carboxylase (ACC); steroid response element binding proteins (SREBP1, SREBP2)) [37–41] (Figure 1). Simultaneously, PPARα blocks Erk1/2 activation [42]. Therefore, the possibility exists that PPARα agonists could block Her-2/neu expression without a danger of proangiogenic stimulation of VEGF expression. This might encourage new clinical applications for PPARα ligands against those cancer cells, which are characterized by the overactive FAS.

Lipid metabolism deregulation manifested by hiperlipidemia has been described as a significant risk factor for colorectal cancer development [43]. Increased serum triglyceride and cholesterol level were observed in patients with familial adenomatous polyposis coli. An interesting study by Niho and coworkers [44] showed that APC-deficient mice, the animal model for human adenomatous polyposis coli syndrome, when treated with PPARα ligand and lipid level normalizing drug—bezafibrate, develop

significantly fewer intestinal polyps. This protective action of PPARα agonists against colorectal carcinogenesis seems promising from the therapeutic point of view, suggesting that the patients might benefit not only from normolipidemic activity of PPARα, but also from its antineoplastic effects as well.

2. AMPK AND AUTOPHAGY

In the state of energy depletion, caused for instance by a limited glucose availability, normal cells can switch between energy metabolic pathways to support their survival. AMP-dependent protein kinase (AMPK) plays an integral role in the response to starvation by sensing the rise in AMP/ATP ratio and switching off the ATP-consuming anabolic processes, such as protein and lipid synthesis or DNA replication. AMPK can induce several rescue pathways, which enhance cell survival during glucose deprivation (Figures 1 and 2). One of them includes p53-dependent check point, which blocks cell cycle progression and promotes fatty acid oxidation and autophagy, as an alternative source of energy [45, 46]. Interestingly, p53-deficient cancer cells are very sensitive to the lack of glucose, and being incapable of autophagy, underwent massive apoptosis [46, 47]. It was demonstrated that PPARα acts downstream from AMPK and
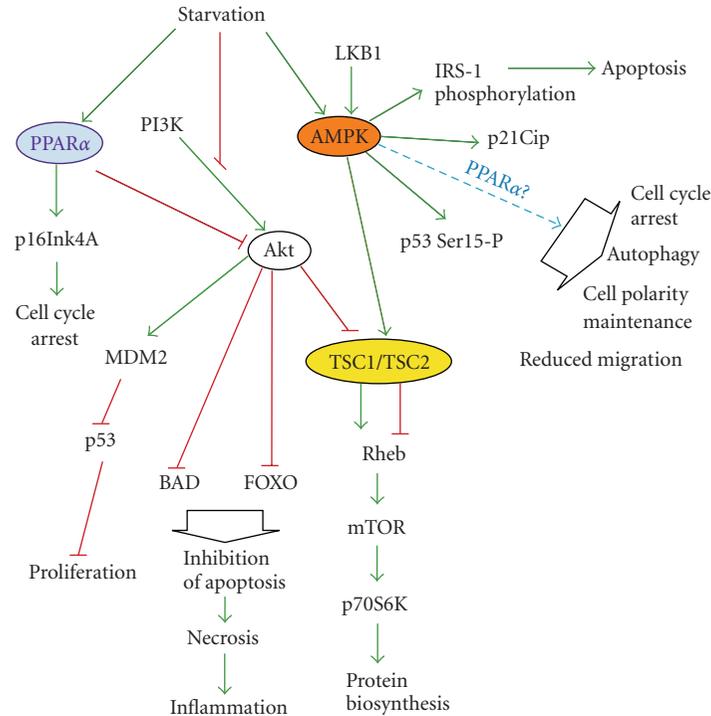
FIGURE 2: PPARα and AMPK activities in the cancer cells exposed to energetic stress. AMPK switches on p53-dependent cell cycle metabolic check point and autophagy and blocks Akt/mTOR protein de novo synthesis pathway. PPARα induces cell cycle arrest and downregulates Akt neutralizing its antiapoptotic actions. For more details, see the text. Arrowheads represent activation/upregulation, and blunted lines indicate inhibition/downregulation of the cellular proteins or processes. IRS-1—insulin receptor substrate-1; mTOR—mammalian target of rpamycin kinase; TSC1—tuberous sclerosis 1 (hamartin); TSC2—tuberous sclerosis 2 (tuberin).

was responsible for AMPK-induced fatty acid oxidation in cardiac and skeletal muscle [48, 49]. This might suggest that PPARα mediates other activities of AMPK. AMPK is a potent inhibitor of PI3K/Akt signaling, especially of Akt-induced glycolysis and protein synthesis [45, 50, 51]. Oncogenic Akt is responsible for increased activity of mammalian target of rapamycin (mTOR) kinase, which phosphorylates downstream regulators of translation such as 4EBP-1 and p70S6 kinase (Rsk) [51, 52]. AMPK antagonizes this Akt-induced mTOR activation by activating tumor suppressor tuberous sclerosis 2 (TSC2, tuberin), which in turn inactivates a small G-protein, Rheb, and in consequence disabled Rheb cannot activate mTOR [53–55]. Some of theses multiple signaling and metabolic connections between PPARα, AMPK, and mTOR are additionally explained in Figures 1 and 2.

We have demonstrated that PPARα activation inhibits Akt phosphorylation and reduces the metastatic potential of mouse melanoma cells [42]. This may provide an interesting synergy between AMPK and PPARα toward mTOR inhibition and the activation of autophagy. Although the mechanism by which fenofibrate attenuates Akt phosphorylation is still under investigation. It has recently been reported that fenofibrate increases plasma membrane rigidity in a manner similar to elevated cholesterol content [56]. In this report, fenofibrate did not change the membrane content of cholesterol but increased plasma membrane rigidity by itself, altering activities of different membrane-bound proteins.

Therefore, one could speculate that fenofibrate, besides its role as a PPARα agonist, may also act in a nonspecific manner by altering membrane-bound growth factor receptors such as IGF-IR or EGFR, which are known to have a strong signaling connection to Akt. Further experiments are required to determine whether similar fenofibrate-mediated changes in the fluidity of the plasma membrane are indeed responsible for the attenuation of the ligand-induced clustering of receptor molecules—a critical step in the initiation of growth and survival promoting signaling cascades.

It has also been demonstrated that omega 3 polyunsaturated fatty acids (n-3 PUFA), which are potent ligands of PPARα, induce fatty acid β-oxidation via AMPK [57]. AMPK is regulated by a tumor suppressor LKB1 and coordinates various cellular responses, which can exert antineoplastic effects [58]. One of them is autophagy, which has been intensively explored in the context of carcinogenesis. Autophagy, also called a type II programmed cell death, is a lysosomal-mediated digestion of different cellular components, including organelles to obtain energy, however, it may also lead to cell death [3, 59, 60]. There is a growing body of evidence that defective autophagy may result in cancer progression [59, 61]. Beclin 1, a protein required for autophagy, is frequently lost in ovarian, breast, and prostate cancers, and beclin 1 +/− mutant mice are prone to increase incidence of tumors derived from epithelial or lymphopoietic tissues [62, 63]. Autophagy is negatively controlled by Akt/PI3K

signaling and specifically by mTOR, which acts as a sensor of growth stimuli and nutrient availability and at the same time is the main target for the rapamycin-mediated antitumor activity [64]. Degenhardt et al. [3] demonstrated that cells transformed by Akt overexpression and by deficiency in proapoptotic genes, BAX, and BAK show a highly invasive phenotype, however, became necrotic when deprived of oxygen and glucose.

Although Akt activation provides a growth advantage, it simultaneously impairs autophagy in response to metabolic stress and condemns cells to necrotic death. Abundant necrosis stimulates inflammation and enhances macrophage infiltration within tumors, which is a poor prognostic factor, and actually accelerates tumor growth [3]. These findings support the notion that loss of autophagy in apoptosis-incompetent cells can have tumor promoting effects. This can happen in cells with constitutively activated Akt, as it triggers a strong antiapoptotic signal, mainly by the inactivation of proapoptotic proteins, BAD, and FOXO [52].

In the state of nutrient deprivation, AMPK induces autophagy in a p53-dependent manner and evokes apoptosis through the serine phosphorylation of insulin receptor substrate (IRS-1), which in turn inhibits PI3K/Akt signaling pathway [65]. It is not known if PPARα is involved in these actions downstream of AMPK, but possibly can support them by the inhibition of Akt [66]. In this respect, inhibition of Akt by PPARα ligand, fenofibrate, significantly suppressed anchorage-independent growth, cell motility and cell migration in vitro; and in the experimental animal model, fenofibrate treatment reduced metastatic spread of hamster melanoma cells to the lungs [42, 67]. This apparent inhibition of cell migration and compromised cell invasiveness was likely associated with alterations in the cytoskeletal structure. Interestingly, AMPK has been implicated in the maintenance of epithelial cell polarity, by affecting actin-fiber distribution during energy deprivation [68]. In particular, AMPK mutations disrupted the polarity of the epithelium and triggered tumor-like hyperplasia, again supporting the notion of a possible cooperation between PPARα and AMPK.

3. PPARα AND INFLAMMATION

The anticancer effects of activated PPARα can be attributed to its well-characterized anti-inflammatory properties. PPARα inhibits expression of variety of inflammatory genes, such as interleukin 6 (IL-6) and inducible cyclooxygenase-2 (COX-2), as well as reduces nitric oxide production in murine macrophages exposed to bacterial lipopolisaccharide (LPS) [69–71]. These events can be ascribed to the PPARα antagonistic action against the main transcription factors mediating inflammatory responses, nuclear factor-κB (NF-κB), and activating protein-1 (AP-1) (Figure 3). NF-κB activity is repressed by inhibition of p50 and p65 nuclear translocation or by I-κB upregulation, which induces p65 phosphorylation and subsequent proteasomal degradation [72–76]. AP-1 is affected by PPARα through inhibition of its binding to the consensus DNA sequence and by suppressing c-Jun activity [77–79]. Inhibition of inflammatory
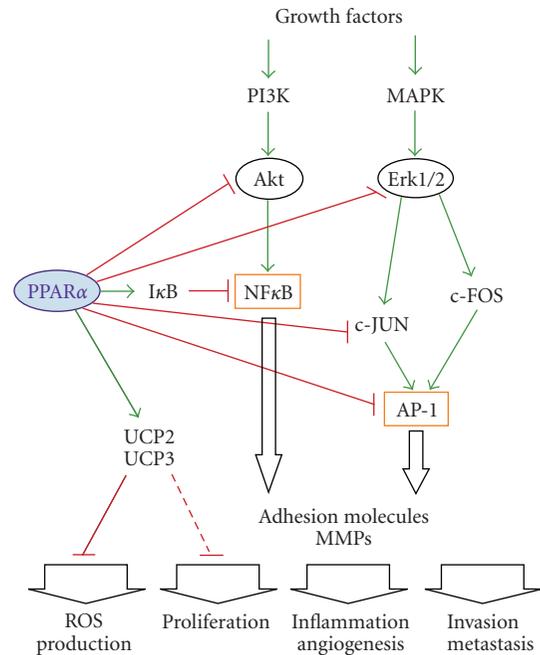

FIGURE 3: PPARα antagonizes main inflammatory signaling pathways through repression of the main inflammatory transcription factors: NFκB and AP-1. Additionally, PPARα reduces ROS-mediated inflammation by upregulation of uncoupling proteins UCP2 and UCP3. See the text for more detailed explanation. Arrowheads represent activation/upregulation, and blunted lines indicate inhibition/downregulation of the cellular proteins or processes. AP-1—activating protein-1; Erk1/2—extracellular signal response kinase 1/2; IκB—inhibitor of NFκB; MAPK—mitogen activated protein kinase; NFκB—nuclear factor κB; ROS—reactive oxygen species.

signaling is important for anticancer therapy in order to reduce mitogenic and angiogenic cytokines and growth factors released by activated immune and stromal cells [80]. Moreover, inhibition of NF-κB, which coordinates a number of antiapoptotic pathways, sensitizes neoplastic cells to nutrient deficiency stress and facilitates apoptosis [81]. NF-κB induces expression of matrix metalloproteinases, such as MMP-9 and urokinase-type plasminogen activator (uPA), and a number of adhesion molecules including ICAM-1, VCAM-1; thus promoting cancer cells' invasiveness and dissemination [82–84]. Therefore, one could speculate that PPARα- mediated inhibition of NF-κB could contribute to the observed reduction of metastatic spread in melanoma-bearing animals treated with fenofibrate [67].

Recently, a completely new image of PPARα in tumor development has been proposed. Kaipainen and coworkers were the first who initiated studies on the role of PPARα expression in host-tumor interaction. They demonstrated that PPARα depletion in the host significantly reduced tumor growth and metastasis [85]. This effect was not correlated with the tumor type and was independent from the presence or absence of PPARα in the tumor cells. The loss of PPARα in the host was associated instead with decreased microvessel density and enhanced granulocyte infiltration in the tumor

tissue and with the elevation of the angiogenesis inhibitor, thrombospondin (TSP-1) [85].

Since necrosis and chronic inflammation within the tumor are associated with intensified macrophage infiltration and poor prognosis [86], it is not entirely clear why granulocyte influx is much more effective in eliminating tumor cells and apparently does not increase the risk of increased tumor vascularization. The possible answer might be a distinct profile of cytokines/chemokines released by macrophages and by granulocytes. The other speculative explanation could be associated with acidic tumor microenvironment, which is known to impair cellular and humoral immune responses. However, it affects differentially macrophages, neutrophils, and lymphocytes, leaving the latter two less prone to this acidic inactivation [87].

4.   CONCLUDING REMARKS

As presented above, PPARα contributes to the maintenance of physiological homeostasis by multiple mechanisms. Particularly interesting is the interplay between PPARα and AMPK, which represents evolutionary conserved sensor of the metabolic equilibrium, governing the balance between cell death and cell survival. The possible involvement of PPARα in the control of autophagy is an exciting direction to explore, which may reveal new aspects of PPARα role in carcinogenesis.

The metabolic, anti-inflammatory and antiproliferative properties of PPARα ligands provide premises for the potential use as supplementary agents in anticancer treatment, and especially antimetastatic therapies. In addition, low toxicity of synthetic PPARα agonists and the abundance of effective natural ligands provide additional encouragement for the anticancer treatment. However, it should be kept in mind that PPARα was first described to promote peroxisome proliferation and hepatocellular neoplasia in rodents which conversely to humans, and the majority of other species, turned out to be particularly sensitive to PPAR ligands.

Finally, role of PPARα in the tumor-host interactions should be thoroughly studied and explained in order to design effective anticancer therapies with minimized risk of unwanted side effects.

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Review Article
A Role for PPARβ/δ in Tumor Stroma and Tumorigenesis

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Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a transcription factor that is activated by endogenous fatty acid ligands and by synthetic agonists. Its role in the regulation of skeletal muscle fatty acid catabolism, glucose homeostasis, and cellular differentiation has been established in multiple studies. On the contrary, a role for PPARβ/δ in tumorigenesis is less clear because there are contradictory reports in the literature. However, the majority of these studies have not examined the role of PPARβ/δ in the tumor stroma. Recent evidence suggests that stromal PPARβ/δ regulates tumor endothelial cell proliferation and promotes differentiation leading to the properly orchestrated events required for tumor blood vessel formation. This review briefly summarizes the significance of these studies that may provide clues to help explain the reported discrepancies in the literature regarding the role of PPARβ/δ in tumorigenesis.

1. INTRODUCTION

Peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) is a transcription factor that is activated by lipid-derived ligands [1, 2]. Major functions of PPARβ/δ are associated with the regulation of intermediary metabolism, in particular energy homeostasis, skeletal muscle lipid catabolism, and glucose metabolism [3]. PPARβ/δ is also important in the control of inflammatory responses as it modulates the function, proliferation, differentiation, and survival of immune cells, notably macrophages and lymphocytes [4]. PPARβ/δ therefore represents a highly relevant drug target for the treatment of major human diseases such as obesity, metabolic syndrome, inflammatory diseases, and arteriosclerosis, which has led to the development of several synthetic drug agonists displaying subtype selectivity and high-affinity binding [5].

Mice lacking PPARβ/δ exhibit embryonic lethality due to aberrant development and malfunction of the placenta, which is, however, modulated by the genetic background [6–8]. In line with these findings, differentiation and metabolic function of trophoblast giant cells in vitro are dependent on PPARβ/δ [8]. *Pparb* null mice also exhibit a defect in wound

healing [9], and consistent with this observation, PPARβ/δ is critical for the AKT-mediated survival of keratinocytes during wound healing in skin [10]. However, in contrast to this prosurvival pathway observed in skin wound healing, PPARβ/δ also stimulates keratinocyte terminal differentiation and inhibits proliferation [6, 11–14], concomitant with a downregulation of protein kinase C and MAP kinase signaling [15]. Differentiation of the digestive tract is also regulated by PPARβ/δ, where it promotes the differentiation of Paneth cells in the intestinal crypts by downregulating the hedgehog signaling pathway [16].

2. PPARβ/δ AND TUMORIGENESIS

Consistent with its functional role in differentiation and proliferation, PPARβ/δ inhibits chemically induced skin carcinogenesis as enhanced skin cancer is observed in mice where PPARβ/δ has been deleted globally in all cells [17]. Since no difference in chemically induced skin carcinogenesis is observed in mice when PPARβ/δ is deleted specifically in basal keratinocytes [18], this suggests that the protective effect of PPARβ/δ in skin cancer may require functional roles in other cell types found in skin. Enhanced tumor

formation has also been observed in a mouse model of Raf oncogene-induced lung adenoma formation, but the precise mechanisms and cell types involved are not known [19]. In the Apc/Min mouse lacking functional APC protein as well as in azoxymethane-induced intestinal carcinogenesis, effects of PPARβ/δ have been described for tumor growth with different outcomes. For example, one study reports that PPARβ/δ is dispensible for intestinal tumorigenesis [7], while other studies suggest that PPARβ/δ attenuates colon cancer by regulating colonocyte terminal differentiation [20–24]. Yet others suggest that PPARβ/δ potentiates colon cancer by promoting cell survival pathways [25–27]. The reason for these discrepancies, and thus the precise function of PPARβ/δ in intestinal tumor cells, remains unclear at present [28]. Importantly, none of these studies addressed the issue as to whether PPARβ/δ might play a role in cells of the tumor stoma, that is host cells recruited by the tumor, such as endothelial cells (ECs), fibroblasts and macrophages [29], and would thus add another level of complexity regarding the interpretation of results obtained with transgenic tumor mouse models. Indeed, recent work suggests that PPARβ/δ also has an essential function in the tumor stroma [30, 31], which is discussed in the following section.

3. A ROLE FOR PPARβ/δ IN TUMOR VASCULARIZATION

Two recent studies showed that the growth of syngeneic tumors is impaired in mice lacking PPARβ/δ. This was seen with two different subcutaneous tumor models, the Lewis lung carcinoma (LLC1) and the B16F1 melanoma [30, 31]. Tumor growth was initially indistinguishable in $Pparb^{+/+}$ and $Pparb^{-/-}$ mice, but halted after approximately 2 weeks selectively in the $Pparb^{-/-}$ mice (Figure 1), while the inoculated $Pparb^{+/+}$ mice invariably succumbed to their tumors within 2-3 weeks, the $Pparb^{-/-}$ mice exhibited a survival rate of >90% after six months. Histological analyses showed that density of functional microvessels is diminished in LLC1 tumors in $Pparb^{-/-}$ mice [30, 31]. In contrast to tumors examined in $Pparb^{+/+}$ mice, the majority of tumor microvessels in $Pparb^{-/-}$ mice exhibited a hyperplastic appearance typified by a thickened endothelial lining and the lack of a lumen (Figure 2(a)). Consistent with this finding, kinetic DCE-MRI analysis showed an obstructed tumor blood flow in the tumors developing in the $Pparb^{-/-}$ mice [31]. These alterations were associated with a striking increase in tumor endothelial cell proliferation in the absence of PPARβ/δ expression (Figure 2(b)), and concomitant with this hyperproliferation, the immature ECs were surrounded by perivascular cells expressing vast amounts of the myofibroblast marker α-smooth muscle actin (Figure 2(c)), a picture that is characteristic of endothelial hyperplasia. These observations strongly suggest that an abnormal organization caused by a hyperplastic response, rather than a lack of ECs, underlies the abundance of abnormal microvessels in $Pparb^{-/-}$ mice. This is consistent with a large body of evidence demonstrating that PPARβ/δ can inhibit cell proliferation in a number of different cell
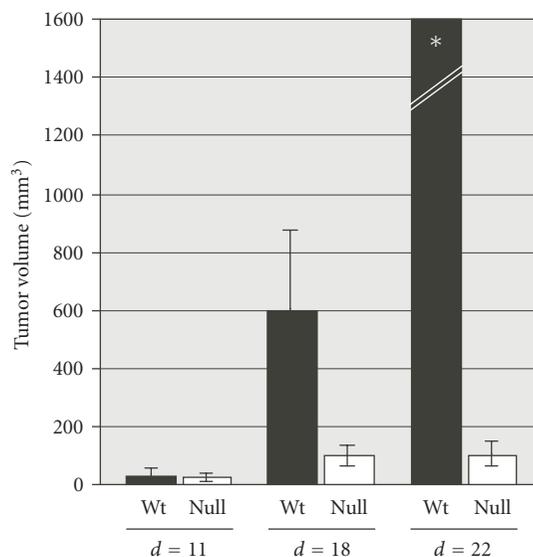

Figure 1: Growth of subcutaneous Lewis lung carcinoma (LLC1) in syngeneic $Pparb^{+/+}$ and $Pparb^{-/-}$ mice. Tumor sizes were determined at the times indicated with a caliper. The calculated volumes are shown as mean $\pm$SD [31]. *All tumor volumes <1000 mm^3.

types [13, 24]. Importantly, PPARβ/δ-dependent tumor vascularization was not restricted to ectopic tumor models, but was also seen with intestinal adenomas in $APC^{+/\min}$ mice which showed disorganized microvessels specifically in a $Pparb^{-/-}$ background (Figure 3). Collectively, these observations point to a general role for PPARβ/δ in the formation or maintenance of tumor blood vessels.

Although a defect in angiogenesis has not been observed during normal development of $Pparb^{-/-}$ mice [6–9], the findings discussed above are consistent with previous findings pointing to a role for PPARβ/δ in terminal differentiation and the control of cell proliferation in different cell types, including keratinocytes [12, 14, 32, 33], trophoblast giant cells [8], and intestinal epithelial cells [16, 22]. This suggests that PPARβ/δ is specifically required by tumor ECs to orchestrate their proliferation and differentiation in an environment providing an abnormally rich source of growth factors and cytokines. A role for PPARβ/δ in tumor vascularization is also supported by several pieces of circumstantial evidence: $Pparb$ is the predominant $Ppar$ subtype expressed in mouse and human tumor endothelial cells, and it is upregulated by angiogenic growth factors of the tumor microenvironment [30, 31].

4. PPARβ/δ TARGET GENES RELEVANT FOR STROMA CELL FUNCTION

Microarray and qPCR analysis led to the identification of a set of genes that are differentially expressed in an in vivo model of growth factor-induced angiogenesis (matrigel plugs) from $Pparb^{+/+}$ and $Pparb^{-/-}$ mice [31]. Consistent with the observed hyperproliferative phenotype in $Pparb^{-/-}$ mice, three of these genes have known inhibitory functions

(a)

(b)

(c)
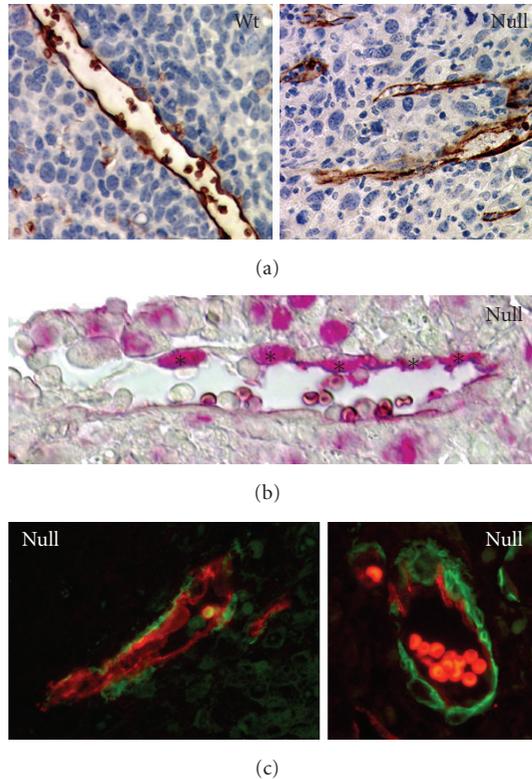
FIGURE 2: (a) Aquaporin-1 immunostaining of endothelial cells and blood vessels in subcutaneous Lewis lung carcinoma (LLC1) 14 days after inoculation into $Pparb^{+/+}$ and $Pparb^{-/-}$ mice (brown stain). Areas of tumor cell necrosis are obvious in the vicinity of the aberrant vascular structures in $Pparb^{-/-}$ mice. (b) PCNA (proliferating cell nuclear antigen) staining of an LLC1 tumor section from a $Pparb^{-/-}$ mouse. The red stain shows a high fraction of proliferating endothelial cells lining the tumor microvascular structures (denoted by asterisks; 38.7% in $Pparb^{-/-}$ mice versus 16.6% in $Pparb^{+/+}$ mice) [31]. (c) Aquaporin-1/α-smooth muscle actin double immunofluorescence of LLC1 tumors from $Pparb^{-/-}$ mice, showing hallmarks of a hyperplastic stroma. Red: aquaporin-1, green: α-smooth muscle actin.


FIGURE 3: Analysis of microvessels in intestinal adenomas from $APC^{+/\min}$ mice in a $PPARb^{+/+}$ or $PPARb^{-/-}$ background (31 ± 3 weeks old mice) by aquaporin-1 immunostaining of paraffin sections (brown). Arrows point to normal microvessels in tumors from $PPARb^{+/+}$ mice, lacking in $PPARb^{-/-}$ mice. Highly aberrant vascular structures lacking a lumen are seen specifically in $Pparb^{-/-}$ mice.

proliferation as CD36 and thrombospondin, suggesting that these molecules may act in concert. It is likely that additional genes with functions in growth control and differentiation will be identified as potential PPARβ/δ target genes in the same experimental system, and it is likely that multiple PPARβ/δ regulated genes are important in the context of tumor stroma development and tumor angiogenesis.

5. CONCLUSIONS

The findings discussed above are consistent with a model where PPARβ/δ is required to modulate the angiogenic response to growth factors during the final stages of tumor angiogenesis, which is characterized by an inhibition of EC proliferation and the acquisition of a fully differentiated phenotype [36]. The lack of PPARβ/δ with the ensuing decreased expression of negative regulators of proliferation may result in a deregulation of angiogenesis with the consequence of tumor endothelial hyperplasia. A similar phenotype of enhanced, but nonproductive, angiogenesis has very recently been described in mice lacking the Notch ligand Delta-Like 4 (Dll4) [37, 38]. In contrast to PPARβ/δ, however, Dll4 is essential not only for tumor angiogenesis but also for embryonic vascular development and arteriogenesis [39], and there seems to be no cross-talk or interaction between both the PPARβ/δ and Notch/Dll4 pathways. This suggests that multiple and presumably mutually independent regulatory mechanisms are required to prevent the deregulation of tumor EC proliferation and the occurrence of nonproductive angiogenesis. The current evidence suggests that PPARβ/δ is such a regulator.

Previous studies addressing the role of PPARβ/δ in tumorigenesis have yielded partly conflicting results leaving it unclear whether PPARβ/δ has tumor-promoting or suppressing properties, in particular in colon cancer models (reviewed in [28]). Our findings provide some insight that may eventually help to resolve this issue. PPARβ/δ may have different functions in tumor stroma and in certain

in angiogenesis (Cd36, Thbs2) or cell cycle control (Cdkn1c) [34, 35]. Thrombospondins attenuate EC proliferation and migrationin vitro and inhibit angiogenesis in vivo, which is strictly dependent on their interaction with the CD36 receptor. In $PPARb^{-/-}$ cells, both ligand (Thbs2) and receptor(Cd36) genes are downregulated, suggesting that an autocrine or paracrine signaling loop with an essential function in modulating angiogenesis is impaired in these cells. Very little is known about the intracellular events that occur after binding of thrombospondin to CD36, so it is difficult to speculate at present about the CD36-triggered signal transduction pathway(s) that is/are affected in ECs lacking PPARβ/δ expression. The third gene identified as a PPARβ/δ target gene in this context is $Cdkn1c$ [31], which codes for the CIP/KIP family member p57$^{\text{KIP2}}$ that it is likely to function as a cyclin-dependent kinase inhibitor [34]. Thus, p57$^{\text{KIP2}}$ would have a similar effect on EC

tumor cells with opposing effects on tumor growth. Clearly, a detailed understanding of these complexities will be a prerequisite for the development of PPARβ/δ directed drugs and their clinical application.

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Review Article

Macrophages, PPARs, and Cancer

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Mononuclear phagocytes often function as control switches of the immune system, securing the balance between pro- and anti-inflammatory reactions. For this purpose and depending on the activating stimuli, these cells can develop into different subsets: proinflammatory classically activated (M1) or anti-inflammatory alternatively activated (M2) macrophages. The expression of the nuclear peroxisome proliferator-activated receptors (PPARs) is regulated by M1- or M2-inducing stimuli, and these receptors are generally considered to counteract inflammatory M1 macrophages, while actively promoting M2 activation. This is of importance in a tumor context, where M1 are important initiators of inflammation-driven cancers. As a consequence, PPAR agonists are potentially usefull for inhibiting the early phases of tumorigenesis through their antagonistic effect on M1. In more established tumors, the macrophage phenotype is more diverse, making it more difficult to predict the outcome of PPAR agonism. Overall, in our view current knowledge provides a sound basis for the clinical evaluation of PPAR ligands as chemopreventive agents in chronic inflammation-associated cancer development, while cautioning against the unthoughtful application of these agents as cancer therapeutics.

1. INTRODUCTION

For many years, the centre of gravity in cancer research was focused on uncovering the activating (oncogenes) and/or deactivating (tumor suppressor genes) mutations in proliferating cells, causing these cells to adopt a cancerous phenotype [1]. By now, it has become increasingly clear that untransformed host cells, in particular cells of the immune system, are equally important in every aspect of cancer, from tumor initiation and progression to metastasis formation. Chronic inflammation, in response to microbial infections or persistent chemical insults, may provoke DNA damage in the surrounding tissue and induce cellular transformation [2–5]. Newly transformed cells can be eliminated or kept in a dormant state under the control of innate and adaptive immune cells, but ultimately the surviving "immunoedited" cancer cells are less immunogenic and more aggressive [6, 7]. Within the organoid context of a tumor, normal physiological functions of stromal cells— including a large fraction of leukocytes—are harnessed in favour of tumour progression, leading to modifications in the local extracellular matrix, neoangiogenesis, stimulation

of cancer cell proliferation, and survival and promotion of cancer cell motility and invasiveness [8]. In each of these aspects of the tumor/immune interface, cells belonging to the mononuclear phagocyte system (including lineage committed bone marrow precursors, monocytes, and macrophages) have been implicated, functioning in different compartments (tumor site, periphery) and, mainly dictated by the context, having the potential to contribute to such diametrically opposed processes as tumor destruction or tumor promotion. The latter stresses the heterogeneity and polyvalency of this type of cells, making them indispensable for development, tissue homeostasis, inflammation, pathogen clearance, and wound healing [9]. As a consequence, drugs with the capacity of modifying macrophage activation are of potential interest in the treatment of different pathologies, including cancer. One such class of drugs is the ligands for peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. The three PPAR isoforms (PPARα, PPARβ/δ, and PPARγ) are encoded by different genes and display differences in their tissue distribution, suggestive of specialized functions. Upon heterodimerization

with retinoid X receptors (RXRs), PPARs bind to specific response elements (PPREs) in the promoter regions of a wide array of PPAR-regulated genes. As a consequence, PPARs have a broad range of effects on metabolism, cellular proliferation, differentiation, and immunity [10]. Of importance in the context of this review, each of the PPAR isoforms is expressed in cells of the monocyte/macrophage lineage and influences the phenotype of these cells [11–13]. This knowledge, in combination with the potential impact of monocytes/macrophages on tumor development, provides a rationale for investigating the modulation of PPAR activity in mononuclear phagocytes as therapeutic strategy in cancer research.

2. PLASTICITY OF MACROPHAGE ACTIVATION

2.1. The M1/M2 conceptual frame of macrophage activation

Macrophages belong to the most versatile cells of the body. Heterogeneity arises as macrophages differentiate from monocyte precursors and is determined by the genetic background as well as by specific tissue-related and immune-related stimuli [9, 14]. In this regard, microbial antigens, tumor products, immune complexes as well as Th1 or Th2 effector T cells and their secretory products influence the heterogeneity and the state of activation of macrophage populations [15, 16]. The better characterized response of macrophages to microbial molecules, cancer cells, and host cytokines is the release of inflammatory/microbicidal/tumoricidal products. This "classical activation" profile occurs in a type I cytokine environment (IFN-γ, TNFα) or upon recognition of pathogen-associated molecular patterns (LPS, lipoproteins, dsRNA, lipoteichoic acid, etc.) and endogenous "danger" signals (heat shock proteins, extracellular matrix components, HMGB1, etc.) [17]. As such, it plays an important role in protection against intracellular pathogens, and under certain conditions also cancer cells. Classically activated macrophages or M1 typically produce high levels of IL-12 and IL-23 [18] combined with low levels of IL-10 and are consequently strong promotors of Th1 immune responses. In addition, these cells exert antiproliferative and cytotoxic activities, resulting partly from their ability to secrete reactive nitrogen and oxygen species (NO, peroxynitrite, hydrogen peroxide, superoxide) and proinflammatory cytokines (TNF, IL-1, IL-6) [19–22]. Although such short-term inflammatory activity could be beneficial for the host in a tumor setting, the persistence of inflammatory processes often results in detrimental tissue and DNA damage contributing to cancer development [2–5]. Therefore, in the course of a response, inflammation is usually counteracted through the development of anti-inflammatory mechanisms. Ideally, this regulation must be spatially and temporally controlled.

Distinct mediators have been reported to inhibit the development of M1 and impart anti-inflammatory properties on macrophages, which were collectively termed "alternatively activated" or M2: Th2 cytokines, such as IL-4 and IL-13, deactivating cytokines, such as IL-10 and TGF-β, hormones, such as the glucocorticoids and vitamin D3, and apoptotic cells [23]. M2 have been reported to actively contribute to the pathology of helminth and protozoan infections, but also cancer [24–28]. The heterogeneity of these anti-inflammatory macrophages, whereby each stimulus induces both unique and overlapping gene expression repertoires, has urged the need for a more refined nomenclature. Gordon and colleagues proposed to restrict the definition of "alternative activation" to IL-4 and/or IL-13-elicited macrophages [29]. Subsequently, Mantovani and coworkers used a high production of IL-10 and low production of IL-12 as unifying theme for M2 [15]. Following this logic, a further subdivision was suggested between M2a, b, and c, representing IL-4/IL-13-stimulated (alternatively activated *sensu strictu*), immune complexes + TLR ligand-stimulated [30], and IL-10-stimulated (deactivated) macrophages, respectively. Though a usefull working scheme, it should be realised that any form of classification underscores the complexity of the in vivo situation, where macrophages are exposed to mixtures of stimuli and will adopt mixed functional profiles accordingly. This is exemplified by the determination of a consensus gene signature for in vivo induced M2 in different pathologies, which contains genes that are not inducible in vitro by any of the known M2 inducing stimuli [31].

2.2. Impact of PPARs on the macrophage activation state

All three isoforms of PPAR have been reported to be constitutively expressed in macrophages, with their mRNAs being upregulated during monocyte to macrophage differentiation [11, 32–34]. Though not all reports are in agreement, PPARγ, but not PPARα or -β/δ, may actually promote macrophage differentiation and contribute to the development of typical macrophage-associated features, such as phagocytosis of apoptotic cells [33–36]. The further regulation of PPARs in M1- or M2-conditioning environments has been thoroughly investigated in the case of PPARγ. PPARγ mRNA and protein are strongly induced in resident peritoneal macrophages and peripheral blood monocytes by the typical M2 inducers IL-4 and vitamin D3, suggesting a preferential association of high PPARγ activity with M2 [33, 37]. Indeed, M1 stimuli such as IFNγ, LPS, TNFα, or IL-1β either have no effect on PPARγ expression or were even inhibitory [37, 38]. On top of a higher level of PPARγ receptors, M2 also produce more endogenous PPARγ ligands, in part as a consequence of IL-4-mediated induction of 12/15 lipoxygenase [37, 39, 40]. This lipid-peroxidating enzyme generates the PPARγ ligands 13-HODE and 15-HETE through the oxygenation of linoleic acid and arachidonic acid, respectively [40, 41]. In addition, both in mouse peritoneal macrophages and in human monocytes, IL-13 is able to increase the production and the nuclear localization of the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) by a mechanism dependent on phospholipase A$_2$ activation [42, 43].

Very few data are available on PPARα gene regulation by pro- or anti-inflammatory stimuli, with only one study demonstrating a relatively unaltered PPARα mRNA content

in macrophages upon LPS treatment [44]. In general, systemic LPS treatment tends to decrease overall PPARα expression levels, though it is unclear whether macrophages account for this phenomenon [45].

In the case of PPARβ/δ, current data suggest that this gene could be upregulated in both M1 and M2 polarizing conditions. On the one hand, PPARβ/δ mRNA is upregulated by LPS in macrophages, suggesting an association with M1 [44]. In keratinocytes, LPS and inflammatory cytokines not only induce PPARβ/δ gene transcription through an AP-1 site in the promoter, but also initiate the production of endogenous PPARβ/δ ligands [46, 47]. Of importance, the anti-inflammatory cytokine TGF-β1 counteracts PPARβ/δ expression in these cells [47]. This dynamic control of PPARβ/δ expression is particularly important in tissue injury and wound control [48]. If extendible to macrophages, these data would imply an enhanced PPARβ/δ transcriptional activity in an M1 context. On the other hand, in a very recent paper, IL-4 and IL-13 were shown to induce macrophage PPARβ/δ expression through a STAT6 binding site on its promoter [49]. Taken together, PPARβ/δ could be unique in its capacity to contribute to both M1 and M2 characteristics.

2.3. PPARs and M1 activation of macrophages

In macrophages, numerous inflammatory signalling pathways downstream of cytokine receptors or pattern recognition receptors orchestrate the inflammatory process. Central players in these signalling cascades are the NF-κB, AP-1, and STAT family of transcription factors, whose binding sites can be found in the promoters of inflammatory cytokines, chemokines, metalloproteinases, iNOS, and other inflammatory genes [50, 51].

2.3.1. PPARγ

PPARγ agonists dose-dependently inhibit the upregulation of inflammatory genes in macrophages in response to Toll-like receptor ligands and interferons. These effects are at least partially PPARγ-dependent and can be attributed to an inhibition of NF-κB, AP-1, and STAT1 transcriptional activities [52–54]. By now, the molecular machinery behind PPARγ-mediated repression of NF-κB-regulated genes has been uncovered and appears to depend on a mechanism termed ligand-dependent transrepression [55, 56]. Under steady-state conditions, some genes (e.g., iNOS) are occupied and actively repressed by the multisubunit NCoR repressor complex. Upon NF-κB activation, the NCoR complex is degraded by the proteasome, NF-κB p65-p50 heterodimers enter the nucleus, bind to NF-κB elements in the promoter, and recruit coactivator complexes to initiate gene transcription. However, simultaneous ligand binding of PPARγ leads to SUMOylation of a fraction of the PPARγ molecules, which bind to NCoR and prevent its clearance from the promoter, leading to a sustained repressed state [57]. The requirement for the NCoR corepressor complex explains why only a subset of LPS-inducible genes is truely PPARγ-regulated [54]. Remarkably, also AP-1-mediated gene transcription depends on the loss of NCoR complexes, suggesting a

similar mechanism of PPARγ-mediated repression of AP-1-regulated genes [58]. Of note, PPARγ agonists such as 15d-PGJ$_2$ and thiazolidinediones suppress a broader range of NF-κB-regulated genes, irrespective of their NCoR dependence, and are even able to do so in PPAR null macrophages [54, 59]. In the case of 15d-PGJ$_2$, this can be explained by a direct, PPARγ-independent modification of critical cysteine residues in the IκB kinase and the DNA-binding domains of NF-κB subunits, inhibiting NF-κB activity [60, 61].

The in vivo relevance of macrophage-expressed PPARγ in attenuating inflammation has been demonstrated in macrophage-specific PPARγ$^{-/-}$ animals. Unstimulated macrophages from these mice display an increased production of inflammatory mediators, indicating that endogenous PPARγ ligands modulate macrophages under steady-state conditions. In addition, PPARγ$^{-/-}$ macrophage recruitment to inflammatory sites is increased, and these macrophages overreact to inflammatory stimuli, resulting in increased severity of DSS-induced colitis [62]. Of importance, thiazolidinediones still improve colitis severity in colonic epithelium-specific PPARγ$^{-/-}$ mice, but not in macrophage-specific PPARγ$^{-/-}$ mice, suggesting that macrophages are the relevant targets of these compounds in this disease [63]. Also in models of insulin resistance and atherosclerosis, macrophage-specific PPARγ was shown to inhibit inflammation and improve insulin sensitivity and reduce atherosclerotic lesion size, respectively [64, 65].

2.3.2. PPARα

PPARα ligands are able to lower the secretion of inflammatory mediators in several cell types, including macrophages [66–70]. Similar to PPARγ, PPARα is able to transrepress NF-κB and AP-1 transcriptional activity, though it does so in a different way. Inhibition of these transcription factors by PPARα is independent of the promoter context but appears to depend on a physical interaction between PPARα and the p65 Rel homology domain or the JNK-responsive part of c-Jun [71]. In addition, ligand-bound PPARα transactivates the IκBα promoter in a DNA binding-independent fashion, as such further attenuating NF-κB activation [72]. Another parallel with PPARγ is the importance of posttranslational modifications in the activity of PPARα. Inflammatory stimuli such as LPS activate protein kinase C (PKC), which subsequently phosphorylates and inactivates PPARα. However, statins inhibit PKC activation, increasing the pool of unphosphorylated transrepression-competent PPARα which is entirely responsible for the anti-inflammatory activity of statins [73]. Also the well-characterised anti-inflammatory potential of glucocorticoids partially depends on PPARα, possibly through a similar impact on PKC [74].

The in vivo significance of macrophage PPARα is illustrated by enhanced atherosclerosis in low-density lipoprotein receptor-deficient mice transplanted with PPARα$^{-/-}$ bone marrow, which is due to an increased inflammatory response of macrophages [75]. In the same vein, PPARα$^{-/-}$ spleno-cytes produce significantly higher levels of inflammatory

cytokines in aged mice, both under basal conditions or in the presence of LPS [76].

2.3.3. PPARβ/δ

In contrast to PPARγ and -α, PPARβ/δ can also be associated with M1 (besides M2) and may contribute to the proinflammatory phenotype of these macrophages. Indeed, under basal conditions PPARβ/δ$^{-/-}$ macrophages display a reduced expression of some (MCP-1, IL-1β, and MMP9), but not all (TNFα, IKKβ) inflammatory mediators. As such, inflammation-driven atherosclerotic lesion formation is significantly reduced in PPARβ/δ$^{-/-}$ bone marrow chimeras. Mechanistically, PPARβ/δ forms a complex with the transcriptional repressor Bcl-6, preventing Bcl-6 from repressing inflammatory genes. However, upon synthetic ligand binding (e.g., GW501516) PPARβ/δ releases Bcl-6 and inflammation is dampened [77]. On top of that, PPARβ/δ activation induces the expression of mediators suppressing inflammatory cytokine/chemokine action (RGS, TIMP-3), altogether explaining the beneficial effects of PPARβ/δ agonists in inflammatory diseases such as atherosclerosis [78, 79].

2.4. PPARs and M2 activation of macrophages

PPARs not only antagonize M1 activation, but actually support M2 activation. Indeed, at least some of the reported anti-inflammatory effects of IL-4 or IL-13 are mediated through enhanced PPARγ activity [80]. IL-4/IL-13 strongly increase the production of different endogenous PPARγ ligands (13-HODE, 15-HETE, and 15d-PGJ$_2$) and PPARγ coactivators (PGC-1β), thereby stimulating the PPARγ transactivating activity [37, 42, 43, 81]. As a matter of fact, some of the hallmark IL-4/IL-13-inducible M2 markers, such as MMR, arginase I, CD36, and dectin-1, depend on PPARγ for full induction [42, 43, 82–84]. Following this logic, administration of PPARγ ligands could be a valuable means of inducing M2 markers in vivo and altering macrophage functions [85]. The significance of these findings was recently established in macrophage-specific PPARγ$^{-/-}$ mice [86]. Although LPS-induced release of IL-6 was not significantly different between w.t. and PPARγ$^{-/-}$ macrophages, only in the PPARγ-deficient cells was IL-4 unable to suppress IL-6, corroborating the notion that a subset of IL-4-dependent anti-inflammatory responses is regulated by PPARγ [86]. These mice are defective in the in vivo generation of M2 to a similar extent as macrophage-specific IL-4Rα$^{-/-}$ mice or STAT6 null mice. As a consequence, these mice are more resistant to Th2/M2-driven pathologies, such as cutaneous leishmaniasis.

Similar to PPARγ, PPARβ/δ ablation was shown to diminish the M2 phenotype in macrophages, notably Kupffer cells and adipose tissue-resident macrophages, in vitro and in vivo (in PPARβ/δ$^{-/-}$ bone marrow chimeras or myeloid-specific PPARβ/δ$^{-/-}$ mice), and to increase inflammation. This results in systemic insulin resistance, increased adipocyte lipolysis, and hepatic dysfunction [49, 87].

Overall, it is clear from previous paragraphs that the regulation of PPARs by pro- or anti-inflammatory signals is one of the important factors that triggers macrophage polarization. It is however important to realize that the exact effects of PPARs on macrophages can depend on the source from which macrophages have been isolated (mouse versus human, different tissues, different pathogenic conditions, in vitro versus in vivo studies, etc.) and on the maturation stage of the macrophage population before PPAR activation.

3. M1 MACROPHAGES IN TUMOR INITIATION

Epidemiological studies clearly established a causal link between chronic inflammation—triggered by microbial infections or autoimmune diseases—and tumor development [2–5, 88]. Consequently, prolonged intake of nonsteroidal anti-inflammatory drugs has been proven to lower cancer incidence [89]. M1 macrophages are central orchestrators of the inflammatory response and are of critical importance in some of the well-known cancer-predisposing malignancies: *Helicobacter pylori* infection for gastric cancer [90], inflammatory bowel disease for colon carcinoma [91], and hepatitis for hepatocellular carcinoma [92]. Hence, inflammatory macrophages are actively involved in de novo carcinogenesis and the first steps of tumor development Figure 1.

3.1. Tumor-initiating role of NF-κB in macrophages

The NF-κB transcription factor is the master regulator of inflammation and has been shown to function as a tumor promoter in inflammation-associated cancers [50, 93]. NF-κB can be activated both in cancer cells and immune cells, in particular M1 macrophages. The presence of such macrophages, bearing activated forms of NF-κB and other inflammatory signaling molecules such as p38 MAPK, is seen in premalignant lesions (e.g., colonic polyps) [94]. Hence, it is of interest to gain insight into the relative importance of the NF-κB cellular context (cancer cell versus macrophage) for carcinogenesis. A number of seminal papers have shed light on this issue in the past few years. In colitis-associated colon carcinoma formation, a prototypical example of inflammation-driven carcinogenesis, tumor formation, was reduced to the same extent in mice with either an enterocyte-specific or a myeloid cell-specific defect in the IKKβ-dependent NF-κB pathway. In the case of the myeloid cells, NF-κB-mediated carcinogenesis depends on the production of inflammatory mediators that act as tumor-promoting paracrine factors [95]. In agreement with these findings, the absence of SIGIRR/TIR8, a negative regulator of NF-κB, aggravates colitis-associated carcinogenesis. SIGIRR/TIR8 functions as a tumor suppressor both in colon epithelium and in bone marrow-derived cells [96]. Surprisingly, even in a model of noninflammatory tumor formation (DEN-induced hepatocarcinogenesis), NF-κB activation in macrophages (Kupffer cells) appears to stimulate tumorigenesis through the secretion of hepatomitogens such as TNFα and IL-6 [97].

Apart from virally or bacterially induced cancers, how does NF-κB get activated in macrophages during carcinogenesis? Recent findings demonstrate an important role
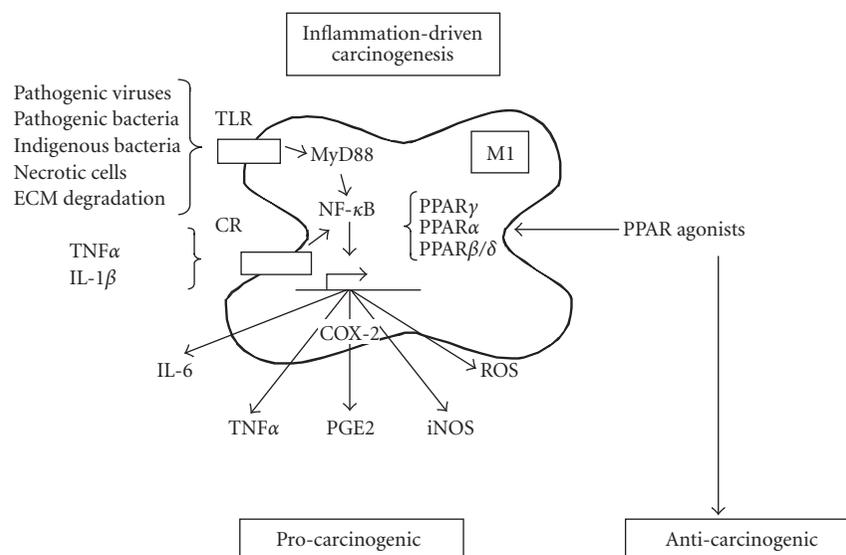
FIGURE 1: *Simplified scheme of the role of M1 macrophages in inflammation-driven carcinogenesis and the potential anticarcinogenic effect of PPAR ligands.* In the context of chronic pathogen infection or chemically induced chronic inflammation, exogenous and/or endogenous ligands for Toll-like receptors are present, which stimulate NF-κB activation via the MyD88 pathway. Also inflammatory cytokines such as TNFα and IL-1β stimulate NF-κB activity through their respective cytokine receptors (CRs). Subsequently, NF-κB transcribes a number of carcinogenic mediators, including IL-6, TNFα, COX-2, and iNOS amongst others. PPAR ligands are able to interfere with the induction of these inflammatory mediators, using different mechanisms. Activated PPARγ tranrepresses NF-κB activity, activated PPARα physically interacts with NF-κB, and activated PPARβ/δ unleashes the transcriptional repressor Bcl-6. Note that the anticarcinogenic actions of the PPAR agonists are only seen in inflammatory tumorigenesis but not in noninflammatory carcinogenesis.

for MyD88, the adaptor molecule in TLR and IL-1R signaling, in inflammation-associated or noninflammatory carcinogenesis alike [98–100]. Interestingly, functional polymorphisms in TLRs can predispose to certain types of carcinoma [101]. TLRs can become activated by endogenous ligands produced during cancer cell necrosis or extracellular matrix degradation, or—as shown in a transgenic model of gastric carcinogenesis—by the indigenous bacterial flora [102]. Another interesting pathway has been suggested by the Coussens lab, Calif, USA.Myeloid cells could become activated in response to immunoglobulins, putting the B cell-myeloid cell axis central in inflammation-associated carcinogenesis [103].

3.2. NF-κB-regulated macrophage products responsible for tumor initiation

A large body of evidence points to inflammatory cytokines as major culprits for tumor stimulation. In the model of DEN-induced hepatocarcinogenesis, the estrogen-regulated difference in IL-6 production by male versus female Kupffer cells entirely accounts for the gender differences in tumor incidence [98]. While IL-6 is a hepatocyte mitogen, TNFα induces hepatocyte NF-κB activation with a strong impact on tumorigenesis. Even under noninflammatory conditions, this carcinogenic TNFα is produced by endothelial cells and Kupffer cells [104]. In addition, carcinogen-stimulated chronic TNFα expression in liver inflammatory cells, presumably Kupffer cells, hyperactivates oval cells through TNF-R1, resulting in liver tumor formation [105]. Comparable

mechanisms are at play in colitis-associated colon carcinoma, where macrophage-derived TNFα interacts with TNF-R1 in an autocrine way, creating an essential inflammatory loop for carcinogenesis [106]. One of the target genes of TNFα-stimulated NF-κB in this model is COX-2 [107]. COX-2, via the production of PGE$_2$, strongly promotes colon carcinogenesis [108]. Importantly, in premalignant lesions of both mice and humans, COX-2 is almost exclusively expressed in macrophages [108, 109]. Similarly, the NF-κB target gene MMP9 is important for skin carcinogenesis and is exclusively produced by inflammatory cells [110]. Finally, other prototypical inflammatory macrophages products, such as nitric oxide and reactive oxygen species, have all been shown to contribute to oncogenesis [97, 111, 112].

3.3. Role of macrophage-specific PPARs in tumor initiation

Considering the importance of inflammatory macrophages as a trigger of carcinogenesis and the anti-inflammatory function of PPARs in macrophages, it seems logical to pursue PPAR ligation as a strategy to block the initial steps of tumor formation. Indeed, some of the most prominent tumor-promoting mediators of macrophages—TNFα, MMP9, iNOS—are known to be repressed by PPARγ ligation [53, 113, 114]. In addition, PPARγ ligands, which had no significant effect on tumor cell lines in vitro, were shown to exert potent inhibitory effects on tumors from the same cells in vivo, suggesting other targets besides cancer cells in the tumor-environment [115].

In line with this rationale, in vivo administration of PPARγ, -α, and β/δ agonists invariably reduces tumor initiation in typical models of inflammation-associated carcinogenesis, such as colitis-driven colon carcinoma [116–118]. The situation is more blurred in colon cancer induced by genetic means (APC$^{\text{Min}}$ mice) rather than by inflammatory stimulation, with contrasting reports describing tumor stimulation or repression upon PPARγ ligation [119–121]. A recent study employed genetic means to assess the role of PPARγ in chemically-induced (inflammatory) versus genetically-induced (noninflammatory) colon carcinogenesis. Haploinsufficiency of the PPARγ gene promotes inflammatory carcinogenesis but has no effect in APC$^{\text{Min}}$ mice [122]. Similarly opposing data exist on the role of PPARβ/δ in tumor formation in APC$^{\text{Min}}$ mice, even between studies looking at APC$^{\text{Min}}$ in a PPARβ/δ null background [123, 124].

Recent studies have studied transplantable tumor growth in PPAR$\alpha^{-/-}$ or PPAR$\beta/\delta^{-/-}$ mice. In both cases, tumor growth was strongly suppressed irrespective of the PPAR status of the cancer cells, indicating that host PPARα and PPARβ/δ are important determinants in tumor formation [125, 126]. In the case of PPARα, absence of the receptor resulted in overt inflammation and neutrophil-mediated tumor clearance [125]. Hence, the level of PPARα stimulation might instruct the anti- or protumor activities of inflammatory cells: (i) absence of PPARα leads to inflammatory cell-mediated tumor destruction, (ii) physiological levels of PPARα stimulation could allow lower, protumoral levels of inflammation, and (iii) strong PPARα stimulation with agonists could shutdown inflammation completely, prohibiting inflammation-driven carcinogenesis. Following this logic, scenarios (i) and (iii) reduce tumor growth, which has indeed been demonstrated experimentally [125, 127].

4. M1/M2 MACROPHAGES IN TUMOR PROGRESSION

Established tumors are often heavily infiltrated by leukocytes, of which tumor-associated macrophages (TAMs) can be a significant portion. The relevance of TAM in tumor biology is underscored by clinical studies showing a correlation between TAM abundance and poor prognosis, data which are particularly strong for breast, prostate, ovarian, and some types of lung cancers [128–130]. In addition, macrophage-deficient mice display reduced progression of tumors to a more malignant phenotype [131, 132]. TAMs are able to promote tumor progression via several mechanisms, including (i) induction of angiogenesis [133], (ii) remodelling of extracellular matrix [129], (iii) stimulation of cancer cell proliferation, migration, and invasion [134], and (iv) inhibition of adaptive immunity [135].

Current knowledge does not allow an unequivocal classification of TAM as prototypical M1 or M2 [28]. While TAMs are generally considered as anti-inflammatory M2, characterized by an IL-10$^{\text{high}}$/IL-12$^{\text{low}}$ cytokine profile and defective NF-κB activation [27, 136, 137], these cells are also known to contribute to angiogenesis and cancer cell aggressiveness via the secretion of the M1-associated and NF-κB-regulated mediators, such as TNFα, IL-1β, and MMP-9 [138–140]. The relative abundance of M1 or M2 markers

in TAM could be related to the phase of tumor progression [141].

In any case, the relative plasticity and diversity of TAM make it difficult to predict the effect of PPAR ligation on these cells and on tumor outcome. In a mouse lymphoma model, we described an increased PPARγ mRNA expression in M2-oriented TAM and splenic macrophages differentiated from a monocytic CD11b$^+$Gr-1$^+$ precursor [135, 142]. Remarkably, stimulation of TAM with PPARγ ligands completely reverses TAM-mediated T-cell suppression, via an as yet unknown mechanism.

5. CONCLUDING REMARKS

In recent years, it has become clear that macrophages and other myeloid cells, such as mast cells and neutrophils, are central orchestrators of both tumor initiation and tumor progression. With the advent of the M1/M2 concept of macrophage activation, it has become clear that inflammatory M1 significantly participate in carcinogenic processes initiated by strong inflammatory stimuli, such as pathogens or certain chemicals. This finding opens a window of opportunity for the use of PPARγ, -α, and β/δ agonists, some of which are already in clinical use for metabolic disorders, in chemoprevention of de novo tumor formation in patients at risk. However, the applicability of these compounds as anticancer agents is confounded by the often confusing findings in mice. In our view, confusion is the consequence of an insufficient insight in the participation of inflammatory cells in the models under study, making it difficult to extrapolate findings from one model to another. Overall, we feel that the usefulness of PPAR agonists is directly correlated with the extent to which inflammation is a driving force for carcinogenesis. Though this might hold true for the initial steps of tumor formation, the situation becomes more complicated in established tumors. Considering the plasticity and heterogeneity of tumor-associated macrophages, with a mixture of M1 and M2 markers and considerable differences between tumor types [28], it is more difficult to envisage a broad applicability of PPAR ligands for the modulation of TAM. However, treatment of certain typical macrophage-driven malignancies, such as breast carcinoma, could potentially benefit from these compounds.

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Review Article

A Role for PPARγ in the Regulation of Cytokines in Immune Cells and Cancer

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Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor and a member of the nuclear receptor superfamily. PPARγ and its ligands appear to serve diverse biological functions. In addition to the well-studied effects of PPARγ on metabolism and cellular differentiation, abundant evidence suggests that PPARγ is an important regulator of the immune system and cancers. Since cytokines are not only key modulators of inflammation with pro- and anti-inflammatory functions but they also can either stimulate or inhibit tumor growth and progression, this review summarizes the role for PPARγ in the regulation of cytokine production and cytokine-mediated signal transduction pathways in immune cells and cancer.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily [1–6]. PPARs exist in three isoforms, PPARα, PPARβ/δ, and PPARγ, which are encoded by different genes and harbour isotype-specific expression patterns and functions. PPARs were initially identified as mediators of peroxisome proliferation in rodent liver, where PPARα plays the major role. However, none of the PPARs could be attributed to peroxisome proliferation in humans [7–10]. Among the various subtypes of PPARs, PPARγ is the best characterized receptor in humans. There are at least two PPARγ isoforms derived from the alternative promoters, PPARγ1 and PPARγ2. PPARγ2 isoform is longer than PPARγ1 by additional 30 N-terminal amino acids [11, 12]. Synthetic ligands including the thiazolidinedione (TZD) class of drugs, L-tyrosine-based compounds, and diindolymethanes as well as natural ligands including a broad range of polyunsaturated fatty acids 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE) and the eicosanoids 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-d-PGJ2) function as efficacious PPARγ activators [13–15].

PPARγ is expressed at high levels in adipose tissue and is an important regulator of adipocyte differentiation, which

functions as a ligand-dependent, sequence-specific activator of transcription. Expression of PPARγ in immune system was initially documented in 1994. Kliewer et al. reported that PPARγ is expressed at high levels in mouse spleen [8]. Greene et al. detected the expression of PPARγ2 in normal neutrophils and peripheral blood lymphocytes by Northern blot analysis in 1995 [9]. Monocytes and macrophages were the first cells of the immune system in which the physical presence and anti-inflammatory properties of PPARs were first described [16, 17]. Subsequently, PPARγ has been reported to exist in other immune cell types of hematopoietic origin, including T lymphocytes [18–22], B lymphocytes [23], NK cells [24], dendritic cells [25–28], eosinophils [29], and mast cells [30–32].

Multiple lines of evidence suggest that PPARs, especially PPARγ, are known to be expressed or overexpressed in several cancers such as epithelial tumor cells, renal cell carcinoma cells, myeloid and lymphoid malignancies, and multiple myeloma cells [33–37]. Ligands of PPARγ have been shown to promote differentiation and to inhibit cell growth and induce apoptosis in several types of human cancer, including colon cancer [38–40], breast cancer [41, 42], lung cancer [43], prostate cancer [44, 45], gastric cancer [46], liposarcoma [47, 48], and leukaemia [49],

supporting a role for PPARγ ligands as potential tumor suppressors in PPARγ-dependent or -independent manner [50, 51], although several murine models suggest that, under certain circumstances, PPARγ ligands may stimulate cancer formation [36].

The cytokines are a large family of secreted molecules consisting of more than 100 peptides or glycoproteins. Each cytokine is secreted by particular cell types in response to a variety of stimuli and produces a characteristic constellation of effects on the growth, motility, differentiation, or function of its target cells. Cytokines can act in an autocrine manner to affect the behavior of the cell that releases the cytokine and/or in a paracrine manner to affect the behavior of adjacent cells. Moreover, some cytokines are stable enough to act in an endocrine manner to affect the behavior of distant cells, although this depends on their abilities to enter the circulation and their half-life in the blood. Cytokines are especially important for regulating immune and inflammatory responses with pro- and anti-inflammatory functions, and have crucial functions in controlling both the innate and adaptive arms of the immune response. Not only do cytokines govern the development and homeostasis of lymphocytes, but they also direct the differentiation of helper T cells and promote the generation of memory cells [52]. During formation and development of tumor, the mixture of cytokines that is produced in the tumor microenvironment has an important role in cancer pathogenesis. Cytokines can either stimulate or inhibit tumor growth and progression [53–57]. Specific polymorphisms in cytokine genes are associated with an increased risk of cancer [58]. Cytokines are produced by immune cells as a host response to cellular stress caused by either exogenous or endogenous agents to control and minimize cellular damage. However, an uncontrolled and sustained generation of cytokines can lead to altered cell growth, differentiation, and apoptosis. Therefore, cytokines are a linker among immunity, inflammation, and cancer [59].

In addition to their antiproliferative and proapoptotic activities on immune cells and cancer cells, effects of PPARs and their ligands in immune system and cancer cells may be mediated through influencing cytokine production or cytokine-mediated signal transduction pathways. Conversely, the expression of PPARs is also modulated by cytokines. In this review, we recapitulate molecular mechanisms on PPARs regulating cytokine production or cytokine-mediated signal transduction and cell responses, and enumerate their physiological and pathological consequences in immune responses, inflammation, and cytokine-responsive tumors.

2. MECHANISM(S) OF CYTOKINE GENE REGULATION BY PPARγ

Like other nuclear receptors, the structure of PPARs is comprised of: an amino-terminal activation function, AF-1 (A/B domain), which can activate transcription in a ligand-independent fashion, the DNA-binding domain (DBD), a hinge region, and a carboxy-terminal ligand-binding domain (LBD) [1–3, 60, 61]. The DBD allows them to bind to and activate target genes, thus defining them as transcription factors. The LBD also contains a second activation function (AF-2) that maps to a surface-exposed hydrophobic pocket, proving a docking site for coregulatory proteins, and modulates their activities, making them hormone-dependent transcription factors. Upon ligand binding, PPARs heterodimerize with retinoid X receptors (RXRs) and form a complex that translocates to the nucleus and regulates gene expression. This heterodimeric complex binds to peroxisome proliferator response elements (PPREs) located within the promoter regions of target genes that consist of a direct repetition of the consensus AGGTCA half-site spaced by one or two nucleotides (DR1 or DR2). In addition to the heterodimer complex, it has been reported that a host of accessory proteins, named "coactivators" or "corepressors," bind to the nuclear receptors PPAR/RXR in a ligand-dependent manner and impact the transcriptional process by either remodeling chromatin structure and/or acting as adapter molecules that link the nuclear receptor complex to key transcriptional machinery. Ligand binding to PPARs appears to trigger conformational changes that permit their dissociation from corepressors and favor their association with coactivators. The coactivators possess or recruit histone acetyltransferase activity to the transcription site. Subsequently, acetylation of histone proteins alters chromatin structure, thereby facilitating the binding of RNA polymerase and the initiation of transcription. In the absence of ligand, PPARγ has the potential to silence genes to which it is bound by recruiting transcriptional corepressor complexes and repress gene expression [1–5, 62–64].

Surprisingly, most of the effects of PPARs on cytokine expression result from crosstalk with other transcriptional factors through nongenomic transrepressive mechanisms. It is well known that some key transcriptional factors such as nuclear factor of activated T cells (NFAT), nuclear factor-kappa B (NF-κB), GATA-3, T-bet, AP-1, or signal transducers and activators of transcription (STAT) regulate the expression of cytokine genes. Transrepression by PPARs can occur either by inhibiting the binding of transcriptional factors to DNA through direct protein to protein interactions or by sequestrating cofactors necessary to their activity. A protein-to-protein interaction between PPARs and other transcription factors completely prevents these transcription factors from binding to their own response elements and therefore blocks their transcriptional activation of cytokine genes [63, 64]. Activation of PPARγ negatively influences the production of inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), Interleukin (IL)-6, and IL-1β by macrophages. A well-established example is PPARγ coassociation with NFAT, a T-cell specific transcription factor, in regulation of IL-2 gene expression [18]. The transcription factor NFAT plays an essential role in gene expression of IL-2 by T lymphocytes and is also involved in the proliferation of peripheral T lymphocytes. Therefore, we evaluated transcriptional activity and DNA binding of NFAT to determine whether NFAT might be a target for negative regulation of T-cell activation by PPARγ ligands. Utilizing the gel-shift experiment, we found that PPARγ ligands significantly inhibited the specific binding of NFAT probe

corresponding to the human IL-2 promoter. The transcriptional activation of the reporter construct directed by the NFAT distal site of the IL-2 promoter was abrogated by 15-d-PGJ$_2$ or ciglitazone in the presence of PPARγ over expression. We further tested for complex formation between PPARγ and NFAT in a coimmunoprecipitation experiment. The NFAT can be coprecipitated with PPARγ in T cells induced by PMA/PHA and 15-d-PGJ$_2$ or ciglitazone. Furthermore, the addition of anti-PPARγ antibody induced high-affinity binding of extracts to the NFAT probes as determined by using an electronic mobility shift assay, demonstrating that removal of PPARγ with this antiserum increases the target specificity of NFAT. This data indicated that a direct physical protein-protein interaction occurs between nuclear receptor PPARγ and transcription factors NFAT, in turn inhibiting transcription of IL-2 in T lymphocytes.

3. CROSSTALK OF PPARγ WITH CYTOKINE-MEDIATED SIGNAL TRANSDUCTION PATHWAYS

Cytokines induce a variety of biological responses by binding to specific cell surface receptors and activating cytoplasmic signal transduction pathways, such as the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, which transmits information received from extracellular polypeptide signals, through transmembrane receptors, directly to target gene promoters in the nucleus, providing a mechanism for transcriptional regulation without second messengers [65–74]. JAKs bind specifically to intracellular domains of cytokine receptor signaling chains and catalyze ligand-induced phosphorylation of themselves and of intracellular tyrosine residues on the receptor, creating STAT docking sites. Phosphorylation of STATs on activating tyrosine residues leads to STAT homo- and heterodimerization. STAT dimers are rapidly transported from the cytoplasm to the nucleus and are competent for DNA binding. Binding of the activated STAT dimer to a target promoter initials formation of a primary transcription complex and dramatically increases the transcription rate from this promoter of target gene. Transcription of target genes induced by the STAT dimers reflects an intrinsic ability of STAT transcriptional activation domains to recruit nuclear coactivators that mediate chromatin modifications and communication with the core promoters [73].

Several lines of evidence indicated that activated PPARγ crosstalks with cytokine-mediated signal transduction pathways in modulation of immune responses and tumor cell growth and apoptosis [75–82]. Interestingly, in the case of interactions between PPARγ and STAT3 [83–87], two structurally distinct PPARγ ligands suppress IL-6 activated-STAT3 through the divergent types of crosstalk including direct or a corepressor SMRT-mediated association (see Figure 1). The 15-d-PGJ2 is a naturally occurring ligand with low affinity of PPARγ, whereas a class of antidiabetic drugs known as thiazolidinediones is a type of high-affinity synthetic ligands of PPARγ. Because the ligand-binding pocket is not static, each PPARγ ligand has the potential to induce a different conformation of the receptor. Additionally,

a non-PPARγ-dependent mechanism may be involved in the difference between the effects of 15-d-PGJ2 and the thiazolidinediones on STAT3. Therefore, it is reasonable that these two structurally distinct PPARγ agonists suppress IL-6 activated STAT3 through diverse molecular mechanisms. The multiplicity of crosstalk between nuclear receptors and other transcriptional factors is an important factor that contributes to both signal diversification and specification.

Direct protein-protein interaction between transcription factors and ligand-activated nuclear receptors has been shown involved in the regulation of some transcription factors. In multiple myeloma cells, we demonstrated that upon 15-d-PGJ2 binding, PPARγ indeed interacted with phosphorylated STAT3 and represses IL-6 signaling by inhibiting the binding of STAT3 to target genes [84]. Ligand-induced activation of PPARγ induces growth arrest by antagonizing the prosurvival signaling cascade induced by IL-6. PPARγ impedes IL-6 signaling by inhibiting the transcription of a number of STAT3-regulated genes such as mcl-1 and c-myc that are important in cell growth and survival. The exact mechanism through which PPARγ represses STAT3 has not been fully elucidated. PPARγ has been shown to physically associate with STAT3, which may inhibit STAT3 from binding DNA or possibly facilitate the export of STAT3 out of the nucleus. However, certain agonists that induced growth arrests of these cells did not induce SMRT to dissociate from PPARγ, suggesting that this nuclear hormone receptor may use numerous mechanisms to inhibit multiple myeloma cell growth.

An alternative mechanism for PPARγ-mediated STAT3 repression has also been suggested, in which PPARγ agonist treatment of multiple myeloma cells induces the corepressor protein SMRT to dissociate from PPARγ; SMRT could then complex with and inhibit the transcriptional activities of STAT3. The corepressor SMRT has also to be demonstrated to mediate PPARγ downregulation of STAT3 in multiple myeloma cells. PPARγ can form weak interactions with the corepressor NCoR/SMRT complex. PPARγ cannot bind to DNA while it is associated with the corepressor complex. After ligand binding, PPARγ disassociates from the corepressor complex, and then binds to DNA through a peroxisome proliferator response element. We first clarified that treatment of MM cells with troglitazone decreased association of SMRT with PPARγ, which results in redistribution of corepressor SMRT from PPARγ to activated STAT3. Furthermore, this interaction between SMRT and IL-6-activated STAT3 can be attenuated by a PPARγ antagonist GW9662, confirming the specificity of the exchange of corepressor SMRT induced by the liganded PPARγ. Recruitment of SMRT, which is associated with histone deacetylase, by STAT3 leads to transcriptionally inactivating STAT3 and consequently downregulating IL-6 mediated MM cell growth and gene expression. These observations support that coactivators or corepressors function is not only for regulation of the ligand-dependent DNA binding and transcriptional activities of nuclear receptors themselves but also acts as a bridge protein to modulate nuclear receptors crosstalk with other transcription factors.
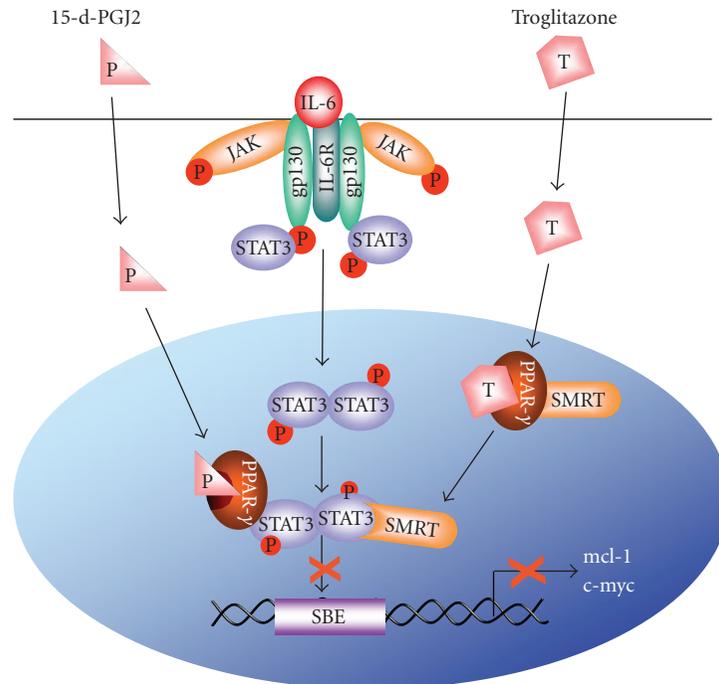
FIGURE 1: *PPARγ crosstalk with IL-6-activated STAT3 signaling pathway.* Upon IL-6 binding, the IL-6R/gp130 dimer induces phosphorylation of JAK1,3, which in turn phosphorylates STAT3. The phosphorylated STAT3 dimerizes and translocates to the nucleues, where they bind to the STAT3 binding element (SBE) in the responsive gene to initiate transcription. Two structurally distinct PPARγ agonists suppress IL-6-activated STAT3 through diverse molecular mechanisms. 15-d-PGJ2 enhances direct physical protein-protein interaction between PPARγ and phosphorylated STAT3 and represses IL-6 signaling by inhibiting the binding of STAT3 to target promoters; Troglitazone inhibits the interaction between PPARγ and the corepressor SMRT, thereby inducing the redistribution of SMRT from PPARγ to activated STAT3, in turn transcriptionally inactivating STAT3 signaling.

4. PPAR REGULATION OF CYTOKINE IN IMMUNE CELLS

The immune response can be classified into two fundamental types: innate and adaptive immunity. The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils, and neutrophils), mast cells, macrophages, dendritic cells, and natural killer cells. The adaptive immune response is slower to develop but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and CD4$^+$ and CD8$^+$ T lymphocytes. Natural killer T cells and γδT cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity [57]. In immune responses innate and adaptive immunity are interlocked and complement each other.

Signaling in the immune system can be either a direct interaction of cells or be mediated by cytokines and antibodies that are carrying signals to all cells with the appropriate receptors. Although PPARγ involvement in the regulation of innate immunere sponses has been studied since the late 1990s [16, 17], only recently it has the role of PPARγ in adaptive immunity been investigated [18–32]. Here, we focus on PPARγ regulation of cytokine-mediated immune responses in immune cells.

4.1. PPAR and IL-2

IL-2 is an autocrine and paracrine growth factor that is secreted by activated T lymphocytes and is essential for clonal T cell proliferation. Although originally described as a potent T cell growth factor in vitro, the main nonredundant role of IL-2 in vivo is now known to be the maintenance of peripheral T cell tolerance. As well as promoting the proliferation and survival of recently activated effector T cells, IL-2 also plays a critical role in regulatory T cell (Treg) homeostasis and has been variously described as promoting the thymic development, peripheral homeostasis and suppressive function of Tregs. These observations, stemming largely from studies on various murine models of IL-2 and IL-2 receptor deficiency, have prompted a greater understanding of the protolerogenic nature of IL-2 dependent signaling.

Greene et al. detected the expression of PPARγ2 in normal neutrophils and peripheral blood lymphocytes in 1995 [9]. In human peripheral blood T cells, we detected inhibition of PHA-induced proliferation and IL-2 production by 15-d-PGJ$_2$ and TZD troglitazone in a dose-dependent manner [18]. When PPARγ2 wild type expression vector was transfected into Jurkat cells, we found that troglitazone and 15-d-PGJ$_2$ inhibited transcription and production of IL-2 in Jurkat cells in a PPARγ-dependent manner. Cotransfection assays with PPARγ and PPRE-driven/IL-2 promoter

luciferase reporter constructs revealed that the inhibitory effects of troglitazone and 15-d-PGJ$_2$ on IL-2 promoter activity are dependent on the expression and activation of PPARγ. Finally, we demonstrated that activated PPARγ inhibited the DNA-binding and activity of transcription factor NFAT regulating the IL-2 promoter in T cells.

Clark et al. described the expression and function of PPARγ in mouse T-lymphocytes [20]. They demonstrated that murine SJL-derived Th1 clones and freshly isolated T cell-enriched splenocytes from SJL mice express PPARγ1 mRNA but not PPARγ2. To test its functional significance, they used two PPARγ ligands, 15-d-PGJ$_2$ and a TZD, ciglitazone. Both ligands could inhibit antigen-induced and anti-CD3 antibody-induced T cell proliferative responses of T cell clones, and the freshly isolated T cell enriched splenocytes. In these studies, it was also demonstrated that the two PPARγ ligands mediated inhibition of IL-2 secretion by the T cell clones, whereas inhibition of IL-2 induced proliferation was not detected.

4.2. PPAR and IL-4

IL-4 is a pleiotropic and multifunctional cytokine produced by activated T cells, mast cells, and basophils [88]. IL-4 plays a critical role in regulating the outcome of an immunere sponse by facilitating the differentiation of CD4$^+$ T cells into IL-4-producing T helper (Th) type 2 cells and suppressing the differentiation of interferon-γ producing Th1 cells, thereby favoring humoral immune responses [89]. Regulation of IL-4 gene expression, therefore, is critically important for the differentiation of Th2 cells and Th2-dependent immune responses [90]. Dysregulated expression of IL-4-producing cells has been linked with autoimmune and allergic diseases [91].

In T cells, IL-4 gene expression is regulated at the transcriptional level by both ubiquitous and cell type-restricted factors, including NF-AT, c-Maf, GATA-3, STAT6, JunB, and other transcription factors [90]. These factors interact with a proximal promoter region composed of multiple regulatory elements that can both positively and negatively affect transcriptional activation. IL-4 gene transcription is mediated by subset-specific transcription factors such as GATA-3 and c-Maf during the differentiation of naive T cells into Th2 cells. A phase of short-term gene transcription, elicited by the interaction of differentiated T cells with antigen, requires the antigen-induced transcription factor NFAT. Treatment of CD4$^+$ T cells with ciglitazone or 15-d-PGJ2 triggered the physical association between PPARγ and NFATc1, resulting in IL-4 promoter inhibition and decreased IL-4 production [92].

Huang et al. [93] reported that IL-4 induces expression of PPARγ and 12/15-lipoxygenase in macrophages, suggesting the potential of coordinated induction of both receptor and activating ligands. Therefore, it appears likely that PPARγ is a key factor in regulating at least some aspects of macrophage lipid metabolism and primarily as a repressor of inflammatory responses. The ways how these two processes are connected, and the contribution of macrophage specific PPARγ-induced gene expression and transrepression to inflammatory responses in vivo remains to be explored.

We reported an interesting PPARγ ligand-mediated immunoregulatory circuit between monocyte/macrophages and T cells [19]. Traditionally, T helper cells can be divided into two functional subsets consisting of Th1 and Th2 cells on the basis of the immunoregulatory cytokines that these T cells produce. Some of these immunoregulatory cytokines possess cross-regulatory properties and can enhance or suppress cytokine production by Th1 or Th2 subset. Thp cells are the pluripotent precursors of Th1 and Th2 cells. Moreover, the development of either Th1 or Th2 helper cells is believed to be determined by the effects of cytokines directly on helper Thp cells. IL-4 is principally produced by helper T cells of theTh2 phenotype. IL-4 has been shown to induce 12/15 lipoxygenase in monocytes/macrophages, which converts arachidonic acid into several metabolic products, including the potential PPARγ ligand 13-HODE [93]. Based on this finding, we tested the relevance of the regulation of soluble mediators (PPARγ ligands) released by IL-4 treated monocytes/macrophages on T cell activation. The medium of macrophages cultured with or without IL-4 was added to T cells stimulated with anti-CD3 or PHA/PMA. We found that T cells with the conditioned medium from IL-4-treated macrophages produced significantly less IL-2. The medium of IL-4-treated macrophages contained a sufficient amount of 13-HODE and anti-13-HODE antibody could neutralize the inhibitory effects of the IL-4-conditional medium on T cell IL-2 production. Using human T lymphocytes and the PPARγ-transfected Jurkat T cells, we demonstrated the specific inhibition by 13-HODE of the transcription factors NFAT and NF-κB, the IL-2 promoter reporter, and IL-2 production. These observations led us to hypothesize that IL-4, produced by Th2 cells, may indirectly affect the production of IL-2 by Thp or Th1 helper cells by inducing the production of these potential PPARγ ligands by macrophage 12/15-lipoxygenase, which in turn interferes with the subsequent development of T helper cells (see Figure 2) [19].

Since many complicated pathological situations cannot be simply explained by the Th1 cell and Th2 cell paradigm, efforts to resolve these issues in recent years have resulted in the discovery of many new T helper cell subsets such as Treg cell and Th17 cell subsets. Therefore, it is interesting to explore further how PPARγ regulates these new Th subsets (see Section 5).

4.3. PPAR and IFNγ

IFNγ plays a central role in inflammatory reactions and is predominately produced by CD4, CD8, and NK cells. IFNγ drives inflammatory reactions by stimulating the release of NO, TNF-α, and IL-1β bymonocytes/macrophages. IFNγ is also a major effector cytokine, responsible for driving cell-mediated immunity and mediating organ-specific autoimmunity. Recent studies have shown that PPARγ ligands inhibit IFNγ production by T lymphocytes; however, the mechanism underlying this observation has not been clarified [94]. Based on previous studies, PPARγ ligands could indirectly decrease IFNγ by inhibiting activation of T cells,
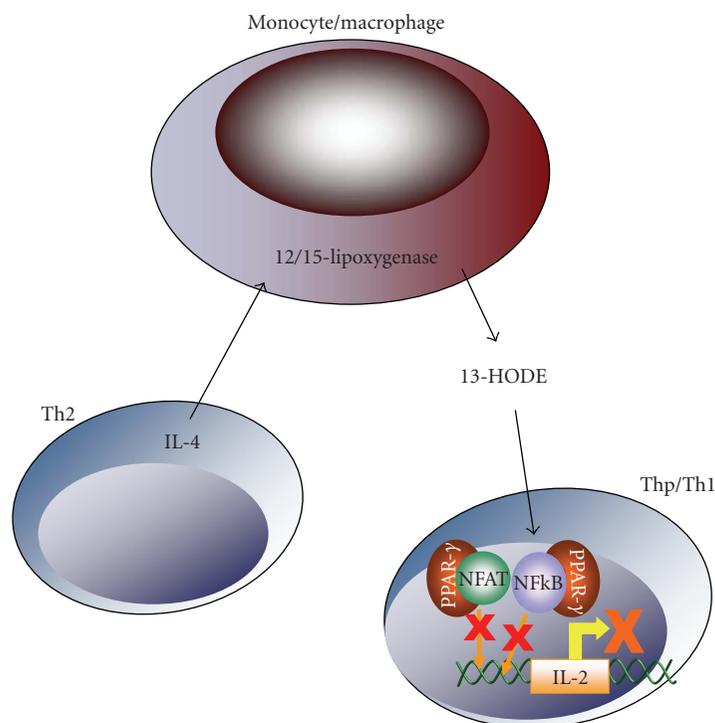
FIGURE 2: *PPARγ regulation of cytokine-mediated immunoregulatory circuit between monocytes/macrophages and T lymphocytes.* T helper (Th) lymphocytes can be traditionally divided into two functional subsets consisting of Th1 and Th2 cells on the basis of the immunoregulatory cytokines that these T cells produce. Thp cells are the pluripotent precursors of Th1 and Th2 cells. IL-4 is principally produced by helper T cells of theTh2 phenotype. IL-4 can induce the upregulation of expression of the enzyme 12/15 lipoxygenase in monocytes/macrophages, providing a potential PPARγ-specific ligands 13-HODE. The mediator secreted by monocytes can be taken up by neighboring Thp or Th1 cells and activate PPARγ in these cells. Since NFAT and NF-κB bind to the promoter region of the IL-2 gene and are needed to activate IL-2 transcription in T cells, the ligand-dependent binding of PPARγ to NFAT and NF-κB correlates the dissociation of NFAT and NF-κB from IL-2 promoter, thus inhibiting gene expression of IL-2 in Thp or Th1 cells.

production of IL-2, or induction of apoptosis, or inhibiting IL-12 production by antigen-presenting cells [95–98].

Cunard et al. demonstrated that PPARγ is expressed in both murine CD4 and CD8 cells and that PPARγ ligands directly decrease IFNγ expression by murine and transformed T cell lines. In contrast, GW9662, a PPARγ antagonist, increases IFNγ expression. Transient transfection studies reveal that PPARγ ligands, in a PPARγ-dependent manner, potently repress an IFNγ promoter construct. Repression localizes to the distal conserved sequence of the minimal IFNγ promoter. They also demonstrate that PPARγ acts on the minimal IFNγ promoter by interfering with c-Jun activation. These studies suggest that many of the observed anti-inflammatory effects of PPARγ ligands may be related to direct inhibition of IFNγ by PPARγ [94].

5. PPAR REGULATION OF CYTOKINES IN TH17 AND REGULATORY T CELLS

Recently, Th17 cells and CD4$^+$ CD25$^+$ regulatory T (Treg) cells have been described as two distinct T helper cell subsets from Th1 and Th2 cells. Th17 cells play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 and, to a lesser extent, TNF-α and IL-6 [99, 100], while Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self components by contact-dependent suppression or releasing anti-inflammatory cytokines [transforming growth factor (TGF)-β1 and IL-10], therefore, the balance between Th17 and Treg may be important in the development/prevention of inflammatory and autoimmune diseases [101, 102].

5.1. PPAR, IL-17 and Th17 cells

Production of IL-17 is a defining feature of a recently identified class of effector T cells termed Th17 cells [99, 100]. Th17 cells act as a distinct effector subset and secrete the signature cytokine IL-17, a proinflammatory cytokine that recruits and activates neutrophils, enhances T cell priming, and promotes the release of inflammatory mediators. Th17 cells provide defense against extracellular bacteria, mediate inflammation, and are critical for many types of autoinflammatory disorders (i.e., experimental autoimmune encephalomyelitis, type II collagen-induced arthritis, inflammatory bowel disease, and psoriasis). The discovery and initial characterization of these Th17 cells have provided a potential explanation for

various chronic disease pathologies that were unclear with an understanding of only the Th1 and Th2 cell subsets.

IL-10-deficient (IL-10$^{-/-}$) mice spontaneously develop inflammatory bowel disease with a Th1-polarized cytokine pattern. In addition to showing high colonic expression of the Th1-derived cytokine IFNγ, IL-10$^{-/-}$ mice also show high expression of IL-17. Lytle et al. observed that rosiglitazone, a high-affinity ligand for PPARγ, had its greatest effect in suppressing IL-17 production in IL-10 knockout mice [103]. Interestingly, the PPARα ligand fenofibrate has been shown to repress IL-17 expression in cultured splenocytes activated by PMA plus ionomycin and by Th17 cells in a pathogenic CD4$^+$ T cell line cultured from C3H Bir mice treated with cecal bacterial antigens [104].

5.2. PPAR, TGFβ and Treg cells

At least two subtypes of CD4$^+$ CD25$^+$ regulatory T cells (Tregs) have been described: thymically derived natural Tregs (nTregs) and inducible Tregs (iTregs) generated peripherally from CD4$^+$ CD25$^-$ T effector cells (Teff) [100, 101]. Induced Treg are more functionally and phenotypically heterogeneous in comparison to natural Treg and can be subdivided into: induced Foxp3$^+$ Tregs, Th3, and Tr1. Which signals drive Treg cell proliferation in the tumor setting? TGFβ is the cytokine that is thought to foster Treg-cell amplification [101]. Both tumor cells directly or "tumor educated" immune cells can locally produce large amounts of TGFβ [102]. Some mouse and rat tumors actively induce myeloid immature dentritic cells to secrete TGFβ and this promotes Treg cell proliferation. There is also substantial evidence that indicates the involvement of TGFβ in Treg cell conversion. Wohlfert et al. have used ciglitazone, a synthetic PPARγ ligands, to characterize the relationship between PPARγ ligands and both iTregs and nTregs. They reported that ciglitazone-activated PPARγ enhances the TGFβ-dependent conversion of naive T cells into Foxp3$^+$-induced Tregs in vitro, although the mechanism by which PPARγ enhances Treg activity remains unknown [105]. Hontecillas and Bassaganya-Riera have used PPARγ deficient CD4$^+$ cells obtained from tissue-specific PPARγ null mice to investigate the role of endogenous PPARγ on Treg and effector CD4$^+$ T cell function. They demonstrated that only PPARγ-expressing Treg was able to completely prevent inflammation induced by effector cells of either genotype, suggesting that PPARγ expression and/or activation by endogenous agonists is required for optimal Treg function [106].

6. PPAR REGULATION OF CYTOKINES IN CANCER CELLS

Cytokines that are released in response to infection, inflammation, and immunity can function to inhibit tumor development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apotosis, and facilitate invasion and metastasis. Proinflammatory cytokines implicated in carcinogenesis include IL-1, IL-6, IL-15, colony stimulating factors, TNF-α,

and the macrophage migration inhibitory factor. A unique immune response signature, consisting predominantly of humoral cytokines, promotes metastasis in hepatocellular carcinoma. Likewise, a signature consisting of 11 cytokine genes in the lung environment predicted lymph node metastasis and prognosis of lung adenocarcinoma with *IL-8* and *TNF-α* as the top 2 genes for predicting prognosis. *IL-8* was originally described as a monocyte-derived neutrophil chemotactic factor that specifically attracted neutrophils and was renamed due to its multiple function. *IL-8* can have angiogenic activities in several cancers including nonsmall cell lung cancer and can function as a positive autocrine growth factor. Both *TNF-α* and *IL-6* contributed to the chemically induced skin tumors and lymphomas in mice. Collectively, cytokines are considered as a linker between inflammation and cancer [55–57].

A considerable amount of research has shown that PPARγ ligands suppress the proliferation rates of many types of cancer cells, particularly those derived from liposarcoma, colon cancer, breast cancer, prostate cancer, myeloid leukemia, glioblastoma, and many others. Various in vitro studies have shown that treatment of many types of cancer cells with TZD resulted in the induction of cell differentiation or apoptosis as well as improvement in levels of various markers for invasion and metastasis. Furthermore, activation of PPARγ by glitazones inhibits angiogenesis and neovascularization both in vitro and in vivo and blocks the release of vascular endothelial growth factor from smooth muscle cells [107, 108]. In addition to the above direct antiproliferative and proapoptotic activities on cancer cells, effects of PPARs and their ligands in cancer cells may function through influencing cytokine production or cytokine-mediated signal transduction pathways. The mechanisms are probably linked to: (1) PPAR ligands may sensitize cancer cells to the antitumor effects of cytokines such as TNFα, (2) PPAR ligands may suppress production of cytokines for tumor cell growth, and (3) PPAR ligands may affect tumor microenvironment by regulation of Treg through influencing associated cytokines. A good example is that PPARγ ligands suppress multiple myeloma through inhibiting IL-6 and IL-6 activated signal pathway in both PPARγ-dependent and -independent manner.

6.1. PPAR and IL-6

Interleukin-6 (IL-6) is a cytokine with multiple biologic activities on a variety of cells. IL-6 plays a major role in the response to injury or infection and is involved in the immune response, inflammation, and hematopoiesis. Its deregulation impacts numerous disease states, including many types of cancer. Consequently, modulating IL-6 may be an innovative therapeutic strategy in several diseases. IL-6 is a pleiotropic cytokine that is involved in the physiology of virtually every organ system. Aberrant expression of this cytokine has been implicated in diverse human illnesses, most notably inflammatory and autoimmune disorders, coronary artery and neurologic disease, gestational problems, and neoplasms. In cancer, high levels of circulating IL-6 are observed in almost every type of tumor studied and predict a poor outcome.

Furthermore, elevated IL-6 levels are associated strongly with several of the striking phenotypic features of cancer. Several molecules have been developed recently that target the biologic function of IL-6. Early results in the clinic suggest that this strategy may have a significant salutary impact on diverse tumors. The field of cytokine research has yielded a deep understanding of the fundamental role of IL-6 and its receptor in health and disease. Therapeutic targeting of IL-6 and its receptor in cancer has strong biologic rationale, and there is preliminary evidence suggesting that targeting of the IL-6 system may be beneficial in the treatment of cancer [109].

One of the most studied tumor types in relation to IL-6 is multiple myeloma, a malignancy of differentiated B-lymphocytes. Multiple myeloma is characterized by accumulation of clonal plasma cells in the bone marrow, accounts for 10% of all hematologic cancers, and remains an incurable hematological malignancy [110–112]. Recently, we investigated how PPARγ ligands suppress IL-6 gene expression through crosstalk between PPARγ and NF-κB or between PPARγ and C/EBPβ [86]. C/EBPβ and NF-κB bind to the promoter region of the IL-6 gene, and their cooperation is needed to activate IL-6 transcription. The nuclear receptor PPARγ can be activated by troglitazone. Predominately, the complex between C/EBPβ and troglitazone-bound PPARγ leads to decreased DNA binding and transactivation of C/EBPβ, inhibiting gene expression of IL-6. In addition, PGC-1, a coactivator, is shared by both PPARγ and NF-κB. After activation by ligands, ligand-bound PPARγ competes for the limited amounts of PGC-1. Therefore, NF-κB dissociates with PGC-1 and decreases NF-κB DNA-binding and transactivation, leading to blocked IL-6 transcription. In the case of 15-d-PGJ2 inhibition of IL-6 transcription, although 15-d-PGJ2 also shares the above ligand-bound PPARγ downregulation mechanisms on C/EBPβ and NF-κB, 15-d-PGJ2, compared with troglitazone, prefers to use PGC-1 as a bridging protein to associate with NF-κB. In addition, 15-d-PGJ2 inactivates NF-κB through decreasing phosphorylation of IKK and IκB in PPARγ-independent manner. The molecular mechanisms of PPARγ ligands on the regulation of multiple transcription factors have proven, not surprisingly, complex. Given that IL-6 is the key growth and survival factor of multiple myeloma cells, and is particularly involved in the origin of all benign and malignant plasma cell expansions as well as MM cell resistance, the effects and targets of the PPARγ ligands on aspects of multiple myeloma biology and bone marrow stromal cells may be clinically relevant.

7. CONCLUSIONS

Most proinflammatory cytokines produced by either host immune cells or tumor cells themselves promote tumor development. By contrast, proapoptotic and anti-inflammatory cytokines usually interfere with tumor development [55]. There is emerging evidence that the nuclear receptor PPARγ interacts with transcriptional factors to modulate cytokine production and action in immunity, inflammation, autoimmune diseases, and

tumors. PPARγ regulation may occur at the levels of gene expression of cytokines themselves and their receptors or cytokine-mediated signaling transduction pathways in immune cells and cancer. The crosstalk between PPARs and cytokine signaling pathways mediating inflammatory effects at the cellular level is also effective to induce the expression of PPAR genes. The molecular basis of this interaction has remained elusive, despite the proposal of several distinct mechanisms. One of the most important mechanistic aspects is protein-protein interaction through a direct or cofactor-mediated indirect manner. On the basis of insights into the mechanisms on interaction between these two distinct families of transcriptional factors activated by different signaling pathways, new targeting drug design and/or therapeutic strategies will be discovered and developed for treatment of cytokine-related diseases ranging from inflammation to cancer.

ABBREVIATIONS

AF: Activation function
AP-1:   Activation protein 1
C/EBP: CCAT/enhancer-binding protein
DBD: DNA binding domain
ER: Estrogen receptor
IFN: Interferon
IL: Interleukin
Jaks: Janus kinases
LBD: Ligand binding domain
MAPK: Mitogen-activated protein kinase
MM: Multiple myeloma
NcoR: Nuclear receptor corepressor
NFAT: Nuclear factor of activated T cells
NF-κB: Nuclear factor-kappa B
PPAR: Peroxisome proliferator-activated receptor
PPRE: PPAR response element
RAR: Retinoic acid receptor
RXR: Retinoid-X receptor
STAT: Signal transducer and activator of
         transcription
SMRT: Silencing mediator of retinoid and thyroid
         receptors
SRC: Steroid receptor coactivator
TGF: Transforming growth factor
Th: T helper cell
TNF: Tumor necrosis factor
Treg: Regulatory T cell
TZD: Thiazolidinedione.

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Review Article

CXCR4 in Cancer and Its Regulation by PPARγ

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Chemokines are peptide mediators involved in normal development, hematopoietic and immune regulation, wound healing, and inflammation. Among the chemokines is CXCL12, which binds principally to its receptor CXCR4 and regulates leukocyte precursor homing to bone marrow and other sites. This role of CXCL12/CXCR4 is "commandeered" by cancer cells to facilitate the spread of CXCR4-bearing tumor cells to tissues with high CXCL12 concentrations. High CXCR4 expression by cancer cells predisposes to aggressive spread and metastasis and ultimately to poor patient outcomes. As well as being useful as a marker for disease progression, CXCR4 is a potential target for anticancer therapies. It is possible to interfere directly with the CXCL12:CXCR4 axis using peptide or small-molecular-weight antagonists. A further opportunity is offered by promoting strategies that downregulate CXCR4 pathways: CXCR4 expression in the tumor microenvironment is modulated by factors such as hypoxia, nucleosides, and eicosanoids. Another promising approach is through targeting PPAR to suppress CXCR4 expression. Endogenous PPARγ such as 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ and synthetic agonists such as the thiazolidinediones both cause downregulation of CXCR4 mRNA and receptor. Adjuvant therapy using PPARγ agonists may, by stimulating PPARγ-dependent downregulation of CXCR4 on cancer cells, slow the rate of metastasis and impact beneficially on disease progression.

1. INTRODUCTION

The regulation of the distribution of motile cells in both normal and disease situations depends upon a variety of peptide and nonpeptide mediators, which stimulate cell movement by both directed (chemotaxis) and nondirected (chemokinesis) mechanisms. Amongst these mediators are the chemokines, a class of peptide mediators that play critical roles in normal development, regulation of the hematopoietic and immune systems in the adult, and in repair processes such as wound healing and inflammation. Among the different chemokines is the stromal cell-derived factor-1 (SDF-1), which is now known as CXCL12. CXCL12 binds principally to the receptor CXCR4, although it also acts through the more-recently-described receptor CXCR7 [1]. This review describes the roles of CXCL12 and CXCR4 in normal tissue functions and in cancer, and suggests that the regulation of CXCR4 expression by PPARγ may emerge to be a unique avenue by which a key receptor involved in cancer

cell metastasis can be suppressed in a way that will assist with disease therapy.

2. CHEMOKINES AND THEIR RECEPTORS IN CELL REGULATION

Chemokines are low-molecular-weight peptide ligands involved in the trafficking of leukocytes and other motile cells [2, 3]. There are four major groups of chemokines, the CXC, CC, C and CX3C chemokines, categorized as such on the basis of their number and spacing of conserved cysteine residues [2, 4]. The nomenclature of chemokines (e.g., "CXCL12") is made up of their subclass (CXC, CC, etc.) followed by "L" for ligand, and a specific number [2, 3].

The receptors for chemokines are cell-surface, seven-transmembrane G protein-coupled receptors [2]. The naming of these receptors (e.g., "CXCR4") is based on the subclass of chemokine that the receptor recognizes, followed by "R" for receptor and a number (which need not correspond

to the number assigned to its cognate ligand(s)). There are 19 well-recognized chemokine receptors (e.g., CXCR1-6, CCR1-10, CX$_3$CR1, and XCR1) [1, 5]. Many chemokine receptors have more than one known ligand, and many chemokines can activate more than one receptor. Thus, there is much promiscuity in chemokine/receptor signaling.

Chemokines bind within the extracellular domain of the chemokine receptor, which comprises the N-terminus and three extracellular loops [3]. The intracellular domain, which consists of three loops and the C-terminus, associates with G proteins that, upon activation, lead to inhibition of adenylyl cyclase activity [3]. Typical cellular consequences of chemokine binding include changes in gene expression, cell polarization, and chemotaxis (directed cell migration) [4].

Chemokines play a major role in regulating the migration of cells of the immune system, leading to the modulation of immune responses. Their exact role depends on the expression pattern of receptors on specific leukocyte subsets [2] but encompasses the regulation of lymphocyte trafficking, lymphoid tissue development, Th1/Th2 modulation, and the effecting of inflammatory reactions. Chemokine receptors are also found on other cell types, and play a part in stem cell recruitment and angiogenesis, in development and wound healing [4]. When such pathways are subverted in neoplastic cells, chemokines take over prominent roles in the metastatic process, both in terms of the dissemination of cells from primary tumors and in growth of the cancer at metastatic sites. As we will see, this is the case for CXCR4.

3. THE CHEMOKINE RECEPTOR CXCR4 AND ITS LIGAND CXCL12 (SDF-1)

The receptor now known as CXCR4 was cloned in 1994, and was originally given the name leukocyte-expressed seven-transmembrane domain receptor (LESTR) due to its abundant expression in several leukocyte populations [6]. It was independently cloned by others and named "fusin" because of its ability to act as a coreceptor for HIV fusion and entry [7]. It further has the designation "CD184" as part of the cluster of differentiation antigens found on activated leukocytes. LESTR/fusin/CD184 was originally considered to be an orphan receptor. However, the chemokine CXCL12, originally termed stromal cell-derived factor 1 (SDF-1), was shown by two independent research groups to be a ligand for LESTR/fusin/CD184, and the name CXCR4 was proposed [8, 9]. The *CXCR4* gene is constitutively expressed, and CXCR4 protein has been detected on many leukocytes, including lymphocytes, monocytes, NK cells, and dendritic cells; as well as on vascular smooth muscle cells, endothelial cells, cells lining the gastrointestinal tract, microglia, neurons, and astrocytes [10–13]. Until recently, CXCR4 was considered to be the only receptor for CXCL12, but the previous orphan receptor RDC1 is now recognized as an additional CXCL12 receptor, for which the name CXCR7 has been given [1]. CXCL12 itself is widely expressed at different levels in many tissues [14].

4. CXCL12 AND CXCR4 IN NORMAL TISSUE FUNCTION

The interplay between CXCL12 and CXCR4 is critical to normal development. Indeed (and unlike mice deficient in other chemokine/receptors) mice lacking CXCL12 or CXCR4 die in utero or shortly after birth [2, 15–17]. CXCL12/CXCR4 signaling is required during the development of the hematopoietic, cardiac, vascular, and nervous systems. Absence of this axis in embryonic life leads to defects in bone marrow myeloid cell formation, cardiac dysfunction due to impaired ventricular septum formation, and developmental defects in the cerebellum and in the vasculature of the gastrointestinal tract [15–17].

In the normal adult, CXCL12 and CXCR4 are involved in the homing and retention of hematopoietic progenitor cells in the bone marrow. These progenitor cells express high levels of CXCR4, and are attracted to CXCL12 produced by stromal cells in specialized bone marrow niches [18]. Activating mutations of the *CXCR4* gene lead to aberrant retention of myeloid cells within the bone marrow [19]. CXCL12 also acts as a chemoattractant for stem cells and some differentiated cells in the pathological contexts of inflammation and tissue regeneration/repair [20–24]. It is this function of controlling cell migration and homing that is subverted in cancer.

5. CXCL12 AND CXCR4 IN CANCER METASTASIS AND GROWTH

In many ways, the process of metastasis is similar to leukocyte and stem cell trafficking, processes which involve the CXCL12/CXCR4 axis [20]. Cancer cells that express CXCR4 exploit the same signaling pathway, leading to homing and retention in tissues that are rich in CXCL12.

The foundation for our appreciation of the role that CXCR4 and CXCL12 may play in cancer metastasis was set in 2001, when a landmark study by Albert Zlotnik's group demonstrated the importance of the CXCL12/CXCR4 axis in site-specific metastasis of breast cancer [25]. In that study, it was found that CXCR4 expression was low or undetectable in normal epithelial cells, but consistently upregulated in breast cancer cell lines and primary breast cancer cells at both the mRNA and protein level. Human breast carcinoma cells that expressed high levels of CXCR4 underwent morphological changes and migrated directionally in response to CXCL12, indicating that the CXCR4 receptor was active. Crucially, the ligand CXCL12 was highly expressed in tissues taken from human organ sites to which breast cancer cells metastasize, including lymph nodes, lung, liver, and bone marrow, but expressed at low levels in tissues that represent rare sites of metastasis, including the kidney, skin, and muscle. The ability of MDA-MB-231 human breast cancer cells (a cell line that is metastatic in experimental models) to migrate towards protein extracts of lung and liver, or to produce lung and lymph node metastasis after tail-vein injection or orthotopic implantation, was inhibited by neutralizing anti-CXCR4 and/or anti-CXCL12 antibodies. These findings were the first to show the biological importance of this

chemokine/receptor pair in the evolution and spread of cancer.

Since that time, the CXCL12/CXCR4 axis has been shown to be important in the progression and spread of more than 25 different cancers. Our present knowledge is based on (i) studies in cellular and animal experimental models, (ii) surveys of human tissues at different stages of cancer progression, and (iii) population-based studies of morbidity and survival. A summary of present data is shown in Table 1.

CXCR4 has been shown to be expressed at high levels on cells of all of the major adult solid epithelial cancers (breast, colorectal, lung, ovary, prostate, etc.). The ability of the cells to colonize other tissues by gaining advantage from CXCR4-dependent mechanisms depends on the presence of CXCL12 in the tissue fluid. Various studies have shown significant CXCL12 concentrations in the fluid-filled cavities through which many cancers disseminate, and at tissue locations in which metastases characteristically develop. Biologically, significant CXCL12 levels have been found in peritoneal ascites from ovarian cancer patients [26], pleural effusions in lung cancer [27], lymph nodes, bone, and lungs as well as other tissues [25, 28, 29].

Detailed studies of the cellular interactions involved in the metastasis of prostate cancer cells to bone [29] have shown that the interaction of CXCL12 with CXCR4 plays a major role in successive steps in the metastatic process. Human osteoblasts express CXCL12 mRNA and protein, whereas prostate cancer cells express CXCR4 mRNA and receptor. Prostate cancer cells that have become disseminated into the circulation respond to the CXCL12-CXCR4 pathway by enhanced adherence to the bone marrow endothelium and migration across endothelial barriers and basement membranes, ultimately adhering to components of the bone marrow in response to a CXCL12 gradient [29]. CXCL12 from osteoblasts has also been shown to act on CXCR4 to induce release of IL-6 from human squamous cell carcinoma cells to promote osteoclastogenesis [30].

As well as promoting the migration of cancer cells and their invasion through physical barriers as well as adherence to target structures, CXCL12 can act upon CXCR4 on the cancer cells to promote cancer cell growth along with other mitogenic factors. This has been shown in cells from colorectal [31], prostate [32], and ovarian [33] cancers. Furthermore, CXCL12 can promote cancer dissemination indirectly by enhancing the vascular supply, since the CXCL12/CXCR4 axis may also promote tumor angiogenesis. Vascular endothelial growth factor (VEGF) and CXCL12 have been shown to increase angiogenesis synergistically in an in vivo Matrigel assay and to promote proliferation and migration of human umbilical vein endothelial cells (HUVECs) in vitro [34].

6. THE EFFECT OF CXCL12 ON CELLULAR PROCESSES

Activation of CXCR4 produces specific cellular changes that are consistent with a migratory and invasive cell phenotype. Exposure of cells to CXCL12 produces upregulation of matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 [25, 26, 29, 35–39]. In addition, CXCL12 enhances adhesion to components of the extracellular matrix such as fibronectin, laminin, and collagen types I/III [37, 40], or to other cell types (e.g., endothelial or bone marrow stromal cells) [29, 41, 42]. These changes are mediated in large part by integrin signaling [29, 43, 44]. Many signaling pathways are activated by CXCL12 downstream of CXCR4 in cancer cells. For example, CXCL12 has been shown to increase ERK1/2 phosphorylation [30, 31, 49, 0.70, 76.78, 79], Akt phosphorylation [50, 77.88], and PI3K activation [45].

7. CXCR4 IN BREAST CANCER

CXCR4 is expressed at a low level in normal breast epithelium but becomes more strongly expressed in the early stages of carcinogenesis, showing both a more intense immunohistochemical staining pattern and an altered cellular localization in studies of human ductal carcinoma in situ (DCIS) [46, 47]. An extensive tissue microarray study of 1808 invasive breast carcinomas and 214 pre-invasive breast samples linked to clinical data has shown that the level of CXCR4 expression can be linked to tumor progressivity (tumor grade and lymph node status) and to other prognostic factors such as HER2 expression and hormone receptor (ER and PR) negativity, as well as to patient survival [46]. These observations in human tissues have led to the view that CXCR4 provides a selective advantage to newly formed neoplastic cells in the early primary breast tumor as well as being important to later invasion and metastasis [13, 46–48]. This is consistent with observations in mouse models of breast cancer in which interventions affecting CXCR4 reduced both growth of the primary tumor and metastasis [49].

Prominent CXCR4 expression is a feature of all major histological forms of invasive breast cancer, including ductal, lobular, mucinous [46], and the distinctive and highly aggressive inflammatory form of the disease [50]. Several independent studies have shown that the extent and pattern of CXCR4 expression is related to axillary lymph node involvement in different forms and stages of breast cancer [28, 51–53]. CXCR4 positivity has also been noted as a key feature of breast carcinoma metastasis to bone [54] and brain [55]. The power of CXCR4 as a marker for lymph node metastasis can be greatly increased by concurrently examining the expression of additional markers such as VEGF, MMP-9, and CCR7 [38, 56]. Furthermore, CXCR4 is also one of a subset of markers (the others are uPAR, S100A4, and vimentin) that define highly aggressive and invasive breast carcinoma cells that are associated with malignant pleural or peritoneal effusions in breast cancer patients [57]. CXCR4 expression is therefore a general marker for the spread of breast cancer to its secondary sites, and for aggressive stages of the disease.

There is evidence not only for the use of CXCR4 as a general marker for the progression and metastasis of breast cancer, but also for the identification of individual tumor cells as they are homing from the primary tumor to secondary sites as patients develop metastatic disease. Individual CXCR4-expressing tumor cells have been found

TABLE 1: Involvement of CXCL12/CXCR4 in different cancers.

Cancer	Comments	References
Acute lymphoblastic leukemia	Levels of CXCR4 are elevated on lymphoblasts. Elevated levels of CXCR4 are associated with increased infiltration in liver and spleen	[58]
Acute myelogenous leukemia	High CXCR4 expression is associated with relapse and reduced survival	[59]
Brain cancer	CXCR4 expression is demonstrated in tissues and cell lines derived from glioblastoma, medulloblastoma, and astrocytoma. Cell lines respond to CXCL12 with increased proliferation, survival and migration. Gliomas expressing CXCR4 are associated with increased tumor size and reduced survival	[41, 60–64]
Breast cancer	High CXCR4 expression is noted in breast cancer tissues compared to normal tissues and cell lines with invasive characteristics. CXCR4 expression is associated with more extensive lymph node metastasis and with liver metastasis, although CXCR4 expression in lymph node metastases may be lower than primary cancers. CXCR4 co-expression with HER2/neu is an indicator of more extensive lymph node involvement	[25, 28, 65–67]
Cervical cancer	CXCR4 expression is associated with increased tumor size, stromal invasion, lymph node metastasis, and reduced survival	[68]
Chronic lymphocytic leukemia	Malignant B cells express 3- to 4-fold higher cell-surface CXCR4 levels than normal B cells. High CXCR4 expression on B cells is associated with reduced survival in patients with familial chronic lymphocytic leukemia	[69, 70]
Colorectal cancer	CXCR4 is over-expressed in colorectal carcinoma tissues compared to normal tissues, and on certain established cell lines. In patients with liver metastasis, higher CXCR4 expression is found on liver metastases compared to the primary tumor. In patients with stage I/II disease, high CXCR4 mRNA expression in tumor samples is associated with increased disease recurrence. In patients with stage IV disease, patients with high CXCR4 have decreased overall survival. High CXCR4 expression is associated with increased lymph node involvement and distant metastasis, as well as reduced 3-year survival	[40, 71–75]
Endometrial cancer	Endometrial adenocarcinoma tissues and human cell lines express CXCR4 protein. CXCL12 induces proliferation of endometrial carcinoma cells	[76]
Esophageal cancer	CXCR4 expression is associated with reduced survival and increased lymph node/bone marrow metastasis	[77]
Gastric cancer	A majority of primary gastric tumors and many human gastric carcinoma cell lines express CXCR4. Primary tumors that express CXCR4 protein are associated with peritoneal carcinomatosis	[78]
Head and neck squamous cell cancer	CXCR4 expression is found in tissues and cell lines. High CXCR4 expression is associated with increased occurrence of distant metastases and reduced survival	[79, 80]
Hepatocellular carcinoma	CXCR4 is correlated with tumor progression, metastasis, and reduced survival	[81]
Melanoma	CXCR4 protein is expressed on human melanoma cell lines, as well as on cells isolated from melanoma surgical specimens. CXCL12 enhances cell adhesion to fibronectin, the binding of murine melanoma cells to endothelial cells, and invasion of human melanoma cells across basement membranes. CXCR4 expression is associated with reduced disease-free survival and overall survival	[35, 43, 82, 83]
Multiple myeloma	Multiple myeloma cells isolated from bone marrow and multiple myeloma cell lines express cell-surface CXCR4 protein. CXCL12 enhances adhesion to fibronectin and stimulates cell migration	[84]

TABLE 1: Continued.

Cancer	Comments	References
Nasopharyngeal cancer	Most primary human nasopharyngeal carcinoma biopsy samples and metastatic lymph nodes stain positively for CXCR4 protein. Nasopharyngeal carcinoma cell lines also express CXCR4 mRNA	[85]
Non-Hodgkin's lymphoma	Most tissue samples and cell lines express high levels of CXCR4 mRNA and cell-surface protein. CXCR4 is implicated in transendothelial migration and proliferation of non-Hodgkin's lymphoma cells	[86]
Nonmelanoma skin cancer	CXCR4 is expressed on invasive squamous cell carcinoma and basal cell carcinoma tissues. Expression on invasive squamous cell carcinoma is increased compared to normal skin	[87]
Non-small cell lung cancer	CXCR4 mRNA is upregulated in NSCLC tissues compared to normal tissues, and levels are higher in tissue samples taken from patients with metastasis than from those without metastasis. Overexpression of CXCR4 in NSCLC cells leads to enhanced migratory, invasive, and adhesive responses to CXCL12. Nuclear CXCR4 staining is associated with longer survival and reduced incidence of metastasis	[88, 89]
Osteosarcoma	CXCR4 mRNA is expressed in most human osteosarcoma samples, and two of three osteosarcoma cell lines. CXCR4 expression is higher at metastatic sites than in the primary tumor	[90, 91]
Ovarian cancer	CXCR4 mRNA is expressed in ovarian cancer cell lines, as well as in biopsies from primary tumors and ovarian cancer ascites. High levels of CXCL12 are present in ascitic fluid taken from patients with ovarian cancer. CXCL12 stimulates the growth of ovarian cancer cells. CXCR4 expression is associated with increased recurrence and reduced survival	[26, 33, 92]
Pancreatic cancer	Most human pancreatic cancer tissues stain positively for CXCR4 expression, and more than half of pancreatic cancer cell lines express CXCR4 mRNA and cell-surface protein. CXCL12 induces chemotaxis of human pancreatic carcinoma cells, as well as stimulates proliferation and promoted survival	[42, 93]
Prostate cancer	Prostate cancer cell lines express CXCR4 mRNA and protein, and approximately half of prostate cancer tissues stain positively for CXCR4. Treatment of cells with CXCL12 increases their adherence to osteosarcoma cells and bone marrow endothelial cells, transendothelial migration, and invasion into Matrigel. CXCR4 expression is a positive predictor of bone metastasis, particularly in patients with elevated prostate specific antigen (PSA) levels. High CXCR4 expression is associated with increased cancer-specific mortality	[29, 36, 94, 95]
Renal cell cancer	One of four human renal cell cancer lines express CXCR4 mRNA, which is upregulated in renal cell cancer tumor samples compared to normal tissue. High CXCR4 expression is associated with poor tumor-specific survival, independent of tumour stage and differentiation grade	[96, 97]
Rhabdomyo sarcoma	Several rhabdomyosarcoma cell lines express cell-surface CXCR4 protein. CXCL12 increases cell motility, induces chemotaxis, increases adhesion to extracellular matrix, and stimulates secretion of MMP-2	[37]
Small cell lung cancer	CXCR4 mRNA and cell-surface protein are detected in cell lines. CXCL12 induces proliferation, increases adherence and motility, and induces morphological changes such as filopodia formation	[98]
Thyroid cancer	Human thyroid carcinoma cell lines express CXCR4 protein, and CXCR4 is upregulated in primary papillary thyroid carcinomas compared to normal thyroid tissue. CXCL12 increases proliferation, inhibits apoptosis, and increases migration and invasion of human thyroid cancer cells	[99, 100]

Table 2: Rosiglitazone downregulation of CXCR4 on HT-29 cells and suppression by PPARγ antagonists. HT-29 cells were treated with the PPARγ antagonists (I) GW9662 at $1 \mu M$ or (II) T0070907 at 100 nM for 30 minutes before exposure to rosiglitazone (10 nM). Cell-surface CXCR4 protein expression was measured after 48 hours. The data are mean values ± SE ($n = 4$). The table is taken from [101] with permission.

Experiment	PPARγ antagonist	Treatment		Decrease due to rosiglitazone (%)
		Control	Rosiglitazone	
I	Control	2.53 ± 0.14	0.95 ± 0.09***	63
	GW9662	2.47 ± 0.22	2.43 ± 0.27 n.s.	2
II	Control	1.90 ± 0.17	0.81 ± 0.11**	57
	T0070907	2.74 ± 0.17	3.07 ± 0.18 n.s.	0

Significant change due to rosiglitazone, ***$P < .001$; **$P < .01$; n.s.: not significant.

in the peripheral blood of breast cancer patients [102], and CXCR4 expression in breast cancer has been associated with the presence of individual tumor cells in the bone marrow of patients [103].

8. CXCR4 IN COLORECTAL CANCER

CXCR4 is abundantly expressed by colorectal carcinoma cells [104, 105]. The involvement of CXCR4 expression in colorectal cancer progression was first shown by Roos and colleagues [71]. CT-26 mouse colon carcinoma cells were transfected with CXCL12 extended with a Lys-Asp-Glu-Leu (KDEL) sequence. The KDEL receptor functions to retain resident endoplasmic reticulum (ER) proteins, which contain a C-terminal KDEL sequence, in the ER. With this "intrakine approach," CXCL12-KDEL binds to the KDEL receptor and is retained in the ER, and CXCR4 protein which binds to CXCL12 is also retained in the ER, preventing its expression at the cell-surface [71, 106]. This approach was first developed as a strategy to reduce HIV infection [107]. After intrasplenic injection, CXCL12-KDEL-transfected CT-26 cells, which had reduced cell-surface CXCR4 protein expression, did not form liver metastases, whereas control cells did [71]. The incidence of lung metastasis was also reduced with CXCL12-KDEL-transfected cells, and survival was increased. Interestingly, unlike Zlotnik's group, who had suggested that CXCR4 expression was necessary for the movement of tumor cells to secondary sites [25], Zeelenberg and colleagues found that CXCR4 expression was not required for migration of CT-26 colorectal tumor cells to the lungs, but rather for tumor expansion at secondary sites [71]. Therefore, these authors concluded that CXCR4 is necessary for the outgrowth of colon cancer micrometastases.

Ottaiano and colleagues found that CXCR4 was overexpressed in human colorectal carcinoma tissues compared to normal tissues [40]. Cell-surface CXCR4 protein was also expressed at high levels on SW620, SW48, and SW480 colorectal carcinoma cells, and at moderate levels on Caco-2 and LoVo cells. CXCL12 enhanced the chemotaxis of SW480 cells as well as their adhesion to fibronectin and collagen type I/III, and both effects were blocked with an anti-CXCR4 neutralizing antibody. CXCL12 also induced cytoskeletal changes, proliferation, and ERK1/2 phosphorylation in SW480 cells. Similarly, Schimanski and colleagues

found that SW480, SW620, and HT-29 colorectal carcinoma cells expressed CXCR4 protein, as did colorectal carcinoma tissue samples [72]. CXCL12 induced the chemotaxis of SW480 and SW620 cells. Kim and colleagues found that in patients with colorectal cancer with liver metastases, higher CXCR4 expression was found on metastatic tissues compared to the primary tumor [73]. Furthermore, elevated CXCR4 expression in colorectal cancer is associated with disease progression and reduced survival [40, 72, 73, 75].

9. THE UTILITY OF CXCR4 AS A MARKER OF TUMOR PROGRESSION

CXCR4 expression has been associated with disease progression, increased recurrence, and reduced survival in many cancer types, as listed in Table 1. As pointed out earlier, CXCR4 protein expression is detectable in the majority of cases of DCIS of the breast, whereas CXCR4 levels are very low in adjacent normal breast epithelium [46]. This suggests that the acquisition of CXCR4 expression may occur very early in malignant transformation, suggesting its potential as a biomarker. As indicated earlier, it has been suggested that CXCR4 expression may be useful as an indicator of prognosis [56, 73].

Although mutations in the *CXCR4* gene have not been reported in the context of cancer, patients with a single nucleotide polymorphism in the $3'$ untranslated region of the *CXCL12* gene had reduced incidence of long distance metastasis of epidermoid non-small cell lung cancer (NSCLC) [108].

10. PRECLINICAL EFFICACY OF ANTI-CXCR4 TREATMENTS

Several studies have demonstrated the efficacy of strategies designed to reduce CXCR4 expression or inhibit its activity in preclinical models of cancer development and metastasis. A neutralizing anti-CXCR4 antibody prevented metastasis of MDA-MB-231 breast cancer cells in mice [25] and in another study reduced tumor growth after intraperitoneal (IP) injection of Namalwa non-Hodgkin's lymphoma cells [86]. Interestingly, a neutralizing antibody against CXCR4 also inhibited the growth of subcutaneous tumors derived from pancreatic cancer cells that did not themselves express

CXCR4, probably because of the ability of the antibody to block CXCR4 on tumor vasculature [109].

CXCR4 peptide antagonists have also proven effective in preclinical cancer models. The CXCR4 peptide antagonist 4F-benzoyl-TN14003 inhibited lung metastasis of MDA-MB-231 breast cancer cells [110], and 4F-benzoyl-TE14011 reduced pulmonary metastasis of B16-BL6 melanoma cells [111]. Murakami and colleagues assessed the contribution of CXCR4 to the metastatic process by transducing B16 murine melanoma cells with CXCR4, followed by IV injection in syngeneic B57BL/6 mice [112]. CXCR4 expression in this context led to increased pulmonary metastasis, which was reduced with the CXCR4 peptide antagonist T22. Liang and colleagues showed that TN14003 itself, which is a 14-mer peptide CXCR4 antagonist, inhibited in vitro invasion of MDA-MB-231 breast cancer cells and lung metastasis after tail vein injection of these cells, without causing any toxicity [113].

Small molecule (nonpeptide) inhibitors of CXCR4 have also been tested in preclinical cancer models. Rubin and colleagues showed that the noncompetitive CXCR4 antagonist AMD3100 inhibited tumor growth after intracranial implantation of Daoy medulloblastoma cells and U87 glioblastoma cells [63] and also inhibited peritoneal carcinomatosis and ascites formation after IP inoculation of NUGC4 human gastric carcinoma cells [78]. In a different approach, blocking the mammalian target of rapamycin (mTOR) pathway downstream of CXCR4 was shown to suppress processes involved in the peritoneal dissemination of gastric cancer [114].

Liang and colleagues also showed the preclinical efficacy of anti-CXCR4 treatments using an RNA-silencing molecular approach [115]. MDA-MB-231 breast cancer cells transfected with siRNA oligonucleotides to knock down CXCR4 were injected into the tail veins of SCID mice. Mice received twice-weekly IV injections of siRNA oligonucleotides to maintain CXCR4 knockdown. The control mice all developed lung metastases, whereas only one of six mice receiving CXCR4 siRNA-transfected cells and followup injections with CXCR4 siRNA developed metastases. Stable knockdown of CXCR4 expression in 4T1 murine breast carcinoma cells using short hairpin RNA reduced orthotopic tumor growth and lung metastasis [49]. Similarly, MDA-MB-231 cells that had undergone stable knockdown of CXCR4 did not form tumors or lung metastases after orthotopic injection into mammary fat pads of SCID mice, whereas CXCR4-positive cells were tumorigenic [116]. NSCLC 95D lung cancer cells in which CXCR4 was knocked down using antisense technology also formed lung metastases in fewer mice after SC injection compared to CXCR4 positive cells [88]. Finally, manipulations of CXCR4 expression have become possible using microRNAs (miRNAs), which are endogenous short RNAs with the ability to repress the translation of target mRNAs [117–119]. The approach of expressing a synthetic miRNA against CXCR4 mRNA to knock down CXCR4 expression has been used successfully in MDA-MB-231 breast cancer cells, HeLa cervical carcinoma cells, and U2-OS osteosarcoma cells [118, 120, 121]. Reduced CXCR4 expression in the breast cancer model was accompanied by

reduced migration and invasion of the cells in vitro and fewer lung metastases in vivo [121]. These studies show the importance of CXCR4 expression in both primary and secondary tumor growth.

11. CLINICAL ASSESSMENT OF CXCR4-TARGETED REAGENTS

The bicyclam compound AMD3100 was developed as a small molecule CXCR4 antagonist [122]. Although this compound has not yet been fully assessed in clinical trials to determine its therapeutic potential in cancer, it has been examined in small trials in the context of HIV treatment and hematopoietic progenitor cell mobilization [123–128]. One trial with AMD3100 reported one patient with thrombocytopenia, two patients with premature ventricular contractions, and several patients with paresthesias [126]. AMD3100 did not reduce viral load in HIV patients [122], but did effectively increase hematopoietic progenitor cell mobilization [124, 125, 127, 128]. However, the mechanisms of action are under debate and may be unrelated to inhibition of CXCR4 as was first presumed.

12. REGULATION OF CXCR4 EXPRESSION BY FACTORS WITHIN THE TUMOR

Zeelenberg and colleagues found that CT-26 murine colon carcinoma cells grown in vitro expressed CXCR4 mRNA, but cell-surface protein levels were not detectable [71]. When the same cells were freshly isolated from lung or liver metastases or from intrasplenic tumors, cell-surface expression was strongly upregulated. This elevated expression was lost after 2–4 days in culture, indicating that it was not due to selection of a subpopulation of cells with a high CXCR4 expression. The authors concluded that CXCR4 expression was induced by the in vivo tumor microenvironment. Although others have shown that metastatic cells maintain high CXCR4 expression when cultured in vitro [129], and indeed CXCR4 has been suggested as a cancer stem cell biomarker [130], as discussed below there is substantial evidence indicating that CXCR4 expression is nevertheless influenced by the tumor microenvironment. Additionally, aberrant activation of signaling pathways within cancer cells, such as those initiated through HER2, can also contribute to elevated CXCR4 expression [131].

Multiple features and factors present in the tumor microenvironment have been shown to regulate CXCR4 expression on tumor cells and other cell types. One such feature is hypoxia [97, 132]. Solid tumors tend to be hypoxic due to structural abnormalities in their vasculature [133]. Staller and colleagues were the first to demonstrate the involvement of hypoxia in the regulation of CXCR4 expression [97]. Their goal was to identify genes regulated by the von Hippel-Lindau tumor suppressor protein (pVHL) in renal cell carcinoma cells. pVHL is often inactivated in renal cell cancer (RCC) leading to constitutive activation of hypoxia-inducible factor-1 (HIF-1) target genes. In a microarray analysis, they found that CXCR4 mRNA expression was suppressed by the reintroduction of functional

pVHL into pVHL-deficient A498 RCC cells, an effect that was due to inactivation of HIF-1. CXCR4 protein was also downregulated, resulting in reduced migration of RCC cells towards CXCL12. Hypoxia increased CXCR4 mRNA expression in HEK-293 human embryonic kidney cells and primary human proximal renal tubular epithelial cells, and a hypoxia response element (HRE) was identified within the CXCR4 promoter [97]. The authors speculated that intratumoral hypoxia may lead to increased CXCR4 expression in diverse types of solid tumors, increasing metastasis to distant organs. Shioppa and colleagues found that hypoxia increased CXCR4 mRNA and cell-surface protein expression in several cell types, including monocytes, human monocyte-derived macrophages, tumor-associated macrophages, HUVECs, CAOV3 ovarian carcinoma cells, and MCF-7 breast carcinoma cells, leading to increased migration towards CXCL12 due to the activation of HIF-1 [132].

The hypoxic environment within tumors also leads to high extracellular levels of adenosine (adenine-9-β-D-ribofuranoside), a nucleoside that is involved in energy metabolism and comprises the core structure for adenine nucleotides. The concentration of adenosine in the extracellular fluid of solid tumors is about 100-fold that of adjacent normal tissue [134]. Adenosine concentrations in tumors reach levels that can act on any of four subtypes of adenosine-selective, G-protein-coupled receptors: A1, A2a, A2b, and A3 [135]. Adenosine receptors of all four known subtypes are expressed differentially on different cell types within the tumor, including stromal cells, endothelial cells, and infiltrating leukocytes. We have shown that through such receptors, adenosine can have protumor effects directly on cancer cells and also indirectly via other supporting/infiltrating cells [136–139]. Adenosine also acts through A2a and A2b adenosine receptors on human colorectal carcinoma cells to upregulate CXCR4 mRNA expression up to 10-fold, and selectively increase cell-surface CXCR4 protein up to 3-fold [31]. This increase in cell-surface CXCR4 enables the carcinoma cells to migrate toward CXCL12 and enhances their proliferation in response to CXCL12.

One of the further major factors that allows tumor expansion is vascular endothelial growth factor (VEGF), which is also produced in response to hypoxia and which promotes neovascularisation of the tumor. The angiogenic effect of VEGF increases the supply of nutrients and blood-borne growth factors to allow growth of the tumor. There is significant interplay between the roles of VEGF and CXCR4 in tumor expansion. Concomitan high expression of CXCR4 and VEGF has been observed in colorectal [74, 75], breast [38], and ovarian [34] cancers, as well as in glioma [140] and osteosarcoma [91], in each of which it has been linked to increased angiogenesis, invasion, and/or metastasis. Clinical studies have shown that although VEGF and CXCR4 both predispose to lymphatic involvement and nodal metastasis in colorectal cancer, they work through different regulatory strategies [74]. Their collaborative role in angiogenesis parallels a similar joint action in noncancer processes involving neovascularisation (e.g., [141]), and it has been suggested in the context of tumor angiogenesis that

their actions may be synergistic [34]. It is not surprising that these two entities are closely linked; VEGF receptors and CXCR4 have common regulatory pathways. For example, interference with Notch signalling leads to downregulation of both VEGF receptor 2 and CXCR4 [142].

The relationship between VEGF and CXCR4 is complex. Firstly, VEGF can promote CXCR4 pathways. VEGF is present in high levels in tumors and may upregulate CXCR4 expression on tumor cells, as has been demonstrated in glioma [143] and breast cancer [144]. In the case of tumor cells, this upregulation of CXCR4 by VEGF can happen through an autocrine mechanism [144]. VEGF can also upregulate CXCR4 on the endothelial cells that may be involved in angiogenesis during tumor expansion [145, 146].

Conversely, the ability of CXCR4 to signal through PI3K/Akt and ERK1/2 provides a route through which VEGF expression may be regulated by CXCR4 [147–149]. Binding of CXCL12 to CXCR4 has been shown to increase cellular secretion of VEGF in ovarian cancer [150], breast cancer [147], prostate cancer [149, 151], and malignant glioma [152]. This phenomenon parallels the ability of the CXCL12/CXCR4 axis to stimulate VEGF secretion in normal lymphohematopoietic cells [153]. One might therefore expect a large part of the antitumor activity of CXCR4 antagonists to be mediated through reduced secretion of VEGF. Indeed, interference with the CXCL12-CXCR4 pathway has been shown to cause downregulation of expression of VEGF [39]. However, blocking the CXCL12/CXCR4 axis in vivo can inhibit tumor growth and angiogenesis without producing alterations in VEGF pathways [109].

Other growth factors whose levels are elevated in tumors may also enhance CXCR4-dependent mechanisms. Tumors have high levels of tumor necrosis factor-α (TNF-α), derived primarily from tumor-associated macrophages (TAMs) [154–156]. TNF-α itself, or macrophages that serve as a source of TNF-α, are able to increase CXCR4 mRNA and cell-surface protein expression on ovarian cancer cells [157] and astroglioma cells [158]. A significant correlation between TNF-α and CXCR4 expression was found in ovarian cancer biopsies [157]. The increase in CXCR4 at a cellular level appears to be due to TNF-α-induced activation of NF-κB signaling and is associated with enhanced migration towards CXCL12 [157]. Therefore, TAMs may contribute to increased CXCR4 expression on cancer cells via production of TNF-α.

Finally, polypeptide growth factors that are associated with the extracellular matrix, and indeed components of the extracellular matrix itself, can upregulate CXCR4 on cancer cells. Transforming growth factor-β (TGF-β) increases cell-surface CXCR4 protein expression on human melanoma cells [35] and we have recently found that FGF-2 upregulates CXCR4 on human colorectal cancer cells (Bseso B and Blay J, manuscript in preparation). Furthermore, type-I collagen and the preparation Matrigel, which is a secreted ECM rich in laminin [159], also increase levels of CXCR4 on melanoma cells [35]. Therefore, interactions with matrix proteins within tumors may also increase CXCR4 expression.
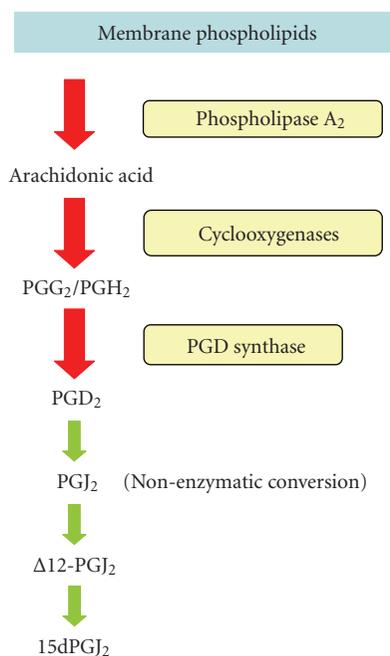
FIGURE 1: Production of PGD_2 and conversion to its metabolites. Prostanoids follow an initial common pathway in which arachidonic acid is released from membrane phospholipids by phospholipase A_2 and then converted to the short-term intermediates PGG_2 and PGH_2 by cyclooxygenases. Prostaglandin D synthase forms PGD_2 itself, but subsequent nonenzymatic reactions in aqueous media lead to the sequential production of prostaglandin J_2 (PGJ_2), 9-deoxy-Δ^9, $\Delta^{12\text{-}13,14}$-dihydro-PGD_2 (Δ^{12}-PGJ_2), and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2).

13. THE ROLE OF CYCLOOXYGENASE-2 AND PGE_2 IN CANCER

The shift to malignancy in epithelia and indeed the progression to invasion and metastasis are associated with increased expression of the enzyme cyclooxygenase-2 (COX-2) [160–163]. High COX-2 expression is in cancer is often associated with reduced patient survival [163]. The immediate effect of high COX-2 expression is increased prostaglandin synthesis, particularly prostaglandin E_2 (PGE_2) [164], which in experimental models is associated with the production of vascular loops and arches and evidence of abnormal vessel function [165], a phenotype consistent with tumor angiogenesis. Observations of increased expression of angiogenic regulatory genes, including VEGF, ang-1, and ang-2 are consistent with this view [166]. Furthermore, nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit cyclooxygenases, reduce both tumor incidence and microvessel density in COX-2-expressing mice [166] and reduce cancer progression in preclinical models and clinical trials [167]. Indeed, NSAIDs and COX-2 inhibitors reduce the relative risk of developing colorectal cancer by 40–50% [167–169].

Tumor-promoting effects of COX-2 overexpression appear to be due in large part to increased PGE_2 production [170–173]. Associated with the increase in COX-2, there is a decreased expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), an enzyme involved in the inactivation of PGE_2, in cancer compared to normal tissues [174], as well as upregulation of cytosolic PLA_2 (cPLA_2), which increases the supply of arachidonic acid substrate for COX-2 [175–177]. In addition to promoting angiogenesis, PGE_2 also stimulates cancer cell proliferation [178, 179], promotes cell migration [180], and causes transactivation of polypeptide growth factor receptors [181].

14. OTHER PROSTAGLANDINS IN CANCER

Prostaglandins together with the thromboxanes are classed as prostanoids, and belong to a larger group of compounds referred to as eicosanoids [182]. The main prostanoids apart from PGE_2 are prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), prostaglandin I_2 (PGI_2 or prostacyclin), and thromboxane A_2 (TXA_2). As well as reflecting changes in COX-2, cPLA_2, and inactivating enzymes, the levels of different prostanoids in tumors can be modulated by altered expression of specific prostaglandin synthases [183]. Prostaglandins can also be metabolized nonenzymatically to form a range of products both in the body and in cell culture. PGD_2 can be converted to cyclopentenone J-series prostaglandins, including prostaglandin J_2 (PGJ_2), 9-deoxy-Δ^9, $\Delta^{12\text{-}13,14}$-dihydro-PGD_2 (Δ^{12}-PGJ_2), and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2); PGE_2 can be converted to prostaglandin A_2 (PGA_2) [184–186]. The tumor microenvironment therefore has a rich and varied content of eicosanoid mediators.

15. PROSTAGLANDIN EFFECTS ON CANCER CELLS

Although the major focus of attention has been on PGE_2, a range of eicosanoids acts to restrain tumor growth. Indeed the PGE_2 metabolite PGA_2 reduces cell number and induces apoptosis and cell cycle changes in both human breast cancer cells and human epithelial cervical carcinoma cells [187].

More notably, PGD_2 and its series of derivatives have anticancer effects. PGD_2 itself can reduce the growth of carcinoma cells [188]. However, other studies have shown that the nonenzymatic breakdown of PGD_2 to sequential metabolites (Figure 1) may be required for growth inhibition and that the latter metabolites are the active eicosanoids [189–194]. PGD_2 therefore can act independently of its DP receptors by its metabolism through a dehydration reaction to prostaglandin J_2 (PGJ_2), Δ^{12}-PGJ_2, and then to 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2(15dPGJ_2) [184]. This reaction occurs in cell culture media, both in the presence and absence of serum [184, 189, 195]. Therefore, it is possible that many effects noted in vitro with PGD_2 are actually due to the formation of J-series prostaglandins. Frequent replacement with fresh medium containing PGD_2 in such circumstances can eliminate the response, while the addition of the metabolite(s) themselves leads to growth inhibition in a shorter timeframe than PGD_2 itself [189]. Some workers have proposed that Δ^{12}-PGJ_2 is the key metabolite [189]; but in fact all of the successive J-series prostaglandins, that is, PGJ_2, Δ^{12}-PGJ_2, and 15dPGJ_2, are able to reduce proliferation and induce apoptosis of cancer cells [190].

Furthermore, the end metabolite 15dPGJ$_2$ is active against many cell types, including colorectal carcinoma cells [191, 192], prostate carcinoma cells [193], and Burkitt lymphoma cells [194], suggesting that 15dPGJ$_2$ may be the crucial mediator.

16. THE ROLE OF 15dPGJ$_2$ AND ITS ACTION ON PPARγ

15dPGJ$_2$ is an agonist for the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) [196, 197], and activation of PPARγ may account for the growth inhibitory effects of 15dPGJ$_2$. PPARγ activation results in its heterodimerization with the retinoid X receptor (RXR), binding to peroxisome proliferator response elements (PPREs) on DNA, and subsequent activation of target gene expression [198]. PPARγ is aberrantly expressed in some cancer types [199], and in many cases its activation leads to cell death or differentiation [191, 200, 201]. This action of 15dPGJ$_2$, and by extension its precursors PGD$_2$, PGJ$_2$, and Δ^{12}-PGJ$_2$, may underlie the major action of these eicosanoids on cell growth. For example, 15dPGJ$_2$ reduces the growth of PC-3 human prostate cancer cells through the activation of PPARγ [202]. However, in addition to direct growth-inhibitory effects, 15dPGJ$_2$ may also exert anticancer effects by reducing expression of protumor proteins. For example, 15dPGJ$_2$ inhibits phorbol ester-induced VEGF and COX-2 expression in SW620 human colorectal carcinoma cells [203].

17. 15dPGJ$_2$ CAUSES DOWNREGULATION OF CXCR4 ON CANCER CELLS

In our studies of the possible effects of these different prostaglandins on CXCR4, we focused upon the expression of the mature protein and furthermore restricted our quantitation exclusively to the receptor that is displayed to the external environment at the cell surface [31]. Cell-surface CXCR4 reflects functional receptor that is coupled to cellular responses [31] rather than the very large intracellular pool of inaccessible receptor protein [72].

Although PGF$_{2\alpha}$ (to some extent) and PGE$_2$ (as well as its product PGA$_2$) have some ability to modulate CXCR4 levels, by far the most potent prostaglandins in this regard are PGD$_2$ and its derivatives [204]. Prostaglandin D$_2$ and the J-series prostaglandins used at low micromolar concentrations cause substantial loss of CXCR4 from the surface of HT-29 human colorectal carcinoma cells [204]. In particular, 15dPGJ$_2$ completely eliminates cell-surface CXCR4 at a concentration of 10^{-5} M in vitro, and has significant effects after a single dose of 300 nM, about 100-fold less than for PGF$_{2\alpha}$ [204]. The time course of the decline in cell-surface CXCR4 protein is slow, reaching a maximum only after 48–72 hours (Figure 2). The concentrations of prostaglandins that are needed to cause downregulation after a single dose likely grossly overestimate the steady-state levels that would cause such a response, as we have found in other studies with labile metabolites [31, 138]. We estimate that the effect of 15dPGJ$_2$ on CXCR4 is achievable with concentrations of 15dPGJ$_2$ present in vivo.
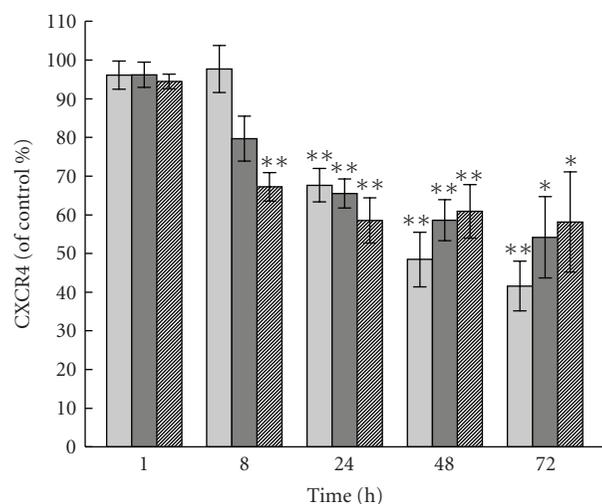

Figure 2: Time course of changes in cell-surface CXCR4 protein expression on HT-29 cells by PGD$_2$ and its metabolites. HT-29 cells were treated with vehicle or with $10\,\mu$M PGD$_2$ (light gray bars), $10\,\mu$M PGJ$_2$ (dark gray bars), or $3\,\mu$M 15dPGJ$_2$ (hatched bars), and cell-surface CXCR4 protein expression was measured at the indicated time points. The data shown are expressed relative to the level of CXCR4 receptor on cells treated with vehicle alone at that time point. Values have also been corrected for any possible changes in cell number. The data are mean values $\pm$ SE ($n = 4$). Significant decrease due to prostaglandin, $^{**}P < .01$; $^{*}P < .05$. The figure is taken from [204] with permission.

As can be seen in Figure 2, the response to 15dPGJ$_2$ occurs more rapidly than that to PGJ$_2$, which in turn has a more rapid onset than PGD$_2$. We further found that each of these prostaglandins does suppress CXCR4 mRNA expression and that the effect of 15dPGJ$_2$ again occurs earlier than that of PGD$_2$ [204]. The different relative kinetics of the downregulation of CXCR4 for the J-series prostaglandins are consistent with data on the conversion of PGD$_2$ through to 15dPGJ$_2$ [189] pointing to 15dPGJ$_2$ as the key factor in controlling the levels of functional CXCR4. PGD$_2$ produces similar downregulation of CXCR4 in other cell types such as the T47D human breast carcinoma cell line (Richard CL, Blay J, unpublished observations), suggesting that this may be a common phenomenon. The downregulation of CXCR4 expression by 15dPGJ$_2$ differs from 15 dPGJ$_2$-mediated downregulation of other proteins, including cyclin D1 and estrogen receptor α, which has been shown to occur through protein degradation rather than through changes in transcription [205].

18. 15dPGJ$_2$ DOWNREGULATES CXCR4 PRIMARILY VIA PPARγ

The main target for 15dPGJ$_2$ is the nuclear receptor PPARγ [196, 197]. We found that the ability of 15dPGJ$_2$ to downregulate CXCR4 occurred primarily through this pathway. The effect of 15dPGJ$_2$ was mimicked by PPARγ agonists such as rosiglitazone (Table 2, [206]), and antagonized or blocked by the PPARγ antagonists GW9662 and T0070907
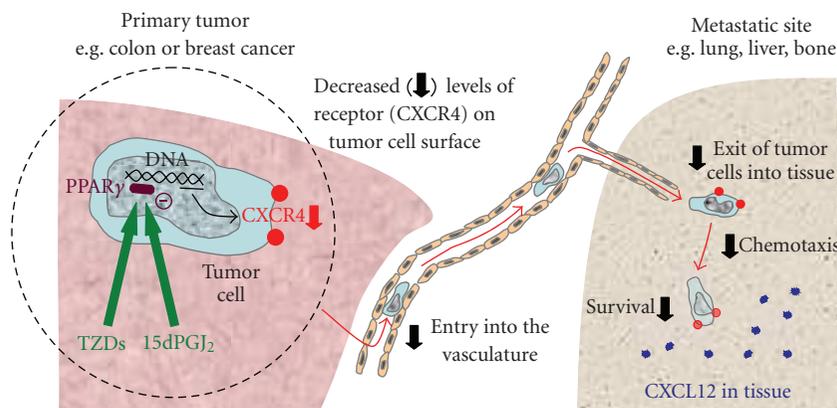
FIGURE 3: How PPARγ downregulation of CXCR4 may act to decrease metastasis. Tumor cells typically have high levels of CXCR4 at their cell surface. During metastasis, cancer cells that find their way into the bloodstream lodge in tissues that have high concentrations of CXCL12 (e.g., lungs, liver, and bone marrow). CXCL12 both encourages the entry of cells into the tissue and promotes growth of the cell population. Downregulation of CXCR4 by PPARγ activation (endogenous 15dPGJ$_2$ or thiazolidinedione drugs, TZDs) will interfere with this process and may impede metastasis.

[204], which are irreversible inhibitors of PPARγ [207, 208]. A minor part of the downregulatory activity of 15dPGJ$_2$ was due to the inhibition of NFκB since the 15dPGJ$_2$ analogue CAY10410 (9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$) [209, 210], which retains the ability to act on PPARγ but lacks the ability of 15dPGJ$_2$ to inhibit NFκB, was less potent than 15dPGJ$_2$ [208]. It is the cyclopentenone structure of 15dPGJ$_2$ (not present in CAY10410) that confers an ability to inhibit NFκB [211]. Consistent with a role for this structure, cyclopentenone itself (but not cyclopentane or cyclopentene) caused downregulation of CXCR4 [204]. Furthermore, since PGA$_2$ possesses the cyclopentenone configuration [212], this explains the ability of PGA$_2$ (and that of PGE$_2$) to downregulate CXCR4, although it does not contain the α, β-unsaturated ketone moiety necessary to activate PPARγ signaling [210].

The existence of a mechanism of 15dPGJ$_2$-induced CXCR4 downregulation may, in evolutionary terms, be an extension of the anti-inflammatory effects of 15dPGJ$_2$. Late in the inflammation process the prostaglandin profile shifts from a PGE$_2$-rich state to a PGD$_2$-rich (and therefore 15dPGJ$_2$-rich) state, leading to the resolution of inflammation [213]. Reduced CXCR4 expression may be an additional mechanism by which 15dPGJ$_2$ attempts the resolution of inflammation.

It is clear that this mechanism is not operative in the context of metastatic tumors, because CXCR4 levels are characteristically high (Table 1). Unlike PGE$_2$ which is present in elevated concentration in tumors [170–173], 15dPGJ$_2$ levels are likely low in tumors compared to normal tissue. Levels of its precursor PGD$_2$ are low in tissues of familial adenomatous polyposis, a condition that predisposes to colorectal cancer [172], and have been negatively correlated with hepatic metastasis in tumor tissues taken from patients with colorectal cancer [188]. The enzyme involved in PGD$_2$ synthesis, PGD synthase (PGDS), is decreased in cerebrospinal fluid of brain cancer patients compared

to patients without disease [214]. There is a contested report of levels of 15dPGJ$_2$ being decreased during breast cancer progression, with the lowest levels being detected in metastatic disease [173]. Finally, mechanisms to sequester or eliminate 15dPGJ$_2$ may be upregulated in cancer [215, 216]. Overall, it seems that the predominant prostaglandin within tumors is PGE$_2$, and 15dPGJ$_2$ may not be present in high levels at all. Thus, 15dPGJ$_2$-dependent suppression of CXCR4 seems to be a restraint mechanism that is not operative in a cancer situation.

19. SYNTHETIC PPARγ AGONISTS DOWNREGULATE CXCR4 ON CANCER CELLS

As indicated above, the PPARγ agonist rosiglitazone also decreased CXCR4 expression on human colorectal cancer cells, congruent with an effect of 15dPGJ$_2$ through PPARγ. This effect was seen at both the mRNA and protein level, and was more durable than the effect of 15dPGJ$_2$, as it would be expected for a more chemically stable ligand [101, 204]. Moreover, we found that other glitazone agents also downregulate CXCR4, with a rank order of potency (rosiglitazone > pioglitazone > ciglitazone > troglitazone) consistent with their potencies for interaction with PPARγ [206, 217, 218]. Further confirming that these agents were acting through their expected target, PPARγ, and that this target is linked to elimination or reduction of CXCR4 at the cell surface, we showed that the ability of rosiglitazone to decrease CXCR4 was blocked by the PPARγ antagonists GW9662 and T0070907 (Table 2), or by shRNA knockdown of PPARγ expression in the cancer cells [101].

Therefore, rosiglitazone and its analogues act through PPARγ to cause substantial and persistent suppression of CXCR4 on cancer cells. Since these agents are the same chemicals as the thiazolidinedione (TZD) class of drugs that have been used clinically for the treatment of diabetes (although recent concerns regarding side effects have limited

their utility), it opens up the possibility that we may already have a means to manipulate CXCR4 levels in cancer. Given that CXCR4 expression is linked to metastasis, judicious use of TZDs may allow us an opportunity to influence the metastatic process (Figure 3). Recent studies have shown that a unique population of CXCR4+ stem cells may be crucial for expansion of tumor cell populations [130]. We suggest that TZD therapy, by stimulating PPARγ-dependent downregulation of CXCR4 on cancer cells, may slow the rate of metastasis and may impact beneficially on disease progression.

ABBREVIATIONS

CAY10410:	9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$
CXCL12:	CXC chemokine ligand 12
CXCR4:	CXC chemokine receptor 4
DCIS:	Ductal carcinoma in situ
GW9662:	2-chloro-5-nitro-N-phenylbenzamide
HIF-1:	Hypoxia-inducible factor-1
HRE:	Hypoxia response element
LESTR:	Leukocyte-expressed seven-transmembrane domain receptor
NF-κB:	Nuclear factor-κB
NSAIDs:	Nonsteroidal anti-inflammatory drugs
NSCLC:	Non-small cell lung cancer
PPARγ:	Peroxisome proliferator-activated receptor γ
PPRE:	Peroxisome proliferator response element
pVHL:	Von Hippel-Lindau tumor suppressor protein
RCC:	Renal cell cancer
RXR:	Retinoid X receptor
SDF-1:	Stromal cell-derived factor 1
T0070907:	2-chloro-5-nitro-N-(4-pyridyl)benzamide
TAM:	Tumor-associated macrophages
VEGF:	Vascular endothelial growth factor
15dPGJ$_2$:	15-deoxy-$\Delta^{12,14}$-PGJ$_2$
15-PGDH:	15-hydroxyprostaglandin dehydrogenase
Δ^{12}-PGJ$_2$:	9-deoxy-Δ^9,$\Delta^{12-13,14}$-dihydro-PGD$_2$.

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Review Article

Activated PPARγ Targets Surface and Intracellular Signals That Inhibit the Proliferation of Lung Carcinoma Cells

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. Their discovery in the 1990s provided insights into the cellular mechanisms involved in the control of energy homeostasis, the regulation of cell differentiation, proliferation, and apoptosis, and the modulation of important biological and pathological processes related to inflammation and cancer biology, among others. Since then, PPARs have become an exciting target for the development of therapies directed at many disorders including cancer. PPARs are expressed in many tumors including lung cancer, and their function has been linked to the process of carcinogenesis. Consequently, intense research is being conducted in this area with the hope of discovering new PPAR-related therapeutic targets for the treatment of lung cancer. This review summarizes the research being conducted in this area, and focuses on the mechanisms by which a member of this family (PPARγ) is believed to affect lung tumor cell biology.

1. INTRODUCTION

Lung carcinoma is one of the most common malignant tumors in the world, and is the leading cause of carcinoma death in USA [1]. Primary malignant cancers of the lung are classified into small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC). NSCLC accounts for 80% of malignant lung cancer, and SCLC constitutes the remainder [2]. Based on the cellular phenotype, NSCLC is further subdivided into squamous cell carcinoma, adenocarcinoma, and large cell carcinomas [3]. Despite advances in understanding the mechanisms involved in carcinogenesis, the development of new surgical procedures, and the use of new radio and chemotherapeutic protocols, the five-year survival rate for lung cancer patients is poor and remains less than 15% [1]. This underscores the desperate need for novel strategies for early detection, prevention, and treatment of this malignancy.

Since their discovery in 1990, peroxisome proliferator-activated receptors (also known as PPARs) have captured the attention of investigators interested in learning about the intracellular pathways that control signal transduction and gene transcription. PPARs were originally cloned in an attempt to identify the molecular mediators of peroxisome proliferation in the liver of rodents. Today, PPARs are recognized as versatile members of the ligand-activated nuclear hormone receptor superfamily of transcription factors that includes receptors for steroids, thyroid hormone, retinoic acid, and vitamin D, among others [4]. PPARs are considered to play key roles in diverse physiological processes ranging from lipid metabolism to inflammation, and have been implicated in diseases such as cancer, atherosclerosis, and diabetes [4, 5]. Although information about the function of PPARs in lung is scarce, data implicating these molecules in key processes in lung biology are rapidly emerging.

Three subtypes of PPARs have been identified and cloned: PPARα, PPARβ/δ, and PPARγ. These subtypes are distinguished by their tissue distribution, and to a lesser degree, by their ligand specificity. PPARα has been implicated in hepatocellular carcinoma in rodents, whereas activation of PAPRβ/δ promotes human lung carcinoma cell proliferation through PI3-Kinase/Akt activation [6]. However, of the

three PPARs identified to date, PPARγ represents the most promising target in view of the many reports implicating this molecule in lung carcinoma cell growth both in vitro and in vivo. This review focuses on PPARγ, its role in lung carcinogenesis, and the potential therapeutic role of PPARγ agonists in lung cancer.

2. ELUCIDATING THE FUNCTION OF PPARγ

PPARγ was discovered based on its similarity to PPARα, and it is the most intensively studied ligand-dependent transcriptional regulator. By utilizing three different promoters, a single PPARγ gene encodes three isoforms, namely, PPARγ1, PPARγ2, and PPARγ3 [7]. Analysis of PPARγ1 and γ3 transcripts revealed that they both translate into the same PPARγ1 protein [8]. PPARγ2 protein contains additional 30 amino acids at its N-terminus compared to PPARγ1. PPARγ is highly expressed in adipose tissue, and it is a master regulator of adipocyte differentiation [9, 10]. In addition to its role in adipogenesis, PPARγ serves as an important transcriptional regulator of glucose and lipid metabolism, and it has been implicated in the regulation of insulin sensitivity, atherosclerosis, and inflammation [11, 12]. PPARγ is also expressed in multiple other tissues such as breast, colon, lung, ovary, prostate, and thyroid, where it was demonstrated to regulate cellular proliferation, differentiation, and apoptosis [13, 14]. As will be discussed later, PPARγ activation inhibits tumor progression in NSCLC [15, 16]. Several leukocyte populations, including monocytes/macrophages, lymphocytes, and dendritic cells, have also been shown to express PPARγ suggesting a role for this molecule in the regulation of immune responses [17]. PPARγ has been described as a negative regulator of macrophage function since its activation suppresses the production of inflammatory cytokines, chemokines, metalloproteases, and nitric oxide [18, 19]. These PPARγ-mediated anti-inflammatory effects are not restricted to monocytes, as the treatment with PPARγ agonists results in inhibition of cytokine/chemokine production in several epithelial and stromal cells [15].

Several natural and synthetic compounds have been identified as activators of PPARγ. The insulin-sensitizing antidiabetic drugs known as thiazolidinediones (TZDs) were the first compounds identified as PPARγ agonists [20]. The TZDs' rosiglitazone and pioglitazone are currently in clinical use for the treatment of type II diabetes, while troglitazone was withdrawn from clinical use because it was linked to idiosyncratic liver toxicity [21]. Other non-TZD synthetic ligands include certain nonsteroidal anti-inflammatory drugs such as isoxazolidinone JTT-501 [22] and tyrosine-based GW7845 [23]. Naturally occurring compounds that activate PPARγ include long-chain polyunsaturated fatty acids which are found in fish oil (e.g., n-3-PUFA and n-6-PUFA), eicosanoids (e.g., 15d-PGJ2), lipid hydroperoxides (e.g., 9(s)-HODE and 13(s)-HODE), as well as linoleic acid, 15-deoxy-$\Delta^{12,14}$ prostaglandin J$_2$ (15d-PGJ$_2$), 12/15 lipoxygenase products of 15-hydroxyeicosatetraenoic acid (15-HETE), and 13-hydroxyoctadecadienoic acid [24–26]. In addition, compounds from several medicinal plants such

as Saurufuran A from *Saururus chinensis* [27], flavonoids like chrysin and kaempferol [28], phenolic compounds from *Glycyrrhiza uralensis* [29], and curcumin from *Curcumin longa* [30, 31] are also shown to activate PPARγ.

The synthetic ligands and some natural ligands have been used to elucidate the role of PPARγ in cellular functions both in vitro and in vivo. However, several caveats should be taken into consideration when interpreting such studies. First, the natural ligands that regulate PPARs in vivohave not been completely elucidated. Second, not all PPARγ ligands exert their effects through PPARγ since there is strong evidence for the activation of PPARγ-independent signals, particularly with the natural ligand 15d-PGJ$_2$. Third, high-affinity ligands for PPARγ (e.g., TZDs) may exert partial agonist/antagonist activity [32]. The latter might be due to the fact that individual TZDs induce different PPARγ conformations that influence the recruitment of different coactivator/corepressor molecules. Thus, the activity of the PPARγ transcriptional complex is influenced by the context of a given gene and its promoter, and by the relative availability of pertinent coactivator/corepressor molecules in the cell or tissue of interest.

3. PPARγ IN LUNG CANCER

Among the three subtypes, the role of PPARγ has been investigated the most in lung carcinogenesis. PPARγ is expressed in many cancers including colon, breast, and prostate cancers, and with few exceptions, PPARγ ligands are antiproliferative in these cancers. Similarly, PPARγ is expressed in SCLC and NSCLC [33]. Furthermore, PPARγ ligands induce growth arrest and promote changes associated with differentiation as well as apoptosis in a variety of lung carcinoma cell lines although most of the knowledge available in this area has been generated in NSCLC [34, 35]. The exact mechanisms linking modulation of PPARγ with cancer growth inhibition remain unclear. However, current evidence suggests that PPARγ ligands affect a number of mechanisms including regulation of the intracellular machinery involved in cell signaling and cell cycle control, inhibition of mitogenic factors and tumor promoters, prevention of tumor cell recognition of extracellular mitogenic signals, breakdown of nicotine-induced cell survival, and modulation of the expression of angiogenic factors needed for the development of the vascular networks that supply tumor cell (see Figure 1). These mechanisms are discussed below as they relate to the action of PPARγ ligands in lung cancer.

Several studies demonstrate that PPARγ ligands affect cell cycle control in tumor cells. For example, PPARγ ligands have been found to inhibit the growth of A549 adenocarcinoma cells due to G0/G1 cell cycle arrest through the upregulation of mitogen-activated protein kinases ERK1/2 and the downregulation of G1 cyclins D and E [15]. Troglitazone inhibits NSCLC proliferation in part by stimulating the expression of the GADD 153 (for growth arrest and DNA damage-inducible gene-153) [36]. Also, troglitazone was found to induce apoptosis in NCI-H23 cells via a mitochondrial pathway through the activation of ERK1/2
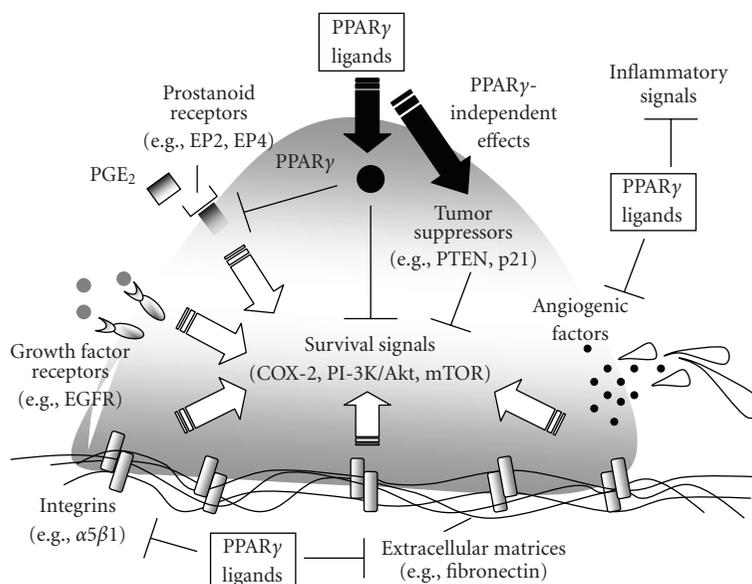
FIGURE 1: *Anticancer actions of PPARγ ligands*. In addition to genetic abnormalities, lung carcinoma cells receive mitogenic and antiapoptotic signals that promote their progression and metastasis through the activation of key intracellular pathways (e.g., COX-2, Akt, and mTOR). Lung carcinoma cells recognize these signals via diverse receptors for growth factors (e.g., EGFR), prostanoids (e.g., EP2 and EP4), and extracellular matrices (e.g., integrins), among others. In addition, angiogenic factors assist in the vascularization of tumors, while inflammatory signals further promote tumor progression. PPARγ ligands inhibit tumor growth in animal models, but the mechanisms responsible for these effects appear to be multidimensional. In vitro studies reveal that PPARγ ligands affect tumors by inhibiting the expression of key prostanoid and integrin receptors, by reducing the expression of fibronectin, a matrix glycoprotein that stimulates tumor cell proliferation, and by inhibiting the production of angiogenic and inflammatory signals. In addition, PPARγ ligands increase the expression and/or activity of tumor suppressors like PTEN and p21. Although many of these anticancer effects are mediated through PPARγ, others appear to be independent of this nuclear transcription factor (e.g., via targeting TSC2, AMPK, and ROS production and ERK activation, and interacting with CRE, AP-1).

[37]. Others have shown similar results using CRL-202 cells, and further demonstrated that troglitazone downregulated the expression of the antiapoptotic molecules Bcl-w and Bcl-2, as well as decreasing the activity of SAPK/JNK [38]. PPARγ ligands also induce the expression of death receptor 5 (DR5) and increase DR5 distribution at the cell surface in addition to reducing c-FLIP levels in human lung cancer cells. These agents cooperated with TRAIL to enhance apoptosis in human lung carcinoma cells [39]. One recent report found that PPARγ ligands 1-[(trans-methylimino-N-oxy)-6-(2-morpholinoethoxy)-3-phenyl-(1H-indene-2-carboxylic acid ethyl ester (KR-62980)] and rosiglitazone induce NSCLC apoptotic cell death mainly through PPARγ-dependent reactive oxygen species formation via increased expression of proline oxidase, a redox enzyme expressed in mitochondria [35].

Tumor suppressor genes are also affected by PPARγ ligands. For example, PGJ$_2$ and ciglitazone stimulated the expression of p21 mRNA and protein expression in NSCLC, and this coincided with a reduction in cyclin D1 mRNA expression [40]. Of note, p21 antisense oligonucleotides significantly blocked lung carcinoma cell growth inhibition observed with PPARγ ligands, thereby establishing an important role for p21 in this process. These findings are consistent with those of others showing that the proliferation

of A549 cells injected subcutaneously into nude mice was inhibited significantly by treatment with ciglitazone, and this coincided with increased expression of PPARγ and p21 and with downregulation of cyclin D1 [41]. A connection between another tumor suppressor gene (p53) and PPARγ ligands has also been demonstrated by showing that 15-deoxy-PGJ$_2$, together with docetaxel, stimulates apoptosis in NSCLC through inhibition of Bcl2 and cyclin D1 and overexpression of caspases and p53 [34].

Other reports implicate alterations in the mammalian target of rapamycin (mTOR) signaling pathway in the antitumor effects of PPARγ ligands. Rosiglitazone, for example, was reported to reduce the phosphorylation of Akt, an upstream positive modulator of mTOR, and to increase PTEN, a negative modulator of mTOR, in NSCLC H1792 and H1838 cells; this resulted in inhibition of cell proliferation [42]. Although the effects of rosiglitazone on Akt and PTEN were blocked by the selective PPARγ antagonist GW9662 and restored by transient overexpression of PPARγ, cell growth was not entirely restored suggesting the involvement of additional PPARγ-independent mechanisms of action. Further work revealed that rosiglitazone increased the phosphorylation of AMPKα, a target of LKB1, and TSC2, another potential tumor suppressor and upstream down-regulator of mTOR. The latter pathway was independent

of PPARγ since GW9662 and PPARγ siRNA did not affect it [42, 43]; others have shown similar increases in PTEN expression induced by rosiglitazone [44].

More recently, we found that rosiglitazone and dietary compounds such as fish oil (which contain certain kinds of fatty acids like ω3 and ω6 polyunsaturated fatty acids known to work as PPARγ ligands) inhibit integrin-linked kinase (ILK) expression through PPARγ signaling and the recruitment of a PPARγ coactivator, PGC-1α (Han et al., unpublished data). ILK is a unique intracellular adaptor and kinase that links cell-adhesion receptors, integrins, and growth factors to the actin cytoskeleton and to a range of signaling pathways that are implicated in the regulation of anchorage-dependent tumor cell growth/survival, cell cycle progression, invasion and migration, and tumor angiogenesis [45]. This effect was associated with activation of p38 MAPK followed by induction of transcription factor AP-2α. In turn, this resulted in inhibition of NSCLC cell proliferation (Han et al., unpublished data).

Several studies suggest that PPARγ ligands also exert antitumor effects by blocking access to mitogenic agents such as PGE$_2$, a major cyclooxygenase metabolite that plays important roles in tumor biology. The functions of PGE$_2$ are mediated through one or more of its receptors: EP1, EP2, EP3, and EP4 [46]. Human NSCLC cell lines express EP2 receptors, among other EP receptors, and the inhibition of cell growth by PPARγ ligands like GW1929, PGJ$_2$, ciglitazone, troglitazone, and rosiglitazone is associated with a significant decrease in EP2 mRNA and protein expression. Notably, the inhibitory effects of rosiglitazone and ciglitazone, but not PGJ$_2$, were reversed by a specific PPARγ antagonist GW9662, suggesting the involvement of PPARγ-dependent and PPARγ-independent mechanisms [46]. Also, a recent study showed that ciglitazone suppressed cyclooxygenase-2 (COX-2) mRNA expression and COX-2 promoter activity, while upregulating peroxisome proliferators' response element (PPRE) promoter activity in NSCLC cells, further suggesting a negative modulator role for PPARγ ligands in the COX-2/PGE$_2$ pathway in NSCLC [47].

Nicotine, a major component of tobacco, stimulates NSCLC cell proliferation through nicotinic acetylcholine receptor- (nAChR-) mediated signals. A recent case-control study of 500 incident lung cancer cases and 517 age and sex frequency-matched cancer-free controls suggested that PPARγ polymorphisms in Chinese smokers may contribute to the etiology of lung cancer [48]. Monocytes and monocyte-derived macrophages from healthy smokers showed increased PPARγ expression as compared to those from healthy nonsmokers, which were reproduced by nicotine in vitro [49]. Interestingly, concomitant administration of PPARγ agonists can effectively attenuate the effects of nicotine on alveolar type II cells [50]. We recently found that rosiglitazone reduced nicotine-induced NSCLC cell growth through downregulation of α7 nAChR-dependent signals including ERK and p38 MAPK; this effect appeared to be PPARγ-independent (Han et al., unpublished data). If confirmed, this may unveil a novel mechanism by which rosiglitazone inhibits human lung carcinoma cell growth.

Other studies suggest that PPARγ ligands might prevent the interaction of tumor cells with their surrounding stromata, thereby interfering with host-derived and tumor-derived factors and mitogenic and prosurvival effects. An example of this is fibronectin, a matrix glycoprotein residing in the lung stroma that is increased in most, if not all, chronic forms of lung disease [51]. This is true for tobacco-related lung disorders and fibrotic disorders—all associated with increased incidence of lung cancer [52]. Several studies suggest that fibronectin serves as a mitogen and survival factor for NSCLC [53], and fibronectin has been recently shown to stimulate tumor cell expression of matrix metalloproteinases, proteases implicated in metastatic disease [54]. These observations support the idea that tumor cell interactions with fibronectin through surface integrin receptors are advantageous for tumors since they stimulate proliferation, survival, and metastases [53]. This idea remains to be proven in vivo, but if found to be true, this might unveil a new target for anticancer strategies. In this regard, PPARγ ligands were shown to inhibit fibronectin expression in NSCLC cells by inhibiting transcription factors involved in regulation of fibronectin gene expression [55]. PPARγ ligands (rosiglitazone and GW1929, but not PGJ$_2$) have been also recently reported to inhibit the expression of the gene encoding for the α5 integrin subunit resulting in reduced expression of the integrin α5β1, a fibronectin receptor that mediates fibronectin's mitogenic effects in NSCLC cells and nontumor lung cells [56]. Thus, by inhibiting the expression of fibronectin and its integrin α5β1, PPARγ ligands might reduce tumor cell recognition of fibronectin with consequent changes in cell proliferation and apoptosis.

PPARγ might also regulate the generation of the complex vascular network that supplies tumor cells. This idea is supported by studies showing a reduction in blood vessel density in the lung tumors generated by the injection of A549 cells into the flanks of SCID mice treated with PPARγ ligands [57]. In in vitro studies, the treatment of A549 cells with troglitazone or their transient transfection with a constitutively active PPARγ construct blocked the production of angiogenic molecules such as ELR+CXC chemokines IL-8 (CXC-8), ENA-78 (CXCL5), and Gro-alpha (CXCL1) [57]. Furthermore, PPARγ activation inhibited NF-κB, a transcription factor known to regulate the expression of many of the proangiogenic factors mentioned above. Similarly, rosiglitazone was shown to inhibit mouse lung tumor cell growth and metastasis in vivo through direct and indirect antiangiogenic effects [16]. It is important to note that PPARγ signaling has also been associated with tumor promoter activities in some tumor cells such as colon and breast, and that this effect was linked to increased beta-catenin and c-Myc expression [58, 59] (Table 1). These findings need to be confirmed and tested in other tumors. However, these data suggest that activation of specific PPARγ-related pathways may differ depending upon the cells and tumors examined. More than one pathway was involved in the effect of PPARγ ligands in one cell line which was not observed in others. Internal genetic variations and other factors may be responsible for these outcomes, and these

TABLE 1: PPARγ-dependent signals in mediating the effects of PPARγ ligands.

(1) PPARγ ligands inhibit cancer cell growth and induce apoptosis via:
↓ PGE$_2$ receptors (e.g., EP2 and EP4)
↑ Tumor suppressors (e.g., PTEN, p21)
↓ Inflammatory factors (e.g., NF-κB, MCP-1, COX-2)
↓ Angiogenic factors (e.g., VEGF)
↓ Survival factors (e.g., PI3-K/Akt, mTOR)
↑↓ Other kinase signals (e.g., ERK, p38 MAPK)
↓ Growth factor receptors (e.g., EGF-R, PDGF-R)
↓ Extracellular matrices (e.g., fibronectin, MMP-9)
↓ Integrin receptors (e.g., $\alpha5\beta1$)
↑↓ Others (e.g., cytokines (e.g., IL-13, IL-21, TGF-β1) and chemokines (e.g., MIP-1β))

need to be explored further followed by confirmation in in vivo models of cancer.

4. IMPLICATIONS FOR THERAPY

The studies mentioned above suggest that PPARs are involved in lung cancer cell biology. However, their roles remain uncertain and much needs to be learned before they are targeted for therapeutic intervention, especially when considering PPARγ. Nevertheless, activation of PPARγ is strongly associated with decreased lung carcinoma cell proliferation both in vitro and in vivo. Furthermore, in primary NSCLC, the expression of PPARγ has been correlated with tumor histological type and grade, and decreased PPARγ expression was correlated with poor prognosis [60]. Because of this and the fact that synthetic agonists of PPARγ with good safety profiles are currently in use in the clinical arena, PPARγ has emerged as a reasonable target for the development of novel antilung cancer therapies. Synthetic and natural PPARγ activators might be useful as well. For example, arachidonic acid inhibits the growth of A549 cells, and this effect is blocked by the synthetic PPARγ inhibitor GW9662 [61]. MK886, a 5-lipoxygenase activating protein-directed inhibitor, stimulates apoptosis and reduces the growth of A549 cells through activation of PPARγ [62]. These and related drugs can be used alone or in combination with other drugs for synergistic effects. This was observed when using low doses of MK886 in combination with ciglitazone and 13-cis-retinoic acid on A549 and H1299 cells [62]. Also, dramatic synergistic anticancer effects have been reported for lovastatin (an HMG-CoA reductase inhibitor) and the PPARγ ligand troglitazone in several cell lines including lung cancer cells [63]. An enhancement of the antitumor effects of gefitinib by rosiglitazone on A549 cell growth has been recently noted, suggesting that combination strategies using selective nuclear receptor activators in conjunction with epidermal growth factor receptor inhibitors might be effective [64].

A recent study demonstrated that combining the PPARγ ligand rosiglitazone with carboplatin dramatically reduced lung tumor growth in vivo [65]. More tantalizing data were derived from retrospective analysis demonstrating that thiazolidinedione (TZD) use was associated with reduced risk of lung cancer. This study revealed 33% reduction in lung cancer risk among thiazolidinedione users as compared to the nonusers after adjusting other variables [66]. Interestingly, similar risk reduction was not observed for colorectal and prostate cancers [66].

Despite the above, enthusiasm for the use of PPARγ ligands as anticancer agents should be tempered by the fact that PPARγ ligands stimulated PPARγ transactivation in lung adenocarcinoma cell lines, while few to no effects were noted in squamous cell or large cell carcinomas. Also, it is important that we better define PPARγ-independent pathway to avoid unforeseen events and to identify new targets for intervention [64, 67] (Table 2). Furthermore, a novel splice variant of human PPARγ1 which is expressed strongly in tumor tissues of primary human lung SCC has been recently identified. This splice variant exhibits dominant-negative properties in human lung tumor cells, and its overexpression renders transfected cells more resistant to chemotherapeutic drug- and chemical-induced cell death [68]. This suggests that the decreased drug sensitivity of PPARγ1-expressing cells may be associated with increased tumor aggressiveness and poor clinical prognosis in patients. Thus, a better understanding of the mechanisms of action of activated PPARs in tumors (and host cells) is required since the dissection of these pathways might unveil better targets for therapy. Nevertheless, the data available to date regarding PPARγ are promising and justify engaging in clinical studies to determine the true role of PPARγ ligands in lung cancer, while further work should be performed to identify more selective and effective strategies.

5. CONCLUSION

In summary, although its exact role in controlling lung tumor growth and apoptosis remains undefined, PPARγ has been implicated both as a tumor suppressor (in most cases) and as a tumor promoter (in rare cases). Hence, targeting this receptor for therapeutic purposes while minimizing side effects represents a great challenge. Nevertheless, it is clear that selective PPARγ modulation of desired gene sets can be achieved by targeting corepressor interactions, separating transactivation from transrepression, and favoring specific subsets of coactivators. Although the exact mechanisms

Table 2: PPARγ-independent signals triggered by PPARγ ligands.

(2) PPARγ ligands stimulate cancer cell growth and reduce apoptosis via:
↑ Wnt signaling and oncogenes (e.g., cyclin D1, β-catenin, c-myc)
↑ Tumor suppressors (e.g., LKB1, AMPK, TSC2)
↑ ROS production and ERK activation (note that this also occurs in PPARγ-dependent pathways)
↓ Effects on transcription factors (e.g., AP-1, NF-κB, Smads, Sp1, CRE)

mediating this effect remain incompletely elucidated, data available to date regarding this member of the PPAR family are promising and justify engaging in prospective, randomized clinical studies to determine the true role of PPARγ ligands in lung cancer biology.

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Review Article

The Role of PPAR-γ and Its Interaction with COX-2 in Pancreatic Cancer

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In recent years, the study of the peroxisome proliferators activated receptor gamma (PPAR-γ) as a potential target for cancer prevention and therapy has gained a strong interest. However, the overall biological significance of PPAR-γ in cancer development and progression is still controversial. While many reports documented antiproliferative effects in human cancer cell and animal models, several studies demonstrating potential tumor promoting actions of PPAR-γ ligands raised considerable concerns about the role of PPAR-γ in human cancers. Controversy also exists about the role of PPAR-γ in human pancreatic cancers. The current review summarizes the data about PPAR-γ in pancreatic cancer and highlights the biologically relevant interactions between the cyclooxygenase and PPAR system.

1. INTRODUCTION

Despite advances in surgical techniques, imaging modalities, and intensive care, pancreatic cancer is still an almost universally lethal disease with annual mortality figures virtually equaling incidence numbers. An estimated number of 37 170 patients hse been diagnosed with pancreatic cancer in 2007, and 33 370 patients have succumbed to that disease in the same year [1]. Absence of specific symptoms, lack of early detection markers, aggressive tumor growth, and virtual resistance to conventional chemo- and radiotherapy conspire to culminate in a median overall survival of less than nine months. Currently, surgical removal of the tumor offers the only hope of long-term survival with 5-year survival rates approaching 25–30% in large-volume centers in the US [2]. Although an adjuvant treatment regimen after surgical resection seems to prolong survival, the precise treatment protocol including drug-of-choice is still debated and the focus of several ongoing clinical trials [3]. Only a disappointing 10–15% of patients at the time of diagnosis are candidates for surgical resection and even patients who have undergone "curative" resection often die of recurrent tumor. The majority of pancreatic cancer patients unfortunately present with locally advanced or metastatic tumors which render them ineligible for surgical resection. Gem-citabine,

an S-phase nucleoside cytidine analog, has been the standard chemotherapeutic drug for locally advanced and metastatic pancreatic cancer for more than ten years, but the improvement of overall survival is unacceptably small, often approaching only a few weeks [4]. Currently, several trials are underway that investigate gemcitabine-based combination therapiesin patients with advanced pancreatic cancers. Capecitabine, an oral fluoropyrimidine carbamate and 5-fluorouracil prodrug, and erlotinib, an inhibitor of the epidermal growth factor receptor, are two promising agents which seem to improve survival in combination with gemcitabine compared to gemcitabine monotherapy [4]. The encouraging results from a large, double-blind, placebo-controlled, international phase III trial led to the approval of erlotinib for the treatment of locally advanced and metastatic pancreatic cancer in combination with gemcitabine [5]. Although certainly noteworthy, the improvement of overall survival with the combination regimen, however, was only marginal compared to gemcitabine monotherapy [5], strongly emphasizing the need for the identification of novel targets and the development of more efficacious therapeutic agents.

Although several environmental risk factors for the development of pancreatic cancers, including tobacco smoking and dietary factors, have been described, detailed insights

into the pathogenetic mechanisms are virtually lacking [6]. Dietary intake of high-caloric, high-fat diets with ensuing obesity and metabolic syndrome has been correlated with an increased risk of pancreatic cancer [7, 8]. An important molecule in fatty acid sensing and metabolism is the peroxisome proliferator activated receptor gamma (PPAR-γ), a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor [9]. There is now a large body of evidence demonstrating an important role of PPAR-γ in the metabolic syndrome [10–13]. The thiazolidinedione (TZD) class of PPAR-γ ligands has been used for the treatment of hyperglycemia and insulin resistance in type 2 diabetes for the past ten years [14]. In addition, TZDs may also show beneficial effects on cardiovascular complications associated with type 2 diabetes and the metabolic syndrome [14–17]. More recently, the role of PPAR-γ in various human cancers has been studied. There is now strong evidence that PPAR-γ is overexpressed in a variety of cancers, including colon, breast, prostate, stomach, lung, and pancreas [18–20]. However, the biological significance of PPAR-γ is still controversial [21, 22]. Although several reviews highlight the antiproliferative actions of PPAR-γ ligands in cell culture and animal models of human cancers [23, 24], more recent studies illustrating a tumor-promoting effect of PPAR-γ, in particular in colon and breast cancer models, raise considerable concern about the significance and safety of PPAR-γ ligands as anticancer drugs [25–29]. This review will summarize and discuss the data concerning the role of PPAR-γ in pancreatic cancer.

2. PPAR-GAMMA IN PANCREATIC CANCER

Reports from several groups have shown that the thiazolidinedione (TZD) class of PPAR-γ ligands attenuates the growth of pancreatic cancer cells in vitro by induction of terminal differentiation and G1 phase cell cycle arrest [30, 31], and by an increase in apoptotic cell death [32]. Furthermore, thiazolidinediones attenuated pancreatic cancer cell migration and invasion by modulation of actin organization and expression of matrix metalloproteinase-2 and plasminogen activator inhibitor-1, respectively [33, 34]. However, many growth-inhibitory effects of PPAR-γ ligands are independent of PPAR-γ [35]. To date, several non-PPAR-γ targets have been implicated in the antitumor activities of certain TZDs, for example, troglitazone and ciglitazone, including intracellular Ca^{2+} stores, mitogen-activated protein kinases, cyclin-dependent kinase inhibitors p27kip1 and p21WAF/CIP1, the tumor suppressor protein p53, and Bcl-2 family members [36]. There is increasing evidence that TZDs directly affect mitochondrial function which impairs oxidative respiration leading to increased reactive oxygen species (ROS) production and ATP depletion, which in turn can activate AMP kinase [37]. An increase in ROS and activation of AMP kinase can lead to PPAR-γ independent reduction in inflammation and cell growth [37]. In addition, it has been shown in pancreatic cancer cells that 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazolide (CDDO-Im), a partial PPAR-γ agonist, induces apoptosis directly by targeting mitochondrial glutathione

[38]. Furthermore, 3,3'-diindolylmethane (DIM), another PPAR-γ agonist, induced apoptotic cell death in pancreatic cancer cells through activation of the endoplasmic stress response [39]. Overall, the potential to elicit PPAR-γ-independent effects may be ligand- and cell context-dependent. Our own studies have demonstrated that PPAR-γ is expressed in the nucleus of six human pancreatic cancer cells and that treatment of these cells in vitro with 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-PGJ2) and ciglitazone dose- and time-dependently decreases cell growth by induction of caspase-3-dependent apoptosis [40]. In addition to their antiproliferative actions, both ligands, 15-PGJ2 and ciglitazone, reduced the invasive capacity of pancreatic cancer cells in vitro by a PPAR-γ-mediated decrease of urokinase-type plasminogen activator and elevation of plasminogen activator inhibitor-1 expression that resulted in an overall reduction in urokinase activity [41]. Taken together, there is a strong evidence today from cell culture models that PPAR-γ ligands potently reduce the growth of human pancreatic cancer cells. The discrepancy of the reported underlying mechanisms, however, may be caused by the use of different cell lines, culture conditions, and experimental settings. In contrast to the notion of PPAR-γ ligands being potent antitumor drugs in pancreatic cancers, we have reported that treatment of human pancreatic cancer cells in vitro with 15-PGJ2 and troglitazone dose-dependently increases the secretion of the vascular endothelial growth factor (VEGF), which is widely recognized as a potent stimulus for tumor angiogenesis [42]. In addition, the culture medium of troglitazone-treated human pancreatic cancer cells enhanced migration of endothelial cells, another step in the angiogenic cascade (own finding). These findings are already observed at submicromolar concentrations of the PPAR-γ ligands, which are usually considerably lower than the typical ligand concentrations needed for the antiproliferative effects in pancreatic cancer cells. Our in vitro data suggest that PPAR-γ ligands may have a tumor-promoting effect in vivo by enhancing tumor angiogenesis. Although the precise role of PPAR-γ in tumor angiogenesis is still debated and controversial, there is accumulating evidence that activation of PPAR-γ stimulates VEGF production and neoangiogenesis also in other cell models [43, 44].

In addition to the effects of PPAR-γ ligands on the growth of established pancreatic cancers in preclinical cell culture and xenograft mouse models, dietary intake of 800 ppm pioglitazone for 22 weeks correlated with an improved serum lipid profile and a decreased incidence and multiplicity of pancreatic tumors in the N-nitrosobis(2-oxopropyl)amine (BOP) model of pancreatic carcinogenesis in Syrian golden hamsters, suggesting a potential chemopreventive role of TZDs [45].

There are very few data concerning the significance of PPAR-γ in clinical pancreatic cancer specimens. In a recent study, PPAR-γ was expressed in the majority of human pancreatic cancer specimens, positively correlated with higher tumor stage and grade, and interestingly was associated with shorter patient survival, suggesting a potential role in pancreatic cancer progression [20].
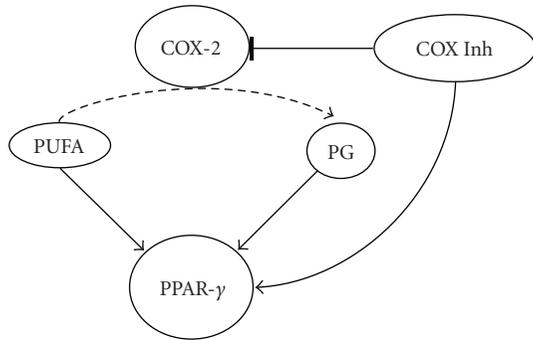
FIGURE 1: Possible interactions between the COX-2 and PPAR-γ pathways: polyunsaturated fatty acids (PUFAs) are substrates for cyclooxygenase-2 (COX-2) enzymes leading to the formation of various prostaglandins (PGs). Certain PUFAs and PGs can also activate PPAR-γ. Selective and nonselective COX-2 inhibitors (COX Inh) block PG formation by COX-2 but can also at higher concentrations activate PPAR-γ. Solid arrows indicate activation; dashed arrow indicates metabolic pathway; blocked arrow indicates inhibition.

3. INTERACTION BETWEEN THE PPAR-GAMMA AND COX-2 PATHWAYS

Besides the TZD class of antidiabetic drugs, various intracellular lipids and lipid mediators are capable of activating PPAR-γ. Among those, polyunsaturated fatty acids (e.g., arachidonic acid (AA) and eicosapentaenoic acid (EPA)) and eicosanoids (e.g., 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15-PGJ$_2$)) are also substrates and products, respectively, of intracellular cyclooxygenase (COX) enzymes strongly suggesting relevant interactions between the PPAR and COX pathways (see Figure 1).

3.1. COX products as PPAR-γ activators

COX activity leads to the formation of an unstable hydroxy-endoperoxide, prostaglandin H$_2$, which can be further converted to various prostanoid species by tissue specific isomerases [46]. While parent prostaglandins (e.g., PGE$_2$, PGF$_{2\alpha}$, and PGD$_2$) transduce their signals through binding to G-protein coupled cell surface receptors [47], cyclopentenone prostanoids (e.g., PGJ$_2$) are known ligands of PPAR-γ [48]. In fact, there is evidence suggesting that COX-2 is preferentially located on the nuclear membrane allowing cyclopentenone prostaglandins to directly enter the nucleus and bind to ligand-activated transcription factors [49]. In this regard, human pancreatic cancer cells seem to express COX-2 preferentially in a perinuclear localization [50]. 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15-PGJ$_2$), a nonenzymatically formed dehydration product of PGD$_2$, is detectable in COX-2 expressing human pancreatic cancer cells (own observation) and able to activate PPAR-γ in these cells [42]. Furthermore, a selective COX-2 inhibitor at a concentration that inhibits COX-2 activity and consequently prostanoid production reduces PPAR-γ activity, presumably by decreasing the levels of cyclopentenone prostaglandins (own observation).

3.2. COX substrates as PPAR-γ activators

Certain polyunsaturated fatty acids (PUFAs) (e.g., arachidonic acid (AA; 20 : 4 $n-6$) and eicosapentaenoic acid (EPA; 20 : 5 $n-3$)) are substrates for COX enzymes and also known PPAR-γ ligands [51]. Both PUFAs are released from the *sn*-2 position of major membrane phospholipids by phospholipase A$_2$ (PLA$_2$) enzymes, particularly by the cytoplasmic PLA$_2$, which upon activation seems to preferentially locate to the nuclear membrane [52, 53]. Once released, the PUFAs can be metabolized by COX enzymes or enter the nucleus to activate PPAR-γ. Our own studies demonstrated that EPA decreased the growth of human pancreatic cancer cells through COX-2 dependent and independent mechanisms (manuscript in press). The COX-2 independent mechanism involved activation of PPAR-γ by EPA as the growth-inhibitory effect of EPA was abolished by a pharmacological PPAR-γ antagonist. Furthermore, EPA and to a lesser extent AA can activate PPAR-γ transcriptional activity in human pancreatic cancer cells (own observation). This effect is less pronounced in pancreatic cancer cells that express COX-2 presumably because EPA is rapidly metabolized by COX-2 in these cells. The overall efficacy of PUFAs to activate PPAR-γ may therefore be dependent on the cellular expression and activity of COX-2.

3.3. COX inhibitors as PPAR-γ activators

In addition to COX-2 substrates and products, certain nonselective and selective COX-2 inhibitors have also been shown to activate PPAR-γ independent of their ability to inhibit COX-2 enzymatic activity [54], although the precise molecular mechanisms are still unknown. There is a compelling evidence today that the inducible COX-2 isoform plays an important role in pancreatic cancer development and growth and that selective COX-2 inhibitors may be efficacious for pancreatic cancer prevention and therapy [50]. Our own studies demonstrated that dietary intake of a selective COX-2 inhibitor delayed the progression of recognized pancreatic cancer precursor lesions in a genetically engineered mouse model of pancreatic cancer development [55]. Furthermore, a selective COX-2 inhibitor decreased the growth of COX-2 positive human pancreatic cancers in a xenograft mouse model by induction of apoptosis in cancer cells and by inhibition of tumor angiogenesis [42]. In contrast, the selective COX-2 inhibitor enhanced the growth of xenografted human pancreatic cancers that lacked or had very little COX-2 protein expression. This tumor-promoting effect was associated with an increase in intratumoral VEGF levels and tumor angiogenesis [42]. Additional studies showed that the tumor-enhancing effect of the selective COX-2 inhibitor in COX-2 negative or weakly COX-2 expressing human pancreatic cancers was abolished by GW9662, an irreversible pharmacological PPAR-γ antagonist, suggesting biologically important interactions between the COX-2 inhibitor and PPAR-γ [42]. Further studies demonstrating enhanced PPAR-γ binding activity in tumors that were treated with a selective COX-2 inhibitor confirmed that interaction [42]. The findings obtained in vivo were

corroborated by in vitro experiments. Human pancreatic cancer cells treated with relatively high concentrations of selective COX-2 inhibitors showed an increased production and secretion of VEGF, which was inhibited by a pharmacological PPAR-γ antagonist and a dominant-negative PPAR-γ receptor [42]. Additionally, the selective COX-2 inhibitor at that concentration stimulated PPAR-γ transcriptional and DNA-binding activities [42]. These data clearly indicated that a biologically significant interaction between selective COX-2 inhibitors and PPAR-γ exists and that activation of PPAR-γ by these drugs may have detrimental, that is tumor-promoting, effects on pancreatic cancer growth. It is important to note that the tumor-promoting effects of selective COX-2 inhibitors were only observed at relatively high concentrations (much higher than needed to inhibit COX-2 enzymatic activity) in tumors that had no or only very little COX-2 expression [42]. Although the selective COX-2 inhibitor stimulated VEGF production by pancreatic cancer cells through a PPAR-γ mediated mechanism also in COX-2 expressing pancreatic cancers, the potential proangiogenic and tumor-promoting effect in COX-2 positive cancers was masked by a significant reduction of COX-2 generated proangiogenic and protumorigenic prostanoids [42].

4. CONCLUSION

While several in vitro studies demonstrate that PPAR-γ activation decreases pancreatic cancer cell growth, the finding that PPAR-γ ligands can stimulate VEGF production by pancreatic cancer cells raises serious concerns that PPAR-γ activation in vivo may lead to enhanced angiogenesis and tumor growth. Further detailed studies using pancreatic cancer animal models and specific PPAR-γ ligands are necessary to evaluate possible proangiogenic and protumorigenic properties of PPAR-γ activation in vivo. Unfortunately, information about the role of PPAR-γ in pancreatic carcinogenesis is almost nonexistent. The use of the recently developed genetically engineered mouse models of pancreatic cancer development that closely recapitulate our current knowledge of pancreatic cancer development on a histological and genetic level should shed some needed insights into the role of PPAR-γ in pancreatic carcinogenesis.

There is now clear evidence of a biologically relevant interaction between the COX and PPAR-γ pathways. Our data suggest that activation of PPAR-γ by selective and nonselective COX-2 inhibitors may have tumor-promoting effects in vivo by enhancing tumor angiogenesis. The effect of COX-2 inhibitors on PPAR-γ activation seems to be observed only at relatively high concentrations of the inhibitors and the overall biological phenotype of that interaction is dependent on the cellular expression and activity of the COX-2 protein. Although the role of PPAR-γ in pancreatic cancer development and growth has begun to be elucidated in recent years, a precise knowledge of molecular targets downstream of PPAR-γ, a more comprehensive elucidation of PPAR-γ-independent actions of PPAR-γ ligands, and a detailed understanding of crosstalks between PPAR-γ and other intracellular signaling pathways seem to be absolutely necessary and needed to eventually clarify the role of PPAR-γ in human cancer development and progression.

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Review Article

Mitochondria, PPARs, and Cancer: Is Receptor-Independent Action of PPAR Agonists a Key?

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Before the discovery of peroxisome proliferator activated receptors (PPARs), it was well known that certain drugs considered as classical PPAR-alpha agonists induced hepatocarcinoma or peroxisome proliferation in rodents. These drugs were derivatives of fibric acid, and they included clofibrate, bezafibrate, and fenofibrate. However, such toxicity has never been observed in human patients treated with these hypolipidemic drugs. Thiazolidinediones are a new class of PPAR activators showing greater specificity for the γ isoform of PPARs. These drugs are used as insulin sensitizers in the treatment of type II diabetes. In addition, they have been shown to induce cell differentiation or apoptosis in various experimental models of cancer. PPAR-α ligands have also been shown to induce cancer cell differentiation and, paradoxically, PPAR-γ drug activators have been reported to act as carcinogens. The confusing picture that emerges from these data is further complicated by the series of intriguing side effects observed following administration of pharmacological PPAR ligands (rhabdomyolysis, liver and heart toxicity, anemia, leucopenia). These side effects cannot be easily explained by simple interactions between the drug and nuclear receptors. Rather, these side effects seem to indicate that the ligands have biological activity independent of the nuclear receptors. Considering the emerging role of mitochondria in cancer and the potential metabolic connections between this organelle and PPAR physiology, characterization of the reciprocal influences is fundamental not only for a better understanding of cancer biology, but also for more defined pharmacotoxicological profiles of drugs that modulate PPARs.

1. INTRODUCTION

Since the discovery of the first PPAR by Issemann and Green in 1990 [1], the role of this fascinating class of nuclear receptors in normal physiology and pathophysiology has become progressively more important. The potential biological activities attributed to PPARs have been expanding ever since they were identified as potential mediators of the hypolipidemic effect of fibrates in humans and as participants in peroxisome proliferation and hepatocarcinogenicity in rodents. So far, PPARs have been implicated in diverse processes including lipid and carbohydrate metabolism, energy expenditure, immune and inflammatory processes, vascular homeostasis, tissue remodeling, and cell differentiation and proliferation in normal and neoplastic tissues [2–8].

It is evident, but too often ignored, that important interrelationships must exist among PPARs, mitochondria, and cancer. Regardless of the precise nature of these interconnections, PPARs undoubtedly have a significant energetic, plastic, and signaling role in the pathophysiology of cancer cells purely by virtue of their central role in lipid metabolism. This role necessarily involves interaction with mitochondria. Mitochondria are not only the main site of lipid oxidative metabolism, but they are also the cellular powerhouses that coordinate cellular metabolism and serve as the origin of important anabolic fluxes and signal transduction pathways [9, 10].

Thus, the role of mitochondria in cancer is under a critical re-evaluation, particularly in light of the so-called Warburg effect: most cancer cells exhibit increased aerobic glycolysis, and use this metabolic pathway for generation of ATP as a main source of their energy supply. Too often this does not justify the complex metabolic alterations present in different types of neoplasia [11–13].

Similarly, the interrelationships between PPARs and cancer are not entirely clear. Some studies show that PPARs

have antineoplastic and/or cancer differentiating activities, while others show that they have important carcinogenic properties [6, 14–20].

These reports highlight the multifaceted role of PPARs in neoplastic cells. Hence, the roles of PPAR in normal physiology and pathophysiology should be clarified, since this may benefit our understanding of how cancer occurs and how it can be treated.

Discussion of the interrelationships among PPARs, mitochondria, and cancer should first involve careful evaluation of some misleading factors that have contributed to confusion about PPAR biology.

2. PPARs IN PATHOPHYSIOLOGY AND MISLEADING FACTORS

2.1. Synthetic ligands

Initially, the physiological ligands of PPARs were unknown and PPARs were classified as "orphan receptors." Their function was studied using synthetic ligands of PPAR-α, the first PPAR discovered. These synthetic ligands were a heterogeneous class of molecules ranging from trichloroacetic acid to plasticizers such as di-2-ethylhexyl phthalate (DEHP) and mono-2-ethylhexyl phthalate (MEHP) [1]. The structural heterogeneity of the ligands seems to reflect the conformation of the PPAR ligand binding domain (LBD), which forms a large, Y-shaped hydrophobic pocket with relatively low ligand specificity [2, 7]. For PPAR-α, the ligands used most often in experiments were fibrates such as clofibric acid, bezafibrate, and gemfibrozil. Studies on fibrate binding to PPAR-α showed that these drugs caused a hypotriglyceridemic effect by inducing transcription of several genes related to oxidative metabolism of lipids, which occurs primarily in the mitochondria [2–4]. However, some discrepancies soon emerged. In fact, previous PPAR research failed to provide thorough explanations of the drugs' other important biological activities, such as peroxisome proliferation and hepatocarcinogenicity in rodents or, most importantly, their side effects. These side effects include angina crisis; elevation of serum aminotransferase, which indicates liver damage; increases in serum creatine phosphokinase concentrations, which can initiate myositis, myopathy and, in rare cases, rhabdomyolysis; increases in serum creatinine concentration; and acute renal failure in rare cases [21].

Some of these biological activities had already led investigators to propose that these ligands had effects independent of their binding to PPAR. If true, neglecting these "extrareceptor" functions may interfere with our understanding of PPAR pathophysiology. For example, studies in the 1980s established that fibrates (clofibric acid, bezafibrate, and gemfibrozil) can pass freely through red blood cell membranes and bind to human hemoglobin at the level of the hydrophobic pocket of the alpha chain interfaces. This binding lowers the oxygen affinity of hemoprotein more strongly than does the natural allosteric effector 2,3-biphosphoglycerate [22], which could, for example, lead to an angina crisis (by perturbation of microcirculation in ischemic areas caused by abrupt changes in the level of

oxygen released from the blood) at the usual therapeutic plasmatic drug concentrations [23]. Around the same time, other researchers showed that the agents acting as peroxisome proliferators hampered mitochondrial respiration, with potentially significant clinical implications [24–26]. Indeed, treatment with fibrates was found to lead to some histological and biochemical features characteristic of hepatic, muscular, and renal toxicities. This result led to the hypothesis that disruption of the mitochondrial electron respiratory chain in conjunction with other genetic or acquired predisposing factors may contribute to these toxic effects independently of PPAR activation. Moreover, molecular analysis of the interactions between human hemoglobin and fibrates indicated that particular physicochemical aspects of the drug molecules, specifically their carboxylic group and their significant hydrophobicity, might be responsible for their biological activity. Interestingly, these physicochemical characteristics of fibrates fit well with the milieu of mitochondria in general, namely the difference in pH between the more alkaline matrix and the more acidic intermembrane space, as well as with structural features of complex I (NADH cytochrome c reductase) in the mitochondrial electron respiratory chain, which is a large, hydrophobic protein component in the mitochondria [10, 27].

All of these considerations led to studies that examined how mitochondria were affected by fibrate administration, and whether these effects might have clinical implications [18, 28]. The results clearly showed that fibrates could disrupt the mitochondrial electron respiratory chain at the level of NADH cytochrome c reductase [18]. This effect was even more pronounced for ciglitazone, which was one of the first thiazolidinediones to be synthesized. Thiazolidinediones are a class of molecules that are chemically related to fibrates (Figure 1). Drug-induced mitochondrial dysfunction causes a series of compensatory metabolic mechanisms, which, in addition to PPAR agonist activity, may be partially responsible for some of the pharmacological and toxicological properties of this class of molecules. In fact, the resulting shut-down of mitochondrial NADH oxidation drives cells to change their oxidative metabolism in a way that is strictly correlated to the degree of complex I inhibition. Specifically, upon treatment with fibrates, which are less potent inhibitors of complex I, cells tend to use those components of the electron respiratory chain that remain efficient (e.g., complex II). This leads the cell to use $FADH_2$ oxidation to obtain energy. In other words, compensatory mechanisms come into play, which are probably sustained by glycerol catabolism viamitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase or by fatty acid β-oxidation viaelectron-transferring flavoprotein (ETF). These changes have a significant hypotriglyceridemic effect and a slight hypoglycemic effect. In contrast, using more potent complex I inhibitors (i.e., thiazodilinediones) greatly reduces NADH dehydrogenase activity, thus reducing the use of β-oxidation and increasing reliance on glycolysis, resulting in a stronger hypoglycemic effect and a much weaker or null hypotriglyceridemic effect (Figure 2) [18].

Based on these findings, PPAR-α activation may be due, at least in part, to a shift in the metabolic state: preferential
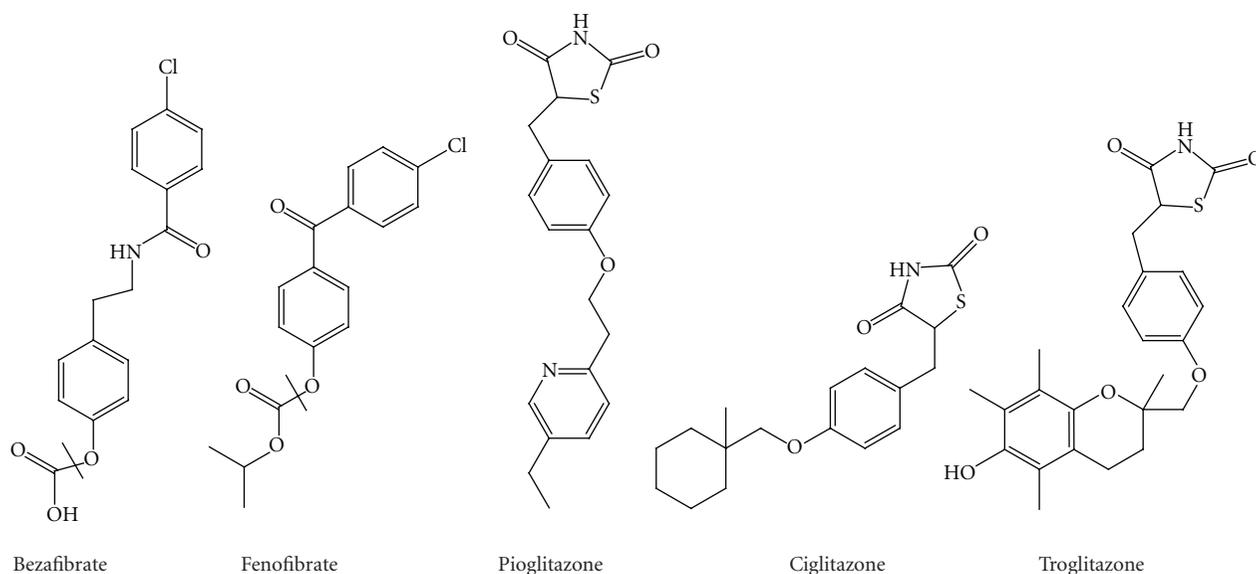
Figure 1: Structure of some drugs acting as PPAR ligands.
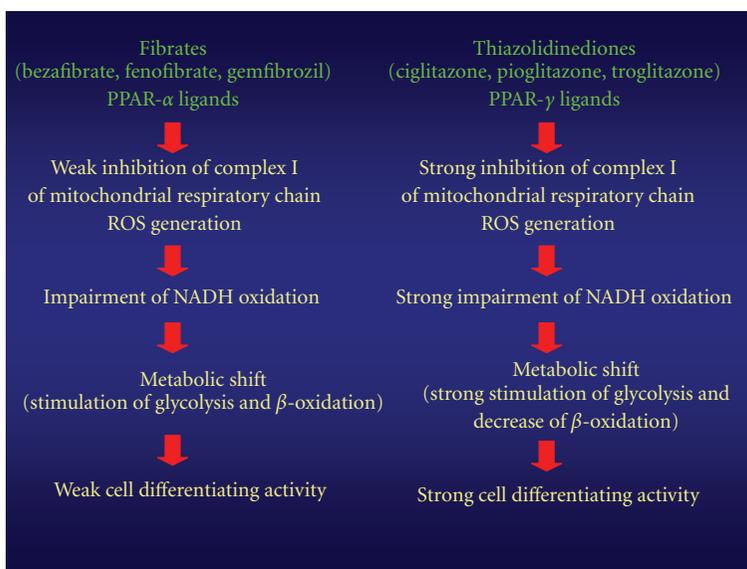
Figure 2: Flow chart of possible molecular mechanisms caused by a mitochondrial dysfunction induced by fibrates (PPAR-α ligands) and thiazolidinediones (PPAR-γ ligands).

use of lipids through glycerol catabolism via mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase and fatty acid β-oxidation via ETF. In this way, as suggested by Kersten et al. [3], fatty acids would stimulate their own metabolism. Interestingly, this mechanism of switching to lipid metabolism in response to fibrate-induced complex I inhibition may explain the reported activation of genes in the cytochrome P450 IV family. This family of proteins is responsible for microsomal ω-oxidation of long chain and very long chain fatty acids [29]. This pathogenic mechanism may also provide a better explanation of the peroxisomal proliferation observed in rodents given synthetic PPAR-α ligands. In fact, the lipid component of these animals' diet

contains a particularly high proportion of polyunsaturated fatty acids [30]. Hence peroxisomal β-oxidation, which is normally more active in rodents than in humans or other primates, may be further enhanced by inhibition of mitochondrial NADH oxidation.

Moreover, the increase in free radical oxygen species resulting from stimulated peroxisomal β-oxidation may further increase the oxidative stress that results from complex I inhibition [31, 32] and thereby contributes significantly to the observed carcinogenic properties of PPAR ligands in rodents, particularly in the liver.

From a clinical point of view, thiazolidinediones, which are stronger inhibitors of NADH cytochrome c reductase

[18, 33–36] than fibrates, strongly disrupt NADH oxidation such that they may prevent the induction of β-oxidation, which could otherwise serve as a compensatory energy source. This renders metabolism almost exclusively dependent on glycolysis. Interestingly, damage to the pathways of energy production, particularly in organs that are rich in mitochondria (e.g., liver, muscle, heart, and kidney), may explain (i) the prevalent hypoglycemic activity of γ-ligands, despite minor or absent hypolipidemic effects, (ii) the weight gain due mainly to water retention typically observed in patients treated with PPAR-γ ligands, (iii) the differentiation of adipose cells, and (iv) the dramatic cardiac and hepatic toxicities often observed following administration of thiazolidinediones [37–40].

Interestingly, similar mitochondrial impairment by PPAR agonists was observed by Nadanaciva et al. [41], who used a phosphorescent oxygen-sensitive probe and an immunocapture technique to evaluate the mitochondrial respiration and the activity of individual oxidative phosphorylation complexes on isolated rat liver mitochondria. By this dual approach, the authors were also able to obtain a rank order of the mitochondrial toxicity of thiazolidinediones, fibrates, and statins. These results could be important as they suggest the possibility of a screening strategy to evaluate potential mitochondrial toxicity, reducing in such a way the incidence of clinical side effects.

Moreover, a novel mitochondrial target protein has recently been identified for the thiazolidinediones [42]. This protein, called mitoNEET, is an iron-containing outer mitochondrial membrane protein that seems to play a role in regulating mitochondrial oxidative processes. Recently, Wiley et al. [43, 44] showed that cardiac mitochondria isolated from mitoNEET-null mice demonstrated a reduced oxidative capacity, confirming that mitoNEET is a protein involved in the control of maximal mitochondrial respiratory rates.

These results underline the importance of carefully defining the direct interrelationships between pharmacological PPAR ligands and mitochondria, also to better clarify the physiology and pathophysiology of PPARs.

In conclusion, many PPAR ligands possess extrareceptor biological activities that can complicate interpretation of the results of experiments investigating the pathophysiology of PPARs. This caveat has serious consequences, not only for our ability to correctly understand the metabolic roles of PPARs, but also for our ability to determine their roles in the (de)differentiation of cancer cells.

2.2. Metabolic and genetic studies of PPARs in rodents

Most of the available data on the pathophysiology of PPARs has been obtained from metabolic studies in rodents. However, species-specific differences in metabolism and diet can be an obstacle when applying the results of animal studies to human patients [35, 45].

Another source of data that may be misleading is genetic studies on knock-out rodents for PPARs or their transcriptional coactivators (i.e., PGC-1α). Conclusions drawn from these studies about the metabolic roles of the different PPARs

neglect the interaction between PPARs and their coactivators in mitochondrial biogenesis in general, and in mitochondrial lipid metabolism in particular [5, 46–49].

3. PPAR AND CANCER: WHAT IS THE ROLE FOR MITOCHONDRIA?

Various molecular links between PPARs and cancer have been considered in other articles. Here, we highlight some intriguing observations related to PPARs and cancer that seem to indicate a role for mitochondria in this disease of cell proliferation and differentiation. Since the aim of this review is to discuss the potential molecular link between PPARs and cancer from a mitochondrial point of view, we focus on the particular "extrareceptor" interrelationships between PPARs and fibrates or their thiazolidinedione derivatives.

3.1. PPARs, mitochondria, and carcinogenesis

PPARs and their pharmacological ligands were originally considered to be "oncopromoters." Specifically, PPAR ligands were considered to be nongenotoxic carcinogens. It is well known that these molecules can induce hepatocarcinoma in rodents; however, their administration first provokes hepatomegalia and induces expression of a series of antioxidant enzymes, such as catalase, superoxide dismutase, and glutathione peroxidase. In this way, these molecules may create an imbalance in oxidative metabolism in general and oxidative stress in particular [50, 51]. The ability of PPAR ligands to induce oxidative stress has since been confirmed for thiazolidinediones as well [52–54]. Interestingly, reactive oxygen species (ROS) and cellular oxidative stress have long been implicated in carcinogenesis, despite the fact that the precise pathogenic molecular mechanisms are complex, debated, and at times paradoxical [55]. More specifically, the following hold.

(i) In normal cells, mutations in nuclear or mitochondrial genes encoding components of the mitochondrial electron transport chain (ETC) or xenobiotics capable of disrupting the mitochondrial electron flux can lead to an increase in the generation of ROS, particularly superoxide. This radical is rapidly dismuted by superoxide dismutase to yield hydrogen peroxide (H_2O_2), which can diffuse to the nucleus and attack DNA before cellular antioxidant defenses adjust to the new level of oxidative stress. This oxidative damage may contribute to genetic instability in congenital and/or acquired predisposed subjects [56, 57].

(ii) Cancer cells generally generate more ROS than normal cells. This difference may relate to the greater number of metabolic and proliferative activities that often occur in a transformed cell, or to a qualitative or quantitative imbalance between cellular antioxidant defenses and the oxidative environment [55, 58–60].

(iii) In cancer cells, the levels of expression of some components of the antioxidant system are amplified

independently by drug treatment (e.g., thioredoxin, DJ-1 protein, peptidyl-prolyl cis-trans isomerase A, cyclophilin A, protein disulfide isomerase A3, ERP 60/GRP58) [55, 61]. Interestingly, the increase in thioredoxin activity in cells with elevated oxidative stress may relate to its essential role in facilitating transcription in an environment where increased oxidative stress signaling in the cytosol is required for stimulating cell proliferation. Furthermore, drug-induced cancer cell differentiation typically reduces the expression of these antioxidant proteins [62].

(iv) Numerous studies implicate increased oxidative stress in the cell death induced by diverse chemotherapeutic agents. Anthracycline derivatives, newer redox cycling agents, and, more recently, histone deacetylase inhibitors and proteasome inhibitors all appear to increase oxidative stress in cells. Although the mechanism responsible for the increase has not been established, mitochondria are fundamental in ROS generation and seem to be involved, either directly or indirectly [55, 63–65].

This puzzling picture suggests that PPAR-related rodent hepatocarcinogenesis depends on strong stimulation of ROS generation, mainly in mitochondria dysregulated by the PPAR ligand in question. Moreover, this oxidative stress may be reinforced by the specific membrane composition and the abundant H_2O_2 production from peroxisomal lipid metabolism in rodents, as already discussed.

Could a similar pathogenic mechanism have a role in human carcinogenesis?

A direct role for PPARs in carcinogenesis is hardly credible, considering their fundamental physiological role in cell metabolism. The altered expression of different PPAR isoforms observed in some neoplasias may be the result of secondary metabolic changes in transformed cells relative to normal cells [6, 66].

On the other hand, the question of whether the synthetic PPAR ligands play a role in human carcinogenesis is still open and intriguing. In fact, one of the first large clinical studies on gemfibrozil, a classic PPAR-α ligand, [67] showed a small but significant increase ($P = .032$ by the Fisher exact test) in the incidence of basal cell carcinoma in patients taking gemfibrozil; this finding, unfortunately has been largely ignored by investigators. Moreover, in an intermediate follow-up study [68], cancer occurred at equal rates in both the untreated group and the group treated with gemfibrozil, but the cancer in the latter led more often to mortality, primarily during the last 1.5 years of follow-up. To be sure, results recently obtained from an 18-year mortality follow-up of this study do not seem to confirm this increase in cancer mortality, but the follow-up design failed to address certain possible interpretations and also in the Autors opinion of this cited study some of the follow-up data can be misleading [69].

Nevertheless, the data obtained in the original study are intriguing considering the peculiar molecular epidemiology and pathogenesis of basal cell carcinoma and the relatively short (5-year) period of drug exposure used in the study. These findings, together with the demonstrated ability

of fibrates and thiazolidinediones to alter mitochondrial oxidative metabolism and induce ROS generation, indicate that care should be taken when this class of drugs is used in the treatment of nutrition-sensitive tumors [70, 71].

3.2. PPARs, mitochondria, and inhibition of tumor growth

It is well established that activation of PPARs (α, β/δ, and γ) by natural or synthetic agonists can inhibit growth and induce differentiation or death of tumor cells. The original observation was of PPAR-γ ligands and liposarcoma, consistent with the physiological function of PPAR-γ [3, 72, 73]. Subsequently, PPAR-γ and PPAR-α ligands were shown to promote the differentiation of various tumor cell lines, including breast, lung, prostate, leukemia, colon, melanoma, and liver. This differentiation was often independent of the relative expression levels of the different PPAR isoforms [6, 15, 16, 66, 70, 72]. These studies also suggested extrareceptor activities of fibrates and thiazolidinediones as the basis of their ability to induce cancer cell differentiation [15–18, 72]. Moreover, a recent study by Panigrahy et al. [74, 75] on endothelial and mesenchymal tumor cells and mice showed that PPAR-α ligands such as fenofibrate directly suppress tumor growth through receptor-dependent and -independent pathways, and that they indirectly suppress tumor growth by inhibiting angiogenesis and the inflammatory response in the microenvironment of the tumor. Therefore, the noncancerous host tissue could be an important target for cancer treatment with pharmacological PPAR ligands.

These data illustrate the extreme complexity of the interrelationships among PPARs, mitochondria, and cancer. Nevertheless, the most important aspects of these interrelationships are the activities of the synthetic PPAR ligands, particularly their extrareceptor activities. Mitochondria are becoming increasingly important as targets for these drug-induced extrareceptor activities, as discussed in recent reviews [35, 76, 77]. To better understand the interactions among PPAR ligands, mitochondria, and cancer, it may be useful to describe our work, which parallels that of other groups. Curiously, the differentiating activity of fibrates was originally hypothesized in binding studies of fibrates and hemoglobin. The physicochemical properties of fibrates and their toxicological profile allow them to interact with some hydrophobic components of the mitochondrial electron respiratory chain. The resulting oxidative metabolic stress may induce differentiation of cancer cells, similar to the effects of heat shock [15]. Importantly, this effect does not depend on PPAR agonism, but it is related to the physicochemical properties (pKa, log P, log D, water solubility, and pH profile) of the molecules. These properties should favor permeation, accumulation, and interaction with components of the internal mitochondrial membrane [10].

For example, therapeutic doses of bezafibrate inhibited proliferation of human leukemia cell lines HL-60, U-937, and K-562 in a dose-dependent manner. In HL-60 cells, growth inhibition was associated with an increased number of cells in the G0/G1 phase and a significant decrease

in the number of cells in the G2/M phase. Analysis of cell differentiation markers (CD) showed a dose-dependent increase in expression of CD11b and CD14 in HL-60 cells and of CD14 in U-937 cells. Functional assays confirmed that the phenotypes of these cells were more mature. Both HL-60 and U-937 cells showed a dose-dependent restoration of the respiratory burst stimulated by PMA and zymosan. K-562 erythroleukemia cells showed a dose-dependent increase in hemoglobin synthesis. Similar cellular differentiation was observed following treatment with two other fibrate derivatives, clofibric acid and gemfibrozil. Interestingly, fibrate-induced differentiation was partially inhibited by antioxidants including acetylcysteine (NAC), and electron microscopy revealed that fibrate-treated cells had mitochondrial damage [15]. Functional evaluation of this drug-induced mitochondriopathy showed that fibrates and ciglitazone specifically inhibited NADH cytochrome c reductase activity in a dose-dependent manner in HL-60, TE-671 human rabdomyosarcoma, and Hep-G2 human hepatocarcinoma cell lines, whereas the activity of other mitochondrial respiratory chain enzymes remained unchanged [18, 33, 36]. The impairment of NADH oxidation induced a cellular metabolic shift towards anaerobic glycolysis and/or β-oxidation, as shown by the dose-dependent increases of certain metabolites (lactate, alanine, glycolytic, and nonglycolytic derived acetate) [18].

A fundamental observation from this research was the correlation of mitochondrial dysfunction, metabolic shift, and differentiation activity in tumor cells treated with increasing concentrations of PPAR ligands. Furthermore, quantitative comparison on a molar ratio basis between these PPAR ligands (bezafibrate, clofibric acid, gemfibrozil, and ciglitazone) for inhibition of NADH cytochrome c reductase activity, metabolic adaptations, differentiation potency, and antiproliferative index confirmed a strict correlation between these parameters [18]. These results suggested that inhibition of mitochondrial NADH dehydrogenase could contribute to both the pharmacological and toxicological profiles of fibrate derivatives (strong hypolipidemic/weak hypoglycemic effect, liver and muscle toxicity) and thiazolidinediones (hypoglycemic/insulin sensitizer effect, liver and heart toxicity) [17, 18, 25, 26, 33–40].

In terms of mitochondrial oncology, these data suggest a possible molecular mechanism for the peroxisome proliferator activity and carcinogenicity of fibrates typically observed in rodents. These data also indicate a strict correlation among fibrate- and thiazolidinedione-induced cellular respiration dysfunction, stimulation of glycolysis, and cancer cell differentiation that strongly implicates mitochondria and oxidative metabolism in the pathophysiology of cancer.

Importantly, these results confirm and extend the results of other studies focusing on nongenomic activities of fibrates and thiazolidinediones [16, 17, 78–81]. Furthermore, these observations explain some contradictory data related to the role of PPARs in cancer cell differentiation [3, 6, 72, 77]. Above all, the intriguing data concerning the induction of differentiation associated with a shift towards aerobic glycolysis (a paradoxical Warburg effect) confirms the need to reconsider cancer cell metabolism in general and the

Warburg effect in particular [11, 13, 82]. To that end, our understanding of the role of PPARs in cancer should assume a new level of complexity that takes into account their fundamental functions in lipid metabolism, in inflammation and, directly or indirectly, in angiogenesis [74, 75].

The molecular link among the synthetic PPAR ligands, mitochondria, and cancer indicates the need for a careful evaluation of some aspects of cancer cell pathophysiology, such as the following.

(i) The possible existence of a transduction pathway master signal as the basis of the complex cellular differentiation program related to PPAR. ROS, nitric oxide (NO), and reactive nitric oxide species (RNS) should form an important branch of this program. In addition, there should be a role for the NADH/NAD+ ratio.

(ii) The role of some oncogenes/oncosuppressors in cancer pathogenesis, given that mitochondrial respiratory chain dysfunction can induce a more differentiated phenotype in tumor cells and thereby influence their activity [15–18].

(iii) The significance of the modulation of the expression of proteins with oncogenic and antioxidant functions (stathmin 1, DJ-1 protein, peroxiredoxin 2, nucleoside diphosphate kinase A, etc.) in PPAR-related cancer cell differentiation. This is an important topic given the potential pathophysiological role of PPAR in cancer [62, 83].

At last, an understanding of the molecular mechanisms involved in the interrelationships between mitochondrial respiration and PPAR-related cancer regression may have important clinical implications for cancer diagnosis, prognosis, and therapy.

4. CONCLUSION

Many molecular mechanisms have been proposed to explain how PPARs, directly and/or indirectly, may induce cancer cell cycle arrest and induction or cancer cell differentiation or dedifferentiation. In spite of this, the molecular interrelationships between the mechanisms of functional modulation of PPAR and these important cellular phenotypic changes are still debated. It is clear that the various molecular modifications observed in different studies (decrease in cyclin D1, inhibition of IkB, induction of TSC22, NF-kB, GADD153, PTEN, etc.) may depend on the particular cell and cell functional status and that a potential master signal should be investigated.

Here, we have briefly described the molecular link between PPARs and cancer from a mitochondrial point of view. In our opinion, the most important factor linking cancer to PPARs is represented by their "synthetic ligands," which are characterized by other important and debated extrareceptor activities. Specifically, these agents can induce oxidative stress, which has an ambiguous role in cancer, leading it to act as a double-edged sword.

In this sense, mitochondria plays a critical role as one of the most important organelles for generating reactive species. The metabolic stress and energetic failure that result from fibrate- and thiazolidinedione-induced mitochondrial impairment may also play an important part in cancer regression, especially in cells that require active and complete anabolic pathways to sustain cancer growth, an aspect that has not always been completely or correctly evaluated.

Considering their physicochemical properties discussed above, it is worthy of note that tumor regression induced by PPAR ligands may be a useful approach for the treatment of neoplasias of the central nervous system, which are classically difficult to treat with conventional chemotherapy. Specifically, interesting results have already been obtained in terms of decrease of cell proliferation, apoptosis induction, and expression of markers typical of a more differentiated phenotype in glioblastoma and astrocytoma cell lines [84–90], in primary cultures of human glioblastoma cells derived from surgical specimens [91], and above all, in patients with high-grade gliomas (glioblastoma or anaplastic glioma) [92].

Moreover, given that pharmacological modulation of PPAR in cancer cells typically arrests the cell cycle in the G0/G1 phase, combination therapy with a PPAR agonist and an antimitotic antitumor agent deserves careful consideration.

Furthermore, it may be useful to distinguish between real differentiating agents, which show low cytotoxicity indices relative to their differentiation activity (thiazolidinediones, fibrates, retinoids) and spurious differentiating agents, which show low differentiating activity and high cytotoxicity indices (old and new HDAC inhibitors) [93, 94].

A mitochondrial approach to analysis of the molecular link between PPARs and cancer certainly adds new levels of complexity to the already complicated picture. However, an optimal definition of all molecular mechanisms relating PPARs, mitochondria, and cancer may be fundamental to our understanding of the real therapeutic index of pharmacological modulation of these nuclear receptors. This is important not only in cancer, but also in the other diseases in which PPARs play a significant role, including atherosclerosis, hyperlipoproteinemias, metabolic syndrome, diabetes mellitus, and obesity. Moreover, a complete understanding of the pharmacotoxicological profile of these agents may reduce the incidence of dangerous side effects that have already dramatically afflicted patients treated with PPAR ligands [37–40].

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Research Article

Rosiglitazone Suppresses the Growth and Invasiveness of SGC-7901 Gastric Cancer Cells and Angiogenesis In Vitro via PPARγ Dependent and Independent Mechanisms

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Although thiazolidinediones (TZDs) were found to be ligands for peroxisome proliferators-activated receptorγ (PPARγ), the mechanism by which TZDs exert their anticancer effect remains unclear. Furthermore, the effect of TZDs on metastatic and angiogenesis potential of cancer cells is unknown. Our results in this paper show that rosiglitazone inhibited SGC-7901 gastric cancer cells growth, caused G1 cell cycle arrest and induced apoptosis in a dose-dependent manner. The effects of rosiglitazone on SGC-7901 cancer cells were completely reversed by treatment with PPARγ antagonist GW9662. Rosiglitazone inhibited SGC-7901 cell migration, invasiveness, and the expression of MMP-2 in dose-dependent manner via PPARγ-independent manner. Rosiglitazone reduced the VEGF induced angiogenesis of HUVEC in dose-dependent manner through PPARγ-dependent pathway. Moreover, rosiglitazone did not affect the expression of VEGF by SGC-7901 cells. Our results demonstrated that by PPARγ ligand, rosiglitazone inhibited growth and invasiveness of SGC-7901 gastric cancer cells and angiogenesis in vitro via PPARγ-dependent or -independent pathway.

1. INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the ligand-inducible nuclear receptor superfamily. After activation, PPARγ associates with the 9-cis retinoic acid receptor (RXR) to form functional heterodimers, which binds to the PPAR response element of the target genes and regulates the expression of these genes. Previous documents have shown that the PPARγ/RXR signal pathway plays critical role in a variety of biological processes, including adipogenesis, glucose metabolism, inflammation as well as inhibition of normal and tumor cells growth [1].

Thiazolidinediones (TZDs) are synthetic agonists for PPARγ. These PPARγ ligands were clinically used as antidiabetic drugs which could attenuate the insulin resistance associated with obesity, hypertension, and impaired glucose tolerance in humans [2]. Recent studies have suggested that PPARγ is a potential molecular target for anticancer drug development, due to the increased expression of PPAR in several cancer cells. It has been reported that TZDs could inhibit growth and induce apoptosis in a variety of cancer cell lines. More importantly, TZDs exhibited antitumor activities in vivo in the prevention of prostate, liver, and pituitary cancers. Although increasing evidence showed that TZDs are potential anticancer agents [3], the mechanisms underlying the antitumor effects are not well understood. TZDs were initially thought to inhibit the cancer cells proliferation through regulation of expression of PPARγ-mediated target genes. However, recent evidence revealed that the antitumor effects of TZDs exist via PPARγ-independent mechanisms in various types of cancers [4–6].

We previously found the expression of PPARγ decreased in primary and metastatic gastric carcinoma, compared with normal gastric tissues [7]. Recent studies in gastric cancer cells demonstrated that TZDs treatment resulted in significant growth arrest both in cultured cell and in nude mice models [8–12]; however, the effects of PPARγ ligands on invasiveness and angiogenesis of gastric cancer are still

unclear. Therefore, this work was undertaken to investigate the effects of PPARγ agonists, such as rosiglitazone, on cell growth and the invasiveness in human cell line SGC-7901, as well as on angiogenesis in vitro.

2. METHODS

2.1. Cell culture

Human gastric cancer cell line, SGC-7901, was obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were purchased from the Keygen Technology Company (Najing, China). SGC7901 cells and HUVECs were cultured in RPMI-1640 medium (GIBCO, Carlsbad, Calif, USA) containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, Sigma-Aldrich, Mo, USA).

2.2. RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, Calif, USA) according to the manufacturer's instructions. Reverse transcription reaction was performed with random hexamer primers and a SuperScript Reverse transcriptase kit (Invitrogen, Carlsbad, Calif, USA). The sequences of specific primers were as follows: PPARγ mRNA, forward, 5′-TCT CTC CGT AAT GGA AGA CC-3′, and reverse, 5′-GCA TTA TGA GAC ATC CCC AC-3′. MMP-2 mRNA, forward, 5′-GGC CCT GTC ACT CCT GAG AT-3′, and reverse, 5′-GGC ATC CAG GTT ATC GGG GA-3′. VEGF mRNA, forward, 5′-GAC AAg AAA ATC CCT GTG GGC-3′, and reverse 5′-AAC GCG AGT CTG TGT TTT TGC-3′. β-actin mRNA, forward, 5′-CTT CTA CAA TGA GCT GCG TA-3′, and reverse, 5′-TCA TGA GGT AGT CAG TCA GG-3′. PCR conditions were 94°C, 30 seconds, 55–57°C (depending on the primer set), 30 seconds, and 72°C, 1 minute with 35 cycles using Taq PCR MasterMix (Tianwei, Beijing, China). The resultant PCR products were 474 bp (PPARγ), 243 bp (β-actin), 474 bp (MMP-2), and 169 bp (VEGF). PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining.

2.3. Quantitative real-time RT-PCR analysis

The PCR reactions were performed in a Brilliant SYBR Green QPCR master mix (Stratagene, Calif, USA) according to the manufacturer's instructions. The sequences of specific primers were the same as for RT-PCR. After 10 minutes at 95°C to denature the cDNA, the cycling conditions were 95°C, 1 minute, 55–57°C (depending on the primer set), 30 seconds, and 72°C, 1 minute with 40 cycles. The LightCycler software constructed the calibration curve by plotting the crossing point (Cp), and the numbers of copies in unknown samples were calculated by comparison of their Cps with the calibration curve. To correct differences in both RNA quality and quantity between samples, the data were formalized to those for β-actin.

2.4. Western blotting

The cells proteins were extracted according to NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, Ill, USA). Protein concentration of each sample was assayed using BCA Protein Assay Reagent according to manufacturer's instructions (Pierce Biotechnology, Rockford, Ill, USA). Twenty micrograms of proteins of different groups were separated in 10% SDS-PAGE, and transferred onto PVDF membrane (Invitrogen, Carlsbad, Calif, USA). Five percent of milk (blocking solution) was loaded over the membrane and incubated for 1 hour at room temperature with agitation. The membranes were then incubated with the mouse antihuman PPARγ antibody at a dilution of 1:200 (Santa Cruz, Calif, USA), the mouse antihuman MMP-2 antibody (1:400, Neomarker, Calif, USA), the rabbit antihuman VEGF antibody (1:200, Zymed, Calif, USA), and the mouse antihuman β-actin (1:200, Xiaxin, China) overnight at 4°C with agitation. After being washed with 0.1% Tween 20 in Tris-saline, three times, the membranes were incubated with biotin-labeled antirabbit or mouse IgG for 1 hour at room temperature with agitation. Reactive protein was detected using ECL chemiluminescence system (Pierce, Rockford, Ill, USA).

2.5. ELISA of secreted VEGF

The effect of RGZ on VEGF release in tumor cells was measured by ELISA. Cells grown in 90 mm plates were exposed to various concentrations of RGZ (1–20 μM) or vehicle with or without GW9662 (2.5 μM, pretreated 1 hour) for 24 hours. VEGF concentration in the supernatant was measured using a VEGF ELISA kit (R & D systems, Minneapolis, Minn, USA).

2.6. Cell viability

The viability of the cells was assessed by MTT assay. Briefly, cells grown in 96-wells were exposed to various concentrations of RGZ with or without GW9662 (2.5 μM, pretreated 1 hour), for 24, 48, or 72 hours. Then, 20 μL of MTT (5 mg/mL) was added to each well, and cells were incubated continuously at 37°C for 4 hours. After removal of medium, the crystals were dissolved in DMSO, and absorbance was assessed at 570 nm with a microplate reader.

2.7. Cell cycle and apoptosis analysis

Cells treated with RGZ (1–20 μM) or vehicle with or without GW9662 (2.5 μM, pretreated 1 hour) for 48 hours were collected and fixed in cold 70% ethanol. Then, the samples were treated with RNase, stained with 50 mg/mL propidium iodide (PI), and analysed by EPICS Elite flow cytometer (Coulter Electronics, Fla, USA).

2.8. Invasion assay

The ability of cells to invade through a Matrigel-coated filter was measured in transwell chambers (Corning, NY, USA). Polyvinylpyrrolidone-free polycarbonate filters

(pore size 8μm) were coated with basement membrane Matrigel (50μL/filter) (BD, Bedford, Ohio, USA). The membrane was washed in PBS to remove excess ligand, and the lower chamber was filled with 0.6 mL of RPMI-1640 medium containing 10% fetal bovine serum (FBS). Cells were serum-starved overnight (0.5% FBS), harvested with trypsin/EDTA, and washed twice with serum-free RPMI-1640 medium. Cells were resuspended in migration medium (RPMI-1640 medium with 0.5% FBS), and 0.1 mL migration medium containing 1×10^5 cells was added to the upper chamber. After incubation with RGC ($1–20 \mu$M) with or without GW9662 (2.5μM, pretreated 1 hour) at $37°$C for 24 hours, the cells on the upper surface of the membrane were removed using a cotton swab. The migrant cells attached to the lower surface were fixed in 10% formalin at room temperature for 30 minutes and stained with hematoxylin. The numbers of migrated cells were counted under a microscope.

2.9. Scratch wound-healing motility assays

Gastric cancer cells were seeded on 60 mm plates and allowed to grow to confluence. Confluent monolayers were scratched with a pipette tip and maintained under RGZ ($1–20 \mu$M) with or without GW9662 (2.5μM, pretreated 1 hour) for 24 hours. Plates were washed once with fresh medium to remove nonadherent cells and then photographed. The cell migration was evaluated by counting cells that migrated from the wound edge.

2.10. In vitro Angiogenesis assay

The angiogenesis assays were performed as per the manufacturer's instructions, that is, transfer 50μL of ECMatrixTM solution to each well of a precooled 96-well tissue culture plate on ice. Incubate at $37°$C for 1 hour to allow the matrix solution to solidify. Harvest human umbilical vein endothelial cells (HUVECs) resuspend and Seed 5×10^3 cells per well onto the surface of the polymerized ECMatrixTM. Incubate with RGC ($1–20 \mu$M) with or without GW9662 (2.5μM, pretreated 1 hour) at $37°$C for 12 hours. Inspect tube formation under an inverted light microscope at 100 X magnification.

2.11. Zymography

Cells were cultured for 24 hours in serum-free medium, washed twice, and finally treated with RGZ ($1–20 \mu$M) with or without GW9662 (2.5μM, pretreated 1 hour) for a further 48 hours. The supernatants were collected and concentrated, using centrifugal filter devices (Millipore Corp., Bedford, Mass, USA) and the protein content was determined using BCA Protein Assay Reagent. Equal amounts of protein (20μg) were mixed with SDS sample buffer without reducing agents and incubated for 40 minutes at $37°$C. For gelatinolytic activity, the assay samples were separated on polyacrylamide gels containing 1 mg/mL gelatin. After electrophoresis, the gels were stained for 1 hour in a 45% methanol/10% acetic acid mixture containing

coomassie brilliant blue G250 and destained. Zymograms were photographed after 10 hours of incubation at $37°$C.

2.12. Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments, each done in triplicate. Differences between control and experiment groups were analyzed using the t-test. $P < .05$ was considered statistically significant.

3. RESULTS

3.1. RGZ inhibited proliferation and induced apoptosis in SGC-7901 cells through PPARγ-dependent mechanism

In SGC-7901 cells, the expression of PPARγ was observed by RT-PCR and western blot (not shown).

RGZ ($0.1–100 \mu$M) treatment for 24, 48, and 72 hours inhibited cells growth in a dose- and time-dependent manners in SGC-7901 gastric cancer cell line as determined by MTT assay. Pretreatment with the highly selective PPARγ antagonist GW9662 (2.5μM) reversed the effect of RGZ on cell viability (see Figure 1(a)).

To explore whether the growth inhibition of RGZ in SGC-7901 cells was caused by apoptosis, we analyzed the sub-G1 population of the cells after treatment with RGZ ($1–20 \mu$M) for 48 hours. RGZ induced apoptosis in a dose-dependent manner, which was also reversed completely by 2.5μM GW9662 treatment (see Figure 1(b)).

Furthermore, to determine whether the inhibitory effect of RGZ on cell viability is associated with the arrest of the cell cycle, we analyzed the cell cycle progression after treatment with RGZ ($1–20 \mu$M) for 48 hours. RGZ treatment increased the number of cells in the G1-G0 and decreased the number of cells in the S phases in dose-dependent manner. The effects of RGZ on cell cycle of SGC-7901 cells were also reversed by 2.5μM GW9662 (see Figure 1(c)).

3.2. RGZ inhibited SGC-7901 cells migration and invasiveness through PPARγ-independent mechanism

After treatment with RGZ ($1–20 \mu$M) for 48 hours, the number of cells migrated to the scratched area was 60 ± 3.1 cells/mm^2, 58 ± 2.7 cells/mm^2, 49 ± 2.8 cells/mm^2, 27 ± 2.9 cells/mm^2, and 20 ± 1.9 cells/mm^2, respectively, which were significantly lower than those in control group (84 ± 3.4 cells/mm^2). GW9662 treatment had no effects on the cells migration with inhibition induced by RGZ. The number of the cells migrated to the scratched area treated with GW9662 and RGZ ($1–20 \mu$M) for 48 hours was 61 ± 1.8 cells/mm^2, 53 ± 3 cells/mm^2, 47 ± 2.5 cells/mm^2, 29 ± 2.8 cells/mm^2, 18 ± 3.2 cells/mm^2, respectively, which were not different from those in the groups treated with RGZ alone (see Figure 2(a)).

The effect of RGZ on the cells invasion through reconstituted basement membranes was analyzed using Matrigel-coated invasion chambers. After treatment with RGZ ($1–20 \mu$M) for 48 hours, the cells attached to the lower surface of

(a)

(b)

(c)
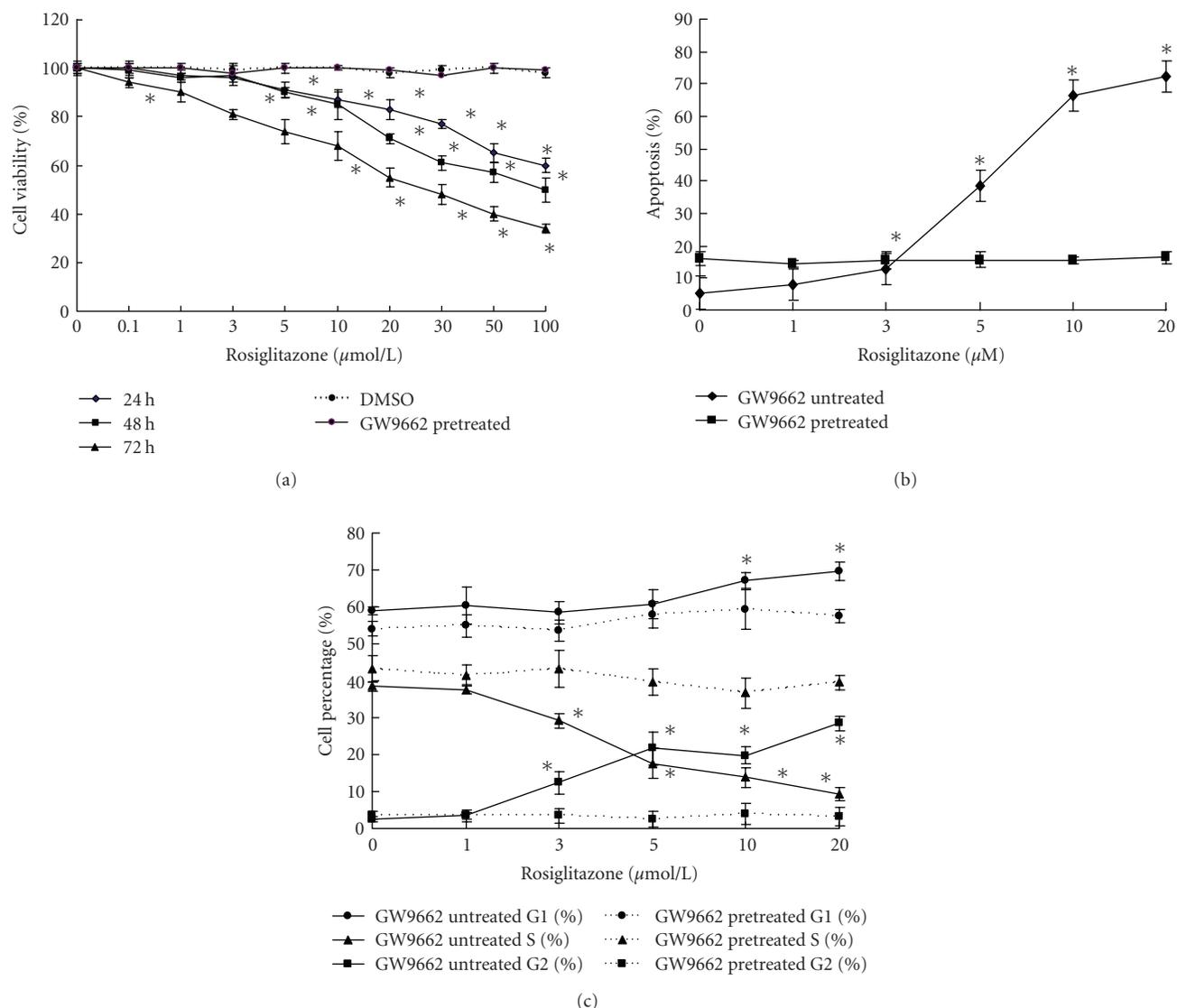
FIGURE 1: (a) RGZ (0.1–100 μM) treatment for 24, 48, and 72 hours inhibited cell growth in a dose- and time-dependent manners in SGC-7901 gastric cancer cell line, as determined by MTT assay, which was reversed completely by 2.5 μM GW9662 pretreatment for 1 hour. Cell viability was expressed as the percentage of cells under control conditions (0 μM of RGZ or GW9662). (b) RGZ induced apoptosis in a dose-dependent manner, which was also reversed completely by 2.5 μM GW9662 pretreatment for 1 hour. (c) RGZ treatment increased the number of cells in the G1-G0 and decreased the number of cells in the S phases in dose-dependent manner, which was reversed completely by 2.5 μM GW9662 pretreatment for 1 hour. Values are the means ± SD of three representative experiments. *Statistical significance ($P < .05$ or higher degree of significance) versus vehicle-treated controls.

the filters were 256 ± 9 cells/mm², 248 ± 7 cells/mm², 219 ± 12 cells/mm², 174 ± 11 cells/mm², and 154 ± 10 cells/mm², respectively, which were significantly lower than those in control group (279 ± 9 cells/mm²). After cotreatment of the cells with GW9662 and RGZ, the cells attached to the lower surface were 251 ± 29 cells/mm², 238 ± 12 cells/mm², 220 ± 7 cells/mm², 166 ± 16 cells/mm², and 148 ± 12 cells/mm², respectively, which were not different from those in the groups treated with RGZ alone (see Figure 2(b)).

Metalloproteases (MMPs) have been demonstrated to play a significant role in tumor cell invasion [13]. In this study, our results showed that RGZ inhibited the mRNA and protein expression levels of MMP-2 in a dose-dependent

manner (see Figures 3(a), 3(c), and Tables 1, 2). Moreover, the gel zymography results demonstrated that the activity of MMP-2 decreased after RGZ (1–20 μM) treatment for 48 hours in dose-dependent manner (see Figure 4(a)). The inhibitory effects of RGZ on MMP-2 were not affected by GW9662 treatment (see Figures 3(b), 3(c), and 4(b)).

3.3. Effects of RGZ on angiogenesis in vitro

Matrigel-plated HUVECs elongated and migrated in the presence of VEGF and formed tubular networks. RGZ markedly suppressed the formation of the tube-like structures of HUVEC cells in a dose-dependent manner

(see Figure 5(a)), which was completely antagonized by GW9662 (see Figure 5(b)). These results suggested that rosiglitazone exhibits antiangiogenic activity via PPARγ-dependent mechanism.

To further determine whether the effect of RGZ on angiogenesis is due to the down regulation of the tumor-secreted growth factors, we measured the expression levels of VEGF in SGC-7901 cell cultured medium, after treatment with various concentrations of RGZ. Our results demonstrated that RGZ (1–20 μM) did not change the expression of mRNA and protein of VEGF in SGC-7901 cells (see Figures 3(a), 3(c), and Table 1), but also the results were confirmed by ELISA (see Figure 6).

4. DISCUSSION

As a potential molecular target for anticancer drug development, PPARγ and its ligands have been extensively studied in the past several years. Previous studies have shown that PPARγ is expressed in several human gastric-cancer cell lines, including MKN-7, MKN-28, MKN-45, and AGS. TZDs could inhibit these cancer cell lines growths in vitro and in vivo [9, 12]. Also, the growth inhibitory effects of TZDs on MKN45 cells depend on the PPARγ expression levels. The growth inhibition of TDZs was more significant in the higher PPARγ expressing cells. Moreover, Lu et al. [10] found that PPARγ (+/−) mice were more susceptible to MNU-induced gastric cancer than wild-type (+/+) mice, and troglitazone significantly reduced the incidence of gastric cancer in PPARγ (+/+) mice but not in PPARγ (+/−) mice. All these results indicated that TZDs inhibit the cancer cells growth via PPARγ-dependent mechanism. Our results demonstrated that RGZ, the most potent and selective synthetic ligand of PPARγ, inhibited SGC-7901 gastric cancer cells growth, caused G1 cell cycle arrest, and induced apoptosis in a dose-dependent manner. The effects of RGZ on SGC-7901 cancer cells were completely reversed by treatment with PPARγ antagonist GW9662. These results indicated that RGZ suppressed the SGC-7901 cancer cells growth in a PPARγ-dependent mechanism.

In this study, we found that the RGZ inhibited invasion, migration, and the secretion of MMP-2 of SGC-7901 cells. The inhibitory effects of RGZ on metastases and MMP-2 activity were not directly mediated by PPARγ activation, since these effects were not reversed by GW9662 treatment. Our results were consistent with the previous works on human adrenocortical cancer cell line H295R [14], pancreatic cancer cells [15], and human myeloid leukemia cells [16], which showed that PPARγ ligands act independently of PPARγ activation in the invasion suppression and down-regulation of MMP-2 activity. Recent papers showed that PPARγ regulated E-cadherin expression and inhibited growth and invasion of prostate cancer [17], and PPARγ ligand troglitazone inhibited transforming growth factor-beta-mediated glioma cell migration and brain invasion [18]. But some studies have contrasting results that the PPARγ, ciglitazone, induced cell invasion, through activation of Pro-MMP-2, activation via the generation of ROS, and the activation of ERK [19], and that PPARγ antagonists


(a)

(b)
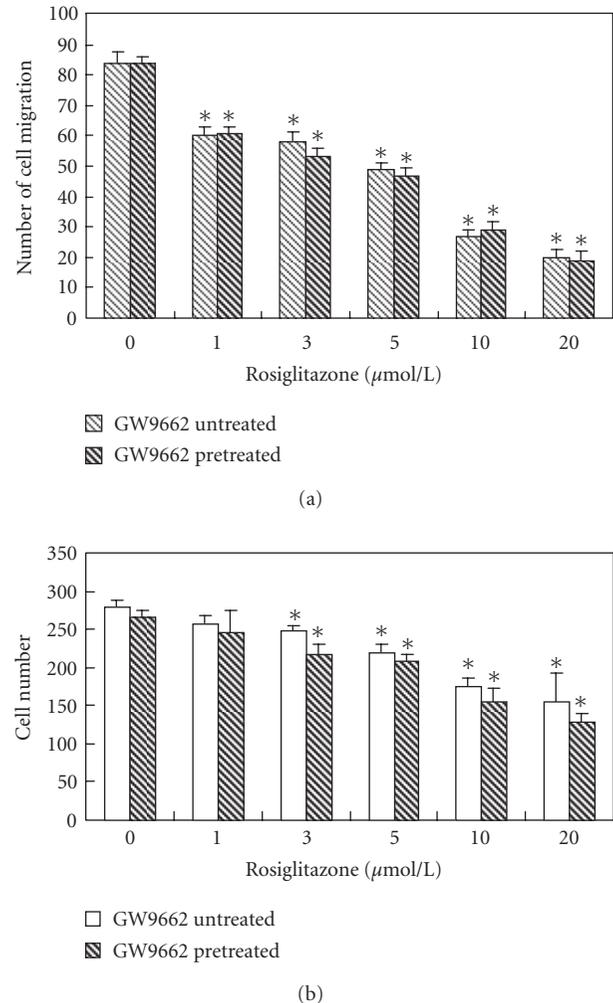
Figure 2: (a) Effect of RGZ on the migration and (b) invasion of SGC-7901 gastric cancer cells, which was reversed completely by 2.5 μM GW9662 pretreatment for 1 hour. Values are the means ± SD of three representative experiments. *Statistical significance (P < .05 or higher degree of significance) versus vehicle-treated controls.

induced vimentin cleavage and inhibited invasion in high-grade hepatocellular carcinoma [20]. Further studies are needed on the mechanism of PPARγ in cancer and invasion.

Recent investigations suggested that PPARγ ligands had inhibitory effects on tumor cell lines, but the effects appear not to be entirely elicited by the direct action on tumor cells. Inhibition of the neovascularization may be another target of TZDs to suppress the growth of cancers. PPARγ is expressed in endothelial cells, and the PPARγ ligands can inhibit the proliferation of these cells induced by growth factors, or cause their apoptosis in vitro [21–23]. It has been reported that PPARγ ligands could inhibit choroidal, retinal, and corneal neovascularization when administered intraocularly [24–26]. In addition, systemic administration of rosiglitazone and troglitazone inhibits FGF2-induced angiogenesis; thereby inhibiting primary tumor growth and metastasis [27]. We observed that RGZ inhibited the angiogenesis of

(a)

(b)

(c)
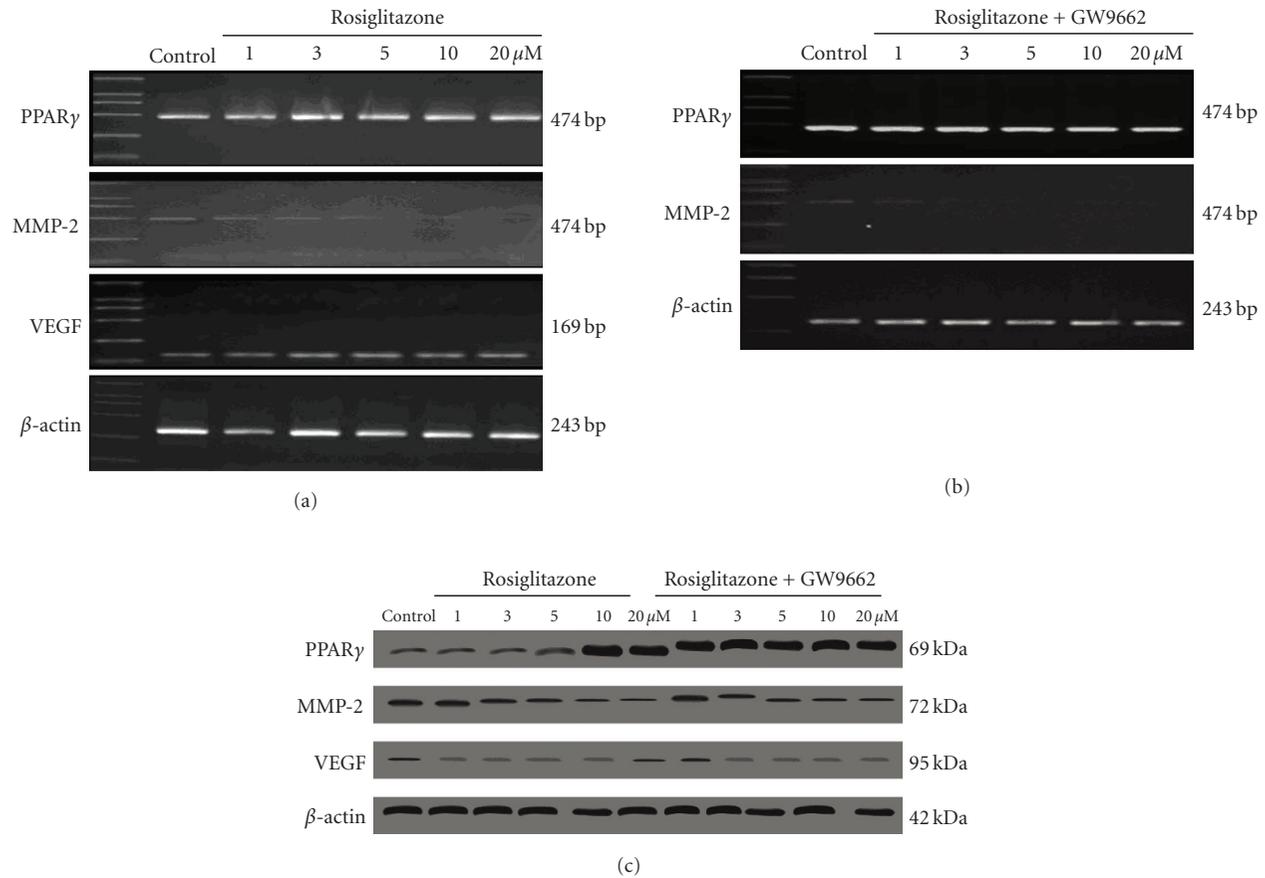
Figure 3: (a) RGZ (1–20 μM) inhibited the mRNA and (c) protein expression levels of MMP-2 in a dose-dependent manner, which were not affected by 2.5 μM GW9662 pretreatment for 1 hour (b), (c). RGZ (1–20 μM) did not change the expression of VEGF in SGC-7901 cells (a), (c).

Table 1: Expression of MMP-2 and VEGF after RZD treatment in SGC-7901 gastric cancers by real-time PCR.

Rosiglitazone (μmol/L)	PPARγ	MMP-2	VEGF
0	0.132127 ± 0.045513	0.008912 ± 0.000133	0.61132 ± 0.078921
1	0.121878 ± 0.034219	0.006003 ± 0.000331*	0.620255 ± 0.054671
3	0.130134 ± 0.0521137	0.005486 ± 0.000541*	0.60728 ± 0.036799
5	0.137778 ± 0.046222	0.005048 ± 0.000346*	0.599438 ± 0.076541
10	0.141171 ± 0.038741	0.001924 ± 0.000189*	0.624165 ± 0.038966
20	0.143889 ± 0.061237	0.001298 ± 0.000267*	0.604246 ± 0.065679

*Statistical significance ($P < .05$ or higher degree of significance) versus vehicle-treated controls.

Table 2: Expression of MMP-2 and VEGF after RZD and GW9662 cotreatment in SGC-7901 by real-time PCR.

Rosiglitazone (μmol/L)	PPARγ	MMP-2
0	0.14161 ± 0.055389	0.00975 ± 0.000533
1	0.137738 ± 0.030102	0.008974 ± 0.000113*
3	0.134614 ± 0.029881	0.006003 ± 0.000401*
5	0.141156 ± 0.564569	0.00564 ± 0.000246*
10	0.135666 ± 0.034887	0.002182 ± 0.000364*
20	0.129278 ± 0.019262	0.001712 ± 0.000178*

*Statistical significance ($P < .05$ or higher degree of significance) versus vehicle-treated controls.

(a)

(b)
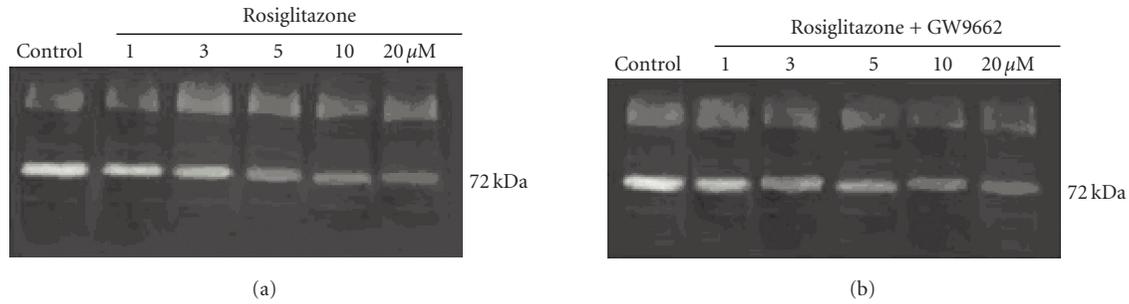
Figure 4: (a)The activity of MMP-2 was decreased after RGZ (1–20 μM) treatment for 48 hours in dose-dependent manner. (b) The inhibitory effects of RGZ on MMP-2 were not affected by 2.5 μM GW9662 pretreatment for 1 hour.


(a)

(b)
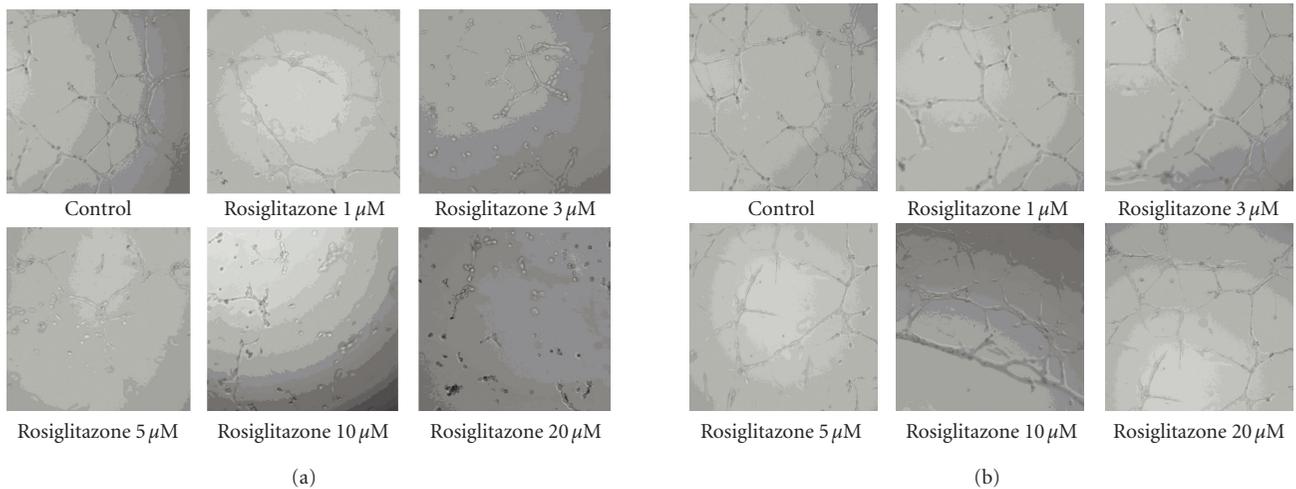
Figure 5: (a) RGZ markedly suppressed the formation of the tube-like structures of HUVEC cells in a dose-dependent manner, (b) which was completely antagonized by 2.5 μM GW9662 pretreatment for 1 hour.
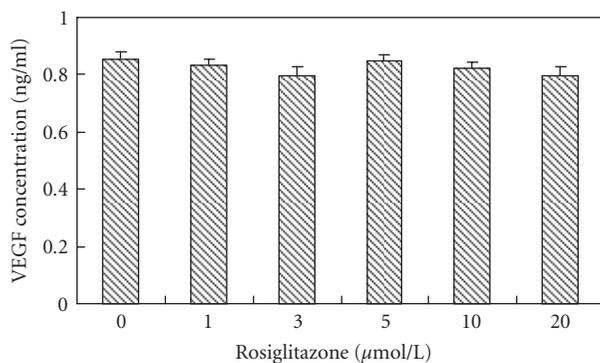

Figure 6: RGZ had no effect on the secretion of VEGF of SGC-7901 cell.

HUVECs in dose-dependent manner via PPARγ pathway. The effects RGZ on the endothelium suggest that RGZ may regulate tumor growth by targeting non-cell-autonomous mechanisms.

Previous studies [5] showed that suppression of angiogenesis could result from a decrease in the local levels of stimulators (e.g., VEGF and FGF2) and/or an increase of endogenous inhibitors of angiogenesis (e.g., thrombospondin) produced by tumor cells. PPARγ ligands suppressed VEGF production in colon carcinoma [28], human breast cancer [29], and human renal cell carcinoma cells [30]. However, contradictory results have also been reported in bladder and prostate cancer cells in which PPARγ ligands increased VEGF production [31, 32]. Inconsistent with the above documents, our results showed that RGZ did not change the secretion of VEGF from SGC-7901.

Taken together, our results demonstrated that RGZ inhibited growth and invasiveness of SGC-7901 gastric cancer cells and angiogenesis in vitro via PPARγ-dependent or -independent pathway. Further study is needed to elucidate the mechanisms by which RGZ exhibits different manner.

ACKNOWLEDGMENTS

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Review Article

The Critical Role of PPARγ in Human Malignant Melanoma

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The past 30 years have only seen slight improvement in melanoma therapy. Despite a wide variety of therapeutic options, current survival for patients with metastatic disease is only 6–8 months. Part of the reason for this treatment failure is the broad chemoresistance of melanoma, which is due to an altered survival capacity and an inactivation of apoptotic pathways. Several targetable pathways, responsible for this survival/apoptosis resistance in melanoma, have been described and current research has focused on mechanism inactivating these pathways. As PPARγ was shown to be constitutively active in several tumour entities and PPARγ agonists extent strong anticancer effects, the role of PPARγ as a possible target for specific anticancer strategy was investigated in numerous studies. However, only a few studies have focused on the effects of PPARγ agonists in melanoma, showing conflicting results. The use of PPARγ agonists in melanoma therapy has to be carefully weighted against considerable, undesirable side effects, as their mode of action is not fully understood and even pro-proliferative effects have been described. In the current review, we discuss the role of PPARs, in particular PPARγ in melanoma and their potential role as a molecular target for melanoma therapy.

1. MALIGNANT MELANOMA AND MOLECULAR TARGETS IN MELANOMA THERAPY

Cutaneous malignant melanoma is the most aggressive form of skin cancer. Despite attempts to treat melanoma using a large variety of therapies, including immuno-, radio, and chemotherapies, survival remains very poor once the disease has spread to distant sites (median survival: 6–8 months) [1]. Systemic therapy, immunotherapy, or even biochemotherapy have failed to improve the survival of these patients. Until now, the only drug approved by the FDA for treatment of metastatic melanoma is the alkylating agent dacrabazine (DTIC), which results in clinical response only of 5–10% of cases when given as a single agent [2]. This treatment failure is mainly due to the notorious chemoresistance of melanoma cells. In contrast to other cancer cells, this chemoresistance of melanoma cells seems not to be acquired selectively following drug therapy, but to be more intrinsic in melanoma cells. Alteration of survival capacity and inactivation of apoptotic pathways are the molecular mechanisms responsible of conventional drug resistance in melanoma cells (see Figure 1). One example for a targetable pathway is the mitogen-activated protein kinase (MAP-kinase) pathway, which plays a crucial role in cell proliferation, invasion, and enhanced survival in diverse cancers [3]. A key player in the MAP-kinase pathway is B-RAF, a serin/threonine protein kinase acting as an oncogene [4]. The recent identification of activating mutations in B-RAF in over 60% cases of melanoma has offered the first opportunity for a rationale treatment program [3] and early clinical trials using the RAF kinase inhibitor BAY 43-9006 have been encouraging, being the first positive example of how targeted therapy can work in malignant melanoma. Other examples of targetable pathways in melanoma are the phosphoinositide-3-kinase (PI3K)/Akt pathway, which can be activated either through growth factors or loss of negative regulators of this pathway [5]. One of the most critical regulators of Akt (also known as protein kinase B) is the phophatase and tensin homologue (PTEN), which degrades the products of PI3K, preventing the activation of Akt [6]. In several studies it has been shown that up to 30% of melanoma, cell lines have lost PTEN expression [7].
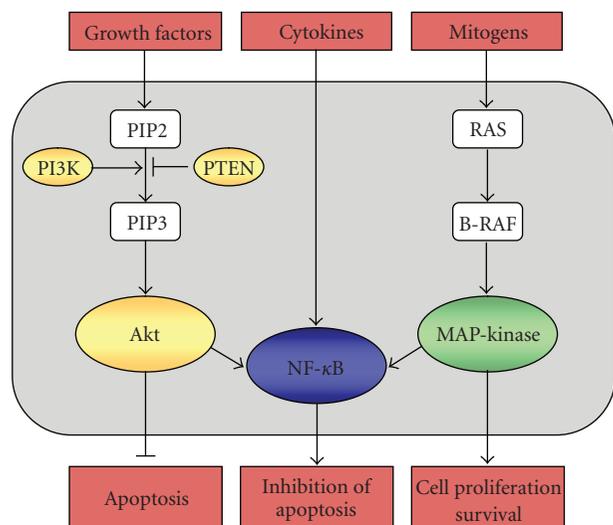
Figure 1: Activating pathways known to be constitutively active and contributing to the chemoresistance of melanoma cells. Over 60% of melanomas have activating mutations of B-RAF, 30% have lost PTEN expression, majority with NF-κB activation.

Finally, Huang et al. investigated that the NFκ-B signaling pathway, acting as a key regulator of survival in cancer cells, is constitutively activated in melanoma cells [8]. In addition, a recent study has demonstrated that inhibiting NFκ-B activity, using the proteasome inhibitor bortezomib, reduced melanoma cell growth in vitro [9]. Although these targets seem to be attractive ones for melanoma therapy in the future, most of the findings in this area do not give a comprehensive picture which would warrant a review. As several studies have shown an antiproliferative effect of PPARγ agonists on several tumour entities including melanoma, this review focuses on the role of the PPARγ as a possible target in melanoma therapy.

2. PPARγ AND PPARγ AGONISTS

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, belonging to the nuclear receptor superfamily [10]. Activated PPAR forms complexes with the retinoid receptor, which bind as a heterodimer to peroxisome proliferator response elements (PPREs) on the DNA, initiating transcription of downstream genes. The PPAR subfamily comprises three isoforms, PPARα, PPARβ/δ, and PPARγ, each showing a distinct distribution and ligand preference. While PPARα is predominantly expressed in metabolically active tissue, like liver, kidney, skeletal muscle and brown fat [11], PPARδ is expressed ubiquitously. PPARγ is highly expressed in adipocytes, where it functions as a key regulator of adipocyte differentiation [12] and insulin-dependent glucose utilization [13]. Prostaglandin15-deoxy-$\Delta_{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$) is the most potent naturally occurring ligand for PPARγ and the thiazolindinedione (TZD), also called glitazones, a class of antidiabetic, insulin-sensitizing drugs, are specific exogenous ligands for PPARγ. The TZD family of PPARγ agonists includes rosiglitazone, pioglitazone, ciglitazone, and troglitazone, rosiglitazone being the most potent agonist (Kd = 40 nM). In general, TZDs are selective for PPARγ in concentrations of 10 μM or less [14]. Recently, expression of PPARγ has been demonstrated in tumor cells originating from various malignancies, including breast, colon, lung, gastric, pancreatic, prostate, and bladder cancer and its activation through PPARγ agonists led to a significant decrease in proliferation of tumor cells in vitro [15–21], however, the exact mecannisms underlying this effect are still being explored. As a consequence, PPARγ has become a molecular target for potential anticancer drug development.

3. PPARγ AND MELANOCYTES

Until now, there is little information on the PPAR subtypes and relative levels of PPAR protein expressed in human skin. The three PPAR subtypes have been investigated in human keratinocytes [22], and PPARγ ligands have been shown to induce the expression of genes associated with keratinocyte differentiation in vitro [23]. In addition, Kang et al. showed the expression of all three PPAR subtypes in human melanocytes [24]. Immunocytochemistry showed that PPAR staining was mostly confined to the cytoplasm. Furthermore, proliferation of melanocytes was inhibited through administration of PPARα (WY-14643) and PPARγ (ciglitazone) agonists but not through PPARβ/δ (bezafibrate) agonists in a dose dependent manner at concentrations ranging between 0 and 100 μM. The inhibitory effect of ciglitazone seemed to occur through induction of apoptosis, which was observed by the TUNEL method and flow cytometry [25]. Moreover, Lee et al. showed that pigmentation in melanocytes was accelerated with PPARα and PPARγ agonists, suggesting a possible role for PPARα and PPARγ in modulating melanocyte proliferation and differentiation (pigmentation) [26]. Eastham et al. investigated the expression of mRNA for PPARα, PPARβ/δ, and PPARγ in human melanocytes [27]. In addition, the natural PPARγ agonist 15d-PGJ$_2$ and the synthetic PPARγ agonists ciglitazone and troglitazone inhibited the cell growth of human melanocytes, whereas the PPARα agonists WY14643 and Leukotriene B$_4$ had no effect on the proliferation of human melanocytes.

4. PPARγ AND MELANOMA CELLS

Only a few studies have focussed on PPARγ expression and effects of PPARγ agonists in melanoma cell lines (summarized in Table 1). Mössner et al. investigated the expression of PPARγ in four human melanoma cell lines MM-358, MM-201, MM-254 (established from lymph node metastasis of cutaneous malignant melanoma), and KAII (derived from a cutaneous nodular melanoma) [29]. In accordance with the immunocytochemistry of the melanocytes, staining was predominantly localized in the cytoplasm. In addition, the PPARγ agonists 15d-PGJ$_2$, troglitazone, and rosiglitazone dose-dependently inhibited the cell proliferation of all melanoma cells at concentrations between 0 and 50 μM. As shown by flow cytometry, this antiproliferative effect was not mediated through induction of apoptosis, but rather

TABLE 1: Effects of PPARγ agonists on melanoma cell growth.

Cell line	PPARγ agonist	Concentration	Results	Mechanism of action	Reference
UISO-Mel6, MV3, MeWo, G361, Lox	Rosiglitazone, pioglitazone, ciglitazone, troglitazone	0.3–300 μM	– Growth inhibition of all cell lines at 30–300 μM, – increase in cell proliferation at 3 μM	– Independent from apoptosis	Freudlsperger et al. [28]
MM-358, MM-201, MM-254, KAII	15d-PGJ$_2$, troglitazone, rosiglitazone	0.1–50 μM	– Growth inhibtion of all cell lines at 20–50 μM	– Independent from apoptosis, – induction of G$_1$ phase cell cycle arrest	Mössner et al. [29]
SK-mel28, A375	Ciglitazone, troglitazone, 15d-PGJ$_2$	0–10 μM	– Growth inhbition only of A375 at 10 μM	– Not investigated	Eastham et al. [27]
WM35, A375	Ciglitazone, 15d-PGJ$_2$	10–15 μM	– Growth inhibition of all cell lines at 10–15 μM	– Induction of apoptosis	Placha et al. [30]
A375	Ciglitazone, 15d-PGJ$_2$,	0–32 μM	– Growth inhibtion of A375 at 16 μM of 15d-PGJ$_2$ – no growth inhibition by ciglitazone	– Induction of apoptosis	Núñez et al. [31]

by induction of G$_1$ phase cell cycle arrest. Eastham et al. investigated the expressions of PPARα, PPARβ/δ, and PPARγ in human melanoma cells SK-mel28 and A375 [27]. Both melanoma cell lines express PPARα protein levels 20–47% higher and PPARγ protein levels 40–50% higher, respectively, than the normal human melanocytes. However, mRNA levels and protein levels for these receptors did not match. In addition, the natural PPARγ agonist 15d-PGJ$_2$ and the synthetic PPARγ agonists ciglitazone and troglitazone inhibited the cell growth of the human melanoma cell line A375 in concentrations of 0–10 μM, whereas the SK-mel28 cells were not affected in this concentration range. The PPARα agonists WY14643 and leukotriene B4 had no effect on the cell proliferation of both cell lines. Placha et al. investigated PPARγ expression in the melanoma cell lines WM35, derived from a primary tumour site, and A375, derived from a solid metastatic tumour. Furthermore, an antiproliferative effect of the PPARγ agonist ciglitazone and 15d-PGJ$_2$ in both melanoma cell lines was observed in concentrations of 10–15 μM [30]. The antiproliferative effect of ciglitazone was mediated through induction of apoptosis, as evidenced by fluorescence microscopy. Núñez et al. showed an antiproliferative effect of 15d-PGJ$_2$ on the melanoma cell line A375 at concentration of 16 μM or higher, which was mediated through induction of apoptosis, while ciglitazone showed no growth inhibitory effect [31]. Our own results showed expression of PPARγ in six different human melanoma cells MV3, Lox, MeWo, G361, FemX-1, and UISO-Mel6, which were established from primary malignant melanoma or metastatic melanoma lymph node [28]. Similar to the findings of Mössner et al., immunocytochemical staining of PPARγ was mostly confined to the cytoplasm. The

PPARγ agonists rosiglitazone, pioglitazone, troglitazone, and ciglitazone all showed a dose-dependent antiproliferative effect on all melanoma cell lines tested at concentrations of 30 μM or higher. This antiproliferative effect was due to a mechanism independent from apoptosis, which was shown by assessment of the nuclear morphology or by molecular analysis of DNA fragmentation. Interestingly, all four PPARγ agonists showed an increase in cell proliferation of all six melanoma cell lines at concentrations of 3 μM.

5. PPARγ-DEPENDENT OR -INDEPENDENT EFFECTS OF PPARγ AGONISTS IN MELANOMA CELLS

Several studies have documented various mechanisms for the antiproliferate effect of PPARγ agonists, both being dependent or independent of PPARγ activation, which holds also true for melanoma cells. Using a reporter gene assay, Eastham et al. showed that the PPARγ agonists 15d-PGJ$_2$ and ciglitazone stimulated PPRE reporter gene activity in a dose-dependent manner in B16 melanoma cells. This activity correlated with their ability to inhibit cell proliferation, hence a PPARγ-dependent mechanism was postulated [27]. Simularely, Placha et al. investigated, that the apoptosis inducing effect of ciglitazone in human melanoma cells was clearly associated with the strong induction of transcription by endogenous PPARγ through PPRE target sequences, as shown in the reporter gene assay system [30]. On the other hand, PPAR agonists have been reported to have nonreceptor mediated effects too. In our own studies, quantitative analyses of PPARγ protein showed no correlation between the amount of the PPARγ protein and the respective susceptibility of the melanoma cell lines towards PPARγ

agonists. Therefore, a PPARγ-independent effect of PPAR-γ agonists was assumed [28]. In other cancer cells, resistance pathways which are constitutively activated in melanoma cells (Figure 1) were affected through PPARγ agonist independent from PPARγ activation. For example, 15d-PGJ$_2$ has been shown to alter NF-κB activity in hepatocellular carcinoma cells where 15d-PGJ$_2$ induces apoptosis via caspase-dependent and -independent pathways [32]. In addition, Straus et al. showed a PPARγ-independent repression of NF-κB by 15d-PGJ$_2$ through two mechanisms, inhibition of IκB kinase (IKK) and inhibition of NF-κB DNA binding [33]. The inhibition of NF-κB by PPARγ agonists through PPARγ-independent mechanisms could be a possible way for the antiproliferative effect in melanoma cells, especially for the combination of the PPARγ agonist rosiglitazone with bortzezomib, a potent inhibitor of NF-κB, has led to an augmented antiproliferative effect on melanoma cells [34]. In addition, Han and Roman investigated that rosiglitazone inhibited the cell growth of human lung carcinoma cells through inactivation of PI3K/Akt pathway and increase of PTEN expression [35]. These changes were inhbited by GW9662, a potent antagonist of PPARγ, suggesting that they depend upon PPARγ activation. If this inactivation of the PI3K/Akt pathway by rosiglitazone also contributes to the antiproliferative effect of PPARγ agonists in melanoma needs to be further elucidated.

6. CONCLUSION

The rapid increase in incidence of malignant melanoma has not been accompanied by better therapeutic options [36]. The past few years have seen great leaps in our understanding of the mechanism of drug resistance and cell survival in melanoma. Many reports have indicated the central role of PPARγ in the control of malignant cell growth in various tumour entities including melanoma. In addition, evidence has been accumulated that PPARγ is expressed in human melanoma cells and that PPARγ specific agonists dose-dependently inhibited proliferation of melanoma cells [27–31]. However, these studies are inconsistent regarding the concentration of PPARγ agonists initiating an antiproliferative effect and the mechanism underlying these growth inhibitory effects. In contrast to PPARγ, PPARα agonists were not shown to have antiproliferative effects on melanoma cells. Significant inhibition of melanoma cell proliferation did not occur until 20 μM or higher concentrations of PPARγ ligands were used. However, many natural and synthetic PPARγ ligands loose their receptor selectivity at these concentrations [37]. Furthermore, conflicting evidence exists on the ability of PPARγ agonists to promote tumour growth, depending on the cell model. For example, the PPAR-γ agonist troglitazone increased cell proliferation of breast cancer cells in low concentrations (<5 μM), while higher concentrations of troglitazone (100 μM) inhibited cell growth [38]. These investigations corroborate our published data in melanoma cells. The PPARγ agonists rosiglitazone, troglitazone, pioglitazone, and ciglitazone showed an increase in cell proliferation of all six melanoma cell lines tested in low concentrations (3 μM), however, in higher con-

centrations (>30 μM) a significant growth-inhibitory effect was observerd [28]. In addition, Lucarelli et al. reported that troglitazone promoted the survival of osteosarcoma cells at concentrations of 5 μM, through the activation of the PI3-kinase/Akt survival pathway (see Figure 1) [39]. Therefore, the administered dose of PPAR-γ ligands in clinical trials for melanoma therapy needs to be carefully defined and monitored closely.

In conclusion, the current studies concerning the role of PPARγ in melanoma proliferation and progression report conflicting results. The concentrations inducing growth inhibitory effects in melanoma cells seem to be different depending on the PPARγ agonist used and the melanoma cell employed. In particular, it remains to be further explored whether activation of PPARγ itself or PPARγ-independent effects of PPARγ agonists contribute to the inhibition of melanoma cell growth. Although the antiproliferative effect of PPARγ agonists in certain concentration ranges in melanoma is undisputable, more detailed information concerning the exact mechanisms seems to be necessary. However, PPARγ might be a promising approach for target specific anticancer strategy in the treatment of melanoma.

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Review Article

PPARγ in Neuroblastoma

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Neuroblastoma (NB) is the most common extracranial tumor in children and accounts for around 15% of all paediatric oncology deaths. The treatment of NB includes surgery, chemotherapy, and radiotherapy. Unfortunately, most children with NB present with advanced disease, and more than 60% of patients with high-risk features will have a poor prognosis despite intensive therapy. Agonists of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) have been shown to have pleiotropic effects, including antineoplastic effects. The studies that addressed the role and the possible mechanism(s) of action of PPARγ in NB cells are reviewed.

1. INTRODUCTION

Neuroblastoma (NB), the most common extracranial solid tumor in children, accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all paediatric oncology deaths [1]. The disease has a heterogeneous clinical presentation and course [2]. First of all, NB is a disease of the sympaticoadrenal lineage of the neural crest, and therefore tumors can develop anywhere in the sympathetic nervous system. The majority of NB is developed within the abdomen and at least 50% of these tumors arise in the adrenal medulla [2]. Other frequent localizations include the neck, chest, and pelvis [3]. The clinical presentation of the disease may be also highly variable and depends on the site of the primary tumor as well as on the presence or absence of metastatic disease (mostly haematogeneous dissemination to cortical bone, bone marrow, liver, and noncontiguous lymph nodes) or paraneoplastic syndromes. The diagnosis of NB is based on histopathological assessment of tumor tissue or on the detection of cancer cells in a bone marrow aspirate/biopsy, together with the presence of increased levels of urinary catecholamines [2]. Imaging studies for the localization of the disease include computed tomography, magnetic resonance, ^{99}mTc-diphosphonate, or metaiodobenzylguanidine (using ^{123}I) scintigraphy for the detection of bone metastases.

The treatment of NB includes surgery, chemotherapy (i.e., cisplatin, etoposide, doxorubicin, cyclophosphamide, vincristine) [4], and radiotheraphy. Unfortunately, although substantial improvement in outcome of certain subsets of patients has been observed during the past few decades [2], most children with NB present with advanced disease and more than 60% of patients with high-risk features will have a poor prognosis despite intensive therapy [5, 6]. Thus, research efforts to understand the biological basis of NB and to identify new and more effective therapies are essential to improve the outcome for these children. In the last years an expanding number of new agents have been developed for use in high-risk patients affected by recurrent disease. Cytotoxic agents, such as the topoisomerase 1 inhibitors topotecan and irinotecan, have an acceptable toxicity profile and are effectively used in early relapsing NB [7–10]. The delivery of radioactive molecules that are selectively concentrated in NB cells, such as metaiodobenzylguanidine, somatostatin analogues, anti-G_{D2} (a disialoganglioside) antibodies, has been used in clinical trials [11–22]. G_{D2}-targeted therapies using monoclonal antibodies are under investigation in phase III trials [19, 23, 24], and other immunotherapeutic strategies (i.e., vaccination or cellular immunotherapy using engineered cytolityc T lymphocytes) are currently investigated [25, 26]. Similarly, angiogenesis [27–33] and tyrosine kinase [34–38] inhibitors appear as an

attractive therapeutic option and clinical trials are ongoing. Retinoids have been shown to interfere with cell growth and to induce apoptosis in NB cells [39, 40] and preliminary clinical trials with retinoids in NB resulted in improved event-free survival in high-risk patients, with limited toxic effects [41, 42]. Thiazolidinediones (TZDs) are a class of molecules that activate the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) [43] and promote association with the 9-*cis* retinoic X receptor (RXR) to form functional heterodimers that recognize its cognate DNA response element within target genes [44, 45]. TZDs have been shown to have antineoplastic effects, as extensively discussed in this issue of the journal, in agreement with the demonstration that PPARγ/RXR signalling exerts an important role in inhibiting cell proliferation and/or in inducing apoptosis [46]. It has been also shown that PPARγ and RXR ligands may have a synergistic effect in inducing cell differentiation [47, 48] and in inhibiting cell growth in different tumors, such as colon, lung, and breast cancer [49–51]. There is evidence that also PPARα and PPARβ ligands may play a role in counteracting tumoral cell growth and in promoting cell differentiation, including neuroblastoma cells [52, 53]. However, most of the reports covering this issue, that have been published in the literature so far, deal with PPARγ agonists. Therefore, the role of PPARγ ligands as a possible therapeutic option in NB is reviewed and discussed here.

2. PPARγ AND PPARγ AGONISTS IN NEUROBLASTOMA

The first demonstration that PPARγ is expressed in NB cells was provided by Han et al. in 2001 [54]. Using RT-PCR the authors showed that LA-N-5 NB cells express also PPARβ, but not PPARα. Similarly, in sections from human primary NB immunostaining for PPARγ was detected in the nucleus and occasionally in the cytoplasm of cells, particularly in those showing ganglionic differentiation. Sato et al. [55] addressed the possibility that the amount of expression of PPARγ in NB might be correlated to patients' outcome. To this purpose, the level of mRNA was measured by semiquantitative RT-PCR in NB samples from 17 patients under the age of one year. In this subset of patients, spontaneous differentiation and regression are often observed [56], and some investigators suggested to observe these patients without surgery until there is an increase of vanilmandelic acid (VMA) or tumoral growth occurs [57, 58]. PPARγ mRNA was present in 12 samples. No difference between the expression of PPARγ and histology, age, staging, DNA ploidy was observed, yet a correlation with the change in urinary VMA was found. In fact, in samples resected from patients, who showed a reduction of VMA in the period of time preceding surgery (2–7 months), higher PPARγ expression was detected compared to those patients in which VMA increased. The authors hypothesized that PPARγ might play a role in the decrease of VMA and hence in the regression of early-onset NB. Thereafter, several studies addressed the potential role of endogenous or synthetic PPARγ ligands in counteracting NB cell growth.

5-*Deoxy*-$\Delta^{12,14}$-*prostaglandin* J_2 (15-deoxy-PGJ$_2$) is a naturally occurring downstream metabolite of PGD$_2$, that is produced by degradationof PGD$_2$ [59]. In contrast to classic prostaglandins, which act after binding to cell surface G-protein coupled receptors(GPCRs), 15-deoxy-PGJ$_2$ is a high-affinity endogenous ligand of PPARγ. A pro-apoptotic effect of 15-deoxy-PGJ$_2$ in SH-SY5Y NB cells, that was reverted by the caspase inhibitor Z-VAD, was reported by Rohn et al. [60]. A subsequent study confirmed that 15-deoxy-PGJ$_2$ was able to inhibit cell growth and to induce apoptosis via the activation of ERK2 in two additional NB cell lines (i.e., SK-N-SH and SK-N-MC). An increase of the expression of the pro-apoptotic proteins caspase-3, caspase-9, and Bax, together with the decrease of the anti-apoptotic protein Bcl-2, was also observed [61]. The PPARγ antagonist GW9662 reverted the effects of 15-deoxy-PGJ$_2$, including the activation of ERK2. The authors concluded that 15-deoxy-PGJ$_2$ induced apoptosis in a PPARγ-dependent manner through the activation of ERK pathway. Another study showed that the mechanism by which 15-deoxy-PGJ$_2$ arrests cell growth may vary depending on the content of lipids in the culture medium [62]. In particular, the delipidation of fetal calf serum, which removes known serum lipid mitogens including lysophosphatidic acid [63] and sphingosine 1-phosphate [64], potentiated the degree of 15-deoxy-PGJ$_2$-induced growth inhibition via PPARγ-dependent apoptosis in the NB cell line IMR-32. Conversely, growth inhibition in the presence of complete medium occurred through programmed cell death typeII (autophagy).

PPARγ-independent effects of 15-deoxy-PGJ$_2$ have been also described. Jung et al. reported that this PPARγ ligand was able to increase NGF-induced differentiation of PC-12 NB cells, as assessed by neurite extension and expression of neurofilament [65]. Pretreatment with the PPARγ antagonist bisphenol A diglycidyl either did not alter the differentiating activity of 15-deoxy-PGJ$_2$. The fact that PC-12 cells do not express PPARγ further supported the hypothesis that the biological effects elicited by 15-deoxy-PGJ$_2$ were not mediated by this receptor. Conversely, 15-deoxy-PGJ$_2$ enhanced NGF-induced p38 MAP kinase expression and phosphorylation as well as the activation of transcription factor AP-1, that on turn were counteracted by a specific inhibitor of p38 MAP kinase (SB203580). Altogether, these data suggested that the promoting effect of 15-deoxy-PGJ$_2$ on cell differentiation may be mediated by the activation of p38 MAP kinase in conjunction with the AP-1 signalling pathway.

Other studies addressed the role of *synthetic PPARγ ligands* in counteracting cell growth in NB. In the already mentioned work by Han et al., in which the presence of PPARγ in NB cells was described for the first time, the authors also demonstrated that the synthetic PPARγ agonist GW1929 induced the differentiation of LA-N-5 cells and inhibited cell proliferation [54]. A subsequent study of the same group showed that the prodifferentiating effect of GW1929 is mediated by PPARγ, because it was inhibited by the cotreatment with specific antagonists [66]. The antiproliferative effects of the TZDs ciglitazone, pioglitazone, troglitazone, and rosiglitazone in different NB cell lines (i.e., LAN-1, LAN-5, LS, IMR-32, SK-N-SH, SH-SY5Y) were

determined by Valentiner et al. [67]. In these cell lines, which express PPARγ, the four ligands were able to markedly inhibit cell growth at the highest doses that were used (10 and 100 μM). Ciglitazone determined the strongest inhibitory effect (more than 90% inhibition). The potency of the different PPARγ ligands was not related to the amount of expression of PPARγ in NB cell lines. Thus, the authors hypothesized that the effects of the molecules that were used seem to be independent of the amount of PPARγ protein in one particular cell line. Conversely, they concluded that the response to PPARγ ligands may rather depend on various cellular conditions, which are associated with the function of the receptor, such as its activation, translocation to the nucleus and binding to PPAR response elements (PPRE). The role played by PPARγ transactivation was confirmed by the finding that growth inhibition determined by 15-deoxy-PGJ$_2$ and ciglitazone in NB cells was counteracted by the repression of PPARγ transactivation via retinoblastoma protein overexpression [68]. Further studies investigated whether the inhibitory effect of TZDs on cell growth was mediated, at least partially, by a stimulatory effect on apoptosis. Kato et al. found that in NB-1 cells troglitazone induced PPARγ-dependent apoptosis [69]. Similar data were reported later on by Schultze et al. [70], who showed that in SHEP NB cells the pro-apoptotic effect of the death ligand TRAIL is reinforced by troglitazone. However, troglitazone-induced sensitization to TRAIL appeared to be PPARγ-independent, because it was achieved at concentrations that failed to activate PPARγ. Conversely, the authors highlighted the fact that troglitazone may induce apoptotic death by various PPARγ-independent mechanisms. In particular, troglitazone led to a marked downregulation of the antiapoptotic protein Survivin, as well as to an upregulation of the agonistic TRAIL receptor TRAIL-R2.

Overall, these data strongly indicate that PPARγ ligands are able to effectively counteract cell growth and to induce apoptosis in NB cells. Undoubtedly, the role of PPARγ in eliciting these responses would be further clarified by studies designed for instance to manipulate gene expression (i.e., by small interfering RNA or dominant negative strategies). To our knowledge, there are only two reports from one Korean group showing, in contrast to the current opinion, that a PPARγ agonist (i.e., rosiglitazone) protects NB (SH-SY5Y) cells against the neurotoxins acetaldehyde and 1-methyl-4-phenylpyridinium ion, through inhibition of apoptosis [71, 72].

3. DIFFERENTIAL PPARγ TRANSACTIVATION IN NEUROBLASTOMA CELL LINES WITH A DIFFERENT PHENOTYPE: RELATIONSHIP WITH THE RESPONSE TO ROSIGLITAZONE

NB is a phenotypically heterogeneous tumor, displaying cells of neuronal, melanocytic, or glial/schwannian lineage. This cellular heterogeneity is also present in vitro, where cells of neuroblastic (N) or stromal (S) type may be identified. It has been hypothesized that the sensitivity to PPARγ ligands may be, at least partially, dependent on the different cell phenotype. To this purpose, Servidei et al. examined the

response of 8 different NB cell lines with N (SH-SY5Y, LA-N-5, SMS-KCNR, SK-N-DZ), mixed (SK-N-FI, LA-N-1), or S (SH-EP1, SK-N-AS) phenotype to PPARγ agonists [73]. All the cell lines investigated expressed a functionally active PPARγ. 15-deoxy-PGJ$_2$ and rosiglitazone inhibited cell growth in all cell lines, and the sensitivity appeared to be more related to the cell phenotype than to PPARγ expression. In particular, the N type cells appeared the most sensitive to treatment. In this experimental setting, the cotreatment with PPARγ ligands and the RXR ligand 9-cis retinoic acid did not determine any synergistic effect on growth inhibition. The more evident response of N type cells to PPARγ ligands was in part related to their higher capability to undergo apoptosis, although only 15-deoxy-PGJ$_2$ appeared to effectively induce the apoptotic cascade in these cells. It has to be said that in this study some experimental observations (i.e., apoptosis and cell viability) were not performed in all the investigated NB cell lines.

In order to further clarify the mechanisms underlying the response of NB cells to PPARγ agonists, we compared the response of two cell lines (SH-SY5Y, N type, and SK-N-AS, S type) to rosiglitazone. In contrast to the above-mentioned findings, we observed that micromolar concentrations of rosiglitazone inhibited cell proliferation and reduced cell viability more effectively in SK-N-AS than in SH-SY5Y [74]. The PPARγ antagonist BADGE reverted the effect of rosiglitazone, thus suggesting a direct role of PPARγ in mediating the effects of this agonist on cell proliferation and viability. In addition, we found that SK-N-AS cells were more sensitive to rosiglitazone in terms of reduction of cell adhesion and invasiveness. The latter effect was in agreement with rosiglitazone-dependent reduced expression of matrix metalloproteinase-9 (MMP-9). In addition, rosiglitazone determined a trend toward increased expression levels of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). MMPs, which promote the invasion of extracellular matrix by tumoral cells, have been related to the progression of different tumors, including NB [75, 76]. In our study, we also addressed the possible role of rosiglitazone in inducing apoptosis. We demonstrated that micromolar concentrations of this molecule were able to induce caspase-3 activation in SK-N-AS, but not in SH-SY5Y (up to 50 μM). Therefore, all our data indicated that rosiglitazone played an effective antitumoral role in the S type SK-N-AS, yet not in the N type SH-SY5Y NB cells. Although our study was limited to two cell lines, this apparent prevalent effect on a particular cell phenotype may have clinical resonance. In fact, it is known that in NB, following cytotoxic therapy, the residual tumor often shows a reduction of the neuroblastic elements and the persistence of stromal components [77]. Hence, a molecule that appears to have S type NB cells as a preferential target might be of interest in the setting of residual disease.

A further aim of our study was to determine the reason underlying the peculiar sensitivity to rosiglitazone displayed by SK-N-AS cells. Both SK-N-AS and SH-SY5Y expressed a similar amount of PPARγ. However, in transient transfection experiments, in which a PPRE-thimidine kinase luciferase reporter plasmid was inserted, we observed that in SK-N-AS 20 μM rosiglitazone induced a near three-fold increase of the
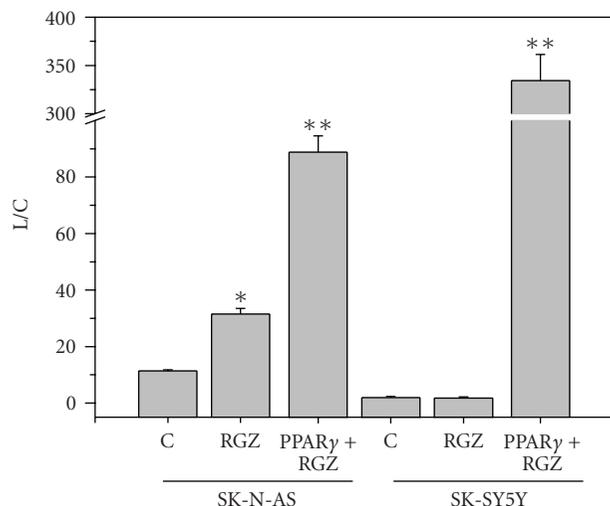
FIGURE 1: PPARγ transcriptional activity in control untreated NB cells (C), in cells treated with rosiglitazone (RGZ) (20 μM), and in cells transfected with PPARγ and treated with RGZ. L/C: peroxisome proliferator response element-n7$_3$-tk-luciferase reporter activity, normalized for CAT activity. * = $P < 0.05$ *versus* C. ** = $P < 0.05$ *versus* C, and *versus* RGZ-treated cells, in the absence of PPARγ transfection (from [74], modified).

reporter activity compared to untreated cells. Conversely, no effect was elicited in SH-SY5Y. Only when these cells were co-transfected with a human PPARγ expression plasmid, the response to rosiglitazone was present. These data indicated that the original lack of response showed by SH-SY5Y was due to a very low or absent transactivation potential of the endogenous PPARγ (Figure 1). The different efficacy of PPARγ as a transcriptional activator in the two cell lines might be hypothetically due to the presence of a PPARγ gene mutation. However, no mutation was found in the entire coding region of the gene. Conversely, we found that the amount of phosphorylated PPARγ was markedly lower in SK-N-AS than in SH-SY5Y cells (Figure 2). There is evidence that phosphorylation reduces the activity of the receptor [78]. Therefore, our conclusion was that the higher efficacy of rosiglitazone in SK-N-AS cells was due to a reduced phosphorylation status, hence to increased activity, of PPARγ. To our knowledge, this was the first demonstration that the response of NB cells to TZDs may be dependent on PPARγ transactivation.

4. PPARγ AGONISTS IN NEUROBLASTOMA XENOGRAFT MODELS

To our knowledge, no study on the in vivo effect of TZDs in neuroblastoma has been published so far. However, our very recent preliminary in vivo observations on CD-1 athymic nude mice, in which SK-N-AS cells were subcutaneously inoculated, appear to confirm our previous in vitro observations [74]. Rosiglitazone (150 mg/kg/day, in agreement with the average dose used in other in vivo studies addressing different tumors) was administered by gavage for 4 weeks.


FIGURE 2: Detection of total (anti-PPARγ antibody) and phosphorylated (anti-P-Ser antibody) PPARγ, by Western blot analysis after PPARγ immunoprecipitation (from [74], modified).

Tumoral growth was markedly reduced compared to control mice, treated with the vehicle alone. At the end of treatment, the weight of the tumor in rosiglitazone-treated animals was about 60% less than in control animals [Cellai et al; unpublished data]. An extensive molecular characterization of tumor specimens is currently ongoing, in order to elucidate the mechanisms underlying the growth inhibitory effect of rosiglitazone observed in vivo in our xenograft model.

5. CONCLUSIONS

In the last few years in vitro studies have shown that PPARγ agonists may inhibit NB cell growth by stimulating cell differentiation and/or by inducing apoptosis. The different molecules that have been tested have generally produced similar results. However, the mode of action may change depending on the agonist and/or on the different cell line used. In addition, both PPARγ-dependent as well as PPARγ-independent effects have been described. Our recent data suggest that PPARγ transactivation, determined at least in part by the phosphorylation status of the receptor, may play an important role in determining the response of NB cells to PPARγ agonists. However, the exact mechanisms of action and the possibility to predict the success or failure of the treatment of NB with these molecules are, at this time, matter of further in vitro as well as in vivo research.

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Review Article

PPAR Gamma Activators: Off-Target Against Glioma Cell Migration and Brain Invasion

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Today, there is increasing evidence that PPARγ agonists, including thiazolidinediones (TDZs) and nonthiazolidinediones, block the motility and invasiveness of glioma cells and other highly migratory tumor entities. However, the mechanism(s) by which PPARγ activators mediate their antimigratory and anti-invasive properties remains elusive. This letter gives a short review on the debate and adds to the current knowledge by applying a PPARγ inactive derivative of the TDZ troglitazone (Rezulin) which potently counteracts experimental glioma progression in a PPARγ independent manner.

Gliomas are the most common primary tumors in the central nervous system, with glioblastomas as the most malignant entity [1]. Despite multimodal therapy regimens including neurosurgical resection, radio- and polychemotherapy, the prognosis of glioma patients remains poor. Less than 3% of affected patients survive more than five years after diagnosis [2]. Rapid proliferation, tumor-induced neurodegeneration, and brain edema [3] as well as diffuse brain invasion are pathological hallmarks of these tumors and are likely to determine unfavorable prognosis. Because local invasion of neoplastic cells into the surrounding brain is perhaps the most important aspect in the biology of gliomas that preclude successful treatment, pharmacological inhibition of glioma cell migration and brain invasion is considered as a highly promising strategy for adjuvant glioma therapy.

Today, there is increasing evidence that PPARγ agonists, including thiazolidinediones (TDZs) and nonthiazolidinediones, block the motility and invasiveness of glioma cells and other highly migratory tumor entities. GW7845, an investigational non-TDZ PPARγ ligand, binds and activates human PPARγ at low nanomolar concentrations and thus possesses a higher potency than TDZs such as pioglitazone (Actos), troglitazone (Rezulin), rosiglitazone (Avandia), and the experimental PPARγ agonist ciglitazone, respectively, which require submicromolar doses [4]. Grommes et al. [5] demonstrated that 30μM concentrations of GW7845 reduced the viability of rat (C6) and human glioma cells (U-87 MG, A172), which could be attributed to a G_1 cell cycle arrest and increased cell death. Besides its antiproliferative and cytotoxic properties, the authors demonstrated for the first time that GW7845 counteracts migration and invasion of C6 rat glioma cells in vitro (spheroid outgrowth, Boyden chamber assay). A subsequent study revealed that the FDA-approved TDZ pioglitazone exhibits antiglioma properties similar to GW7845 [6]. Alike GW7845, micromolar doses of pioglitazone (30μM) counteract C6 rat glioma cell invasiveness in vitro (Boyden chamber assay). In this study, Grommes et al. [6] demonstrated profound in vivo antiglioma properties of pioglitazone. Following C6 glioma cell implantation into the striata of adult rats, oral or intracerebral drug application effectively decelerated glioma progression, resulting in an improved clinical

outcome and 80% reduction of tumor volume at 3 weeks after tumor implantation. Immunohistochemical analyses of pioglitazone-treated animals revealed that protein levels of MMP-9 (*matrix metalloproteinase 9*), which has shown to be intimately involved in glioma migration and invasion [7], were substantially reduced in the bulk tumor and the tumor margins. However, the data regarding the antiglioma properties of pioglitazone are somewhat contradictory in a mouse glioma model (GL261 glioma cells, C57Bl/6 mice). Grommes et al. demonstrated that oral application of pioglitazone increased the number of surviving animals after 30 days of treatment. By employing the same model, Spagnolo and coworkers observed no effect on survival following oral drug application, while intracerebral injection of pioglitazone increased the mean survival time [8]. We have recently shown that the TDZ troglitazone reduces the viability and proliferation of rat (F98), mouse (SMA-560), and human (U-87 MG) glioma cells slightly but significantly more potent than the remaining TZDs tested (troglitazone > pioglitazone > rosiglitazone > ciglitazone) [9]. By employing an ex vivo glioma invasion model [10], troglitazone effectively blocked glioma progression and brain invasion and consistent with the in vitro data presented by Grommes and coworkers, we confirmed that troglitazone ($30\,\mu$M) antagonized rat F98 glioma cell migration (scratch wound healing, Boyden chamber assay).

Inhibition of cell motility and invasiveness by PPARγ activators has also been described for other neoplastic cells and thus appears not to be restricted to glioma. Liu et al. [14] showed that GW7845 ($5\,\mu$M) as well as the FDA-approved TDZs pioglitazone and rosiglitazone (both $25\,\mu$M) inhibits the invasive properties of human MDA-MB-231 breast cancer cells. In this study, treatment with PPARγ agonists was associated with increased *tissue inhibitor of matrix metalloproteinase 1* (TIMP-1) mRNA and protein levels, which are likely to contribute to the anti-invasive effects observed. Recently, Yang et al. [15] demonstrated that troglitazone (10–$30\,\mu$M) inhibits migration and invasiveness of a human ovarian carcinoma ES-2 cells. Anti-invasive properties were also shown for the TDZ ciglitazone, although with a lower potency. Extended analyses by Yang et al. revealed that troglitazone ($20\,\mu$M) inhibits focal adhesion formation associated with reduced focal adhesion kinase (FAK) activity. FAK, an ubiquitously expressed nonreceptor tyrosine kinase, has shown to be a vitally important regulator of cancer cell migration and invasion. FAK is highly expressed in many tumor entities and activated by autophosphorylation [16], which has shown to be reduced by more than 80% in troglitazone-treated ES-2 cells [15]. Based on these data, the authors concluded that troglitazone may inhibit ES-2 cell migration and invasion by preventing FAK activation. Concordantly, inhibition of FAK kinase activity by the investigational small molecule TAE226 reduced the invasive properties of human U-87 MG, U251, and LN18 glioma cells by more than 50% [17], suggesting that FAK activation decisively promotes migration and invasion also of glioma cells. In all, these data demonstrate that PPARγ activators belonging to different chemical classes effectively diminish glioma progression in vitro, ex vivo, and in vivo

[5, 6, 9], which occurs at least in part by the inhibition of glioma cell migration and invasiveness.

Given the fact that cancer cell migration and invasion are highly complex processes [18], the mechanism(s) by which PPARγ agonists exert their antimigratory and anti-invasive properties requires further investigation. Besides MMP-9, TIMP-1, and FAK, which have been shown to be involved in the antimigratory activities of PPARγ agonists, we recently demonstrated that already low doses of troglitazone block *transforming growth factor beta* (TGF-β) release [9], a cytokine which plays a pivotal role in glioma cell motility [19]. Several in vitro studies revealed that exogenously added TGF-β_1 and TGF-β_2 elicit a strong stimulation of migration in a variety of glioma cells [20–23], while TGF-β gene silencing has shown to reduce glioma cell motility and invasiveness [24]. In agreement with these findings, inhibition of TGF-β signaling by the investigational type I TGF-β receptor antagonist, SB-431542 reduced the invasive properties of human D-54 MG and rat F98 glioma cells by approximately 70% [9, 25]. The role of TGF-β as molecular target for glioma therapy has been facilitated by studies using surgically resected glioma tissues, which revealed an intriguing correlation between tumor grade and the expression of TGF-β ligands and their corresponding receptors I and II. High-grade gliomas express high levels of TGF-βRI, TGF-βRII, and TGF-β ligands, while the expression levels of these molecules have been shown to be weak in low-grade gliomas and normal brain tissue [26–28]. A comprehensive transcriptome-wide study by Demuth et al. [29] using 111 glial tumor samples and 24 normal brain specimens identified the TGF-β signaling pathway to be predominantly enriched in glial tumors compared to normal brain. In all, these data implicate that glioma cells release TGF-β ligands at high doses and fortify their promigratory and proinvasive properties in an autocrine manner, thus promoting glioma progression. Given the fact that $10\,\mu$M doses of troglitazone allay TGF-β release of glioma cells (F98, SMA-560, U-87 MG) by more than 50% [9], we hypothesized that the abrogation of glioma cell motility and invasiveness by troglitazone and other PPARγ activators is primarily driven by the inhibition of TGF-β signaling and thus, troglitazone and related compounds may be considered for adjuvant glioma therapy to counteract TGF-β-mediated brain invasion.

However, the mechanism(s) by which PPARγ activators mediate their antimigratory and anti-invasive properties remains elusive. We have shown that PPARγ inhibition by the investigational antagonist GW9662, either alone or in combination with troglitazone, does not affect rat F98 glioma cell invasiveness in a Boyden chamber assay, suggesting that the effects observed are not mediated by PPARγ [9]. Simultaneously, Yang and coworkers [15] have shown that PPARγ knockdown by siRNA did not counteract the anti-invasive features of troglitazone using human ovarian carcinoma ES-2 cells, underscoring the idea that the PPARγ agonists counteract cancer cell migration by a yet unknown off-target activity. To validate these preliminary findings, we analyzed the effects of a troglitazone derivative, Δ2-troglitazone, which has been shown to be PPARγ-inactive
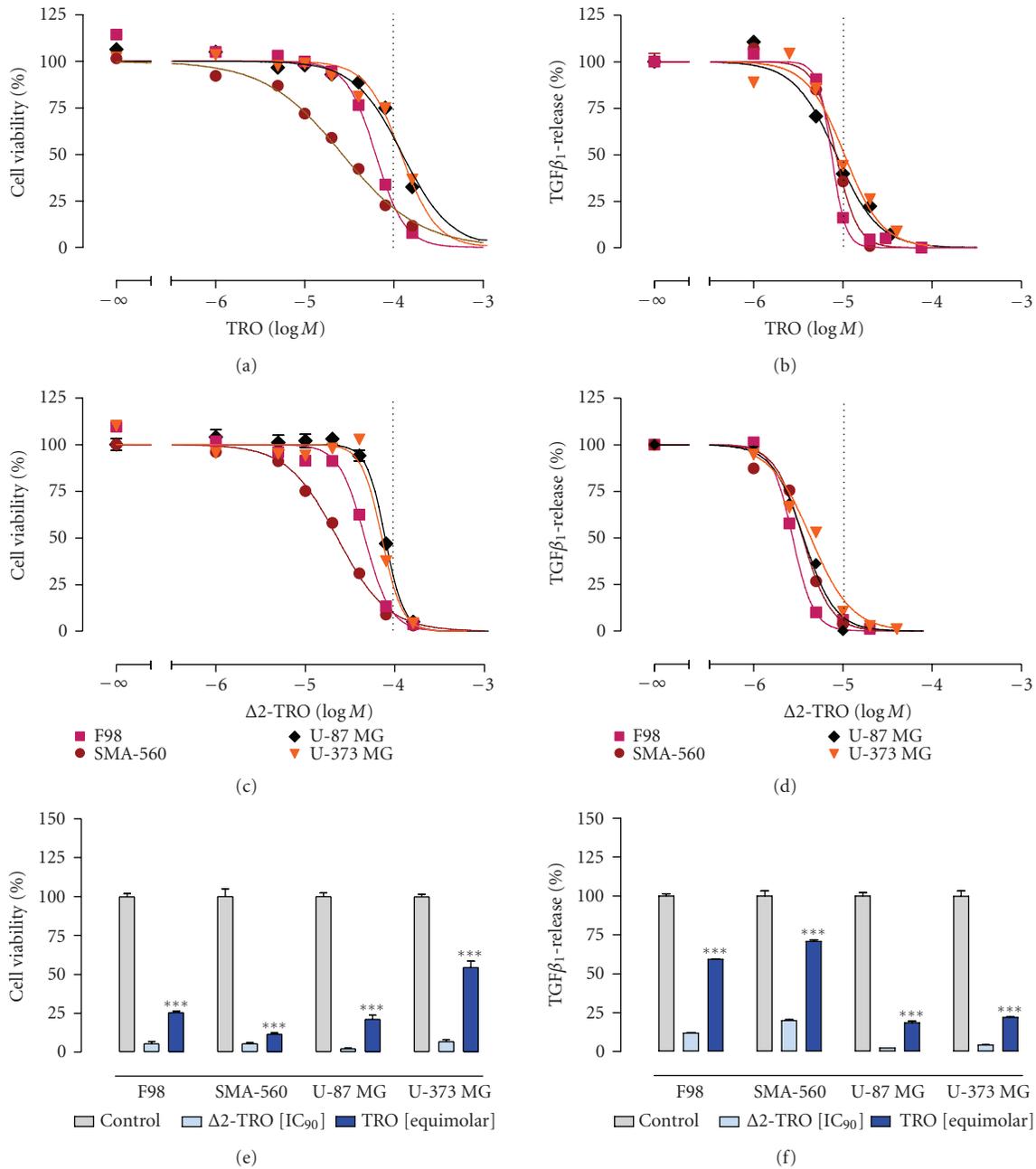
FIGURE 1: *Troglitazone (TRO) and the PPARγ inactive Δ2-troglitazone (Δ2-TRO) reduce glioma cell viability and TGF-β_1 release.* Δ2-TRO was synthesized as previously described in [11]. (a), (c) Concentration-dependent inhibition of glioma cell viability by TRO (a) or Δ2-TRO (c) in the indicated cell lines are given as mean ± SEM percentage relative to time- and solvent-matched controls. Cell viability assays (MTT assay, 96 hours) were performed as described earlier [12, 13]. Inhibitory concentrations IC_{50} and IC_{90}, defined as concentrations shown to inhibit tumor cell viability by 50% or 90%, respectively, were determined by nonlinear regression data analysis: TRO: F98 (62 μM, 166 μM), SMA-560 (26 μM, 407 μM), U-87 MG (120 μM, 324 μM), and U-373 MG (123 μM, 331 μM); Δ2-TRO: F98 (46 μM, 95 μM), SMA-560 (23 μM, 93 μM), U-87 MG (78 μM, 132 μM), and U-373 MG (71 μM, 126 μM). *Troglitazone and the PPARγ inactive Δ2-troglitazone reduce TGF-β_1 release at low micromolar doses*: (b), (d) quantification of TGF-β_1 release by F98, SMA-560, U87-MG, and U-373 MG glioma cell culture supernatants following TRO (b) or Δ2-TRO (d) treatment for 48 hours. TGF-β_1 protein levels in glioma cell culture supernatants were determined as described in [9] using the mouse/rat/porcine/canine or the human quantikine TGF-β_1 ELISA Kit (R&D Systems, Minneapolis, Minn, USA), respectively. Each experiment was repeated at least 3 times ($n \geq 3$). Drug concentrations shown to inhibit TGF-β_1 release by 50% or 90%, respectively, were determined by nonlinear regression data analysis: TRO: F98 (7 μM, 11 μM), SMA-560 (8 μM, 15 μM), U-87 MG (8 μM, 28 μM), and U-373 MG (10 μM, 30 μM); Δ2-TRO:F98 (3 μM, 5 μM), SMA-560 (3 μM, 8 μM), U-87 MG (4 μM, 14 μM), and U-373 MG (4 μM, 14 μM). *Δ2-Troglitazone displays higher potencies than troglitazone*. Using IC_{90} concentrations of Δ2-TRO and equimolar concentrations of TRO, the PPARγ inactive Δ2-TRO displays a significantly stronger effect in both experimental paradigms (*** = $P < .001$, t-test) (e), (f).
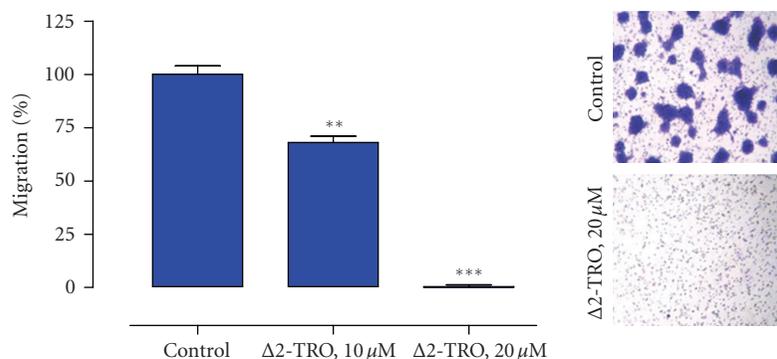
FIGURE 2: *The PPARγ inactive Δ 2-troglitazone (Δ2-TRO) inhibits glioma cell migration.* The glioma cell migration assay (Boyden chamber; QCM-FN Migration Assay, Chemicon, Temecula, Calif, USA) was performed as described recently [9]. Briefly, F98 rat glioma cells, pretreated with the test compound or solvent for 24 hours, were transferred into each Boyden chamber. After 24 hours of incubation, cells which migrated through the fibronectin-coated chamber membranes (8 micron pore diameter) were quantified according to the manufacturer's protocol. Experiments were repeated 3 times ($n = 3$). (** $= P < .01$; *** $= P < .001$; t-test). Right panel: representative microphotographs of F98 glioma cells which migrated though the fibronectin-coated chamber membranes after treatment with Δ2-TRO ($20\,\mu$M) or solvent only.

[11, 31, 32]. In case the antiglioma properties of troglitazone are solely or predominantly due to PPARγ activation, Δ2-troglitazone should display no or a considerably lower inhibitory potency on glioma cell viability than troglitazone. Initially, concentration-dependent inhibition of glioma cell viability by troglitazone and Δ2-troglitazone was investigated using glioma cell lines derived from mouse (SMA-560), rat (F98), and human (U-87 MG, U-373 MG). As shown by MTT assay, both compounds inhibited glioma cell growth in a concentration-dependent manner with similar potencies (Figures 1(a), 1(c)). Even though numerous PPARγ-dependent mechanisms have been identified (for review see Tatenhorst et al., this issue), these data suggest that PPARγ activation is not an imperative prerequisite for the inhibition of glioma cell viability in vitro, which is in line with previous studies using human PC-3 and LNCaP prostate cancer and human A549 lung carcinoma cells [11, 31]. Next, we analyzed the reduction of glioma cell viability using IC_{90} doses of Δ2-troglitazone and equimolar doses of troglitazone. In all four cell lines tested, Δ2-troglitazone displays a slightly but significantly higher potency compared with troglitazone. However, with IC_{90} doses ranging from 93 μM (SMA-560) to 132 μM (U-87 MG), the antiproliferative properties of Δ2-troglitazone can be regarded as moderate.

Next, we analyzed the effects of troglitazone and Δ2-troglitazone on TGF-β release by glioma cells. Hjelmeland et al. [25] have shown that secretion of activated TGF-β_1 is a common attribute of glioma cells (U-87 MG, U-373 MG, D-54 MG, D-270 MG, D-423 MG, D-538 MG), while simultaneous release of TGF-β_2 was found only sporadically (D-54 MG, U-373 MG, D-423 MG). In accordance with these findings, quantification of TGF-β_1 and TGF-β_2 transcript levels by real-time PCR revealed that U-373 MG and SMA-560 glioma cells express both TGF-β_1 and TGF-β_2, respectively, while TGF-β_1 is clearly the predominant isoform in F98 and U-87 MG glioma cells (data not shown). Repeated quantification ($n \geq 7$) of absolute TGF-β_1 levels following cultivation of glioma cells for 48 hours in serum-free medium revealed that all cell lines investigated secrete TGF-β_1 (F98: 8.45 ± 1.59 ng/mL; SMA-560: 2.7 ± 0.54 ng/mL; U-87 MG: 2.55 ± 0.68 ng/mL, U-373 MG: 0.43 ± 0.08 ng/mL), while both troglitazone and Δ2-troglitazone inhibit TGF-β_1 release in a dose-dependent manner (Figures 1(b), 1(d)). The finding that Δ2-troglitazone counteracts TGF-β_1 release indicates that this effect is not PPARγ dependent. Again, Δ2-troglitazone displays a significantly higher potency as compared with troglitazone (Figure 1(f)). In case of Δ2-troglitazone, 90% inhibition of TGF-β_1 release was found at concentrations ranging from 5 μM (F98) to 14 μM (U-87 MG, U-373 MG), whereas troglitazone required 11 μM (F98) to 30 μM (U-373 MG) to achieve the same effects. Strikingly, troglitazone as well as Δ2-troglitazone is approximately 10 fold more potent inhibitors of TGF-β_1 release than of glioma cell proliferation, suggesting that both effects may not be essentially interlinked.

In agreement with the finding that TGF-β_1 promotes glioma cell migration and brain invasion, treatment of glioma cells with micromolar doses of Δ2-troglitazone effectively blocks their migrative properties (Figure 2). Already 10 μM doses of Δ2-troglitazone inhibit F98 glioma cell migration in a Boyden chamber assay, while migration was completely suppressed at 20 μM. An intriguing question is whether inhibition of glioma cell migration alone is sufficient to counteract glioma progression. To address this issue we employed rat organotypic hippocampal brain slice cultures (OHSCs) to monitor glioma progression and brain invasion in the organotypic brain environment [12]. Here, eGFP-labelled F98 glioma cells were implanted into the entorhinal cortex of OHSCs (Figure 3(a)). The tumor infiltration area was quantified up to 12 days by fluorescence microscopy. A continuous increase of the bulk tumor mass was observed in solvent-matched control experiments at all time periods. 12 days after glioma cell implantation, the tumor infiltration area increased approximately 4.5 fold compared to the initial tumor size at day 1 after implantation (Figures 3(b), 3(c)). In contrast, the tumor infiltration size

(a)

(b)

(c)
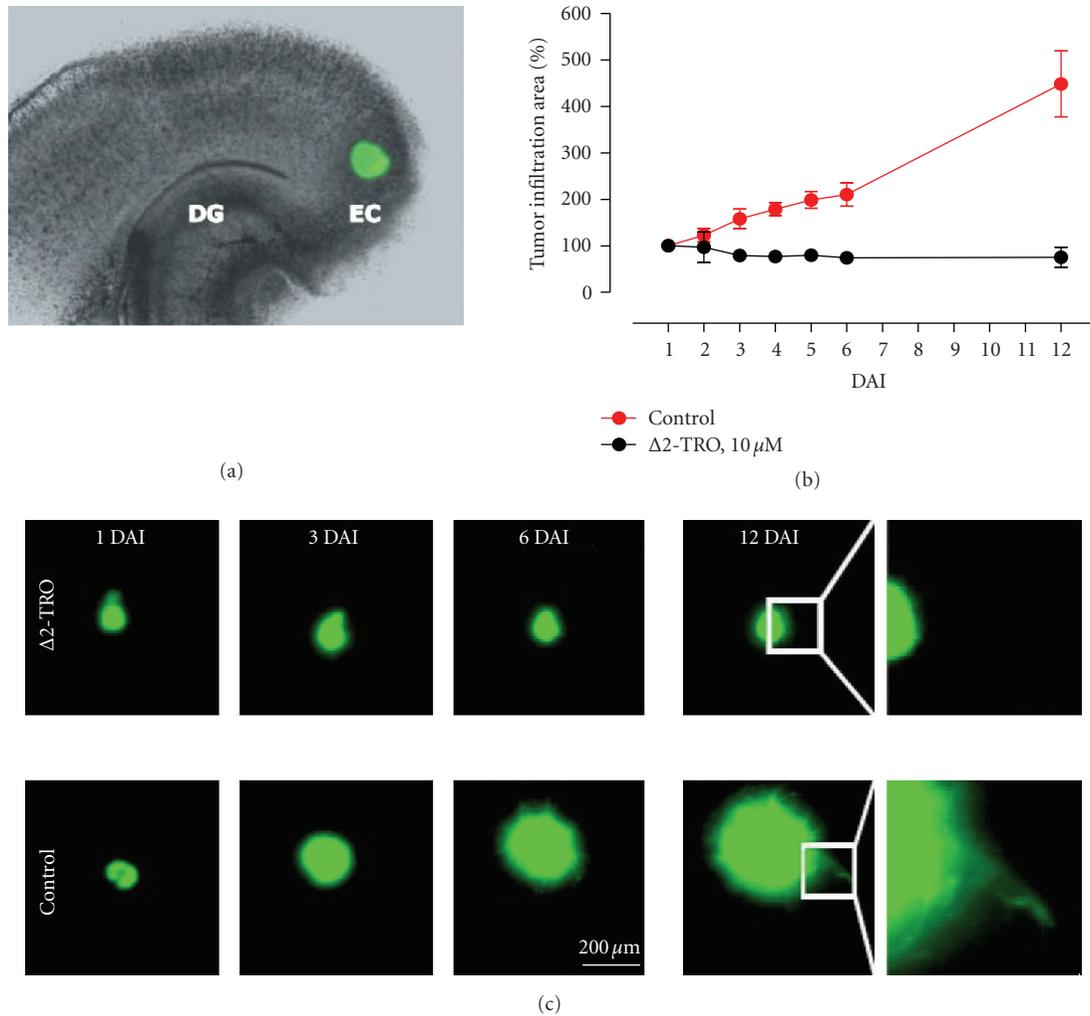
FIGURE 3: *Δ2-Troglitazone inhibits glioma progression in an organotypic glioma transplantation model.* (a) Organotypic hippocampal glioma invasion assay was performed as described earlier [10, 12, 30]. In brief, enhanced green fluorescent protein (eGFP) positive F98 rat glioma cells were transplanted into the entorhinal cortex of organotypic rat brain slice cultures one day after preparation. DAI = days after implantation. DG = dentate gyrus. EC = entorhinal cortex. (b) Tumor progression was monitored by fluorescent microscopy over the time course of 12 days. Quantification of the tumor infiltration area at day 1 to day 12 after transplantation derived from 3 independent experiments is shown. For each experiment, the tumor infiltration area at DAI 1 was defined as 100%. Data are given as mean ± SD percentage. At DAI 12, the tumor infiltration area significantly increased to 448 ± 71 % ($P = .002$, t-test) in solvent-matched controls but remained unchanged following Δ2-TRO treatment (75 ± 22 %; $P = .18$, t-test). Starting from DAI 2, differences in tumor progression (TRO versus Δ2-TRO) reached statistical significance ($P < .01$, t-test) (c) A continuous increase of the bulk tumor masses was observed in solvent-matched controls while $10\,\mu$M concentrations of Δ2-TRO effectively blocked tumor progression. Right column: magnification of the indicated border area between bulk tumor mass and rat brain tissue. In controls, F98 glioma cells have diffusely migrated into the adjacent brain parenchyma, while a sharp tumor border was observed following Δ2-TRO treatment (scale bar: $200\,\mu$m).

remained stable over the period of 12 days after treatment with $10\,\mu$M Δ2-troglitazone. This finding indicates that Δ2-troglitazone is not able to reduce existing tumor masses, but effectively inhibits tumor progression and brain invasion in an organotypic environment. Given the fact that $10\,\mu$M doses of Δ2-troglitazone significantly affect TGF-β_1 release (Figure 1(d)) and glioma cell motility (Figure 2) but not glioma cell viability (Figure 1(c)), these data suggest that glioma cell migration is an essential requirement for glioma progression in a system closely resembling extracellular matrix environment present in the brain.

TGF-β antagonism is considered as a therapeutic strategy including the development of antisense regimens, inhibition of pro-TGF-β processing, scavenging of TGF-β by the TGF-β-binding proteoglycan decorin, and blocking of TGF-β receptor I kinase activity [33]. The finding that troglitazone and its derivative Δ2-troglitazone effectively inhibit TGF-β release suggests readily available PPARγ activators and structurally related PPARγ inactive compounds as candidate drugs for adjuvant glioma therapy. Besides its promigratory and proinvasive activities, TGF-β is considered as one of the most potent immunosuppressive factors released by gliomas

allowing glioma cells to escape from immune surveillance [34, 35]. Friese et al. [24] demonstrated that combined TGF-β_1 and TGF-β_2 knock down in human LNT-229 glioma cells results in a loss of tumorigenicity when xenografted into CD1 nude mice, and natural killer cells isolated from these animals show an activated phenotype. More than 10 years ago, Ständer et al. [36] have shown that inhibition of TGF-β signaling by decorin increases the number of B and T cells (CD45+), T helper cells (CD4+), cytotoxic T cells (CD8+), and, most prominently, of activated T cells (CD25+) infiltrating the tumor in an intracerebral C6 rat glioma model. By employing an SMA-560 mouse glioma model, Tran et al. [37] have shown that inhibition of TGF-β signaling by the TGF-β RI kinase inhibitor SX-007 increased T-cell (CD3+) infiltration into the tumor. Due to the fact that inhibition of TGF-β signaling has been shown to enhance antiglioma immune responses in vivo [24, 36, 37] it appears likely that troglitazone, inhibiting TGF-β_1 release at clinically achievable doses [9, 38], restores immune surveillance. However, the yet-unknown protein/proteins mediating the inhibition of glioma progression by troglitazone and Δ2-troglitazone remain(s) to be identified and may represent future targets for structure-relationship studies. Moreover, PPARγ inactive derivatives of known PPARγ agonists which retain their propensity to counteract glioma progression might be further developed to minimize potential PPARγ mediated side effects in glioma patients.

ACKNOWLEDGMENTS

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Review Article

PPAR Ligands for Cancer Chemoprevention

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Peroxisome proliferators-activated receptors (PPARs) that are members of the nuclear receptor superfamily have three different isoforms: PPARα, PPARδ, and PPARγ. PPARs are ligand-activated transcription factors, and they are implicated in tumor progression, differentiation, and apoptosis. Activation of PPAR isoforms lead to both anticarcinogenesis and anti-inflammatory effect. It has so far identified many PPAR ligands including chemical composition and natural occurring. PPAR ligands are reported to activate PPAR signaling and exert cancer prevention and treatment in vitro and/or in vivo studies. Although the effects depend on the isoforms and the types of ligands, biological modulatory activities of PPARs in carcinogenesis and disease progression are attracted for control or combat cancer development. This short review summarizes currently available data on the role of PPAR ligands in carcinogenesis.

1. INTRODUCTION

Peroxisome proliferators-activated receptors (PPARs) are member of the nuclear hormone receptor superfamily that were initially characterized as molecules that mediated the proliferation of peroxisomes in rodent liver parenchymal cells in response to the hypolipidemic drug clofibrate [1]. Subsequently, PPARs have been shown to regulate the expression of genes involved in a variety of biological processes, including lipid metabolism and insulin sensitivity [2, 3]. Three isotypes of PPAR exist; PPARα, PPABβ/δ or simply δ, and PPARγ which are known and they are encoded by three separate genes and display distinctly different tissue distributions and functions. PPARα regulates numerous aspects of fatty acid catabolism, where as PPARγ controls adipocyte differentiation, systemic glucose levels, and lipid homeostasis [4, 5]. PPARδ is involved in development, embryo implantation, myelination of the corpus callosum, lipid metabolism, and epidermal cell proliferation [6]. The PPARs are ligand-dependent transcription factors that regulate target genes expression by binding to characteristic DNA sequences termed peroxisome proliferators response element (PPREs) located in the 5′-flanking region of target genes [7, 8]. Each receptor binds to its PPRE as a heterodimer with the receptor for 9-cis retinoic acid, the retinoid X receptor (RXR) (Figure 1). Upon binding a ligand, the conformation

of a PPAR is altered and stabilized such that a binding cleft is created, and recruitment of transcriptional coactivators occurs. The result is an increase in gene transcription, therefore PPARs are able to regulate such divers effects as cell proliferation, differentiation, or apoptosis.

2. PPARα LIGANDS AND CARCINOGENESIS

PPARα is the first member of this nuclear receptor subclass to be cloned [9]. PPARα is expressed preferentially in the liver [10] and tissues with high fatty acid catabolism, such as the kidney, heart, skeletal muscle, and brown fat [11–13]. The PPARα isotype is the cellular target for leukotriene B4 (LTB4) fibrates such as bezafibrate and fenofibrate, which are hypolipidemic drugs widely used for reducing triglyceride levels, a risk of cardiovascular diseases. Several studies have established a link between PPARα activation and epidermal differentiation. Fibrates induce differentiation and inhibit proliferation in normal and hyperproliferating mouse epidermis and regulate apoptosis, but are inactive in PPARα-deficient mice [14, 15]. Farnesol also stimulates PPARα-dependent differentiation in epidermal keratinocytes [16]. Topical PPARα ligands have weak preventive effects on tumor promotion in mouse skin, despite upregulation of PPARα in untreated tumors compared with normal epidermis [17]. These observations suggest that the use of
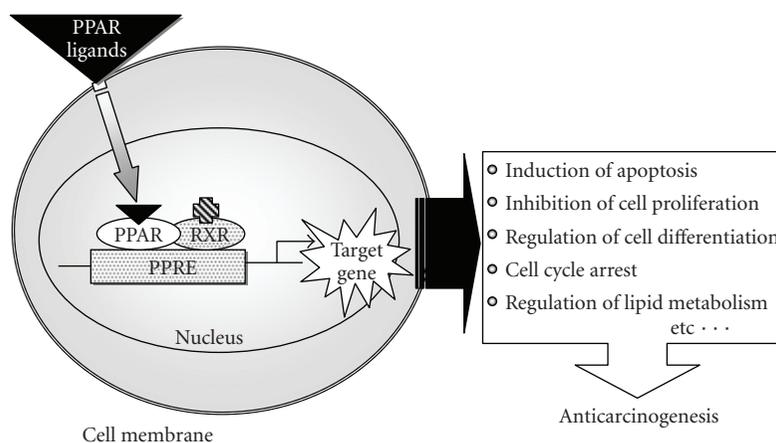
Figure 1: PPAR activation pathway and its target genes.

PPARα activators may have chemopreventive properties in skin carcinogenesis. PPARα expression is also upregulated in human prostate adenocarcinomas [18]. In addition, PPARα ligands suppress the growth of several cancer lines, including colon [19], endometrial [20], and breast [21] in vivo or in vitro. PPARα ligands are able to suppress the metastatic potential of melanoma cells in vivo and in vitro [22, 23]. More recently, a PPARα ligand WY14643 suppresses both endothelial cell proliferation and tumorigenesis in a PPARα-dependent manner [24]. These data suggest that certain PPARα ligands may act as antitumor agents, although the exact mechanisms remain unclear. PPARα activation has been associated with both anti and proinflammatory actions in rodents. PPARα ligands reduce expression of inflammatory markers [25]. In contrast, the expression of the inflammatory mediator cyclooxygenase (COX)-2 in human breast and colon cancer cells is upregulated by PPARα ligands [26]. The increased COX-2 expression is known to link to the risk of epithelial malignancies [27]. These findings indicate that PPARα ligands may be interesting candidates for the chemoprevention of several types of cancers, but we should consider negative face of influence of PPARα ligands on cancer development.

3. PPARδ LIGANDS AND CARCINOGENESIS

A number of reports have described a variety of biological functions of the PPARα and γ isotypes. These two isotypes also have clinical significance in the treatment of dyslipidemia and type II diabetes mellitus [28]. In contrast, less is known about the physiological role of the PPARδ isoform, although there is some evidence supporting its involvement in embryo implantation and development [6, 29], epidermal maturation and wound healing [30], and regulation of fatty acid metabolism [31]. Recently, the effect of PPARδ function on colon carcinogenesis has been reported. However, the role of PPARδ in colon cancer is still unclear, as there are data suggesting that it either inhibits or promotes colon carcinogenesis. PPARδ expression is increased in colon tumor cells with a mutant *Apc (adenomatous polyposis coli)*

allele (min) [32]. The number of polyps was the same among the multiple intestinal neoplasia (Min) mice that were $Ppard^{-/-}$, $Ppard^{+/-}$, or $Ppard^{+/+}$. These findings suggest that PPARδ is not essential for colon carcinogenesis, but PPARδ may affect size and/or growth of polyps [29]. The most striking results were provided by a study demonstrating that in PPARδ deficient ($Ppard^{-/-}$) mice, both *Min* mutants and those with chemically induced cancers, colon polyp formation was significantly greater in those nullizygous for PPARδ [33]. These results suggest that PPARδ attenuates colon carcinogenesis. On the other hand, the following observations strongly suggest that PPARδ enhances colon cancer formation. PPARδ was elevated in colon cancer cells and was repressed by *APC* gene via the β-catenin/Tcf-4 response elements in its promoter [32]. Genetic disruption of PPARδ decreases the tumorigenicity of human colon cancer cells [34]. Nitric oxide donating aspirin is reported to suppress intestinal tumors in *Min* mice and downregulates the expression of PPARδ and enhance apoptosis and perhaps atypical cell death [35]. This suggests that PPARδ contributes to intestinal carcinogenesis.

GW501516 was shown to be a PPARδ subtype-selective ligand using combinatorial chemistry and structure-based drug design [36]. There are some reports describing the effects of PPARδ ligand on colon carcinogenesis. Exposure of $APC^{min/+}$ mice to the GW501516 resulted in activation of PPARδ and significant acceleration of intestinal adenoma growth [37]. Furthermore, PPARδ activation by PPARδ ligand promotes tumor growth by inhibiting epithelial tumor cell apoptosis through activation of a VEGF autocrine signaling loop in $APC^{min/+}$ mice [38]. GW501516 stimulates proliferation of human breast, prostate, and hepatocellular carcinoma cells [39, 40]. In a mouse mammary tumorigenesis model, GW501516 activates 3-phospholinositide-dependent protein kinase-1 that is oncogenic when expressed in mammary ductal cells, and leads to accelerated tumor formation [41]. From these findings, PPARδ selective-ligand tends to exert enhancing effects on carcinogenesis, while its antagonists are expected to prevention and/or treatment of cancer.
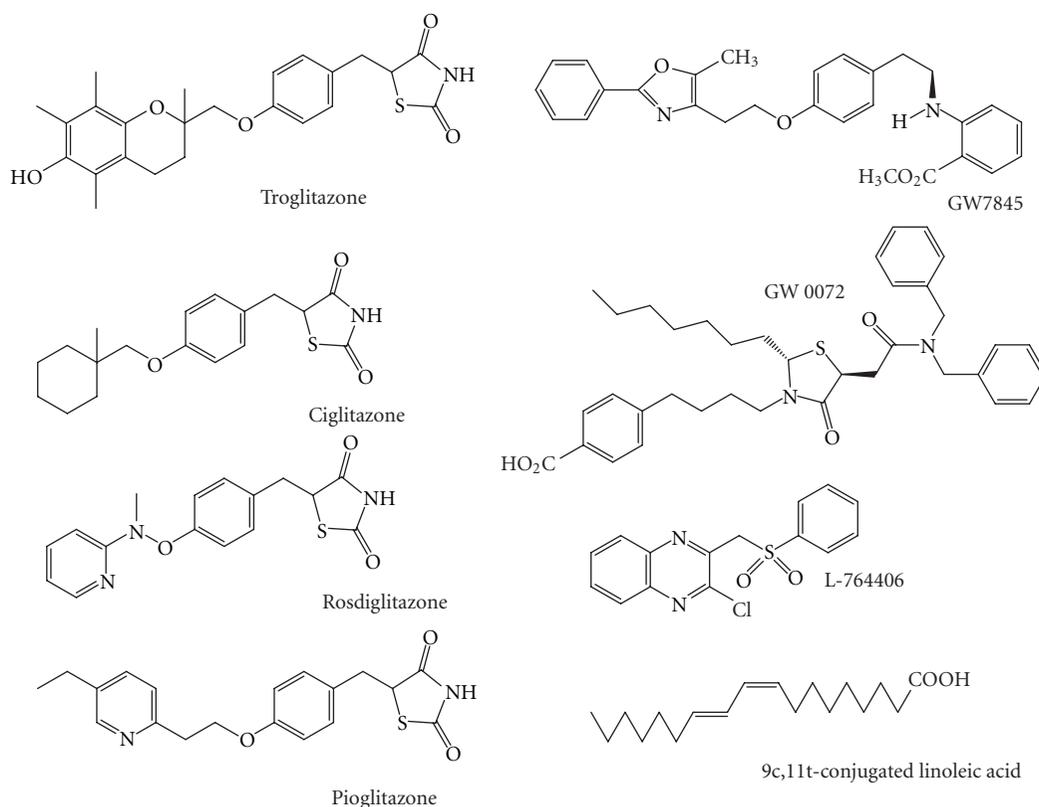
Figure 2: Synthetic and naturally occurring ligands for PPARγ.

4. PPARγ LIGANDS AND CARCINOGENESIS

PPARγ plays an important role in the regulation of proliferation and differentiation of several cell types. PPARγ is known to be expressed in various organs, including adipose tissue [42], mammary glands [43], small intestine [44], lung [45], colon [44], and stomach [46], and is also upregulated in various types of cancer cells.

This receptor has the ability to bind a variety of small lipophilic compounds derived from both metabolism and nutrition. These ligands, in turn, direct cofactor recruitment to PPARγ, regulating the transcription of genes in a variety of complex metabolic pathways. Several specific ligands (Figure 2) have been identified, such as the thiazolidinediones (including pioglitazone, rosiglitazone, and troglitazone), naturally occurring lipid, polyunsaturated fatty acids (PUFA) (including arachidonic, oleic, and linoleic acid) and the cyclopentenone prostaglandin (PG) 15-deoxy Delta$_{12,14}$-PGJ$_2$, a metabolite of PGD$_2$. PPARγ ligands have been reported to induce cell differentiation and apoptosis in several types of cancer [47–51], suggesting potential application as anticancer agents. Furthermore, some reports recently suggested that PPARγ ligands can be used as chemopreventive agents for colon, breast, and tongue carcinogenesis [52–54].

The most widely used synthetic agents belong to the thiazolidinedione class of antidiabetic drugs (also referred to as glitazones). These include ciglitazone, troglitazone, pioglitazone, rosiglitazone, and LY171.833. Pioglitazone, rosiglitazone, and troglitazone have already been used clinically to treat type 2 diabetes, making use of the ability of synthetic PPARγ ligands to sensitize insulin and to lower blood glucose concentration. Recent evidence indicates that certain thiazolidinedione members, especially troglitazone and ciglitazone, exhibit moderate anitproliferative activities against epithelial-derived human cancer cell lines, including those of prostate [55], breast [56], colon [57], thyroid [51], lung [58], and pituitary carcinoma [50]. PPARγ is known to be expressed in a variety of cancer, and the treatment of these cancer cells with PPARγ ligands often induces cell differentiation and apoptosis [47–51], and exerts antiproliferative effects on human colon cancer [59], breast cancer [47], pituitary adenomas [50], gastric cancer [60], and bladder cancer [61]. Furthermore, postulated mechanisms by which PPARγ ligands exert their effects include modulation of the oncogenic Wnt pathway, inhibition of nuclear factor kappaB (NF-κB), and modulation of cell cycle pro and antiapoptotic proteins (Figure 3). Wnt signaling is a complex pathway in which β-catenin binds to transcription factors in the nucleus and plays a role as a central mediator in regulating cell proliferation and differentiation [62]. PPARγ activation causes a decrease in β-catenin expression in adipocytes in vitro and in normal intestinal mucosa in mice [63]. In the cultured human monocytes, PPARγ inhibits NF-κB activation
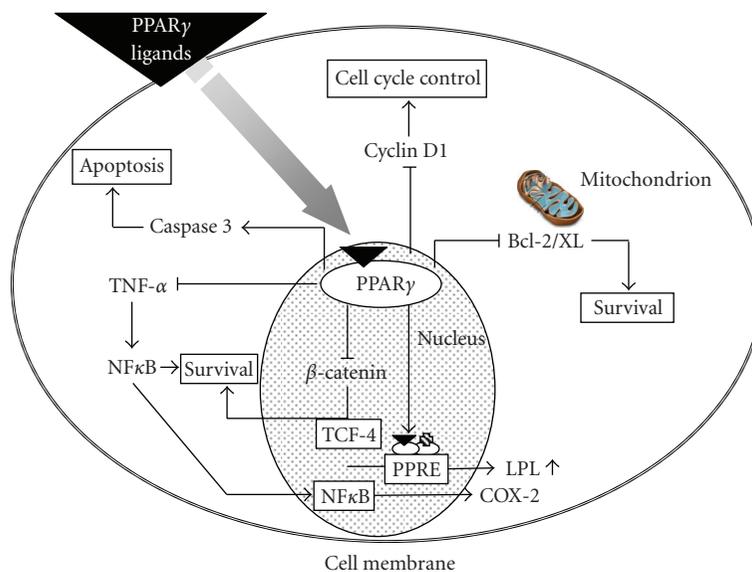
FIGURE 3: Molecular mechanisms for anticarcinogenic and/or chemopreventive effects of PPARγ ligands.

thus influencing the transcription of both survival- and apoptosis-related genes [64]. PPARγ activation also induces the activation of the proapoptotic caspase-3 protein in human liver cancer cell lines and a reduction in antiapoptotic Bcl-2 and Bcl-XL protein level in human colon and gastric cancer cell lines, respectively [65–67]. Furthermore, colon cancer development is related to hyperlipidemia [68], with clear links to high level of serum triglycerides (TGs) [69]. A PPARγ ligand, pioglitazone, suppresses both hyperlipidemia and intestinal polyp formation in the APC-deficient mice in conjunction with elevation of lipoprotein lipase (LPL), which catalyzes TG hydrolysis [70].

We previously investigated the modifying effects of PPARγ or α ligands (troglitazone, pioglitazone, or bezafibrate) on early phase of colon carcinogenesis with or without colitis in male F344 rats [19, 71]. The role of PPARγ in AOM-induced colon tumorigenesis was directly demonstrated by the study showing that the incidence of colonic tumors increased in the hemizygous knockout of PPARγ that received AOM [72]. Although thiazolidinediones inhibit AOM-induced colon carcinogenesis in the wild type mice, the observation using APC-deficient mouse models showed conflicting results regarding the effects of PPARγ ligand treatment [73–76]. This may be caused by use of different PPARγ agonists (troglitazone versus pioglitazone), and different doses (100–2000 ppm in diet) examined. Colonic inflammation is associated with a high risk of colorectal cancer (CRC) [77]. CRC is thus one of the most serious complications of inflammatory bowel disease, such as ulcerative colitis and Crohn's disease [77]. In the experiments, dietary administration of PPARα or γ ligands effectively suppressed azoxymethane (AOM)-induced or dextran sodium sulfate (DSS)/AOM-induced aberrant crypt foci, which are precursor lesions for colon carcinoma (Table 1). Our findings suggested that synthetic PPARγ and PPARα ligands are able to inhibit the early stages of colon tumorigenesis with or without colitis, and the findings were confirmed by the study conducted by Osawa et al. [78]. Furthermore, we demonstrated ligands for PPARγ and PPARα inhibit colitis-related colon carcinogenesis [79] using our AOM/DSS mouse model [80]. In the experiment, dietary administration (0.05% in diet for 14 weeks) with troglitazone and bezafibrate significantly inhibited both the incidence and multiplicity of colonic adenocarcinoma induced by the treatment with AOM/DSS, although bezafibrate feeding did not significantly lower the multiplicity (Table 2). Dietary exposure of troglitazone and bezafibrate suppressed cell proliferation and induced apoptosis and lowered immnore-activity of COX-2, inducible nitric oxide, and nitrotyrosine in the colonic malignancies.

PPARγ receptors are activated by certain lipophilic ligands, such as PUFAs and eicosanoid derivatives. They bind to the PPARγ receptor at micromolar concentrations. The essential fatty acids (arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid) as well as modified oxidized lipids (9-hydroxyoctadecanoic acid and 13-hydroxy-octadecanoic acid) bind to and activate PPARγ [5]. Recently, conjugated linoleic acid (CLA) was shown to act as a high affinity ligand and an activator of PPARγ [81]. Anticarcinogenic activity of CLA is mediated by PPARγ activation in susceptible tumors [81]. When treated with CLA, PPARγ expression is increased, and APC and c-myc proteins are downregulated in the human colon cancer cells, and finally proliferation of cancer cells is inhibited by CLA [82–85]. In fact, feeding with seed oils containing 9c, 11t, 13t-, 9c, 11t, 13c-, and 9t, 11t, 13c-conjugated linolenic acid, which are converted to 9c, 11t- and 9t, 11t-CLA within colonic and liver cells, suppresses AOM-induced colon carcinogenesis by increased expression of PPARγ protein in the colon mucosa [86–89].

TABLE 1: Effects of PPAR ligands on ACF formation in rats.

Treatment (No. of mice)	ACF/colon (% inhibition)	ACs/colon (% inhibition)
AOM alone (12)	83 ± 6[a]	2.0 ± 0.24
AOM + 0.01% troglitazone(8)	68 ± 16 (18%)	1.7 ± 0.21 (15%)
AOM + 0.05% troglitazone(8)	55 ± 13[b] (34%)	1.5 ± 0.13[c] (25%)
AOM + 0.01% bezafibrate (8)	75 ± 8 (10%)	2.0 ± 0.20 (0%)
AOM + 0.05% bezafibrate (8)	53 ± 9[d] (36%)	1.9 ± 0.10 (5%)
None	0	0
1% DSS + AOM (10)	115 ± 22	2.4 ± 0.29
1% DSS + AOM + 0.01% pioglitazone (7)	71 ± 24[e] (38%)	1.8 ± 0.17[f] (25%)
1% DSS + AOM + 0.01% troglitazone(7)	57 ± 14[g] (50%)	1.6 ± 0.14[g] (33%)
1% DSS + AOM + 0.01% bezafibrate (7)	59 ± 18[h] (49%)	1.7 ± 0.16[i] (29%)
None	0	0

[a] Mean ±SD.
[b–d] Significantly different from the AOM alone group: [b] $P < .01$; [c] $P < .005$; and [d] $P < .001$.
[e–i] Significantly different from the DSS/AOM group: [e] $P < .05$; [f] $P < .01$; [g] $P < .001$; [h] $P < .005$; and [i] $P < .002$.

TABLE 2: Effects of PPAR ligands on colon carcinogenesis in mice.

Treatment (no. of mice)	Incidence/Multiplicity (% inhibition)		
	Total	Adenoma	Adenocarcinoma
AOM/DSS	$100\%/5.2 \pm 3.0$[a]	$100\%/2.1 \pm 1.8$	$100\%/3.0 \pm 1.8$
AOM/DSS/0.05% Troglitazone	$90\%/2.5 \pm 1.8$[b] (52%,)	$90\%/1.6 \pm 1.1$ (24%)	40%[c]$/1.2 \pm 2.5$[b] (60%)
AOM/DSS/0.05% Bezafibrate	$80\%/2.6 \pm 2.5$[b] (50%)	$70\%/1.1 \pm 1.0$[b] (48%)	60%[b]$/1.8 \pm 2.6$ (40%)
None	0%/0	0%/0	0%/0

[a] Mean ±SD.
[b,c] Significantly different from the AOM/DSS group: [b] $P < .05$; and [c] $P < .01$.

5. CLINICAL TRIAL FOR PPARγ LIGANDS AGAINST TUMORS

There are several clinical studies on the effects of PPARγ ligands on malignancies (Table 3). The beneficial effects of glitazones on liposarcomas have been demonstrated in a small clinical trial [90]. Three patients with intermediate to high-grade liposarcomas were given troglitazone (800 mg/day orally). In the patients, differentiation of the neoplasms occurred as revealed by histological and biochemical analysis. The clinical outcome of these patients was not reported, but the therapy was well tolarated [90]. However, a phase II study on 12 patients with liposarcoma showed that the PPARγ ligand rosiglitazone did not significantly improve clinical outcome [94]. In prostate, PPARγ immunoreactivity was significantly higher in prostate cancer and prostatic intraepithelial neoplasia than in those with benign prostate hyperplasia and with healthy prostate [98]. A high incidence of prolonged stabilization of serum prostate-specific antigen (PSA) was observed in a phase II clinical study, where patients with advances prostate cancer who had no symptoms of metastasis were treated with troglitazone (800 mg/day orally). Moreover, one patient had a striking decrease in PSA concentration to almost undetectable amounts [91]. In a 75-year-old man with occult recurrent prostate cancer showed a decrease in PSA after oral treatment with toroglitazone (600–800 mg/day for 1.5

years) [92]. Thus, PPARγ is expressed in prostate cancer and activation of PPARγ might offer an additional therapeutic option for treatment of prostate cancer in the near future. At present, most of the available data suggest that PPARγ has antineoplastic effect on malignant neoplasms [99], including colonic malignancies. However, in a clinical phase II study on CRC, orally administrated troglitazone did not lengthen median progression-free survival or median survival in 25 patients with chemotherapy-resistant metastatic colon carcinoma [93]. In phase II study [95] for the use of troglitazone to treat patients with advanced refractory breast cancer, no objective tumor response was observed. However, the study was incomplete because troglitazone was withdrawn from commercial availability after a warning by the US Food and Drug Administration about hepatic toxic effects. On the other hand, it is important to note that neither hormone status of the tumors nor the amount of PPARγ protein is assessed before patients were included in the study. In an open labeled phase II study where ten patients with thyroglobulin-positive and radioiodine-negative differentiated thyroid cancer were enrolled and they were given oral rosiglitazone treatment (4 mg/day for 1 week, then 8 mg per day for 7 weeks), rosiglitazone treatment resulted in a 40% partial response rate, but no complete responses, and the expression level of PPARγ mRNA and protein in the neoplasm appeared unrelated to rosiglitazone treatment response [96]. The findings also suggest that

TABLE 3: Clinical trials on the anticancer effects of PPARγ ligands.

Clinical trials	Drug	Results	Reference no.
Patients with intermediate to high-grade liposarcomas (case reports)	Troglitazone	Histlogical and biochemical differentiation	[90]
Phase II study on patients with histologically-confirmed prostate cancer and no symptomatic metastatic disease	Troglitazone	Lengthened stabilization of prostate-specific antigen	[91]
75-year-old patient with an occult recurrent prostate cancer (case reports)	Troglitazone	Reduced prostate-specific antigen	[92]
Phase II study on patients with metastatic colon cancer	Troglitazone	No significant effect	[93]
Phase II study on patients with liposarcoma	Rosiglitazone	Lengthened mean time of progression	[94]
Phase II study on patients with refractory breast cancer	Troglitazone	No significant effect	[95]
Phase II study on patients with thyroglobulin-positive and radioiodine-negative differentiated thyroid cancer	Rosiglitazone	Induced radioiodine uptake	[96]
Phase I study on patients with solid tumors	LY293111	The recommended oral dose (600 mg/day) for phase II trial	[97]

higher doses and longer duration of rosiglitazone therapy may be useful to better define the role of rosiglitazone as a redifferentiation agent in differentiated thyroid cancer. There is a phase I clinical study of a PPARγ ligand (LY293111) that is not thiazolidinedione members [97]. LY293111 is a novel diaryl ether carboxylic acid derivative and is known as PPARγ agonist and LTB4 antagonist. The study suggested the dose (600 mg) of LY293111 in combination with irinotecan (200 mg/m^2 IV every 21 days for phase II clinical study against solid tumors.

6. CONCLUSIONS

PPARs were originally recognized to be genetic regulators of complex pathways of mammalian metabolism, including fatty acid oxidation and lipogenesis. However, the receptors have been shown to be implicated in carcinogenesis and inflammation. PPARs are involved in cell proliferation and differentiation of a variety of cancer. Numerous reports indicate that PPARs ligands could play an important role in prevention and inhibition of cancer development. Synthetic PPAR ligands used for drugs or those of naturally occurring lipids are promising cancer chemopreventive agents with slight side effects against several types of cancer. We should characterize expression patterns of different isoforms of PPAR in cancerous and precancerous tissues and determine their precise roles in the carcinogenic process for development of PPARs ligands as a novel class of cancer preventive/therapeutic drugs. Based on current data from preclinical and clinical studies, we believe that thiazolidinediones, especially PPARγ agonists, have important role in short-

term prophylactic therapy designed to reduce the number of putative preneoplasia, ACF, in patients who are at high risk for CRC development.

ABBREVIATIONS

AOM: Azoxymethane
APC: Adenomatous polyposis coli
CLA: Conjugated linoleic acid
COX-2: Cyclooxygenase-2
CRC: Colorectal cancer
DSS: Dextran sodium sulfate
LPL: Lipoprotein lipase
LTB4: Leukotriene B4
PG: Prostaglandin
PPARs: Peroxisome proliferators-activated receptor
PPRE: Peroxisome proliferators response element
PSA: Prostate-specific antigen
PUFA: Polyunsaturated fatty acid
RXR: Retinoid X receptor
TG: Triglyceride

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Review Article

Clinical Use of PPARγ Ligands in Cancer

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The role of PPARγ in adipocyte differentiation has fueled intense interest in the function of this steroid nuclear receptor for regulation of malignant cell growth and differentiation. Given the antiproliferative and differentiating effects of PPARγ ligands on liposarcoma cells, investigation of PPARγ expression and ligand activation in other solid tumors such as breast, colon, and prostate cancers ensued. The anticancer effects of PPARγ ligands in cell culture and rodent models of a multitude of tumor types suggest broad applicability of these agents to cancer therapy. This review focuses on the clinical use of PPARγ ligands, specifically the thiazolidinediones, for the treatment and prevention of cancer.

1. INTRODUCTION

The peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor in the superfamily of the steroid/thyroid nuclear hormone receptors, one of three PPAR isotypes (α, β/δ, and γ) [1–3]. PPARs share a highly conserved DNA-binding domain that matches with specific DNA sequences known as peroxisome proliferator response elements (PPREs), binding as a heterodimer with retinoid X receptor (RXR) to initiate transcription of target genes [4–6]. Highly expressed in adipose tissue, PPARγ is best known for its important role in adipocyte differentiation [7–9].

PPARs were first shown to be activated by compounds inducing peroxisome proliferation [10, 11], then by a variety of polyunsaturated fatty acids (PUFAs) in the micromolar range [12]. PPARγ has a more restricted list of activators compared to the other two isotypes, being more selective to PUFAs compared to other fatty acids [13, 14]. Fatty acid derivatives, such as 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) and 13- and 9-hydroxyoctadecadienoic acid (HODE), have also been identified as activators of PPARγ [15–17]. Synthetic ligands for PPARγ include the thiazolidinediones, a class of oral hypoglycemic drugs that reduce hyperglycemia and hyperinsulinemia in insulin-resistant states [18]. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen are also PPARγ ligands, but exhibit much lower binding affinities in comparison to thiazolidinediones [19].

A wealth of preclinical data supports a role for PPARγ ligand therapy in many different types of solid malignancies. Experimental studies of human cancer cells and PPARγ have focused primarily on the γ-activating effects of thiazolidinediones, identifying PPARγ agonists as negative regulators of cell growth and tumor progression. As a putative natural ligand for PPARγ, 15d-PGJ2 also appears to have anticancer effects. Treatment with PPARγ ligands inhibits malignant cell proliferation, with evidence for cell cycle arrest and induction of apoptosis [20–25]. With the pivotal role of PPARγ in adipocyte differentiation, PPARγ ligands have been tested for differentiating effects on malignant cells. Changes consistent with induction of a more differentiated cancer cell phenotype were detected in several tumor model systems, including breast, colon, and liposarcoma [9, 26–30].

The anticancer effects of PPARγ agonists may also be mediated in part via suppression of an angiogenic tumor phenotype or angiogenic, inflammatory tumor microenvironment. PPARγ ligands appear to elicit antiangiogenic effects, via modulation of endothelial cell function and growth [31–35]. The anti-inflammatory actions of PPARγ ligands [36–38], which are of relevance to the treatment of atherosclerosis and cardiovascular disease, may also prove important for the treatment and prevention of cancer given the association of chronic inflammation with increased cancer risk [39].

Approved by the Food and Drug Administration for treatment of type 2 diabetes mellitus, thiazolidinediones have been evaluated for use as investigational cancer therapies. The initial studies utilized troglitazone (Rezulin), which was the first thiazolidinedione in clinical use but ultimately withdrawn from the US market in 2000 because of instances of severe idiopathic liver disease [40]. Rosiglitazone (Avandia) and pioglitazone (Actos) were thereafter used without evidence of similar hepatotoxicity. The majority of clinical trials of thiazolidinediones have been conducted in advanced stages of disease. The following review examines the clinical trial experience to date with thiazolidinediones in cancer.

2. MONOTHERAPY

Searches of the PubMed and www.clinicaltrials.gov databases identified nine clinical trials testing the efficacy of thiazolidinediones as single agent therapy in cancer patients, with the majority conducted in subjects with advanced and/or metastatic stages of disease refractory to treatment. Troglitazone was the drug of choice for human studies initiated prior to 2000, replaced by rosiglitazone or pioglitazone in trials conducted after the withdrawal of troglitazone from clinical use because of rare instances of hepatotoxicity. Thiazolidinediones have been generally administered at the highest recommended dose for treatment of diabetes mellitus, or slightly higher as with troglitazone at 800 mg/day. Rather than mimic the generalized cytoxicity of chemotherapeutic regimens, such doses might selectively target PPARγ-mediated effects such as cellular differentiation, growth inhibition, and induction of apoptosis.

2.1. Liposarcomas

As mesenchymal malignancies that arise from adipose tissue [41, 42], liposarcomas express PPARγ at levels comparable to normal adipose tissue [26]. PPARγ expression is a distinguishing feature of liposarcomas relative to other soft tissue sarcomas [43]. Treatment of primary cell strains derived from human liposarcomas (two well-differentiated liposarcomas and one intermediate grade myxoid/round cell liposarcoma) with PPARγ and/or RXR ligands induced morphologic and molecular changes consistent with adipocyte differentiation [26]. These in vitro findings led to consideration of thiazolidinediones for differentiation therapy of liposarcomas.

Demetri et al. conducted an open label phase II clinical trial to determine the effects of troglitazone (800 mg/day) on tumor differentiation in patients with advanced and/or metastatic liposarcoma [44]. In a report of three study subjects, tumor samples obtained after the six week intervention showed marked cytoplasmic lipid accumulation in comparison to pretreatment biopsies, as well as morphologic changes suggestive of mature adipocytes. The expression of markers of adipocyte differentiation such as adipsin, aP2, and PPARγ by Northern analysis increased following treatment with troglitazone. Furthermore, cell proliferation as assessed by the percentage of liposarcoma cells with Ki67 immunostaining decreased by two- to fourfold with the

study intervention. Three additional patients enrolled in the study also had post-treatment biopsies with histologic evidence of adipocyte differentiation and decreased cell proliferation. The histologic subtypes treated in this study ranged from intermediate to high grade, and the authors raise the possibility that differentiation may prove difficult to assess in more differentiated tumors. This small pilot trial of six subjects demonstrated a differentiating effect of short-term troglitazone therapy in advanced stage liposarcomas providing the rationale for further clinical evaluation of troglitazone and other PPARγ ligands in the management of liposarcomas.

A second phase II trial evaluated the effects of the thiazolidinedione rosiglitazone (8 mg administered as 4 mg twice daily) in nine patients with advanced, unresectable liposarcomas [45]. Analyses of tumor samples obtained at 0, 6, and 12 weeks did not reveal changes in histopathology or gene expression to support a differentiating effect. By quantitative RT-PCR, levels of adipsin, fatty acid-binding protein (FABP), and PPARγ increased in only two of nine tumors although in a somewhat inconsistent fashion. In one of the patients with a dedifferentiated liposarcoma, expression of PPARγ increased at week 12, FABP at week 6 and adipsin at weeks 6 and 12. However, disease progression had been detected in this patient at 10 weeks of treatment, such that the expression of differentiation markers did not correlate with clinical benefit. For the other subject, upregulation of adipsin at week 12 was noted in a myxoid liposarcoma, and treatment continued until progression of disease at six months. Cell proliferation assessed by Ki67 immunostaining did not decrease significantly with the study intervention, although the low baseline levels of proliferation decreased further with rosiglitazone. Taken together the data fail to support a role for rosiglitazone in the treatment of liposarcoma at advanced stages.

The discrepant findings between the two phase II trials of thiazolidinedione therapy may relate to differences in the patient cohorts, tumors, and/or study agents. Whether methodological issues contributed to the differences is not clear. As surgery remains the mainstay of treatment for liposarcomas, with limited therapeutic options for unresectable, advanced disease, and investigation of PPARγ ligands for adjuvant therapy of liposarcomas or control of microscopic or minimal residual disease may still be warranted.

2.2. Colon cancer

Considerable in vitro evidence exists to support the differentiating and antiproliferative effects of PPARγ ligand therapy in colon cancer. PPARγ is highly expressed in human colon tumor specimens and cancer cell lines [27, 46]. In addition to growth inhibition, treatment of colon cancer cells with PPARγ ligands promotes tumor cell differentiation as assessed by increased cytoplasmic to nuclear ratio and higher levels of differentiation markers such as carcinoembryonic antigen (CEA), villin, intestinal alkaline phosphatase, GOB-4, and keratin 20 [27, 29, 47]. Although in vitro and in vivo studies demonstrate a role for PPARγ in modulating

growth and differentiation of human colon cancer cell lines [27], thiazolidinedione treatment with rosiglitazone (BRL49653) or troglitazone stimulated rather than inhibited the development of colon tumors in APC$^{min/+}$ mice [48, 49]. The loss of adenomatous polyposis coli (APC) tumor suppressor gene function, which leads to accumulation of β-catenin and enhanced Wnt pathway signaling, may account for the contradictory findings of enhanced tumorigenesis in this genetic versus xenograft model of colon cancer [50]. Interestingly, treatment with pioglitazone over a wide range of doses suppressed intestinal tumor formation and hyperlipidemia in APC deficient mice (APC$^{min/+}$ and Apc1309) [51, 52]. Whether the use of different thiazolidinediones or experimental conditions led to the discrepant findings is unclear.

Loss of function mutations in *PPARG* in colon cancer may also account for variability in response to PPARγ therapy. Four unique mutations of PPARγ were identified in 55 sporadic colon cancers in exons 3 or 5; one yielding a truncated protein with loss of the ligand binding domain (c.472delA) and three causing defects in the binding of synthetic or natural ligands (Q286P, K319X, and R288H) [53]. A point mutation in exon 6, K422Q, was detected in four human colon cancer cell lines that were resistant to the antiproliferative and differentiating effects of PPARγ ligands [54]. However, K422Q may have limited clinical relevance, as a study of 170 primary human colorectal cancers and 12 liver metastases failed to detect the presence of K422Q mutations [55].

A phase II trial evaluated troglitazone therapy (800 mg/day as a single dose) in 25 colon cancer patients with lung and/or liver metastases [56]. Although well-tolerated, troglitazone did not elicit objective tumor responses. All patients had progression of disease with a median progression-free survival of 1.6 months and median overall survival of 3.9 months. Troglitazone does not seem to have activity in advanced colon cancer, although the lack of response may relate at least in part to the biologically aggressive nature of Stage IV colon cancer that is refractory to cytotoxic chemotherapy (the median number of prior chemotherapy regimens was 3). Although the study was not designed to assess troglitazone for possible tumor promoting effects, Kulke et al. urged caution in the use of PPARγ agonists in colon cancer. In the future, advances in the genomic profiling of colon cancer might allow for identification of susceptible colon cancer subtypes.

Colorectal cancer provides an excellent model of multistep progression in carcinogenesis, based on the acquisition of biochemical, molecular, and genetic alterations that transform normal epithelium to adenomatous polyps to invasive cancer [57]. PPARγ ligands may have a role in chemoprevention of colon cancer, depending on the specific molecular and genetic subtype. Thiazolidinediones suppress the development of aberrant crypt foci (ACF), a putative precancerous lesion of the colon [58]. However, activation of certain oncogenic signaling pathways at later stages of carcinogenesis may override this regulatory role, as suggested by the APC$^{min/+}$ mouse studies [50]. Timing of PPARγ therapy prior to certain initiating events in colon carcinogenesis may

thus prove to be useful for chemoprevention of colon cancer. As suggested by the in vivo studies of thiazolidinediones in min mice, use of PPARγ agonists in colon cancer prevention may require identification of high-risk individuals based on genetic susceptibility as well as aberrant gene expression and signal transduction.

Although the differentiating and antiproliferative effects of PPARγ activation form the basis for evaluating PPARγ ligands for cancer therapy, the anti-inflammatory effects of PPARγ may also be relevant to colon carcinogenesis. Chronic inflammation is linked to carcinogenesis, and diagnoses of inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, carry increased risk for colorectal cancer [59]. PPARγ ligands repress cytokine production by colon cancer cells and inhibit chemically-induced colitis in murine models of inflammatory bowel disease [60–62]. These findings led to an open-label pilot trial of rosiglitazone therapy (4 mg taken twice daily) in patients with active ulcerative colitis for 12 weeks, showing clinical improvement in 8/15 (53%) patients of which 4 (27%) subjects had clinical remission [63]. Two subjects required hospitalization for worsening disease; other serious adverse events included severe pharyngitis requiring hospitalization and nephritic syndrome leading to withdrawal. The increased risk for colon cancer in patients with ulcerative colitis relates to the severity and duration of disease; by suppressing inflammation in the colon, drugs such as the thiazolidinediones or other PPARγ agonists might play a role in colon cancer prevention.

2.3. Breast cancer

In vitro and in vivo research studies support a role for PPARγ agonists in breast cancer therapy. Thiazolidinedione treatment of human breast cancer cells appears to induce changes suggestive of terminal differentiation, resulting in the inhibition of cell proliferation, suppression of keratin 19, mucin-1, and upregulation of maspin [28]. Troglitazone also inhibited breast cancer growth in a xenograft animal model [20]. Another synthetic ligand of PPARγ, GW7845, had inhibitory effects in a carcinogen-induced model of mammary tumorigenesis [64]. Furthermore, PPARγ haploinsufficiency (PPARγ+/−) conferred increased susceptibility to dimethylbenz[a]antracene (DMBA)-induced mammary carcinogenesis [65]. These studies suggest that PPARγ ligands could serve as negative regulators of breast cancer development and progression.

We and others have demonstrated PPARγ immunostaining in human breast cancer specimens, as well as normal and benign proliferative breast tissue [28, 66]. PPARγ appears to be expressed in most if not all breast cancer cell lines, as well as in normal and malignant breast tissue [20, 28, 66]. We did not find that the invasive, metastatic phenotype of the breast cancer cells correlates with PPARγ expression levels, gel shift mobility patterns, or relative sensitivity to PPARγ ligands [67]. All of the cell lines tested (MCF-7, MDA-MB-436, MDA-MB-231, and MDA-MB-435) responded to thiazolidinedione treatment in transactivation assays, which also led to inhibition of cell growth.

However, a Phase II trial of troglitazone in metastatic breast cancer refractory to cytotoxic chemotherapy did not demonstrate beneficial effects on disease progression [68]. In twenty two Stage IV breast cancer patients with an 8-week median treatment duration, troglitazone at 800 mg/day did not prevent disease progression. The median time on study was 56 days, ranging from 11 to 134 days; 19/22 subjects were removed from the study for objective or subjective evidence of disease progression. There were no objective responses, and only 3/21 (14%) subjects completing 8 weeks of troglitazone therapy had stable disease. In patients with elevated serum levels of CEA and/or CA27.29 at the onset of the study, these tumor markers had continued to increase despite 8 weeks of troglitazone. Burstein et al. note that more selective criteria to target PPARγ therapy to the most susceptible tumor type might be helpful. The lack of effect might have also related to an advanced stage of disease that had become refractory to cytotoxic therapies.

To assess the effects of PPARγ ligand therapy on breast cancer, we conducted a pilot study of short-term administration of rosiglitazone (4 mg taken twice a day) to women newly diagnosed with early stage breast cancer (Stages 0 to II) during the two- to six-week period between diagnostic biopsy and surgical removal of the cancer [69]. Thirty eight women completed the study intervention without serious adverse events; thirty four subjects had sufficient pre and posttreatment tumor tissue for correlative analyses. By Ki67 immunostaining, short-term intervention with rosiglitazone did not lead to statistically significant differences cell proliferation. We did not detect any somatic or germline mutations in *PPARG* to account for differential tumor tissue responses or lack of effect. Comparing H&E-stained sections of tumor before and after rosiglitazone therapy also failed to note increased tumor cell differentiation by standard criteria of tubule formation, mitotic activity, and nuclear morphology. PPARγ expression in tumor samples was graded 0 to 3+ for intensity of nuclear and cytoplasmic immunostaining, which did not reflect an overall change in expression following rosiglitazone therapy. Interestingly, nuclear expression of PPARγ was significantly reduced in a majority of the patients whose pre-rosiglitazone PPARγ scores were 1+ or 2+ and therefore assessable for increased or decreased levels of expression, which raised the possibility of a PPARγ mediated response in the tumor tissue.

Serum levels of adiponectin and indices of insulin sensitivity increased with rosiglitazone, based on serum samples obtained from seventeen subjects. Given the association of lower levels of adiponectin and insulin resistance with increased breast cancer risk [70–73], therapy with rosiglitazone or other PPARγ ligands may have a role in breast cancer prevention. Of interest are potential effects of rosiglitazone therapy on nonepithelial components of the mammary tumor microenvironment; unfortunately, pretreatment core biopsy samples in our study had negligible to minimal stromal, noncancerous tissue for any comparative analyses.

Although short-term intervention with rosiglitazone did not alter the endpoint of cell proliferation, this pilot study indicated the potential for other tumor tissue specific effects with evidence for downregulation of PPARγ protein

expression. Breast cancer is a family of diseases with diverse molecular, genetic features, and PPARγ ligands may affect only certain subtypes. Indeed, breast cancer therapy already encompasses molecularly targeted strategies, such as tamoxifen for estrogen receptor positive cancer and trastuzumab for tumors with HER-2/neu overexpression. Variability in PPARγ-mediated effects on cell proliferation could arise from tumor heterogeneity in nuclear receptor cross-talk, complement of cofactors, or mutated or aberrant signaling pathways that override PPARγ signaling, leading to an apparent lack of effect of rosiglitazone [74–77]. For example, in vitro studies indicate that estrogen receptor α (ERα) can repress PPARγ signaling by binding to PPREs and that PPAR/RXR heterodimers can competitively inhibit ER binding at specific estrogen response elements (EREs) [74, 75]. In vitro administration of the PPARγ ligand 15-deoxy-Δ12,14-prostaglandin J2 inhibited estrogen-mediated transactivation of ERE and estrogen-responsive gene expression in MCF-7 breast cancer cells [78]. By immunohistochemical analysis, PPARγ expression has been detected in 42% (99/238) to 58% (101/170) of human breast cancer samples, correlating positively with ER expression and improved clinical outcome [78, 79]. Concomitant administration of other drugs, such as estrogen in postmenopausal women or thyroid hormone in patients with hypothyroidism, may therefore affect the interplay between nuclear receptors. Our sample size did not support subset analyses, and further studies are needed to identify specific molecular subtypes of breast cancer that are susceptible to PPARγ ligand therapy and crosstalk interactions. These data also raise the possibility of PPARγ-mediated modulation of systemic conditions relevant to breast cancer risk, such as serum adiponectin and insulin resistance.

Breast cancer treatment and prevention may benefit from future studies of PPARγ therapy that address issues of susceptible breast cancer subtypes, duration and timing of intervention in the multistep process of mammary carcinogenesis. Combination of PPARγ ligands with other agents may enhance therapeutic efficacy, as with RXR ligands for synergism in PPARγ-RXR heterodimer-mediated signaling [80]. Interestingly, inhibition of HER-2/neu tyrosine kinase activity in a prostate cancer model prevents PPARγ degradation and thereby enhances susceptibility to PPARγ activators such as R-etodolac [81]. Administration of a PPARγ agonist in conjunction with trastuzumab (Herceptin), a humanized monoclonal anti-HER-2/neu antibody used in the treatment of metastatic and high-risk HER-2/neu+ breast cancer, could also represent a novel combination of targeted therapies.

2.4. Prostate cancer

PPARγ is expressed in human prostate cancer cell lines and human prostate cancer specimens [82]. In vitro and in vivo studies also demonstrate the anticancer effects of PPARγ ligands on prostate cancer, including the reduction of prostate specific antigen (PSA) levels in androgen responsive LNCaP cells [82–85].

A phase II clinical study assessed the effects of troglitazone at 800 mg/day in 41 men with androgen-dependent

($n = 12$) or androgen-independent prostate cancer ($n = 29$) that had progressed following local treatment or androgen deprivation therapy yet remained asymptomatic [82]. Four patients with androgen-dependent cancers had a reduction in serum PSA levels after onset of therapy, which were measured every 4 weeks, with a greater than 50% decrease in this tumor marker detected in one patient after 16 months of troglitazone. PSA levels decreased less than 50% in four patients with androgen-independent prostate cancer. The median duration of troglitazone treatment was longer in the androgen dependent (26.8 weeks) versus independent (14.3 weeks) group and could correlate with the more indolent course of disease associated with the former, as well as greater susceptibility of this prostate cancer subtype. As suggested by Mueller et al., variability in response might relate to activated mitogen-activated protein (MAP) kinase signaling leading to phosphorylation and inactivation of PPARγ as well as the possibility of somatic *PPARG* mutations.

Following from this initial study, a randomized placebo-controlled Phase II trial of rosiglitazone (8 mg/day) was conducted in prostate cancer patients with progressive disease evidenced by a rise in serum levels of PSA following local treatment with radical prostatectomy and/or radiation therapy [86]. 106 men participated in this multi-institutional study, with a median duration of 338 and 315 days of treatment for rosiglitazone and placebo, respectively. Based on serum PSA levels obtained every four weeks, rosiglitazone did not significantly increase the amount of time it took serum levels of PSA to double or increase the time before disease progression in men with prostate cancer. This trial does not support a role for rosiglitazone in recurrent prostate cancer, even if detected at the stage of biochemical, nonradiographic progression. Use of PPARγ ligands in prostate cancer may require identification of susceptible tumor subtypes as well as consideration of intervention at earlier stages of disease.

2.5. Thyroid cancer

The thyroid gland is comprised of follicle cells, which produce thyroglobulin as well as synthesize and store thyroxine, triiodothyronine, and parafollicular C cells that produce calcitonin. Papillary and follicular thyroid cancers arise from follicle cells, comprising 80–90% of thyroid cancers. Differentiated thyroid cancers usually retain the ability to take up iodine, which serves as the basis for (1) radioactive iodine treatments to ablate residual or metastatic disease and (2) diagnostic screening via radioiodine scans [87]. These thyroid cancers also produce thyroglobulin, and elevated thyroglobulin levels following definitive therapy can signify recurrent disease.

Normal, benign, and malignant thyroid tissues express PPARγ, with dysregulated expression in thyroid cancers [88, 89]. A PAX 8/PPARγ rearrangement has been noted in half of all follicular thyroid cancers, in which the resulting protein has a loss of PPARγ function [90]. Treatment of human thyroid cancer cells lines with thiazolidinediones inhibits cell proliferation and induces increased expression of markers of

thyroid cell differentiation such as thyroglobulin, sodium-iodine symporter, thyroperoxidase, and TSH receptor [91–93].

These laboratory findings provided the basis for clinical investigation of the differentiating effects of PPARγ ligands in thyroid cancer. Philips et al. conducted a pilot trial of rosiglitazone therapy in five patients with thyroglobulin-positive and radioiodine-negative thyroid cancer, a clinical scenario suggestive of dedifferentiation of the thyroid cancer [94]. After three months of rosiglitazone (4 mg/day for one month, then 8 mg/day for 2 months), thyroglobulin levels increased in 4 of 5 patients but only one subject had faint radioiodine uptake to delineate two lung metastases. Kebebew et al. also administered rosiglitazone (4 mg/day for 1 week, then 8 mg/day for an additional 7 weeks) to patients with recurrent or progressive papillary or follicular thyroid cancer who had elevated serum thyroglobulin levels and negative radioiodine scans [95]. In this open label phase II trial, 4 of 10 (40%) of the subjects had posttreatment radioactive iodine scans showing uptake in the neck ($n = 3$) and pelvis ($n = 1$). At the six month follow-up visit, serum thyroglobulin changes did not reveal a consistent pattern, with decreased ($n = 2$), increased ($n = 5$), and stable ($n = 3$) levels. There were no complete responses, defined as increased radioiodine uptake and decreased thyroglobulin levels. PPARγ expression in pretreatment tumor samples by immunohistochemisty (formalin fixed, paraffin-embedded samples for eight patients) and real time quantitative RT-PCR (frozen samples for four subjects) did not correlate with the biological, biochemical responses. As noted in both reports, serum thyroglobulin level is somewhat problematic as a marker of differentiation in that increases could reflect re-differentiation/apoptosis as well as tumor progression. Future studies will also need to address heterogeneity of stage IV disease (e.g., some patients presented only with elevated thyroglobulin whereas others had diffuse metastases), dose level, duration of therapy, and intervention prior to development of metastases.

3. COMBINATION THERAPY

Despite the wealth of preclinical evidence for the anticancer effects of PPARγ ligands in various types of cancer, thiazolidinediones appear largely ineffective as monotherapy agents for treating advanced, disseminated stages of cancer. However, cancer chemotherapy often involves the concomitant and/or sequential administration of multiple drugs in order to achieve maximal tumor cell kill and improved disease-free and overall survival. Combinatorial drug regimens may allow for additive if not synergistic effects which might also permit the use of lower dosages with decreased side effects. Preclinical studies show the potential benefits of combining PPARγ ligands with other anticancer agents. For example, rosiglitazone treatment of A549 lung cancer cells increases the expression of PTEN (phosphatase and tensin homolog), which enhances the antiproliferative effects of the tyrosine kinase inhibitor gefitinib [96]. The combination of rosiglitazone and platinum-based cytotoxic drugs such as carboplatin and cisplatin synergistically inhibits the growth

of A549 lung cancer cells relative to single-agent therapy [97]. Based on gene array analysis, rosiglitazone appears to mediate the downregulation of metallothioneins, heavy metal binding proteins involved in platinum drug resistance.

Lack of effect of PPARγ ligand therapy may also relate to repression of ligand activation, such as by histone deacetylases (HDAC) which can form complexes with PPARγ to repress gene transcription of specific PPARγ target genes [98]. Removal of HDAC-mediated transcriptional silencing via HDAC inhibitors may allow activation of PPARγ, as suggested by in vitro and in vivo studies of combined therapy of prostate cancer with HDAC inhibitors and PPARγ agonists [85]. Inactivation of PPARγ via phosphorylation could also occur in cancers with high levels of activity of mitogen-activated protein kinase [99], and coadministration of inhibitors of specific kinases might enhance the therapeutic potential of a PPARγ agonist.

Novel treatment regimens combining pioglitazone with the COX-2 inhibitor rofecoxib and low-dose continuous chemotherapy drugs have been tested for efficacy in aggressive solid malignancies at advanced, progressive stages of disease. In a pilot study of 6 patients with advanced malignant vascular tumors that had progressed following surgery, radiotherapy, and/or chemotherapy, treatment with pioglitazone (45 mg/day), rofecoxib (25 mg/day), and metronomic trofosfamide (3×50 mg/day) resulted in 2 subjects with complete responses, 1 with a partial response, and 3 with stable disease [100]. The median progression-free survival was 7.7 months (range of 2 to 15 months), and the average treatment duration was 29.3, 21.7, and 23 weeks for pioglitazone, rofecoxib, and trofosfamide, respectively. Interestingly, one subject experienced regression of an extensive angiosarcoma of the facial skin with initiation of therapy with pioglitazone and rofecoxib alone. The Phase II trial of this treatment regimen in a cohort of patients with progression of previously treated metastatic melanoma ($n = 19$) or soft tissue sarcoma ($n = 21$) led to decreased tumor burden in 5 subjects and stabilization of disease in 6 subjects [101]. More recently, a multi-institutional randomized clinical trial of trofosfamide (50 mg TID) versus trofosfamide (50 mg TID) plus rofecoxib (25 mg/day) and pioglitazone (60 mg/day) of 76 patients with metastatic melanoma demonstrated superiority of metronomic chemotherapy combined with a COX-2 inhibitor and PPARγ ligand, with 0% versus 9% progression-free survival [102]. Assessment of serum C reactive protein (CRP) levels in 48 subjects participating in this melanoma trial showed that those on the combined regimen had a greater than 30% decrease in this proinflammatory marker, suggesting that the anti-inflammatory effects of the drug combination may account for the improvement in progression-free survival. Interestingly, PPARγ appears to suppress the proinflammatory potential of monocytes and macrophages [36, 103, 104], and thiazolidinediones have been shown to decrease CRP levels [105]. Another metronomic, low-dose chemotherapy regimen of either capecitabine (1250 mg/m^2 twice daily for days 1–14 of 21 days) or temozolomide (50 mg/m^2/d) combined with pioglitazone (60 mg/d) and rofecoxib (25 mg/d) was tested in patients who had developed recurrent high-grade gliomas

(10 with gliobastoma, 4 with anaplastic astrocytoma) following chemotherapy, surgery, and/or radiotherapy, with 21.5% overall progression-free survival at six months (20% and 25% PFS for subjects with glioblastoma and anaplastic gliomas, resp.) [106]. By immunostaining the expression of COX-2, PPARγ, and CD31 in these high-grade gliomas did not correlate with patient outcome; however, as recurrence was diagnosed by magnetic resonance imaging, tumor tissue obtained prior to disease progression might not accurately represent target gene expression in the recurrent tumors. The contribution of pioglitazone to the activity of these combination regimens is difficult to assess but may relate to anti-inflammatory effects as suggested by the CRP results in metastatic melanoma. PPARγ ligands are also known to exert suppressive effects on angiogenesis in cancer [34] and on malignant vascular cells [107], which may also enhance the effectiveness of metronomic chemotherapy regimens for certain malignancies.

Combination with ligands to retinoid X receptor (RXR), the heterodimer partner of PPARγ, may also augment the anticancer efficacy of PPAR ligand therapy. Treatment with RXR ligands alone appears efficacious in the treatment of certain malignancies, and bexarotene (Targretin) is a synthetic RXR-selective retinoid utilized in the treatment of refractory cutaneous T-cell lymphoma [108]. A Phase I study of bexarotene in patients with advanced cancer did not show objective tumor responses but indicated the potential for stable disease in 5 of 16 and 1 of 5 lung and head and neck cancer patients, respectively [109]. In a multi-institutional Phase II trial of bexarotene capsules (MINT or Targretin Monotherapy in non-small-cell lung cancer trial) in advanced non-small-cell lung cancer patients who had failed two or more prior chemotherapy regimens, bexarotene (400 mg/m^2/day) did not meet the intended aim of a median survival of 6 months as third-line therapy; however, the subset of subjects with treatment-related hypertriglyceridemia (grade 1 to 4, 51%) and/or skin reactions such as rash, pruritis, and erythema (grade 1 to 4, 28%) had prolonged survival at one year of 30% and 34%, respectively, compared to 18% and 19%, respectively, of patients without these adverse events [110]. A role for bexarotene as monotherapy is also demonstrated in preclinical models of mammary carcinogenesis [111, 112]. A Phase II randomized trial of 200 or 500 mg/m^2/day bexarotene in women with metastatic breast cancer refractory to endocrine therapy or chemotherapy showed clinical benefit in the form of complete/partial response and stable disease ≥6 months in 27 of 146 patients, with an objective response rate of 3 to 6% [113].

To evaluate RXR-PPARγ ligand therapy for synergism in cutaneous T-cell lymphoma, a small uncontrolled study evaluated the addition of rosiglitazone to bexarotene monotherapy in four patients with stable or progressive cutaneous T-cell lymphoma, showing improvement in skin score (50%) and pruritus (75%) [114]. Preclinical studies of solid malignancies such as liposarcomas, breast, and colon cancers also provide support for the greater efficacy of combined regimens of PPARγ and RXR ligands compared to monotherapy [26, 80, 115]. In addition to increased

therapeutic benefits, combination therapy may also allow for lower doses and decreased toxicity.

Novel cancer agents that combine PPARγ agonism with inhibition of other signaling pathways are also of interest. LY293111 is a diaryl ether carboxylic acid derivative that modulates multiple eicosanoid pathways, acting as a leukotriene B4 antagonist, 5-lipoxygenase inhibitor, and PPARγ agonist [116]. Phase I trials have identified the maximally tolerated dose for continuous oral administration of LY293111, both as a single agent and in combination with irinotecan for solid malignancies [116]. Future clinical trials with correlative tissue analyses may provide insight into the specific contribution of PPARγ agonism to the therapeutic potential of LY293111.

4. CANCER PREVENTION

PPARγ may function as a tumor suppressor during early events of carcinogenesis. Thiazolidinediones or other PPARγ ligands may have efficacy during early stages of cancer development, by targeting carcinogenesis prior to onset of initiating events that are no longer subject to PPARγ repression. Indeed, mutant APC function and dysregulated β-catenin signaling in the APC$^{+/\min}$ mice model of colon carcinogenesis appear to abrogate responsiveness to PPARγ activation [50]. Susceptibility to PPARγ ligands is possibly limited to only certain premalignant conditions or individuals, and development of genomic strategies to properly apply this chemopreventive option in an otherwise heterogeneous high-risk population is needed.

In a phase II clinical trial of pioglitazone therapy for dysplastic leukoplakia, a precursor lesion to invasive oral cancer, pioglitazone administered for 3 months at 45 mg/day elicited a response in 70% of subjects (15/21) based on bidimensional measurements of the oral lesions [117]. Although the degree of dysplasia increased in three subjects, pioglitazone did not alter the degree of dysplasia in the majority of patients. These findings raise the possibility that PPARγ ligands may prove efficacious for chemoprevention of oral cancer by decreasing the extent of leukoplakia. The lack of effect on dysplasia suggests that pioglitazone may not function as a differentiating agent for oral leukoplakia or at least within the time frame of a three month intervention.

5. SAFETY AND TOXICITY ISSUES

Even though the risk-benefit ratio of thiazolidinediones for cancer therapy seems favorable for advanced stages of disease, thiazolidinediones appear problematic as candidate agents for chemoprevention rather than for treatment of advanced, metastatic cancer. Use of thiazolidinediones in adjuvant therapy for cancer patients who are otherwise healthy but at increased risk for cancer recurrence would also raise concerns if administered on a long-term basis as for a chronic disease such as diabetes mellitus. Although generally well-tolerated, thiazolidinediones such as rosiglitazone and pioglitazone are associated with body weight gain as a result of increased adiposity and fluid retention, with the latter resulting in peripheral edema, anemia on

the basis of hemodilution, and increased risk of developing congestive heart failure [118–120]. Controversy currently exists regarding the cardiovascular safety of rosiglitazone, with a recent meta-analysis of different treatment trials of rosiglitazone suggesting that rosiglitazone was associated with a significant increase in the risk of myocardial infarction and cardiovascular death [121]. However, other studies have not shown an increased risk of cardiovascular mortality, including the interim analysis of the Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycaemia in Diabetes (RECORD) trial, a prospective trial of rosiglitazone in type 2 diabetes mellitus [122, 123]. For postmenopausal women, rosiglitazone therapy has also been linked to osteoporosis and risk of fracture [124]. Such side effects are concerning for healthy individuals contemplating cancer risk reduction rather than treatment of disease. Defining the risks of thiazolidinediones will allow for avoidance of these drugs in subjects at higher risk for these associated complications.

The carcinogenic potential of thiazolidinediones and other PPARγ agonists is another point of controversy [125, 126]. Upon review of the carcinogenicity of PPARγ and dual PPARα/γ agonists in development, the US Food and Drug Administration has recommended two-year carcinogenicity studies of new PPAR agonist drugs in rodents prior to initiation of clinical trials longer than six months [127, 128]. As discussed above, contradictory in vivo and in vitro findings may relate to specific animal models or molecular, genetic subtypes of certain cancers. Additionally, as diabetes and insulin resistance have been linked to higher risk for certain malignancies such as breast and colon cancer [72, 129, 130], thiazolidinediones might even exert preventive effects on carcinogenesis by ameliorating the chronic inflammation associated with dysregulated metabolism.

Postmarketing experience with pioglitazone and rosiglitazone has not shown significantly increased cancer risk with these drugs. The PROactive Study (PROspective pioglitazone clinical trial In macrovascular events), which randomized 5238 patients with type 2 diabetes mellitus and cardiovascular or peripheral vascular disease to pioglitazone or placebo for an average of 34.5 months, did not detect differences in the overall number of malignancies [131]. Interestingly, the pioglitazone treated group had more bladder tumors (14 versus 6) and fewer breast cancers (3 versus 11); however, the timing of the cancer diagnoses relative to pioglitazone use and the presence of confounding risk factors did not support a causal relationship to the study drug. Use of thiazolidinediones was associated with 33% decreased risk for lung cancer in a large retrospective database analysis of over 87,000 diabetic patients of 10 veteran affairs medical centers, without significant risk reduction for colorectal and prostate cancer [132]. Whether lung cancer is uniquely susceptible to thiazolidinediones or other PPARγ agonists remains to be determined; preclinical studies suggest a potential role for PPARγ ligand therapy in the management of this disease [133, 134]. In the preliminary report of a more recent analysis of this Veteran Affairs database, thiazolidinedione use also correlated with 41 to 55% reduced risk for head and neck squamous cell carcinoma [135]. Conversely, a smaller retrospective study of 1003 diabetic subjects in the

Vermont diabetes information system database showed a possible association of cancer with use of thiazolidinediones and particularly rosiglitazone, based on self reported history of malignancy of any type [136]. Prospective studies with attention to confounding influences such as risk factors or timing and duration of drug use relative to cancer diagnosis are needed to investigate these possible associations and basis for the discrepant findings.

6. CONCLUSIONS

Based on the clinical experience to date, use of PPARγ agonists in cancer therapy will likely require a targeted approach, with restriction to specific types of malignancies and/or stage(s) of disease susceptible to enhanced PPARγ signaling. Studies of PPARγ ligand therapy in cancer patients have focused largely on thiazolidinediones approved for treatment of type 2 diabetes mellitus, administered at the high end of doses tested for tolerability and insulin sensitizing effects. In phase II clinical trials, thiazolidinediones do not have significant activity in most cancers at advanced stages, which may relate to the extent and burden of disease and aggressive tumor phenotypes resistant to cytotoxic therapies. The lack of efficacy of thiazolidinediones in advanced, metastatic disease may relate to the use of noncytotoxic dose levels; alternatively, these drugs may not exert effects at late stages of cancer progression at any dose level. However, the demonstration of differentiating effects in some patients suggests that a subset of patients might benefit from PPARγ ligand therapy. Thiazolidinediones and other PPARγ agonists may ultimately prove effective in combination with standard cytotoxic agents or other molecularly targeted therapies. Interventions at earlier stages of disease, including premalignant or predisposing high-risk conditions, should also be considered. Therapy administered in the adjuvant setting, such as following surgical resection, or in situations of microscopic rather than measurable disease may also have greater efficacy. The use of PPARγ ligands in earlier stages of cancer or cancer prevention will require additional research and/or drug development to address the questions regarding the carcinogenic potential and other adverse effects of these drugs. Genetic and molecular profiling of human cancers may in the future enable selection of a tumor subtype susceptible to PPARγ agonists, as specific genotypes and patterns of nuclear receptor and cofactor expression might predict resistance or responsiveness to PPARγ signal transduction.

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Review Article

PPARγ: The Portrait of a Target Ally to Cancer Chemopreventive Agents

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Peroxisome proliferator-activated receptor-gamma (PPARγ), one of three ligand-activated transcription factors named PPAR, has been identified as a molecular target for cancer chemopreventive agents. PPARγ was initially understood as a regulator of adipocyte differentiation and glucose homeostasis while later on, it became evident that it is also involved in cell differentiation, apoptosis, and angiogenesis, biological processes which are deregulated in cancer. It is now established that PPARγ ligands can induce cell differentiation and yield early antineoplastic effects in several tumor types. Moreover, several bioactive natural products with cancer protecting potential are shown to operate through activation of PPARγ. Overall, PPARγ appears to be a prevalent target ally to cancer chemopreventive agents and therefore pursuing research in this area is of great relevance.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that function as transcription factors regulating the expression of genes involved in lipid biosynthesis, glucose metabolism, as well as cell proliferation, differentiation, and survival [1–4]. Their discovery was driven by search of a molecular target for peroxisome proliferators, a group of agents named after their property to increase peroxisomes in rodent liver [5, 6]. Later on, activity studies helped elucidate the versatile role of these molecules in modulating diverse biological functions such as metabolism, tissue remodeling, inflammation, angiogenesis, and carcinogenesis [7–11]. Three PPAR gene types have been identified: α, β/δ, and γ [12, 13]. Between them, PPARγ is the most intensively investigated [14, 15].

2. THE HUMAN PPARγ GENE

The human PPARγ gene consists of six coding exons located at chromosome 3p25.2 and extends approximately over 100 kb of genomic DNA [16]. Three major transcriptional start sites have identified where three mature mRNAs originate from, differing in their 5′ untranslated regions [17, 18] . Notably PPARγ1 and PPARγ3 mRNAs code for the same protein of 475 amino acids, while PPARγ2 transcript codes for a different protein which contains an additional 28 N-terminal amino acids [19].

2.1. Tissue distribution of different PPARγ isoforms

The PPARγ1 is found in virtually all tissues, such as liver, skeletal muscle, prostate, kidney, breast, intestine, and the gonads. The PPARγ2 is the major PPARγ isoform expressed mainly in adipose tissue where it normally operates as an adipocyte-specific transcription factor in preadipocytes and regulates adipose tissue differentiation, and the PPARγ3 isoform is restricted to adipose tissue and large intestine [18, 20],

2.2. PPARγ protein structure and function

Similar to other members of the nuclear hormone receptors superfamily, PPARγ protein has three functional domains: the N-terminal domain, the DNA-binding domain, and a carboxy-terminal ligand-binding pocket (Figure 1).

PPARγ protein receptor is activated by a number of endogenous and exogenous ligands of various
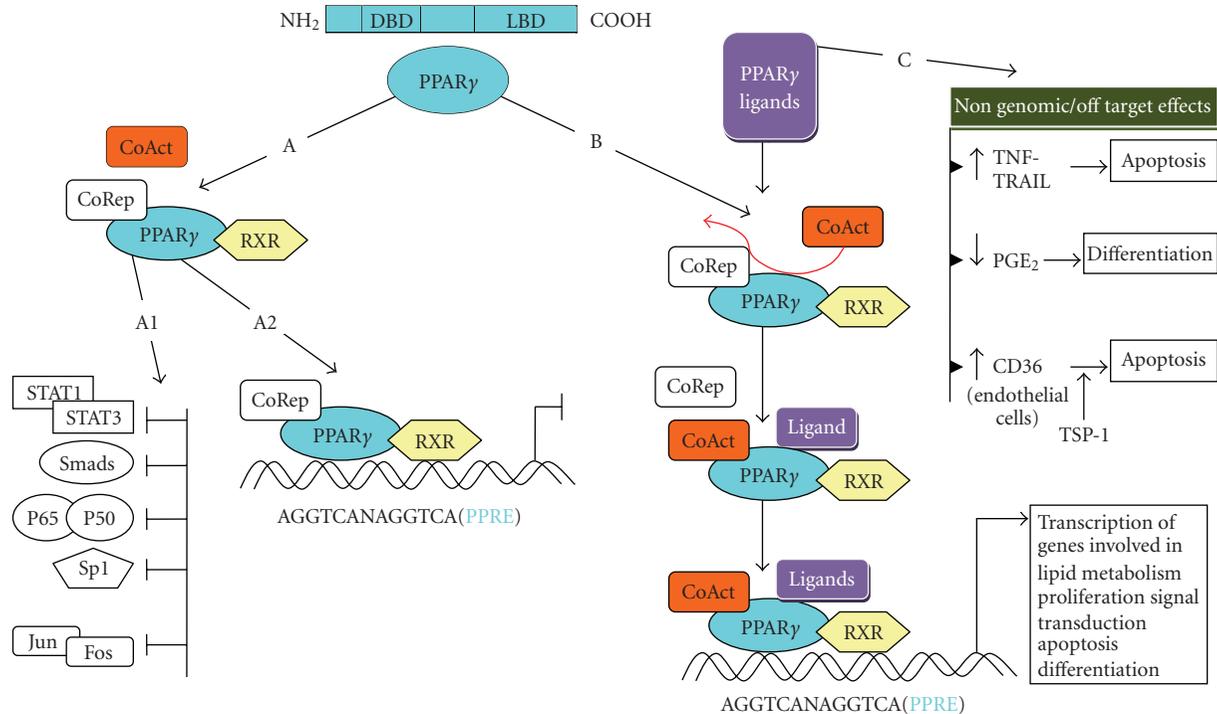
FIGURE 1: *Peroxisome proliferator-activated receptor-γ and ligands: pathways and functions.* PPARγ protein exhibits a structural organization consisting of three functional domains: an N-terminal domain, a DNA-binding domain (DBD) and a carboxy-terminal ligand binding domain (LBD). PPARγ forms heterodimers with a second member of the nuclear receptor family, the retinoic X receptor (RXR). Unliganded PPARγ suppresses transcription (pathway A) either by interfering with key transcription factors (pathway A1) or through recruitment of corepressors (CoRep) on a PPRE element (pathway A2). Ligand binding to PPARγ (pathway B) triggers conformational changes that lead to dissociation of corepressors (CoRep) and subsequent association of coactivators (CoAct). The complex is binding to PPREs and triggers transcription (pathway B). PPARs ligands can also exert their action through PPARγ-independent mechanisms also (pathway C). For instance in NSCLC cell lines activation of TNF-TRAIL induce apoptosis, while PGE$_2$ degradation, trough 15-hydroxyprostagladin dehydrogenase induction, results in enhanced epithelial differentiation. In endothelial cells PPARγ ligands can markedly boost expression of CD36 which functions as the receptor of endogenous antiangiogenic molecule thrombospondin-1, thereby potentiating the apoptotic response. (PFAs: polyunsaturated fatty acids, TZDs: thiazolidinediones, PPRE: peroxisome proliferator response element, TNF: tumor necrosis factor, TRAIL: TNF-related apoptosis-inducing ligand, NSCLC: non-small cell lung carcinoma).

potencies. Among pharmaceutical compounds, thiazolidine-dione (TZD) class of insulin-sensitizing drugs (also called glitazones) are best known to operate as ligands to PPARγ [21, 22] while long-chain polyunsaturated fatty acids are the most well-characterized endogenous ligands [23].

The activated PPARγ protein becomes operational following its heterodimerization with retinoid X receptors (RXR) [24]. The PPARγ/RXR complex translocates to the nucleus where it binds to target genes which contain a peroxisome proliferator response element (PPRE). A PPRE consist of a direct repetition of the consensus sequence AGGTCA separated by a single nucleotide (Direct repetition; DR1) [17]. To initiate transcriptional regulation of PPRE-bearing genes, the PPARγ/RXR complex requires accessory proteins to bind on. These proteins can either trigger (coactivators) or represses gene transcription (corepressors) (Figure 1). It must be noted though that besides their PPARγ-dependent genomic effects, PPARγ ligands can also influence cellular biology via nongenomic, PPARγ-independent events [25] (Figure 1).

As a rule, the transcriptional activity of PPARγ is negatively modulated through phosphorylation by MAPK [26–28]. Phosphorylation of human PPARγ1 protein at Ser-84 site restrains its function [27], and phosphorylation of PPARγ2 modifies the A/B domain and reduces its ligand binding affinity [29]. However, not all phosphorylation events are inhibitory. For example, it has been found that missense mutation which results in the conversion of proline to glutamine at position 115 can render PPARγ2 constitutively active through modulation of the MAPK-dependent phosphorylation status of serine 114 [30] while phosphorylation by protein kinase A (PKA) was shown to enhance its activity [31].

Until now, three molecular processes have been proposed for the termination and downregulation of PPARγ signaling: the phosphorylation of Ser-84/112 of PPARγ1/2 by ERKs [27], the proteasomal degradation of ligand-activated PPARγ [32], and the interaction with MEKs, which promotes its expulsion from the nucleus [33].

3. PPARγ IN CANCER

Early studies portrayed PPARγ as an important regulator of preadipocyte differentiation and glucose homeostasis. Later on, it was identified that PPARγ regulates biological processes which are considered hallmarks of cancer such as cell differentiation, apoptosis, and angiogenesis. This knowledge, coupled with data showing that PPARγ ligands could yield anticancer effects in several cell types, led researches postulate a role for PPARγ in carcinogenesis [11, 34, 35].

Apoptosis is believed to be a fundamental molecular mechanism through which PPARγ activators exert their action against cells which undergo malignant transformation [36–38]. Moreover, apart from their direct inhibitory effects on cancerous transformed cells, PPARγ can also inhibit angiogenesis which is a prerequisite for tumor formation and growth [39–41]. It is suggested that the antiangiogenic activity of PPARγ can be accomplished either by blocking the production the angiogenic ELR+CXC chemokines by cancer transformed cells or by inducing expression of the thrombospondin-1 receptor CD36 in endothelial cells [42–44] In addition, latest exciting data, which showed that PPARγ agonists were able to inhibit the canonical WNT signaling in human colonic epithelium, raises hopes that such agents can possibly block cancer initiation at a stem cell level [45].

It must be underlined herein that despite demonstration of cancer-preventive effects of PPARγ ligands in vitro, clinical trials and animal models failed so far to show significant benefits [46]. The fact that PPARγ ligands have been used in clinic trials at concentrations above those needed to elicit receptor agonistic activity poses questions for receptor-independent off-target effects [47].

3.1. PPARγ and gastrointestinal cancer

PPARγ are heterogeneously expressed throughout the gastrointestinal epithelium, showing significant differences in abundance, distribution, and functions. This protein is principally expressed in differentiated epithelial colonic cells, preferably in the proximal colon [48]. Sarraf et al. showed that PPARγ activation could stimulate a program that is characteristic of colonic cell differentiation [49].

A functional genomics analysis conducted for the identification of PPARγ gene targets revealed that the majority of these genes were transcribed throughout the colon, but their expression varied in cells purified from the proximal colon and in those from the distal colon. Metabolic functions of PPARγ were elicited primarily in the proximal colon, whereas signaling functions were recognized in the distal colon. Interestingly, TZDs transactivated the PPARγ gene targets at the proximal colon but repressed them in the distal colon. TSC22, a TGFβ target gene known to inhibit colon cell proliferation, was also identified as a PPARγ target gene [50]. It is worth mentioning that both TGFβ and PPARγ pathways attenuate during transition from adenoma to carcinoma [51]. From a pharmacological point of view, Yamazaki at al. showed that activation of the RXR/PPARγ

heterodimer by their respective ligands could be considered a useful chemopreventive strategy for colorectal cancer. They found that a combination of the RXR alpha ligand 9-cis-retinoic acid with ciglitazone synergistically inhibited the cell growth and induced apoptosis in Caco2 human colon cancer cells that expressed high levels of p-RXR alpha protein [52].

In the most widely used preclinical model of sporadic colon carcinogenesis, the azoxymethane-treated mice, activation of PPARγ suppressed carcinogenesis but only before damage to the APC/beta-catenin pathway [53]. However, two papers published ten years ago reported that troglitazone and rosiglitazone increased occurrence of colon tumors in mice-caring mutations in the *APC* gene [48, 54]. Moreover, although pioglitazone was later reported to suppresses colon tumor growth in Apc+/− mice [55], biallelic knockdown of PPARγ in colonic epithelial cells was associated with an increase of tumor incidence [56]. It should be reminded, however, that although TZDs are considered pure PPAR agonists, they also wield off-target effects not mediated through linkage to PPAR receptors. An in-depth analysis of the role of TZDs against colon cancer can be facilitated through development of tissue-specific PPARγ knockout mice [57]. Interestingly, a small phase II clinical trial using troglitazone failed to document tumor responses in patients with advance stage metastatic colon cancer [58].

Overall, existing evidence indicates that PPARγ agonists have a potential to inhibit cancer formation in the distal colon, but they are practically inactive in advanced stages of colon cancer.

3.2. PPARγ and lung cancer

Lung cancer is a major global health problem because of its incidence and mortality. It remains the top cancer killer worldwide to which early-detection strategies and development of new therapies failed so far to improve its lethal outcome [59]. This tobacco-related cancer epidemic persists despite public implementation of tobacco control measures because the majority of tobacco-smoke users declare powerlessness to quit. Therefore, the search for potent chemopreventive agents and the development of effective chemoprevention strategies for lung cancer is a viable pursuit highly justified [60, 61].

Several studies have shown that PPARγ agonists can inhibit growth and induce changes associated with differentiation and apoptosis in lung cancer [62–64]. TZDs induced upregulation of PTEN and p21, downregulation of cyclins D and E, and reduced expression of fibronectin and its receptor integrin $α5β1$ in human lung carcinoma cell lines [65–68].

A first evidence of clinical efficacy of PPARγ agonists as cancer chemopreventives in lung cancer was recently published. A retrospective analysis of a database from ten Veteran Affairs medical centers revealed a significant reduction (33%) in lung cancer risk in diabetic patients who were treated with TZDs compared with nonusers of TZDs [69]. However, other studies damped early this enthusiasm by showing that diabetic patients treated with TZDs were at increased risk for cardiovascular complications [70].

It is critical to understand that cancer-protecting effects of PPARγ agonists in lung cancer can be PPARγ dependent but also PPARγ independent [71]. Characteristically, TZDs suppressed the expression of antiapoptotic mediator prostaglandin E(2) in NCLC cells through induction of 15-hydroxyprostagladin dehydrogenase [72] and enhanced TRAIL-induced apoptosis through upregulation of death receptor 5 DR5 and downregulation of c-FLIP in human lung cancer cells [73].

The combination of PPARγ agonists with other chemopreventive agents emerges as a challenging issue in lung cancer chemoprophylaxis. Notably, an amazing synergy of clinically achievable concentrations of lovastatin (an HMG-CoA reductase inhibitor) and troglitazone was recently shown against lung cancer cells [74]. This effect was accompanied by synergistic modulation of E2F-1, p27$\wedge$Kip1, CDK2, cyclin A and RB. In another study, a combination of low-doses of MK886 (5-lipoxygenase activating protein-directed inhibitor), ciglitazone and 13-cis-retinoic acid, also demonstrated synergistic inhibitory activity against lung cancer cells [75]. These studies provide a framework for the development of rationally designed drug combinations aimed to target simultaneously the PPARγ and other cofactors.

3.3. PPARγ and other malignancies

Epidemiological studies suggested that high consumption of carotenoids (known PPARγ activators) could protect women from the development of breast cancer [76, 77]. These findings are also supported by experiments which show that activation of PPARγ can induce terminal differentiation, cell cycle arrest, or apoptosis of preneoplastic and cancerous mammary epithelial cells [78–80]. Unfortunately, this is not the case for advanced breast cancer: a phase II trial of troglitazone in patients with breast cancer metastases failed recently to prove clinical benefits [81].

Prostate cancer appears to be an attractive tumor target for PPARγ agonists because cancerous prostate cells express higher levels of PPARγ compared with their normal counterparts [82]. Moreover, it has been shown that PPARγ1/2 activation suppressed the high level of endogenous COX-2 in normal prostate epithelial cells [83] while TZDs mediated apoptosis in prostate cancer cells through inhibition of Bcl-xL/Bcl-2 functions [84]. In the clinical setting, reduction and prolonged stabilization of prostate-specific antigen levels were demonstrated in patients treated with troglitazone [82, 85]. The above data provide a rationale to consider investigating PPARγ ligands for their role in preventive and possibly therapeutic management of prostate cancer.

In gynecological cancer, Wu et al. reported that rosiglitazone could block or delay the development of hyperplasia and subsequent endometrial cancer. This PPARγ agonist induced apoptosis in both PTEN intact and PTEN null cancer cell lines and decreased proliferation of the endometrial hyperplastic lesions in a PTEN($+/-$) murine model [86].

In human pancreatic cancer cell lines, treatment with TZDs was found to induce cell cycle arrest and increase expression of pancreatic differentiation markers [87, 88].

Moreover, activation of PPARγ together with RXR resulted in suppression of pancreatic cancer cell growth through suppression of cyclin D1 [89].

Among sarcoma tumors, it is liposarcomas which are considered targets for PPARγ agonists because they show a high expression of this nuclear receptor [90]. However, although pioglitazone was found capable to terminally differentiate human liposarcoma cells in vitro, it failed an early phase II trial despite induced changes in relevant target genes [91].

In thyroid cancer, a functional chromosomal translocation of part of *PAX*8 gene which encodes the DNA-binding domain to the activation domain of the PPARγ gene has been detected in patients with follicular type carcinoma [92]. This chimeric fusion protein is resistant to PPARγ ligands, invalidating any anticancer effects of PPARγ ligands in this setting. However, it has been suggested that PPARγ ligands could have activity in combination with retinoids and/or histone deacetylase inhibitors in thyroid tumors which express both PPARγ and also RXRγ [93, 94].

4. PPARγ AS A MEDIATOR TO CANCER PROTECTING NATURAL PRODUCTS

Evidence has accumulated which affirms that bioactive natural compounds can play an important role in cancer chemoprevention through modulation of PPARγ. Preclinical studies and epidemiological data support that tumor growth and metastasis can be restrained or delayed by several herbal products [95–98]. Moreover, it is believed that novel agents derived from bioactive phytochemicals can be used as adjuncts to enhance therapeutic efficacy of standard treatments [99, 100]. Among natural products, triterpenoids, flavononoids, carotenoids, and linoleic acid are the most extensively studied as cancer chemopreventives and have invariably been found to operate as PPARγ activators.

Terpenoids of plant origin have shown antitumor activity which indicates a potential role for these compounds as cancer chemopreventives [100–102]. Specifically, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a synthetic triterpenoid, which was shown to activate PPARγ and induce growth arrest and apoptosis in treated breast cancer cells [103]; also, glycyrrhizin the major triterpene gycoside phytochemical in licorice root and the triterpenoid acid betulinic acid which is found in the bark of several species of plants, both have shown pro-PPARγ activities in cancer cells. These phytochemicals were found to induce expression of proapoptotic protein caveolin-1 and the tumor-suppressor gene Kruppel-like factor-4 (KLF-4) in colon and pancreatic cancer cells [104, 105]. It should though be noted that although caveolin-1 is generally considered a proapoptotic molecule, it has also been associated with drug resistance and possibly metastasis [106]. It is believed that some PPAR-γ agonists induce whilst others repress caveolin-1 [107].

Isoflavones are well known to function as phytoestrogens. They bind to the estrogen-related receptors but also to PPARα and PPARγ [108]. As a result, their biological effects are determined by the balance between activated ERs and PPARγ [109]. Liang et al. investigated apigenin, chrysin,

and kaempferol in mouse macrophages and found that these flavonoids stimulated PPARγ transcriptional activities as allosteric effectors rather than pure agonists [110]. In the clinical setting, purified isoflavones have only been investigated for safety, bioavailability, and pharmacokinetics in men with early-stage prostate cancer [111–114].

Carotenoids are another class of phytochemicals found to activate PPARγ in cancer cells. Hosokawa et al. reported that the edible carotenoid fucoxanthin, when combined with troglitazone, induced apoptosis of Caco-2 cells [115]. Moreover, in epidemiological studies, consumption of carotenoids was shown to protect against breast cancer [76, 77]. Interestingly, Cui et al. unveiled recently the molecular mechanisms which underlie the chemopreventive activity of β-carotene against breast cancer. They found that β-carotene significantly increased PPARγ mRNA and protein levels in a time-dependent fashion, while 2-chloro-5-nitro-N-phenylbenzamide (GW9662), an irreversible PPARγ antagonist, attenuated apoptosis caused by β-carotene in cancer-transformed cells [36].

Linoleic acid, a naturally occurring omega-6 fatty acid which is abundant in many vegetable oils, has been studied comprehensively for its prophylactic effects against cancer formation [116]. Conjugated linoleic acid, which is found especially in eggs and in the meat and dairy products of grass-fed ruminants, was shown to modulate cell-cell adhesion and invasiveness of MCF-7 cells through regulation of PPARγ expression [117]. Moreover α-eleostearic acid (ESA), a linolenic acid isomer, induced apoptosis in endothelial cells and inhibited angiogenesis, also through activation of PPARγ [118]. More recent studies brought up additional evidence and provided insights into molecular mechanisms of the protective effects of linoleic acid against colon cancer. Yasui et al. reported that 9trans-11trans-conjugated linoleic acid inhibited the development of azoxymethane-induced colonic aberrant crypt foci in rats at preinitiation and postinitiation level through activation of PPARγ and downregulation of cyclooxygenase-2 and cyclin D1 [119]. In addition, Sasaki at al. showed that linoleic acid was capable to inhibit azoxymethane-induced transformation of intestinal cells and tumor formation [120]. In most studies, the differentiation-promoting and carcinogenesis-blocking effects were mostly attributed to activation of PPARγ by linoleic acid products [121]. Finally, apart from its direct action as a PPARγ activator, linoleic acid was found to modulate interactions between PPARβ/δ and PPARγ isoforms [122].

Finally, in the class of capsaicinoids, capsaicin, the spicy component of hot peppers, was shown to induce apoptosis of melanoma as well as colon and prostate cancer cells, and was associated with activation of the PPARγ in the case of colon cancer [123–125]. However, controversy exists regarding cancer-preventing and cancer-promoting effects of capsaicin [126, 127].

It must be noted that besides their PPARγ-mediated effects, natural products can also induce transcription of detoxification enzymes glutathione S-transferases (GST) which are known to protect cells from chemical-induced carcinogenesis [128, 129]. Recently, Park et al. examined GSTA2 gene induction by thiazolidinedione and 9-cis-retinoic acid and investigated the molecular basis of PPARγ/RXR-mediated GSTA2 induction in the H4IIE hepatocytes. They found that both PPARγ and RXR agonists could increase the expression of GSTA2 but treatment of cells with a combination of PPARγ and RXR agonists produced synergistic increase [130]. This data suggest that cancer-preventive functions of PPARγ activators may be related to some extent to a parallel induction of GSTA2.

5. CONCLUSION

Existing data suggest that peroxisome proliferator-activated receptor-gamma (PPARγ) is a potential target ally to cancer chemopreventive agents. Although PPARγ was first understood as a key regulator of adipocyte differentiation and glucose homeostasis, it is now recognized that it is also involved in cell proliferation, differentiation, apoptosis, and angiogenesis. Meticulous research for PPARγ agonists with potency to function as cancer chemopreventive agents is highly warranted.

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Review Article

Omega-3 Fatty Acids and PPARγ in Cancer

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Omega-3 (or n-3) polyunsaturated fatty acids (PUFAs) and their metabolites are natural ligands for peroxisome proliferator receptor activator (PPAR)γ and, due to the effects of PPARγ on cell proliferation, survival, and differentiation, are potential anticancer agents. Dietary intake of omega-3 PUFAs has been associated with a reduced risk of certain cancers in human populations and in animal models. In vitro studies have shown that omega-3 PUFAs inhibit cell proliferation and induce apoptosis in cancer cells through various pathways but one of which involves PPARγ activation. The differential activation of PPARγ and PPARγ-regulated genes by specific dietary fatty acids may be central to their distinct roles in cancer. This review summarizes studies relating PUFAs to PPARγ and cancer and offers a new paradigm relating an n-3 PUFA through PPARγ to the expression of the cell surface proteoglycan, syndecan-1, and to the death of cancer cells.

1. INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors comprises three distinct gene products, PPARα, β/δ, and γ, that differ in ligand specificity, tissue distribution, and developmental expression [1–3]. PPARs demonstrate a relatively high level of constitutive transcriptional activity which is further increased upon binding their activating ligands [4–7]. These ligands are primarily long chain unsaturated and polyunsaturated fatty acids (PUFAs) and certain metabolites of these fatty acids [8–10]. Initially, PPARs were thought mainly to govern lipid homeostasis by binding fatty acids and their metabolites to thereby become more active in regulating genes for proteins involved in lipid metabolism [8, 10, 11]. Indeed, PPARα is expressed predominantly in tissues with high fatty acid requirements such as liver, heart, and kidney, while PPARγ isoforms γ1 and γ2 are highly enriched in adipose tissue to regulate adipocyte differentiation and lipid storage [3]. However, expression of PPARγ1, as with PPARβ/δ and PPARα, has now been extended to most other tissues and regulatory roles for PPARs extended to other systemic functions such as carbohydrate regulation, immune modulation, and the

proliferation, survival and differentiation of cells [3]. The latter effects have led to intense interest in the PPARs in relation to cancer.

PPARα and its ligand activators regulate fatty acid and lipoprotein metabolism and promote the development of hepatocellular carcinoma in rodents and reduce the metastasis of melanoma in hamsters [12]. These and other of their effects do not, in general, translate to humans. PPARβ/δ plays a key role in lipid metabolism of peripheral tissues. Its high expression in colon has been shown to promote colon cancer [12, 13], in a mechanism that involves the stimulation of PPARβ/δ by arachidonic acid, PPARβ/δ-dependent upregulation of cyclooxygenase (COX)-2 leading to overproduction of prostaglandin (PG)E$_2$, and PGE$_2$-induced growth of colon cancer cells. There is relatively little documentation of a role for PPARβ/δ in other cancers [14]. By contrast, PPARγ has a broad range of effects on cancer. PPARγ controls fat metabolism by regulating genes involved in lipogenesis, insulin sensitivity, and adipocyte differentiation [3, 15]. These effects underlie the use of thiazolidinediones, which bind and activate PPARγ, to treat insulin-resistant type II diabetes [3, 15]. Although PPARγ activators have been widely shown to inhibit growth in

cultured cancer cells, in vivo effects have proved to be complex: they inhibit but sometimes promote cancer growth [16] probably due to stimulation of antiproliferative and apoptotic signaling pathways or proliferative and antiapoptotic pathways, depending on cellular conditions [3, 12, 15–18]. These findings led to the idea of selective PPARγ modulators (SPARMs), drugs analogous to selective estrogen receptor modulators (SERMs) in which distinct actions of the modulator depend on the cellular context [19] and on distinct receptor conformations, and therefore different gene interactions [20]. Fatty acids may be considered as natural SPARMs since their binding does not necessarily lead to PPAR activation and target gene transcription [11].

The considerations discussed above raise a possibility that managed alterations in the type of fatty acids in tissues, can alter the activity of PPARs and thereby the genes they control for therapeutic benefit. The fatty acid content of tissues is dependent mainly on dietary intake. Omega-3 PUFAs, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) are enriched in the diets of many populations that enjoy a low incidence of cancer [21]. These diets also obtain some modest success ameliorating advanced cancer in humans [22] and have been widely used to inhibit carcinogenesis and tumor progression in animal models. The ability of specific fatty acids to differentially activate PPARs and PPAR-regulated genes may be central to their distinct roles in cancer. This review will focus on PPARγ, its activation by fatty acids, and functional results in cancer cells.

2. FATTY ACID METABOLISM

2.1. Fatty acid types and interconversions

Fatty acids are hydrocarbons with a terminal carboxyl group. The carbons of saturated fatty acids are all connected by single bonds, whereas the chains of monounsaturated and polyunsaturated fatty acids (PUFAs) contain one or more double bonds, respectively. The n-3 and n-6 designation describes the position of the double bond closest to the (omega) carbon at the methyl end of the molecule (Figure 1). Oleic acid (18 : 1) has a single double bond between carbons 9 and 10 from the omega carbon and is designated an n-9 or omega-9 monounsaturated fatty acid. Like the saturated fatty acids, oleic acid can be synthesized de novo in mammalian cells. It can also be obtained from the diet through intake of oils such as olive and canola. By contrast, PUFAs cannot be synthesized de novo in mammals and must be obtained from the diet. The shortest of the n-6 PUFAs is linoleic acid (LA, 18 : 2, n-6). Its 18 carbon, n-3 counterpart is α-linolenic acid (ALA, 18 : 3, n-3). Both LA and ALA are metabolized through a series of elongation and desaturation steps to longer chain PUFAs: LA to arachidonic acid (AA, 20 : 4, n-6) and ALA to EPA (20 : 5, n-3) and DHA (22 : 6, n-3) (Figure 2). The first and rate limiting step in this pathway is the introduction of a double bond by the Δ6 desaturase (for review see [23]). For n-3 PUFAs, ALA is converted to stearidonic acid (SDA, 18 : 4, n-3), elongated, and desaturated by Δ5-desaturase to form EPA. In mammalian cells, the conversion of EPA to DHA follows the Sprecher pathway in which EPA is elongated to docosapentaenoic acid (DPA, 22 : 5, n-3), then to tetracosapentaenoic acid (TPA. 24 : 5, n-3), and desaturated to tetracosahexaenoic acid (THA, 24 : 6, n-3). THA is translocated from the endoplasmic reticulum to peroxisomes, where β-oxidation results in the loss of 2 carbons to form DHA [24]. The PUFAs are also metabolized, most importantly for this review, to PPARγ activators (see Section 2.3).

2.2. Dietary fatty acids

The results of both dietary intake and stable isotope studies have shown that the conversion of ALA to DHA in humans is extremely inefficient (for review see [25]). Most of the ingested ALA is an immediate target for β-oxidation to provide energy, leaving an estimated 8–10% to enter the elongation-desaturation pathway [26, 27]. A kinetic analysis of ^{2}H-labeled fatty acids estimated that conversion of ALA to EPA was only 0.2%, EPA to DPA was 0.13%, and DPA to DHA was 0.05% [28]. There is some evidence of gender-related differences in the activity of the elongation-desaturation pathway that result in a greater efficiency of conversion of ALA to DHA in females than in males [25, 27, 29]. Support for a role of sex hormones in the conversion pathway is provided by data indicating higher DHA in plasma lipids associated with oral contraceptive use [27] as well in males supplemented with estrogen during sex-change procedures [30]. Moreover, testosterone treatment of female-male transsexuals was shown to decrease plasma DHA [30].

Because common enzymes in the elongation-desaturation pathway are responsible for conversion of both n-3 and n-6 PUFAs, background diet is also a factor in efficiency of conversion. LA is the most abundant fatty acid in the Western diet with consumption in US that is ten-fold that of ALA (reviewed in [31]). Studies have shown that a high intake of LA is associated with a low conversion of ALA to EPA [26]. In spite of limited metabolism of ALA to its long chain derivatives in the stable isotope tracer studies, feeding studies have consistently shown that increased consumption of ALA does result in higher levels of EPA in plasma or cell lipids [31]. However, there was no measurable increase in DHA in these pools. Likewise diets supplemented with EPA do not result in a detectable increase in plasma DHA [32]. Thus, the inefficiency of this pathway does not appear limited to one step but rather extends throughout the pathway. The consensus of a number of studies is that the only way to increase plasma and tissue levels of a specific PUFA is to increase the consumption of that fatty acid. This may be of particular importance in the light of recent in vitro studies on the antitumor effects of DHA.

2.3. PUFAs metabolism to PPARγ activators

Tissues metabolize PUFAs to oxygenated products that have quite different impacts on PPARγ than their parent molecules. Moreover, n-3 PUFA inhibit the metabolism n-6 PUFAs and subplant them from their oxygenation pathways to form products [33–35] that have different effects on PPARγ than their n-6 PUFAs oxygenated counterparts.
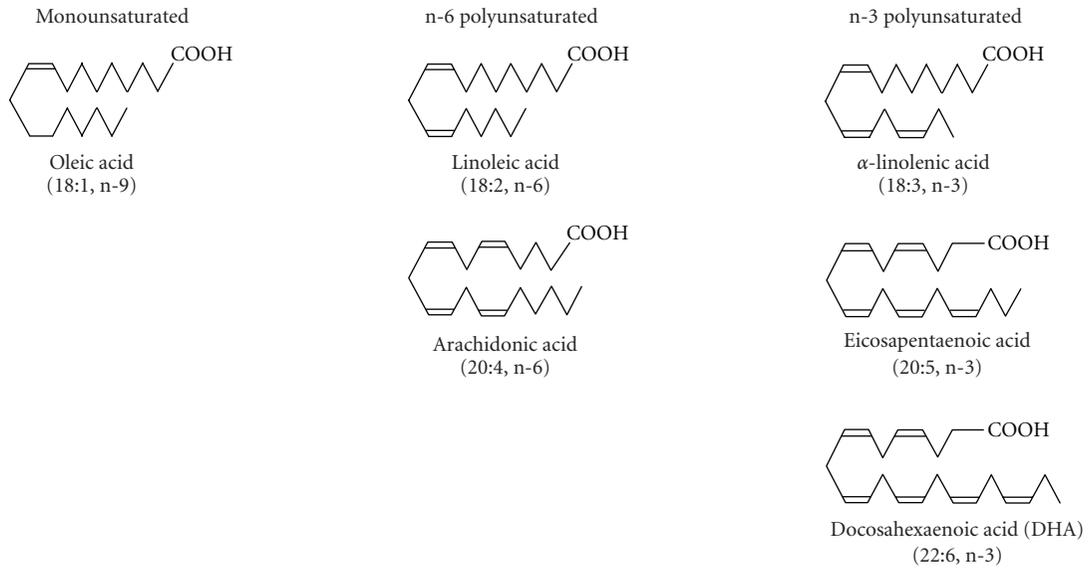
Figure 1: Structures of unsaturated fatty acids: oleic acid (n-9 monounsaturated), linoleic acid and arachidonic acid (n-6 polyunsaturated), α-linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid (n-3 polyunsaturated). The "n" numbers are counted from the methyl or omega terminus.
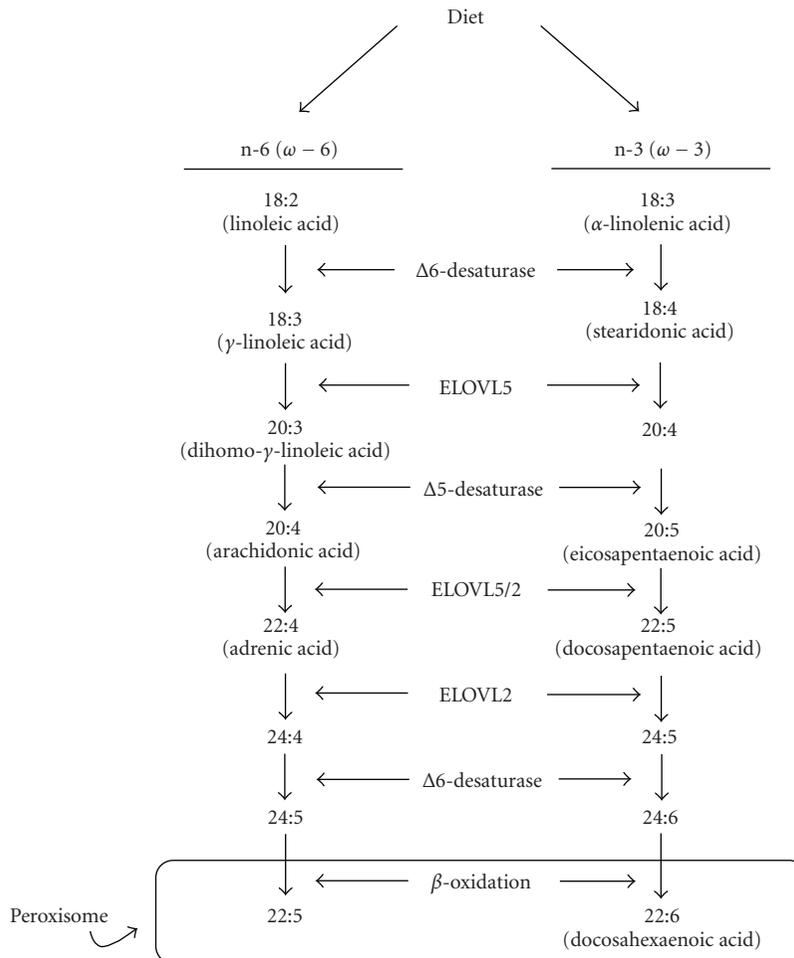

Figure 2: The elongation-desaturation pathway for the metabolism of n-6 and n-3 polyunsaturated fatty acids.

It is therefore important to consider PUFAs oxygenation pathways. LA, AA, and DHA require >10–$30\,\mu M$ to activate PPARγ but are commonly converted to stronger (>0.1–$10\,\mu M$) activators in cells. LA is metabolized (Figure 3, upper panel) by 15-lipoxygenases (LOX)-1/2 to 9(S)- and 13(S)-HODE (hydroxy-octadecaenoate) and by cyclooxygenases (COX)-1/2 to 9(R)- and 13(S)-HODE. The HODEs can be converted to 13-oxo- and 9-oxo-ODE by a dehydrogenase [36–39]. The hydroxy and to a greater extent oxo LA analogs have greater PPARγ-activating potency than LA [36, 40–42]. AA is metabolized (Figure 3, center panel) via 5-LOX to 5(S)-HETE (hydroxy-eicosatetra-enoate) and via 15-LOX-1/2 to 15(S)-HETE. These HETEs can be converted to oxo-ETEs and 5-oxo-15(S)-hydroxy-ETE as shown in Figure 3 [39, 43–50]. 15-HETE has weak and 5-HETE essentially no ability to activate PPARγ. However, their oxo counterparts have appreciable ability to do so with 5-oxo-15(S)-hydroxy-ETE showing the greatest potency in binding and activating PPARγ [43]. AA is also metabolized (Figure 3, center panel) by COX1/2 to PG (prostaglandin) D_2 which as a consequence of successive dehydrations and an isomerization, perhaps by nonenzymatic routes, convert to PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy$\Delta^{12,14}$-PGJ_2(15-d-$\Delta^{12,14}$-PGJ_2); these PGJ_2's have greater ability than PGD_2 to activate PPARγ with 15-d-$\Delta^{12,14}$-PGJ_2 being a most potent (>0.1–$1\,\mu M$) naturally occurring PPARγ activator [9, 43, 51–56]. In one study, the K_d's of 15-d-$\Delta^{12,14}$-PGJ_2, 5-oxo-15-OH-ETE, PGJ_2, 5-oxo-ETE, and 5(S)-HETE in binding to PPARγ were 1.4, 11, 37, 81, and $>1000\,\mu M$, respectively; their potency in activating a cell-based PPARγ reporter paralleled these K_d's [43]. DHA is metabolized (Figure 3, bottom panel) by 15-LOX or other oxygenase to 17-OH- and 7-OH-DHA, products that activate PPARγ with greater potency (ED_{50}'s in activating a cell-based PPARγ reporter of $\sim5\,\mu M$) than DHA [57]. 4-OH-, and 4-oxo-DHA [53], while not yet shown to be made by cancer cells, also activate PPARγ with greater potency (ED_{50}'s of 13.4 and $7.8\,\mu M$ in activating a cellular PPARγ reporter, resp.) than DHA ($ED_{50} > 10\,\mu m$) [53]. Hence, in this DHA series, similar to the 5-HETE series of AA metabolites, the oxo analog exhibits the greatest potency. We note that the more potent PPARγ activators, the oxo-PUFAs, form preferentially in cells undergoing excessive oxidation, free radical, and NADPH/NADH-depleting reactions [43, 44, 48, 57, 58]. This suggests that PPARγ may serve as a sensor for oxo-PUFA thereby monitoring cellular oxidative stress and when this stress is severe, engaging cell death programs [43, 58]. This PPARγ function, we suggest, could contribute to the necrosis that occurs in tumors particularly after chemical and radiation treatment [59].

Cells process PUFAs in other relevant ways. They convert them to nitrates, probably in nonenzymatic reactions, where the nitric oxide made during cell stimulation attacks the PUFAs. Nitrated LA and AA are stronger PPARγ activators than their precursors [60–62]. Cells also convert PUFAs to cannabinoids such as anandamide (ethanolamine amide of AA) and arachidonoylglycerol which also activate PPARγ with greater potency than AA [63–65]. Finally, cells conjugate glutathione to PUFAs that contain an α,β-unsaturated

ketone such as 15-d-$\Delta^{12,14}$-PGJ_2 and 5-oxo-ETE [66–68]. Since the conjugates are rapidly excreted from cells by multidrug-resistance transporters, conjugation inhibits the ability of α,β-unsaturated ketones to activate PPARγ [66]. Cancer cells excrete anticancer drugs through these same transporters and become drug-resistant by overexpressing these transporters [69]. Such mutated cells may also be resistant to α,β-unsaturated ketone activators of PPARγ.

2.4. Low-density lipoproteins (LDL) as deliverers of PPARγ-activating n-3 PUFA

LDL carry esterified PUFAs in glycerolipids and cholesterol. They bind to cell surface LDL receptors and then internalize in endocytic vesicles which merge with lysosomes to de-esterify and release the PUFAs into the cytosol [70]. This route differs from the direct delivery of PUFA: it bypasses cell surface G protein-coupled fatty acid receptors (GPR 40 and 120; see Section 4.3), deposits PUFA in cells more slowly, and thereby avoids stimulation of G protein-coupled receptors and, perhaps, an array of C domain-bearing proteins which are activated by PUFA. This is also an important pathway for delivering PUFA to tumor cells because of a significant increase in LDL receptor activity in neoplastic tissues [71–73]. We have obtained from monkeys fed special diets, LDL enriched with n-6 PUFA (mostly AA and LA) or n-3 PUFA (mostly DHA and EPA). The n-3 but not n-6 PUFA-rich LDL mimicked thiazolidinediones and DHA in inhibiting cancer cell growth [74] and activating PPARγ [75, 76].

3. PPARγ

3.1. Structural considerations

PPARγ1 and γ2 originate from the PPARγ gene through separate promoters and 5' exons. Compared to the ubiquitously expressed PPARγ1, PPARγ2, which is limited mainly to adipose tissue, has 30 additional amino acids at its NH^2 terminus and is a more potent transcription activator [77]. Because they appear to have the same targets, however, the two isoforms are here considered together under the term PPARγ. PPARγ is comprised of four functional parts: the NH$_2$-terminal A/B region bears a ligand-independent transcription-activating motif AF-1; C region binds response elements (PPREs with a DR-1 consensus half-sequence of AGGTCA); D region binds various transcription cofactors; and E/F region has an interface for dimerizing with 9-*cis* retinoic acid receptors (RXRs), an AF-2 ligand-dependent transcription-activating motif, and a ligand-binding domain (LBD) [3, 12, 15, 17]. The LBD has a spacious cavity that binds ligands having a polar head group extending from a hydrophobic tail such as diverse PUFAs and PUFA metabolites [7, 77].

3.2. PPARγ regulation by other signaling pathways

PPARγ is phosphorylated by extracellular signal-regulated kinases (ERK)-1/2 and C-Jun N-terminal kinase; when so
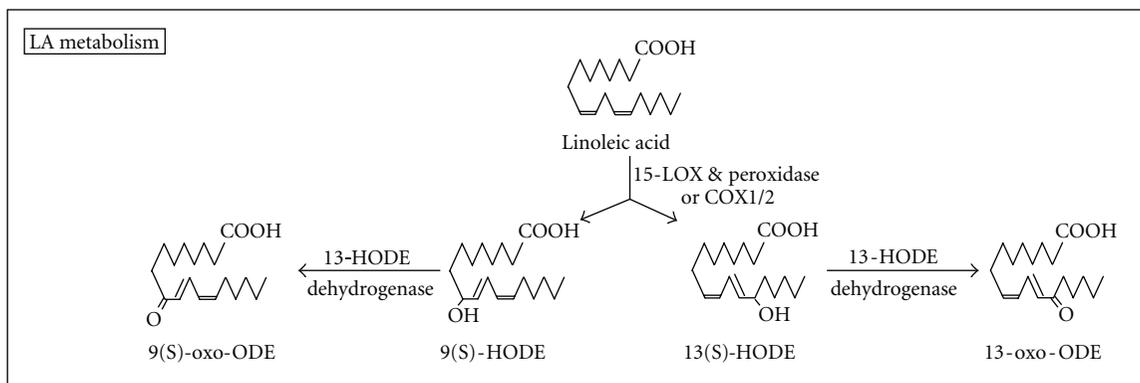
(a)
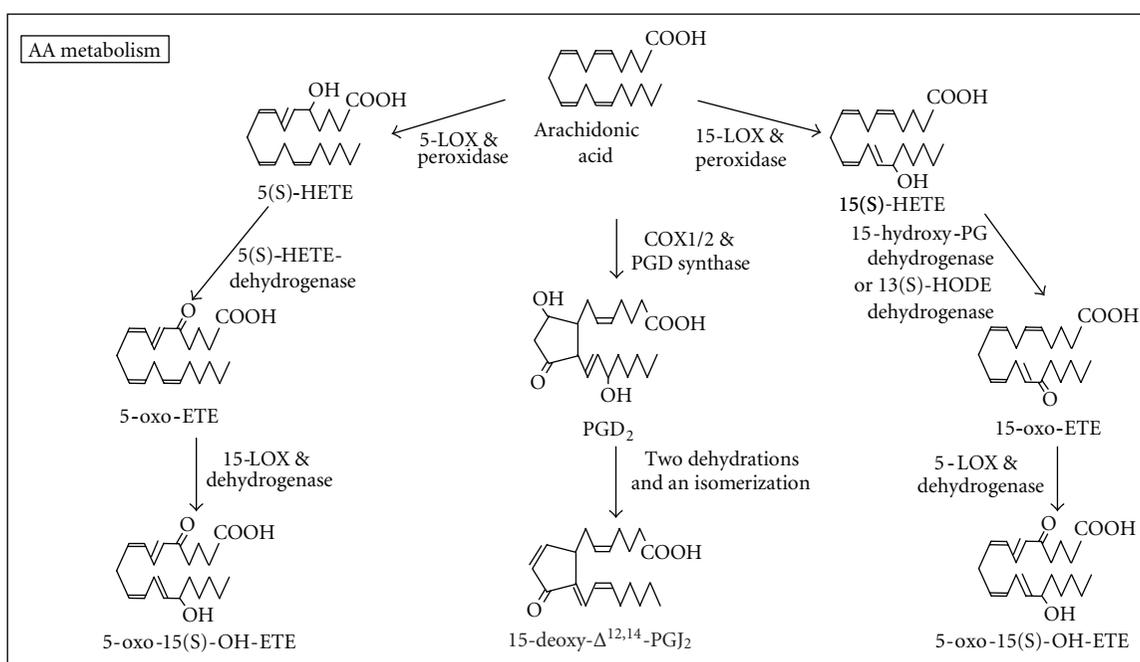
(b)
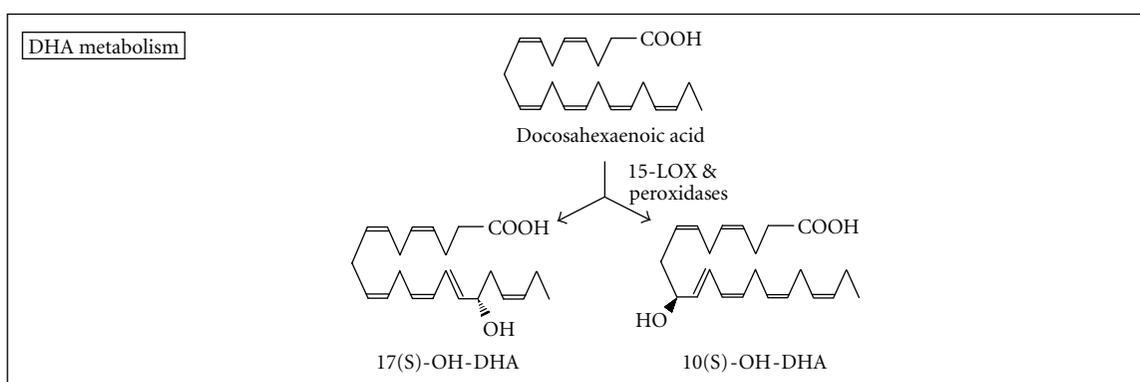
(c)

FIGURE 3: The cellular metabolism of LA, AA, and DHA to more potent activators of PPARγ. ODE is octadecaenoate; HETE is hydroxy-eicosatetraenoate; ETE is eicosatetraenoate; PG is prostaglandin.

phosphorylated, it has less ligand-binding affinity and gene-regulating activity [3, 78, 79]. The phosphorylation and attendant decrease in activity of PPARγ occur in cells treated with PPARγ activators and may cause the activators to show little or no ability to stimulate PPARγ [3, 79–81]. ERK pathways impact PPARγ in another way: the ERK-activating enzyme, MEK, when activated, binds with PPARγ's AF-2 motif. This causes PPARγ to release from PPRE complexes and, bound to MEK and directed by MEK's nuclear export signal, to exit the nucleus [81, 82]. It is important to note that PUFAs and PUFA metabolites can activate the MEK/ERK pathway (see Section 4.3) and therefore may have biphasic effects: they not only directly activate PPARγ but also entrain events inhibiting PPARγ.

PPARγ is targeted for degradation by ubiquitylation and sumoylation. Ligand binding, certain protein kinases, and some transcription cofactors (e.g., p300) promote ubiquitin-dependent degradation of PPARγ in proteasomes [3]. Sumoylation occurs on K107 of PPARγ2 in a ligand-independent fashion to inhibit AF-1 function and on K365 of PPARγ in a ligand-dependent fashion to promote PPARγ's binding of nuclear receptor corepressor [83, 84]. Sumoylation of PPARγ causes its proteasomal degradation. ERK phosphorylation promotes K107 sumoylation. This reaction represents yet another means by which ERKs can inhibit PPARγ [84].

3.3. PPARγ transcriptional cofactors

PPARs bind a specific DNA sequence termed peroxisome proliferator response element (PPRE) in the 5′-flanking region of target genes as a heterodimer with RXR. Studies using various techniques [3, 85, 86] suggest the following model: PPARγ•RXR complexes (the interaction is ligand-independent) exist in nuclei as macrocomplexes associated with various transcription corepressors [3, 87]. Some complexes, ligand-bound or not, may associate with transcription coactivators to control the basal expression of genes. In any event, PPARγ•RXR complexes are highly mobile, rapidly scanning chromatin, although this scanning does not involve their DNA binding domain [86]. Ligands trigger PPARγ•RXR to localize at their cognate PPREs and to exchange corepressors for coactivators such as cyclic AMP response element binding protein (CREB) and p300 [3, 16, 87, 88]. At some gene sites, activators cause PPARγ•RXR to recruit corepressors and thereby cause gene repression [3, 89, 90]. However, the availability of cofactors differs between cell types and within cells over time depending on the cell's history and the association of the cofactors to other genes [3, 15, 16], for example, activation of PPARγ deprives T cell factor/lymphoid enhancing factor (TCF/LEF) of cofactors to thereby inhibit oncogenic signaling by the Wnt pathway [16]. Thus, the effects of PPARγ activation vary depending on context and cofactor availability at each genetic site. It seems at least possible that the PUFA ligands for PPARγ will have differential effects in impacting its interactions with these transcriptional cofactors in a manner similar to the SPARMs model [19].

4. TARGETS OF PPARγ RELEVANT TO CANCER

4.1. Gene targets of PPARγ

Most known target genes of PPARγ regulate lipid metabolism and transport [15] with few cancer-related genes having been confirmed as induced by PPARγ. PPARγ does induce G_0/G_1 switch gene 2 whose product causes growth arrest in 3T3-L1 cells [91, 92]. PPARγ also binds the NFκB promoter of p53 to stimulate expression of p53 and, in consequence, p21$^{WAF1/Cip1}$. It also binds to a promoter in the Fas ligand gene to induce the expression of this member of the extrinsic apoptosis pathway. These effects appear responsible for slowing growth and causing apoptosis in MCF7 breast cancer [93], human umbilical vein endothelial [94], and possibly Reh [95] cells. Recent studies have identified the heparan sulfate proteoglycan, syndecan 1, as a target for PPARγ in human breast [75, 76] and prostate [96] cancer cells. The upregulation of syndecan 1 by PPARγ resulted in apoptosis induction [76].

4.2. Other targets of PPARγ

PPARγ impacts many growth-promoting elements through its secondary actions that, while ligand-dependent, do not directly involve its gene promoters. It interacts with nuclear factor of activated T cells, phosphorylated signal transducer, and activator of transcription (STAT)-3, and nuclear factor κB (NFκB) to block signaling through these pathways [3]. It binds transcription cofactors to alter these cofactors' availability to other transcription factors: ligand bound-PPARγ deprives NFκB of AP-1; deprives STAT-1 of CREB binding protein; and releases SMRT to render it available to repress STAT-3's transcriptional activity [3, 16, 17, 97]. PPARγ activation is also associated with the activation of ERK1/2, protein kinases C, protein kinase A, AMP-activated protein kinase α [17]; induction of p16, p18, and p21 cyclin-dependent kinase inhibitors [3, 17, 18]; decreased expression of cyclooxygenase 2, cmyc, cmyb, D1, and D3 cell cycle control genes, and regenerating gene 1A [17, 18]; decreased secretion of cytokines and growth factors [17, 98]; depression of the Akt survival pathway by upregulating PTEN and inhibiting the phosphorylation of Akt and mTOR [3, 17]; inhibiting retinoblastoma protein (Rb) activity to repress the activities of cyclins D3 and E [3]; and regulating a host of other elements involved in the growth and death of cells [3, 12, 16–18]. It is not clear which if any of these effects are due to the action of PPARγ or PPARγ activators. PUFAs impact many of these same targets but can do so not only by PPARγ-dependent but also PPARγ-independent routes (see the next section).

4.3. Targets of PPARγ-activating Ligands

Studies of PPARγ function depend on challenging cells with PPAR-activating ligands that have numerous side effects impacting cell growth. 15-d-$\Delta^{12,14}$-PGJ$_2$ has a reactive α,β-unsaturated ketone (Figure 3) that covalently binds to cysteine sulfur on PPARγ; this renders its PPARγ binding irreversible [58, 68]. 15-d-$\Delta^{1,14}$-d-PGJ$_2$ also binds to cysteines

in the IKKβ subunit of IκB kinase, thereby inhibiting NFκB activation [99, 100]. Other ligands with an α,β unsaturated ketone (e.g., oxo-ODEs and oxo-ETEs; see Figure 3) have this chemical reactivity [58] and along with 15-d-$\Delta^{1,14}$-d-PGJ$_2$ may exert anticancer effects by covalently attaching to signal molecules like IKKβ [58, 99, 101] or elements needed for expressing the epidermal growth factor receptor (EGFR) and JAK [102, 103].

Naturally occurring ligands have other PPARγ-independent effects. The D and J series of PGs including 15-d-$\Delta^{12,14}$-PGJ$_2$ bind to PGD$_2$ receptors [104], 5-oxo- and 5-oxo-15-hydroxy-ETE bind to the OXE receptor [105], and AA, EPA, and DHA bind to GPR40 and GPR120 receptors [106, 107]. These G protein-coupled receptors regulate signal pathways that effect cancer cell growth. For example, 5-oxo-15-hydroxy ETE acts on OXE to stimulate cells to activate ERK and Akt and proliferate; this stimulation counters its antigrowth activity in various cancer cell types. Indeed, HEK293 cells lack OXE receptors and in contrast to OXE receptor-bearing breast, prostate and ovarian cancer cell lines respond to 5-oxo-ETE and 5-oxo-15-oxo-ETE only by slowing, not speeding, their proliferation [43]. PUFAs activation of GPR120 also causes ERK and Akt activation to increase the survival of serum-starved STC-1 cells [108]. Finally, PUFAs are also metabolized to products that act on G protein receptors to promote cell growth, for example, prostate cancer cells convert AA to PGE$_2$, which acts through its receptors to stimulate the NFκB pathway and thereby the expression of various cytokines and growth factors [109]. The G protein receptor-dependent actions of PPARγ ligands may explain reports that these ligands have biphasic effects in stimulating proliferation and antiproliferation in cancer cells [110].

Thiazolidinediones stimulate cells to activate ERK1/2, p38, and JNK [111–113] by discharging Ca^{2+} from the ER to evoke an ER stress response; this activates Ca^{2+}/calmodulin kinase II, proline-rich tyrosine kinase 2, protein kinases C, c-Src, EGFR, the ERK1/2 and JNK pathways, the double stranded RNA-activated protein kinase, and p38 [111]. Double stranded RNA-activated protein kinase inactivates eukaryotic initiation factor-2 to depress protein translation [111, 114]. Since EPA has recently been shown to have similar effects on ER calcium discharge [111, 115], it seems likely that various other PUFAs activate the ER stress pathway. Nonetheless, PPARγ activators often show very different side effects [42, 103, 116–120]. For example, among three PPARγ agonists, ciglitazone, 9-HODE, and 13-HODE, only 9-HODE induced apoptosis in U937 cells [38], 15d-$\Delta^{12,14}$-PGJ$_2$, but not various other PPARγ ligands, reduced EGFR expression in squamous carcinoma cells [99], 15d-$\Delta^{12,14}$-PGJ$_2$, but not troglitazone, inhibited the stimulated induction of MHC class II molecules in retinal pigmented epithelial cells [112], and DHA, but not EPA, stimulated the target gene, syndecan 1 to inhibit the proliferation and induce apoptosis in breast and prostate cancer cell lines [75, 76, 96]. Numerous other examples of differential effects among PPARγ agonists exist (e.g., [113–116]), but it is worth stressing that n-3 PUFAs inhibit the metabolism of n-6 PUFAs to products that promote the growth of cancer cells

such as PGE$_2$, 5-HETE, and leukotriene B$_4$ [33–35, 45, 113]. This inhibitory effect may make an important contribution to the anticancer effects of n-3 PUFAs.

5. DIETARY FATTY ACIDS AND CANCER

5.1. Human studies

Although there are inconsistencies [121], human population studies have shown that consumption of a diet enriched in n-3 PUFAs may offer protection against a number of cancers including those of breast [122–124], prostate [125, 126], and colon [127–129]. Although many of these studies have relied on dietary intake data from self-reported questionnaires or estimates based on national consumption, a few have used the fatty acid composition of tissues as a measure of exposure to dietary fats. The EURAMIC study is one of the largest to provide evidence that the balance between n-3 and n-6 PUFA may play a role in breast cancer [130]. Adipose tissue aspirates from breast cancer patients and controls demonstrated that the ratio of long chain n-3 to n-6 PUFAs was inversely associated with breast cancer in four of five centers studied. In human prostate tissue, lower EPA and DHA as well as lower n-3 to n-6 PUFAs ratios were associated with cancer compared to benign prostate hyperplasia [131] and with advanced stage compared to organ confined disease [132]. This inverse association of n-3 PUFAs and prostate cancer is supported by analyses of fatty acids in serum and red-cell membranes of patients with prostate disease [133, 134].

5.2. Animal studies

Animal studies provide convincing evidence of a negative relationship with n-3 PUFA diets and a positive relationship with n-6 PUFA diets for breast, prostate, and colon cancer. In studies of breast cancer induced by chemical carcinogens in rats [135–137], and human cancer cell xenografts in nude mice [138–140], tumor growth rate, size, and metastases were all suppressed by dietary n-3 PUFA supplementation. Likewise for colon cancer, antitumor properties of n-3 PUFA diets have been shown in transplantable mouse tumors [141–143] as well as in chemically induced rat tumors [144–151]. Although there have been fewer animal studies of PUFAs in prostate cancer, they are consistent with those in breast and colon cancer. In xenograft models of prostate cancer, n-3 PUFAs enriched diets inhibited tumor growth compared to n-6 PUFA diets [152–154]. Recently, a prostate-specific Pten knockout mouse model was used to demonstrate that a dietary ratio of n-6 to n-3 PUFA lower than 5 was effective in suppressing tumor growth, and extending animal lifespan [155].

5.3. Cell culture studies

Insight into the mechanism(s) responsible for the anticancer properties of n-3 PUFAs have been provided by animal studies as well as by in vitro investigations using human cancer cell lines. A major focus for such studies has been
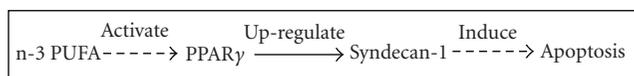
Figure 4: The syndecan-1 pathway for n-3 PUFA induction of apoptosis. Dashed lines indicate that effects may be indirect with involvement of other metabolites and signaling molecules.

the competitive inhibition between n-6 and n-3 PUFAs for the enzymes involved in their metabolism. The desaturation and elongation of LA to AA were shown to be decreased in the presence of high n-3 PUFAs due to enzyme preference for the n-3 substrates [156]. AA and EPA compete for the COX and LOX enzymes, again with preferential n-3 utilization that results in a reduction in the highly reactive eicosanoids generated from AA [157, 158] in favor of less inflammatory n-3 eicosanoids [159]. The decreased growth of prostate xenograft tumors was shown to involve inhibition of COX 2 and PGE$_2$ in the tissues [154]. Thus, the combined human, animal, and cell culture studies indicate that diet is an important regulator of the levels of n-3 versus n-6 PUFAs in tissues, including those that are cancerous. High levels of n-3 PUFAs may directly evoke antitumor events, become metabolized to products with antitumor activity, or suppress the production of tumor-promoting metabolites such as those formed by n-6 PUFAs.

6. n-3 PUFA REGULATION OF SYNDECAN-1

Increasing evidence implicates PPARγ in the divergent effects of n-3 and n-6 PUFAs in cancer cells and point to a growth inhibitory role for PPARγ [160–164]. We recently found that n-3 PUFAs—but not n-6 PUFAs—enriched LDL, inhibited the proliferation, and induced apoptosis in human breast cancer cells [74–76]. The n-3 LDL delivered both EPA and DHA to the cells. When these individual fatty acids were delivered to cells by albumin, DHA but not EPA proved effective in stimulating apoptosis in a pathway that involved activation of PPARγ [75]. The molecular target for both DHA and PPARγ in these cells was shown to be the heparan sulfate proteoglycan, syndecan-1. Syndecan-1 itself was effective in apoptosis induction and when syndecan-1 was silenced, the ability of DHA to induce apoptosis was completely blocked as it was in the presence of a dominant negative PPARγ [76]. Moreover, syndecan-1 siRNA was effective in blocking troglitizone-induced apoptosis. Thus, a novel pathway linking n-3 PUFAs to apoptosis in tumor cells is as follows: DHA activates PPARγ, which results in transcriptional upregulation of the syndecan-1 target gene, and the syndecan-1 protein induces apoptosis (Figure 4). This novel pathway has been confirmed in human prostate cancer cells [96].

Although PPARγ was not a target for EPA in breast and prostate cancer cells, a recent report has demonstrated that EPA was an effective PPARγ transactivator in HT-29 human colon cancer cells [165]. In contrast, both EPA and DHA were shown to reduce PPRE reporter activity in an HCT-116 colon cancer cells [166]. DHA has recently been shown to reduce the growth of human lung cancer cells in a process that was associated with increased PPARγ protein [167]. These conflicting reports are consistent with data showing selective modulation of PPARγ by different ligands in different cells [168]. Several other reasons may be proposed for the differential response to DHA and EPA in the breast and prostate tumor cells including (1) PPARγ activation may be mediated by a unique DHA metabolite rather than DHA itself; (2) there may be a difference in the bioavailability of the two fatty acids following cellular uptake; (3) EPA may be a ligand for or metabolized to a ligand (e.g., 5(S)-hydroxy-eicosapentaenoic acid) for a G protein-coupled receptor that activates ERK and thereby inactivates or in some other way counteracts PPARγ; (4) EPA may directly, or after being metabolized, activate other pathways that counteract PPARγ signaling.

The identification of syndecan-1 as a target gene for PPARγ in the breast and prostate cancer cells was a novel but not unexpected finding. The syndecan-1 promoter contains a DR-1 element that is recognized by a several members of the nuclear hormone receptor superfamily including PPARγ. Although there are conflicting reports of a role for syndecan-1 in cancer, the importance of these studies is the identification of a PPARγ molecular target that is regulated by PUFAs and results in functional response in the tumor cells. As more such targets emerge, we may be able to understand how different dietary fatty acids play divergent roles in cancer.

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Review Article

PPARγ and PPARδ as Modulators of Neoplasia and Cell Fate

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PPARγ and PPARδ agonists represent unique classes of drugs that act through their ability to modulate gene transcription associated with intermediary metabolism, differentiation, tumor suppression, and in some instances proliferation and cell adhesion. PPARγ agonists are used by millions of people each year to treat type 2 diabetes but may also find additional utility as relatively nontoxic potentiators of chemotherapy. PPARδ agonists produce complex actions as shown by their tumor promoting effects in rodents and their cholesterol-lowering action in dyslipidemias. There is now emerging evidence that PPARs regulate tumor suppressor genes and developmental pathways associated with transformation and cell fate determination. This review discusses the role of PPARγ and PPARδ agonists as modulators of these processes.

1. INTRODUCTION

PPARγ and PPARδ are involved in cell cycle regulation, survival and angiogenesis [1–3], and in inflammation through ligand-dependent and independent mechanisms [4]. Several recent reviews have described the role of PPARs in metabolic disease [4–6], cancer treatment [3, 7], and chemoprevention [8]. In addition to their metabolic actions, an emerging area of investigation for PPARγ and PPARδ agonists is their ability to modulate mammary cell lineage and genes associated with tumor suppressor function and cell fate determination. This suggests that PPAR agonists may play a role in stem/progenitor cell proliferation and differentiation to modify tumor response.

2. PPARγ SIGNALING

The PPAR nuclear receptor subfamily consists of the PPARα, PPARγ, and PPARδ/β isotypes that regulate a number of metabolic pathways controlling fatty acid β-oxidation, glucose utilization, cholesterol transport, energy balance, and adipocyte differentiation [4–6]. PPARs function as heterodimeric partners with RXR, and require high-affinity binding of PPAR ligand to engage transcription [7]. PPARs bind to the DR-1 response element (PPRE) consensus sequence AGG(T/A)CA, which is recognized specifically by the PPAR partner [9]. Like other nuclear receptors, PPARs consist of a putative N-terminal transactivation domain (AF-1), a DNA-binding domain (DBD) containing two zinc fingers, a ligand-binding domain (LBD) containing a large hydrophobic pocket, and a C-terminal ligand-dependent transactivation region (AF-2) [10].

There is >97% homology at the protein level, 99% homology within the LBD, and minimal functional differences after ligand-dependent activation between human and mouse PPARγ, [11]. PPARγ is expressed predominantly in white adipose tissue, intestine, endothelial cells, smooth muscle and macrophages [12], and is the major isotype expressed in the mammary gland, and in primary and metastatic breast cancer and breast cancer cell lines [3].

Several mutations and polymorphisms have been identified in PPARγ, such as Lys319X (truncating) and Gln286Pro, in sporadic colon cancer, which are associated with loss of DNA-binding and ligand-dependent transcription by the PPARγ agonist, troglitazone [13]. Similar results were found for PPARγ2 polymorphism Pro112Ala [14], but the polymorphism Ser114Ala resulted in increased transactivation by presumably blocking the inhibitory effect of Ser114 phosphorylation by ERK [15, 16]. However, in a sampling of approximately 400 breast, prostate, colon, and lung tumors

and leukemia's, no mutations of the PPARγ gene were found, suggesting that if indeed this does occur, it is a very rare event [17].

In follicular thyroid cancer, the t(2;3)(q13;p25) translocation results in formation of the Pax8-PPARγ fusion protein, which is pathoneumonic for the majority of cases of this disease [18]. It acts as a dominant-negative receptor of PPARγ [18, 19], and reduces expression of the Ras tumor suppressor, NORE1A [20], which inhibits ERK activation [21]. PPARγ also increases expression of other tumor suppressor genes, such as PTEN [22] and BRCA1 [23] through their respective PPRE promoter regions, suggesting that the antitumor effects of PPARγ agonists may be related to their ability to downregulate multiple tumorigenic signaling pathways. This agrees with the reduction of PTEN and increased nuclear β-catenin and ERK activity in the mammary gland and tumors of MMTV-Pax8PPARγ mice [24] (see Figure 1). Since inactivation of BRCA1 [25] and PTEN [26–28] also increases stem cell proliferation, Pax8-PPARγ may upregulate specific progenitor cell lineages that are more susceptible to tumorigenesis.

PPARs interact with the coactivators C/EBP, SRC-1, and DRIP205, and in the unliganded state with the corepressor SMRT [19, 29–31], and exhibit similar coactivator/corepressor dynamics as other nuclear receptors, such as estrogen receptor-α (ER) [32]. PPARγ can interfere with ER transactivation through its binding to the ERE [33, 34], and preferentially partitions with ER for its canonical response elements [35]; conversely, ER can block PPRE-dependent transcription [36] (see Figure 1). PPARγ also modifies ER signaling by promoting its ubiquitination and degradation [37] as well as by upregulating CYP19A1 (aromatase) activity [38, 39], which can blunt the activity of aromatase inhibitors used to treat patients with ER+ breast cancer. PPARγ agonists block the ER-dependent growth of leiomyoma cells, further suggesting crosstalk between the ER and PPARγ signaling pathways. PPARγ and ER pathways have opposite effects on PI3K/AKT signaling that may also account for the inhibitory action of PPARγ ligands on ER-dependent breast cancer cells [36] (see Figure 1). These findings imply that PPARγ antagonism should upregulate ER expression in responsive tissues, which is precisely the phenotype observed in mammary tumors induced in transgenic mice expressing Pax8PPARγ [24].

Studies using transgenic and knockout mouse models of PPARγ have led to disparate conclusions regarding the role of PPARγ in tumorigenesis. Mice expressing constitutively active VP16-PPARγ in the mammary gland did not exhibit a tumorigenic phenotype but accelerated tumorigenesis when crossed with MMTV-polyoma middle-T antigen mice [40], intimating that the unliganded receptor may have interfered with tumor suppressor transactivation by endogenous PPARγ through corepressor recruitment. Alternatively, the VP16 fusion protein is known to induce many genes that are not indicative of PPARγ activation [41]. In the probasin-SV40 T-antigen prostate tumor model, tumorigenesis was unaffected by a PPARγ null background [42], indicating that oncogenic signaling was already maximally activated. However, in the Apc^{Min} mouse colon tumor model, "glitazone"
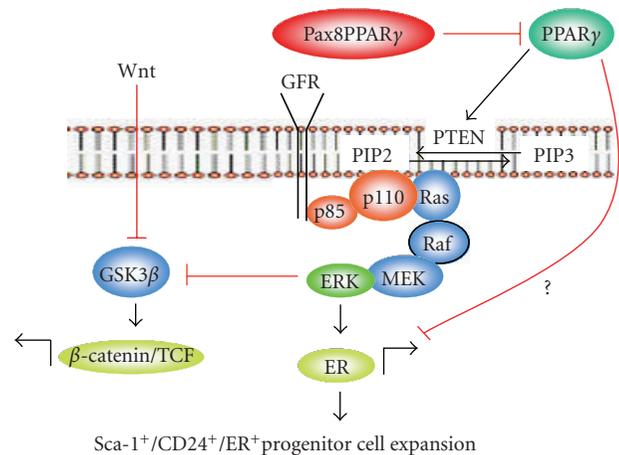

Figure 1: Pax8PPARγ and mammary cell fate determination. Pax8PPARγ acts in a dominant-negative fashion to block PPARγ-dependent transactivation and upregulation of PTEN. MMTV-Pax8PPARγ mice exhibit reduced PTEN and activation of Ras and ERK, presumably through activation of PI3K (p85 and p110). ERK activates ER transcriptionally and posttranslationally, and Pax8PPARγ may interfere with the ability of PPARγ to inhibit ER transactivation. Mammary epithelial cells isolated from the mammary glands of MMTV-Pax8PPARγ mice contain a higher percentage of CD24+/CD29^{hi} stem/progenitor cells, and present with predominantly ER+ ductal carcinomas following carcinogenesis, suggesting a role of PPARγ in cell fate determination.

PPARγ agonists increased the number of colon, but not small intestine polyps [43, 44], as well as colon adenomas [45]. Since the small intestine, and not the colon, is the predominant site of neoplasia in this mouse model, the significance of this observation is unclear. It should also be stressed that PPARγ agonists did not induce malignant changes in wild type mice, indicating their lack of carcinogenicity. Contrary to these results, PPARγ haploinsufficiency produced a greater rate and number of colon tumors following azoxymethane-induced carcinogenesis [46], implying that PPARγ acts as a tumor suppressor rather than as an oncogene. APC$^{+/1638N}$ mice heterozygous for PPARγ did not exhibit changes in polyp formation [46]. This result indicates that the induction of β-catenin in the colonic crypt cells of PPARγ haplosufficient mice, a protumorigenic factor that is constitutively activated in APC mice, is the target of tumor suppression in wild-type mice [47]. A tumor suppressor role for PPARγ is also supported by the inhibitory effect of PPARγ agonists on colon tumor growth [48, 49], and mammary carcinogenesis [50–52]. This effect may be mediated in breast tumors through induction of apoptosis due to reduction of Bcl-2 [53], and in pancreatic and liver tumors through a reduction of cyclin D1 and HB-EGF [54] and an increase of p27^{Kip1} [55–57]. PPARγ agonists may also find utility as modifiers of the response to chemotherapy. CS-7017, a potent thiazolidinedione agonist, synergized with paclitaxel to inhibit the growth of anaplastic thyroid tumors through induction of p21^{Cip1} [58]. Notwithstanding possible "off-target" effects [59, 60], most studies indicate that PPARγ

agonists as a class have antitumor activity, and thus may have efficacy as a relatively nontoxic adjunct to chemotherapy and possibly to radiation therapy through their ability to act as "tumor suppressor enhancers."

3. PPARδ SIGNALING

As with PPARγ, PPARδ is involved in adipocyte differentiation by promoting clonal expansion of preadipocyte progenitor cells [61], possibly through activation of PPARγ expression [62]. The PPARδ agonist GW501516 has been tested clinically as a cholesterol lowering drug in dyslipidemic patients, but the results have been mixed [63]. In animal models, homozygous disruption of PPARδ resulted in a runted phenotype [64] and in 90% embryonic lethality with runted survivors [65], indicating its importance in embryonic development. PPARδ null macrophages exhibited loss of the dominant inhibitory effect by unliganded PPARδ [60], which was previously identified by its ability to block PPARα and PPARγ transactivation through corepressor recruitment [60, 66, 67]. In breast cancer cells, PPARδ expression was greater in ER$^-$ MDA-MB-231 breast cancer cells than in ER$^+$ MCF-7 cells [68], also suggesting a correlation with a more aggressive form of this disease. Indeed, tissue microarray analysis of invasive breast cancers indicated that PPARδ is strongly expressed (see Figure 2, "+3") in 52% of 164 samples, and thus may have value as a prognostic marker and therapeutic target. There are no examples of the development of PPARδ *antagonists* as anticancer therapeutics.

GW501516 accelerated the onset of tumor formation during mammary carcinogenesis, in contrast to the delay of tumor formation by PPARγ agonist GW7845 [52]. PPARδ expression increased in K-Ras-transformed intestinal epithelial cells [69] and PDGF-stimulated vascular smooth muscle cells [70]. Similar findings were reported for conditional expression of PPARδ, where GW501516 increased proliferation of hormone-dependent breast and prostate cancer cells and endothelial cells, and increased expression of genes associated with proliferation and angiogenesis [71]. PPARδ can suppress the antiproliferative effects of PPARα and PPARγ [7] and directly associate with PDK1 [52] to affect its localization and activation [72, 73], which implicate it as a protumorigenic factor, and therefore raise a caution for the general use of this class of agonists [74].

Colon cancer presents an interesting model to exam the role of PPARδ in tumorigenesis since Apc^{Min} mice exhibit constitutive activation of β-catenin/TCF signaling, the pathway believed to activate PPARδ [75]. PPARδ is highly expressed in colorectal cancer cells [75], and somatic cell knockout of PPARδ reduced tumorigenicity in nude mice [76]. Crossing PPARδ null or heterozygous mice with Apc^{Min} mice showed a gene dosage dependent reduction in large intestinal polyps [65], and treatment of Apc^{Min} mice with GW501516 produced an increase in both polyp number and size [77], all suggesting that PPARδ is protumorigenic. However, a study using a different targeting scheme to delete PPARδ reported no change in polyp number or size in the small intestine of Apc^{Min} mice, and a greater

(a)

(b)
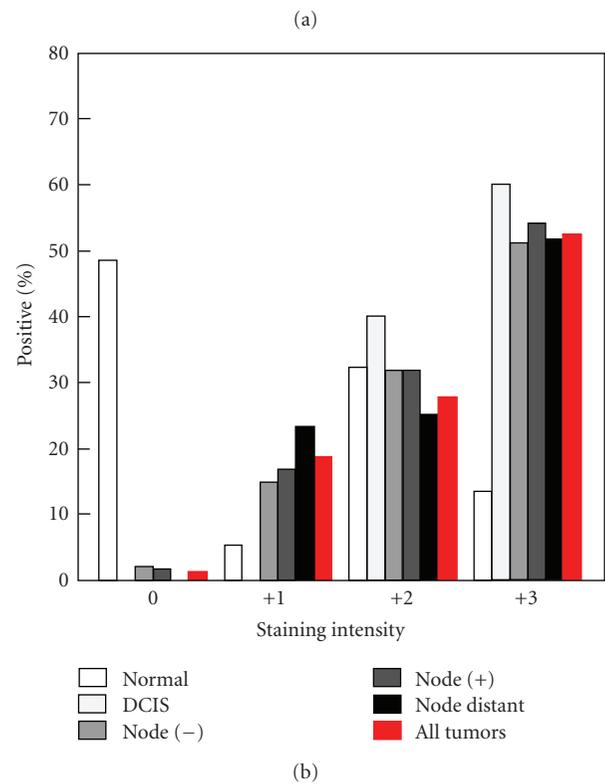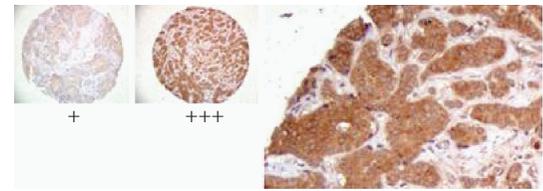
FIGURE 2: PPARδ expression in invasive breast cancer. Representative samples from a tissue microarray analysis of invasive breast cancers are shown. PPARδ staining intensity is indicated as low (+1), medium (+2) or high (+3). The magnified image shows examples of +1 and +3 staining. The bar graph depicts the percentage of samples expressing PPARδ in DCIS, node (+), node (−) and node distant tumors.

number but not size of carcinogen-induced colon tumors in mice with this background [78]. Since the PPARδ knockout mice generated by Barak contained a deletion of exon 4 encoding the hinge region [65], whereas, that generated by Peters et al. [64] contained a deletion of the last exon encoding the AF2 domain, it is possible that the truncated PPARδ may not be as susceptible to corepression as the wild-type receptor. This would explain why their results [79, 80] differ from studies showing that keratinocytes from mice heterozygous or null for PPARδ exhibit less proliferation [81] and those in Apc^{Min} mice in a PPAR null background exhibit increased tumorigenesis [65]. From a mechanistic standpoint, PPARδ is activated in colon cancer cells by prostacyclin (PGI$_2$) [82] and inhibited by the NSAID indomethacin [75], suggesting that its tumor promoting action is related to inflammation, a condition that increases
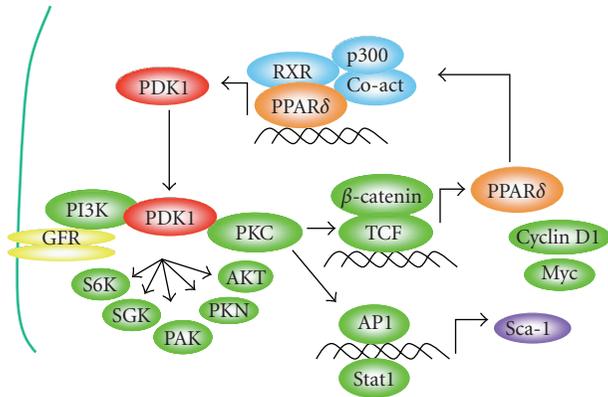
FIGURE 3: PDK1 and PPARδ autoregulatory cascade. Growth factor receptor (GFR) activation activates PDK1 leading to PKCα and β-catenin/TCF activation [88]. TCF target genes include cyclin D1, c-Myc, and PPARδ [75]. PPARδ transactivates PDK1 [72], which in turn perpetuates the oncogenic signaling cascade. Preliminary data suggests that PDK1 maintains the expression of the murine stem/progenitor cell marker, stem cell antigen-1 (Sca-1), which is under the control of AP1 and Stat1 [92].

the risk of colon cancer [83]. NSAIDs downregulate PPARδ and reduce eicosanoid-mediated inflammation [84], and induce apoptosis in colon cancer cells [85], in contradistinction to the anti-inflammatory effects elicited by PPARγ agonists in colitis [86]. Increased expression of PPARδ in tumors may also inhibit PPARγ transcription [60, 66, 67], and reduce its tumor suppressor activity, as mentioned above in colon tumorigenesis. In addition, the tumor promoting effects of PPARδ in the mammary gland relate to activation of β-catenin/TCF signaling [76, 87] (see Figure 3), which is increased in cells transformed by PDK1 [88, 89]. PDK1 is a key regulator downstream of PI3K that is increased by PPARδ in keratinocytes [72, 73]. Mammary tumors formed after administration of GW501516 exhibit an association between PDK1 and PPARδ [52], which further suggests that PPARδ may function as an integrator of proliferative and prosurvival pathways downstream of oncogenic signaling and inflammation [90, 91], which are likely to account for its tumor promoting effects.

PPARs and stem cells

There is evidence that PPARs can modulate stem and progenitor cell expansion and the differentiated or malignant phenotype. PPARγ agonists enhance adipocyte differentiation [5, 6], and its ability to upregulate this process has a negative effect on osteoblast proliferation and bone development from mesenchymal stem cells [93]. To counteract this inhibitory effect in bone stem cells, PPARγ must be transrepressed through corepressor recruitment by the NFκB and Wnt-5a pathways [94]. It is therefore likely that PPARs influence the fate of other stem and progenitor cell populations in normal and malignant tissues. PPARγ agonists have been used as chemopreventive agents [8] to delay mammary carcinogenesis [51, 52]. One aspect to their chemopreventive

action may relate to their influence on specific cell lineages, as in mesenchymal stem cells. Carcinogens target stem cells rather than terminally differentiated cells [95, 96] as well as hormone-responsive lineages [97] during mammary carcinogenesis. Carcinogenesis is markedly attenuated in PR-null mice[98], and is accelerated by progestin treatment of wild-type mice [52, 99–101], where progestins are believed to stimulate the proliferation of stem or early progenitor cells that are intrinsically more susceptible to tumor initiation [102]. The ability of PPARγ and PPARδ agonists to modulate distinct cell lineages during mammary tumorigenesis [52] also suggests that they modulate a complex transcriptional network linked to cell fate [3, 5]. PPARδ agonist GW501516 promoted the development of adenosquamous carcinomas with high expression of the stem cell markers CK19 and Notch1, as well as Proliferin, a growth factor that mediates many of the effects of the stem cell marker, Musashi1, in mammary cells [103]. PPARδ is expressed in the crypt cells of the small intestine and negatively regulates Hedgehog signaling to block differentiation [104], a process that would be expected to promote transformation. PPARδ expression lies downstream of β-catenin/TCF [75], and activation of this pathway increases expression of luminal epithelial and myoepithelial cells [102] as well as mammary tumor cells expressing the stem cell marker Sca-1 [105]. Thus, PPARδ activation may promote expansion of a less differentiated lineage or stem cells that is intrinsically more susceptible to tumorigenesis. The association of Wnt activation with stem cell expansion, activation of β-catenin/TCF signaling by PDK1, the identification of PPARδ as a β-catenin/TCF target gene and PDK1 as a PPARδ responsive gene, as well as the modulation of Sca-1+ stem/progenitor cells by the Wnt pathway, all suggest a common mechanism for the tumor promoting action of PPARδ agonists that may involve stem and progenitor cell proliferation (see Figure 3). This mechanism also suggests that the development of PPARδ antagonists may have utility as cancer therapeutics

PPARγ increases expression of the PPRE-dependent tumor suppressor genes PTEN [22] and BRCA1 [23], suggesting that their chemopreventive effects may be related to the ability of these suppressor genes to promote a more differentiated lineage. On the contrary, inactivation of BRCA1 [25] and PTEN [26–28] should increase stem cell proliferation, which is precisely the case. This effect is similar to what has been described for PPARδ agonists in preventing differentiation and increasing stem cell abundance, and would be expected to complement their tumor promoting activity. Although studies examining the influence of PPARs on cell fate determination are just in their infancy, many of the studies cited imply that their opposing roles in tumorigenesis may be related to their ability to control the programming of specific cell lineages.

4. CONCLUSIONS

The ability of PPAR agonists to modulate the transcriptional activity of this class of nuclear receptors has generated an enormous interest in being able to pharmacologically manipulate entire sets of genes that can modulate

metabolism, inflammation, transformation, differentiation and thus, tumorigenesis. Both genetic and pharmacological approaches to determining the function of PPARγ and PPARδ have yielded some inconsistencies, but that may be explained by the inherent deficiency of either approach. Gene targeting resulting in a truncated gene product may not necessarily recapitulate gene inactivation, and homozygous loss of gene expression can affect the developmental programming of various tissues that can impact directly or indirectly on the outcome of tumorigenesis in a particular organ. By the same token, pharmacological approaches are fraught with the structure-specific and class-specific side effects inherent in most drugs, which may be unrelated to their specific actions on the drug target. Nevertheless, the majority of studies in this field implicate PPARγ activation as an antitumorigenic and prodifferentiation factor, in contrast to the protumorigenic and less differentiated phenotype resulting from PPARδ activation. Although the latter characteristic will likely preclude the clinical development of PPARδ agonists, it will be interesting to see the outcome of current clinical trials utilizing PPARγ agonists as antitumor and chemotherapy modulating therapy.

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Review Article

Role of Peroxisome Proliferator-Activated Receptor Gamma and Its Ligands in the Treatment of Hematological Malignancies

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Peroxisome proliferator-activated receptor gamma (PPARγ) is a multifunctional transcription factor with important regulatory roles in inflammation, cellular growth, differentiation, and apoptosis. PPARγ is expressed in a variety of immune cells as well as in numerous leukemias and lymphomas. Here, we review recent studies that provide new insights into the mechanisms by which PPARγ ligands influence hematological malignant cell growth, differentiation, and survival. Understanding the diverse properties of PPARγ ligands is crucial for the development of new therapeutic approaches for hematological malignancies.

1. INTRODUCTION

In order to understand the influence of PPARγ and its many ligands on hematological malignancies and their normal cell counterparts, we first present background material to orient the reader.

Peroxisome proliferator-activated receptors (PPARs) α, β/δ, and γ are members of the nuclear hormone receptor superfamily of transcription factors that regulate several metabolic pathways in a tissue-selective manner [1]. All PPARs form heterodimers with members of the retinoid X receptor (RXR) subfamily of nuclear hormone receptors and regulate initiation of transcription by binding to the peroxisome proliferator response element (PPRE) in promoters of target genes. Drug classes such as fibrates and thiazolidinediones are used for lowering lipids and improving insulin sensitivity, respectively, thus effectively reducing risk factors that lead to cardiovascular disease [2, 3] and diabetes [4, 5]. PPARγ agonists have both PPARγ-dependent and -independent effects on coagulation, thrombosis, angiogenesis, and tumor growth and metastasis [6, 7]. PPARγ agonists also exert anti-inflammatory and antifibrotic effects by negatively regulating the expression of proinflammatory genes and by inhibiting myofibroblast differentiation [8–10]. Moreover, PPARγ agonists modulate the activity of several transcription factors (e.g., NF-κB, AP-1, and Stat3) [10–13] that regulate inflammation.

1.1. Structure of the human PPARγ gene

The human PPARγ is located on chromosome 3, band 3p25 [14]. This gene gives rise to the two well-known isoforms of PPARγ, PPARγ1, and PPARγ2, which function as transcriptional activators or repressors in a context-dependent
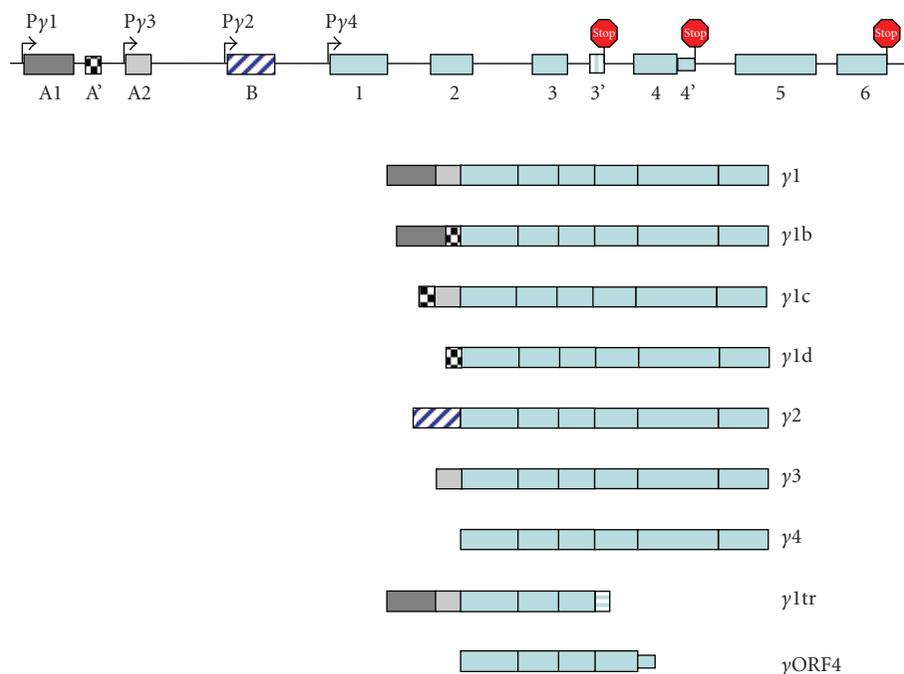
FIGURE 1: *Schematic structure of the human PPARγ gene.* The human PPARγ gene is located on chromosome 3, band 3p25, and is composed of at least 11 exons that give rise to 9 transcript variants. Expression of PPARγ involves differential promoter usage in combination with alternative splicing and polyadenylation site selection. The relative positions of the four known PPARγ promoters are designated as Pγ1-Pγ4. The noncoding exons A1, A′, and A2 are depicted by boxes in different shades of gray or in black and white checked. These exons contribute to the 5′ UTR of transcripts γ1-γ1d, γ3 and γ1tr. The transcript variants 1γ-1γd, γ3, and γ4 encode the PPARγ1 isoform. Exon B (diagonal blue and white hatched box) encodes the 28 additional amino acids found at the amino terminus of human PPARγ2; the mouse PPARγ2 exon B encodes 30 amino acids. Exons 1–6 (light blue boxes) are common in all PPARγ1 transcripts and when they are spliced to exon B encode full-length PPARγ2. Two additional exon regions have been recently identified, exon 3′ (horizontal light blue and white hatched box) and exon 4′ (small light blue box). Inclusion of either of these coding regions in the processed mRNA transcript results in truncated PPARγ1 proteins lacking the ligand binding domain (γ1tr and ORF4, resp.). The sizes of the exon boxes approximate the relative lengths of each exon; however, the introns (depicted as straight lines) are not drawn to scale. The positions of the stop codons are depicted by the hexagonal red stop signs.

manner [15, 16]. Recent evidence suggests that the human PPARγ gene is composed of at least 11 exons that give rise to 9 transcript variants due to the combination of differential promoter usage, alternative RNA splicing, and polyadenylation site selection of the primary transcript (Figure 1). To date, four promoters and three new exons A′, 3′, and 4′ have been identified [14, 17–23]. Similar to exons A1 and A2, exon A′ is noncoding and contributes to the 5′ UTR of several transcript variants (Figure 1). Inclusion of exon 3′ in the processed transcript produces a truncated PPARγ1 protein (γ1tr) [22], as does the read-through of exon 4 to include intron 4 sequences (γORF4) [23]. Both truncated forms of PPARγ1 (γ1tr and γORF4) lack the coding regions for the ligand binding domain and function in a dominant negative manner to wild type PPARγ1. The truncated form of PPARγ (γ1tr) was discovered and cloned from chronic myeloid leukemia K562 cells and enhanced cell proliferation [22]. Similarly, γORF4 protein was found to reside mainly in the nucleus and enhanced cell growth [23]. The complexity in processing the PPARγ primary transcript likely leads to specific regulation of PPARγ functions in a context-dependent manner. This may explain, at least

in part, the pleiotropic functions ascribed to PPARγ1 and PPARγ2 [23–29].

1.2. Posttranslational modifications regulate PPARγ activity

Several reversible posttranslational modifications occur that regulate the transactivation potential of PPARγ (Figure 2). The phosphorylation status and activity of the PPARs are regulated in both ligand-dependent and ligand-independent manners, the details of which have been recently reviewed [30]. Whereas serine phosphorylation of PPARα increases its transcriptional activity in hepatocytes, MAPK/ERK-mediated phosphorylation of Ser[84/112] on PPARγ1/2 leads to attenuation of PPARγ transcriptional activity and its possible relocalization from the nucleus to the cytoplasm [30–33]. Furthermore, both Ser[84/112] phosphorylation [34] and ligand binding [35] contribute to the targeting of PPARγ to ubiquitin-proteasome degradation. In contrast, ERK5 activates PPARγ1 in a phosphorylation-independent manner by directly interacting with the hinge-helix 1 region [36].
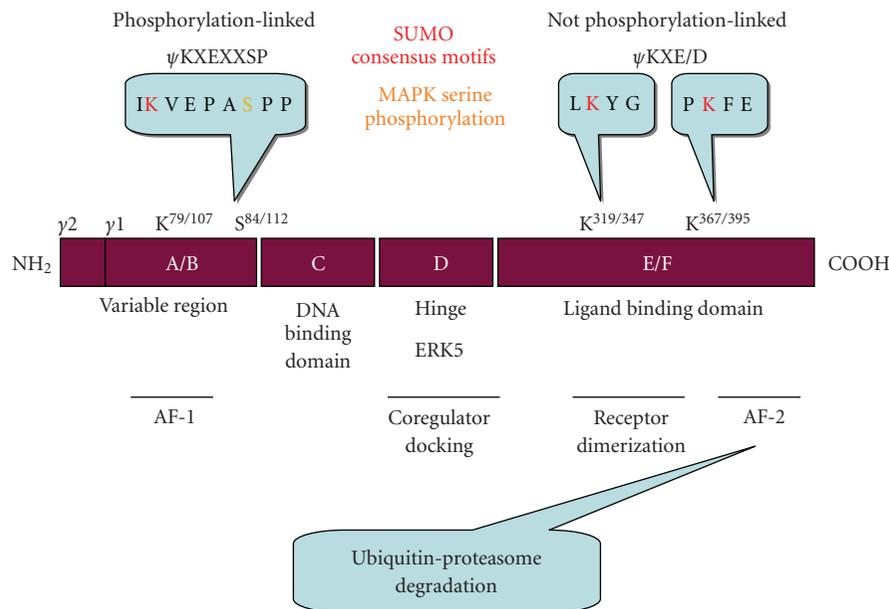
FIGURE 2: *Reversible posttranslational modifications of PPARγ.* The superfamily of nuclear hormone receptors possesses conserved structural and functional domains including PPARγ. The A/B domain is the hypervariable region containing the putative activation function-1 (AF-1) domain. Human PPARγ2 contains a 28 amino acid amino terminal region that arises from differential promoter use and splicing (see Figure 1). The primary structure of the C-domain is the most conserved and contains the DNA binding domain (DBD). The D-domain (Hinge) allows for conformational change following ligand binding to promote coregulator (coactivator or corepressor) docking; binding of ERK5 to the hinge helix 1 region potentiates ligand-dependent PPARγ1 activity. The E/F region contains the ligand binding domain (LBD) of PPARγ and the activation function-2 (AF-2) domain that participates in ligand-dependent degradation mediated by the ubiquitin-proteasome pathway. PPARγ heterodimerizes with its binding partners, RXR family members, through the E/F domain as well. Reversible posttranslational modifications of PPARγ regulate its activation. In addition to proteasome-mediated degradation, PPARγ can be phosphorylated by MAP kinases at $S^{84/112}$ (position of serine in PPARγ1/PPARγ2) or SUMO-1 modification. Two SUMOylation consensus motifs have been described. Whereas SUMOylation at a conserved ψKXEXXSP (where ψ is a hydrophobic amino acid and X can be any residue) is linked to serine phosphorylation events, SUMOylation at ψKXE/D motifs are not generally linked to MAPK phosphorylation. The lysine residues of the three SUMOylation motifs identified on PPARγ1/2 are depicted in red. The serine residue phosphorylated by MAPKs is depicted in yellow. Both serine phosphorylation and SUMOylation negatively regulate PPARγ activity.

In a recent review, Straus and Glass [10] discuss various mechanisms for nuclear hormone receptor-dependent transrepression of target genes by the PPARs, Liver X Receptors (LXRs), and glucocorticoid receptor (GR). Posttranslational modification with small ubiquitin-like modifier (SUMO)-1 converts these nuclear hormones from transactivators to transrepressors of gene expression [10, 37]. SUMOylated PPARγ1 binds to the corepressor complex interfering with its clearance, thereby preventing transactivation of NF-κB target genes [10, 37]. To date, modifications of PPARγ with SUMO-1 occur on three lysine residues ($K^{79/107}$, $K^{319/347}$, and $K^{367/395}$) of PPARγ1/2 [38–40]. Moreover, PPARγ's dimerization partner, RXRα, is also SUMOylated [41]. A summary of PPARγ posttranslational modifications is shown in Figure 2. SUMO competes with ubiquitin for modification of lysines on some proteins, thereby rescuing the protein from ubiquitin-proteasome mediated proteolysis [42]. In addition to increasing protein half-life, SUMOylation plays a role in nuclear-cytoplasmic trafficking, cell-cycle regulation, genome integrity, transcription, and cancer progression and metastasis [43–47].

2. PPARγ LIGANDS

Transcriptional activity of PPARγ is controlled primarily by ligand binding [48]. PPARγ has a large ligand binding pocket, which enables it to bind a variety of ligands [49]. PPARγ ligands include both synthetic and natural molecules [48]. Many of the naturally occurring ligands are fatty acids or fatty acid derivatives obtained through the diet or from intracellular signaling pathways. These include lysophosphatidic acid [50], nitrolinoleic acid [51], 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE) [48, 52], 15-hydroxyeicosatetraenoic acid (15-HETE) [25], prostaglandin D_2 (PGD$_2$), and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$) [25, 48, 49, 53–55]. 15d-PGJ$_2$ is thought to be the most potent endogenous ligand for PPARγ, activating it at low micromolar concentrations [25, 52, 53]. PGD$_2$ and 15d-PGJ$_2$ are derived from arachidonic acid by the catalytic activities of the cyclooxygenase-2 (Cox-2) and prostaglandin D synthase [53, 54, 56]. PGD$_2$ spontaneously undergoes a series of dehydration reactions to form the PGJ family of prostaglandins, including 15d-PGJ$_2$,

and 15d-PGD$_2$, which can also transactivate PPARγ [56–60].

Synthetic PPARγ ligands, including drugs of the thiazolidinedione (TZD) family (e.g., ciglitazone, pioglitazone, rosiglitazone, and troglitazone), have potent insulin-sensitizing properties [3, 25, 49, 56, 61, 62]. Because of this, some are commonly used for the treatment of type 2 diabetes [48, 61]. There also exist TZDs, such as TZD18, that act as dual PPARα/PPARγ agonists [63].

There are also many non-TZD synthetic compounds that can function as PPARγ agonists. Some of these are: L-tyrosine-based GW-7845 and GW-1929 [48, 52], diindolymethane analogs [48, 64], certain nonsteroidal anti-inflammatory drugs (NSAIDs) (i.e., indomethacin, ibuprofen, flufenamic acid, and fenoprofen [25, 27, 65]), and the novel synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its derivatives [48, 66]. CDDO binds to PPARγ with nanomolar affinity [48, 66] and displays antiproliferative and differentiating activities, making it useful as a chemotherapeutic agent. Derivatives of CDDO have more useful pharmacodynamic and pharmacokinetic properties than CDDO itself [67, 68]. Importantly, some CDDO derivatives are orally active and are remarkably well-tolerated in humans [69].

PPARγ ligands, including CDDO, can reduce cell proliferation, migration, cytokine production, expression of costimulatory, and adhesion molecules and can promote apoptosis [48]. These findings suggest that PPARγ ligands may be efficacious in the treatment of hematological malignancies [48]. However, numerous side effects have been observed in patients treated with TZDs [49]. For example, troglitazone has caused hepatotoxicity [49]. TZDs also induce weight gain, edema [70], increased lipoprotein(a) concentrations [3, 49], and probably enhance risk of heart failure and cardiac hypertrophy [48, 71, 72]. Therefore, it is highly desirable to develop PPARγ ligands with improved therapeutic profiles [48].

The identification of "selective PPARγ modulators" (SPPARγMs) has become the object of intense recent interest, with the idea that one might modulate the genes necessary to achieve therapeutic potential, while not affecting the genes involved in producing side effects [49]. This concept is plausible because SPPARγMs take advantage of the large PPARγ ligand binding pocket, which allows a variety of ligands to bind in different orientations [15, 61, 73–76]. SPPARγMs then induce specific conformational changes of the receptor which create different interaction surfaces, favoring the recruitment of only a subset of coregulators [48, 49, 77, 78]. This subset of coregulators will allow the induction of some, but not all target genes [15, 49, 61, 74, 79–83]. The SPPARγM concept has been shown to hold true for some currently recognized PPARγ ligands. For example, CDDO is a more potent inducer of apoptosis than are TZDs [48]. This may be because the PPARγ target genes activated by CDDO are different from those activated by TZDs [48]. CDDO is less effective than rosiglitazone in recruiting coactivators, but it can effectively promote the release of corepressors from PPARγ target genes [48]. A greater understanding of the activities of the various PPARγ ligands will depend on the identification of the specific coregulators recruited to PPARγ target genes in response to binding to specific ligands [25].

3. PPARγ AND THE IMMUNE SYSTEM

One of the earliest indications of an important role for PPARγ in the immune system was the discovery of its expression in mouse spleen [84]. After this finding, our laboratory and others began searching for PPARγ expression and function in immune cells. To date, PPARγ expression has been found in monocytes/macrophages, dendritic cells, granulocytes (i.e., neutrophils, eosinophils, and basophils), mast cells, T cells, and B cells, and most recently our laboratory found PPARγ in human platelets [84–90].

PPARγ ligands have been shown to have anti-inflammatory effects on cells of the innate and adaptive immune system [91–94]. In macrophages, PPARγ has an important role in regulating lipid metabolism, as well as in the generation of macrophage-derived foam cells in atherosclerotic lesions [95–98]. Upon phorbol myristyl acetate (PMA) stimulation, PPARγ ligands can inhibit macrophage activation and production of inflammatory cytokines (e.g., TNFα, IL-1β, and IL-6), inducible nitric oxide synthase (iNOS), gelatinase B, and scavenger receptor A (SR-A) [89, 99, 100]. Moreover, PPARγ activation can skew macrophage differentiation into a more anti-inflammatory phenotype [101]. In dendritic cells, PPARγ activation can inhibit the production of IL-12 and of chemokines involved in the recruitment of Th1 lymphocytes, therefore, favoring a type 2 immune response [90]. PPARγ ligands also enhanced the development of a dendritic cell phenotype that: (1) has increased endocytic activity and (2) induces the expansion of invariant natural killer T (NKT) cells [102].

PPARγ also plays a role in T lymphocyte function, and its levels are upregulated following their activation [103, 104]. PPARγ expression and activation can inhibit T lymphocyte proliferation and reduce the production of IFNγ, TNFα, and IL-2 [92, 105, 106]. These inhibitory effects result from the direct interaction between PPARγ and the transcription factor nuclear factor of activated T cells (NFAT) [107]. Our laboratory demonstrated that mouse and human T cells express PPARγ, and treatment with PPARγ ligands induces apoptosis in malignant T cells [103, 104]. Recent findings reported by Wohlfert et al. could illuminate yet another regulatory role for PPARγ in the immune system [108]. In their study, PPARγ activation enhanced the generation of CD4$^+$ CD25$^+$ regulatory T cells (Tregs). Tregs have been demonstrated to play a key role in negatively regulating autoimmunity and immune responses [109]. There are two different subtypes of Tregs: thymus-derived natural Tregs (nTregs) and inducible Tregs (iTregs), which develop from CD4$^+$ CD25$^-$ effector T cells in the periphery. [109–111]. Wohlfert et al. showed that ciglitazone enhanced the conversion of effector T lymphocytes into inducible Tregs (iTregs). Moreover, PPARγ expression in natural Tregs (nTregs) was required for the in vivo effects of ligand treatment in a murine model of graft versus host disease [108]. These findings suggest that PPARγ ligands enhance

the activity of Tregs while dampening the activation of other T lymphocyte subsets. PPARγ was also shown to have a physiological role in regulating B lymphocyte function. In studies using PPARγ haploinsufficient mice, B lymphocytes exhibited increased proliferation and survival, enhanced antigen specific immune responses and spontaneous NF-κB activation [15, 112]. Our laboratory demonstrated that both normal and malignant B lymphocytes express PPARγ, and that exposure to certain PPARγ ligands inhibits B cell proliferation and can induce apoptosis [85, 93, 113].

In summary, PPARγ activation has antiproliferative and proapoptotic effects and dampens cytokine production in several immune cells. PPARγ ligands can also attenuate several inflammatory diseases such as inflammatory bowel disease [114–119], multiple sclerosis [120–122], rheumatoid arthritis [112, 123], and psoriasis [124–126]. These findings suggest that PPARγ ligands may be useful for the treatment of immunological diseases, which include myelo and lymphoproliferative disorders.

4. PPARγ AND ITS CONTROVERSIAL ROLE AS A TUMOR SUPPRESSOR GENE

As evidence accumulated to support that PPARγ ligands are inhibitors of cell proliferation and inducers of cell differentiation, attention turned to the role of PPARγ in the onset and development of cancer. The potential of PPARγ ligands as anticancer drug therapies has been explored in cells from various malignant tissues, including those of adipose, colon, breast, prostate, lung, pancreas, bladder, and stomach origin [26, 127]. There is emerging evidence for a direct role of PPARγ functional mutations in the initiation of several common human cancers, such as colon, prostate, and thyroid [28, 128–130]. For example, in a study of 55 patients with sporadic colon cancers, four somatic PPARγ mutations were found. [129]. Also, a hemizygous deletion of PPARγ was identified in 40% of prostate cancers [128]. Furthermore, a fusion protein derived from the paired box gene 8 (PAX8) and PPARγ genes (PPARγ-PAX-8) was detected in thyroid cancers, which causes PPARγ not only to be functionally inactive but also to function as a dominant negative form of PPARγ [28, 131]. As described earlier, the PPARγ gene is mapped to human chromosome 3, band 3p25 [14]. Interestingly, 3p deletions have been identified in several hematological cancers, including acute myeloid leukemias (AML), myelodysplastic syndromes (MDS), Philadelphia chromosome-positive chronic myeloid leukemia (CML), acute lymphoblastic leukemias (ALL), chronic lymphoproliferative disorder (CLD), and non-Hodgkin's lymphomas (NHL) [132]. These observations suggest that PPARγ plays a role as a tumor suppressor gene and, as such, may be a therapeutic target for cancer. Studies in liposarcoma [133] and in xenograft models of prostate [134] and colon cancer [135] support this hypothesis. However, a study using a large number of human tumor samples and cell lines ($n = 397$), including those from leukemias, found no detectable abnormalities, either in PPARγ exon 3 (DBD) or in exon 5 (LBD), suggesting that PPARγ gene mutations may occur, but are rare [136].

The expression levels and/or the transactivation of PPARγ may be impaired in certain cancers. In human lung cancer, decreased expression of PPARγ correlated with poor prognosis [29] and well-differentiated adenocarcinomas had more PPARγ expression than poorly differentiated varieties [137]. In addition, a study performed by Jansen et al. demonstrated that the abnormal PML-RARα (promyelocytic leukemia-retinoic acid receptor alpha) fusion protein found in acute promyelocitic leukemia (APL) interferes with PPAR function [138]. Similarly, Hamadani et al. showed that different X-RARα fusion proteins found in APL can inhibit the transactivation of PPARγ, and that this repression can be released by the addition of PPARγ ligands [139, 140]. These findings suggest that (1) PPARγ may be inactive in APL, (2) this may contribute to the undifferentiated phenotype, and (3) PPARγ ligands may help sensitize APL cells to the differentiating effects of all-*trans*-retinoic acid (ATRA).

5. PPARγ AND PPARγ LIGANDS AS POTENTIAL THERAPY FOR HEMATOLOGICAL MALIGNANCIES

5.1. Myeloid malignancies

5.1.1. Acute myeloid leukemia (AML)

Acute myelogenous leukemia (AML) constitutes about 25% of all leukemias in adults in the Western World. It ranks as the second most frequent type of leukemia in adults after chronic lymphocytic leukemia, with more than 13000 new cases, and nearly 9000 deaths from AML in the U.S. in 2007 [141]. Unfortunately, this type of leukemia has one of the lowest survival rates, about 20% [142]. There are several subtypes of AML, including acute promyelocytic leukemia (APL). The most common cause of APL is a translocation between chromosome 15 and 17, t(15;17), that leads to the generation of the PML/RARα fusion gene. The resulting fusion protein arrests the maturation of myeloid cells at the promyelocytic stage and leads to the increased proliferation of promyelocytes [143]. The cell lines typically used to study APL are NB4 and HL-60. NB4 has the t(15;17) translocation, while HL-60 does not [144]. In addition to chemotherapy and stem cell transplantation, treatments for APL also include differentiation therapy using all-*trans*-retinoic acid (ATRA) which has led to long-term disease-free survival in 70–80% of cases of this AML subtype [145].

An early study performed by Fujimura et al. demonstrated that treatment with troglitazone inhibited HL-60 cell growth by a G1 cell cycle arrest and induced their differentiation to monocytes [146]. A similar, G1 arrest was observed in all other hematopoietic cell lines examined. Furthermore, differentiation into the monocytic lineage was observed not only in the myelogenous and promonocytic cell lines, but also in an erythroleukemia cell line [146]. Data shown by Yamakawa-Karakida et al. demonstrated that PPARγ activation by both troglitazone and 15d-PGJ$_2$ inhibits proliferation and induces apoptosis in promyelocytic leukemia cells under serum-free conditions [147]. The induction of apoptosis was caspase-3 dependent, as treatment with a caspase-3 inhibitor completely abolished cell death. Although there

were no changes in antiapoptotic or proapoptotic proteins, the expression levels of the proto-oncogene product *c-myc* were drastically reduced after 24 hours of troglitazone treatment while DNA binding by Tcf-4, a transcription factor responsible for *c-myc* expression, was completely inhibited [147]. Troglitazone and 15d-PGJ$_2$ were found by Liu et al. to significantly induce apoptosis in K562 and HL-60 cells by upregulating the levels of the proapoptotic protein Bax and downregulating antiapoptotic proteins such as survivin and Bcl-2 [148]. Furthermore, these PPARγ ligands downregulated the expression of cyclooxygenase-2 (COX-2), antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, upregulated Bax and activated caspase 3 in human monocytic leukemia cells [149]. Recent observations reported by Han et al. revealed that 15d-PGJ$_2$ was able to sensitize tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-resistant leukemic HL-60 cells to TRAIL-induced apoptosis [150]. This effect of 15d-PGJ$_2$ was PPARγ-independent, as treatment with a PPARγ antagonist did not rescue the cells from apoptosis. These results were consistent with studies performed in other cancer cells where 15d-PGJ$_2$ enhanced TRAIL-induced apoptosis [151]. In a human eosinophilic leukemia cell line, EoL-1, treatment with troglitazone caused a G$_0$/G$_1$ cell cycle arrest that correlated with increased mRNA levels of the cyclin-dependent kinase (cdk) inhibitor, p21WAF1/CIP1. Troglitazone exerted a similar induction of p21 mRNA accompanied by inhibition of cell proliferation in U937 cells and in the KPB-M15 human myelomonoblastic cell line [152]. These findings suggest that this PPARγ ligand inhibits myeloid leukemia cell proliferation at least in part by upregulating p21 [152]. Aside from its growth inhibitory and apoptosis-inducing properties, 15d-PGJ$_2$ has also been shown to decrease the expression of metalloproteinases in AML, therefore, inhibiting leukemic cell adhesion and invasion of the extracellular matrix (ECM) [153].

A recent study investigated the antileukemia effects and the molecular mechanism of action of a novel PPARγ ligand, DIM#34, in AML. DIM#34 can inhibit cell growth and induce apoptosis through PPARγ-dependent and -independent mechanisms. Cell death was associated with defective extracellular signal-regulated kinase (ERK) activity, and inhibition of Bcl-2 phosphorylation [154].

Konopleva et al. demonstrated growth inhibitory, differentiative, and apoptotic effects of PPARγ ligands in cells from a variety of leukemias, including AML [155]. Addition of RXR or RAR ligands (i.e., LG100268 and ATRA, resp.) in combination with PPARγ ligands enhanced the differentiative and growth-suppressive effects. Hirase et al. reported similar findings that the antiproliferative, proapoptotic, and/or differentiating effects of TZDs on HL-60 cells were further enhanced by the addition of the RXR-selective ligand, LG100268 [156]. PPARγ ligands have also been shown to inhibit the clonal proliferation of U937 myelomonocytic leukemia cells by a G1 cell cycle arrest, and that treatment with both PPARγ ligand (troglitazone) and LG100268 had synergistic inhibitory effects on clonal growth [157]. Finally, recent work by Yasugi et al. reported that both pioglitazone and 15d-PGJ$_2$ inhibited cell proliferation in NB4 cells and that combined with ATRA, these PPARγ

ligands also induced myeloid differentiation and lipogenesis [158].

The PPARγ-ligand CDDO and its C-28 methyl ester derivative (CDDO-Me) have also shown prodifferentiative properties in myeloid leukemia cells [159–161]. CDDO-Me induced granulo-monocytic differentiation in HL-60 cells and monocytic differentiation in primary AML cells. Interestingly, while colony formation of AML progenitors was significantly inhibited, normal CD34$^+$ progenitor cells were less affected. The more potent effect of CDDO-Me on leukemic cells compared to normal progenitor cells suggests that CDDO-Me has potential as a new therapeutic agent for the treatment of hematological malignancies [159]. Another group found that low doses of CDDO promoted phagocytosis and granulocytic differentiation in HL-60 cells and primary blasts from AML patients through the regulation of CCAAT enhancer-binding protein (CEBPA) [162]. CEBPA is an important transcription factor for granulocytic differentiation. CDDO upregulated the transcriptionally active p42 CEBPA, while downregulating the inactive p30 CEBPA, thereby enhancing CEBPA-regulated gene transcription. These findings suggest the potential use of CDDO in the treatment of CEBPA-defective AML subtypes.

As proposed earlier, PPARγ transactivation may be impaired in AML, and PPARγ ligands may be able to sensitize AML cells to the prodifferentiation effects of ATRA [138, 139]. In light of this, a recent study revealed that CDDO enhanced ATRA-induced differentiation and apoptosis both in the ATRA-sensitive APL cell line, NB4, and an ATRA-resistant cell line, MR2 [163]. These effects were partially dependent on PPARγ, as inhibition of PPARγ either by a specific inhibitor (T007) or by siRNA diminished CDDO-induced APL differentiation [163].

CDDO induces apoptosis in human myeloid leukemia cells by promoting loss of mitochondrial membrane potential, leading to cytochrome c release and activation of caspases [155, 160, 162, 164]. However, Bcl-xL overexpression only partially inhibited cytochrome c release and caspase activation, indicating that CDDO can activate caspases 3 and 8 in a cytochrome c-independent manner [160]. Similar findings were shown by Konopleva et al. where CDDO activated both caspase-dependent and -independent cell death [164]. CDDO also promotes tumor necrosis factor (TNF)-induced apoptosis in human leukemia cells. CDDO exposure did not inhibit NF-κB translocation into the nucleus, but rather inhibited a step after translocation, such as the NF-κB-dependent resynthesis of the inhibitor of NF-κB, IκBα [165]. Similarly, Shishodia et al. demonstrated that CDDO-Me inhibited both constitutive and inducible NF-κB activity in human leukemic cells. In contrast to the previous study [165], CDDO-Me-induced NF-κB inhibition occurred through suppression of IκBα kinase activation, IκBα phosphorylation, IκBα degradation, p65 nuclear translocation, and NF-κB-mediated reporter gene transcription [166]. These results lead to a downregulation of NF-κB target genes and enhanced apoptosis induced by TNF and other chemotherapeutic agents.

Another CDDO derivative, C-28 imidazole (CDDO-Im), appears to be more potent than CDDO in inhibiting

the growth of human leukemia cells in vitro, as well as murine melanoma and leukemia cells in vivo [167]. The mechanism of CDDO and CDDO-Im-induced apoptosis has been attributed to a disruption of intracellular redox balance by increasing reactive oxygen species (ROS) and decreasing intracellular glutathione (GSH) concentrations [168].

Another subtype of AML is the acute myelomonocytic leukemia (AMML). A well established cell line derived from a child with AMML, THP-1, is often used to study this disease [169]. Several studies have shown that macrophages and myelomonocytic leukemias express PPARγ and that PPARγ agonists can induce differentiation of THP-1 cells into macrophages, as shown by the expression of CD36 scavenger receptors, as well as CD11b, CD14, and CD18 [97]. Another study showed that PPARγ1 expression levels were upregulated by 9-cis retinoic acid (9-cis RA) in THP-1 cells coincident with suppression of cell growth [170]. Moreover, addition of a specific PPARγ ligand enhanced 9-cis RA-induced growth inhibition [170]. A reduction in THP-1 cell migration also occurred in response to PPARγ ligands and was due to downregulation of metalloproteinase-9 expression [171]. These findings suggest that PPARγ ligands may be beneficial in preventing metastasis of monocytic leukemia cells. Indeed, PPARγ ligands also have angiostatic properties because of their inhibitory effects on endothelial differentiation and on vascular endothelial growth factor (VEGF)-induced angiogenesis in vivo [172]. Recently, Ho et al. reported that the pigment epithelium derived factor (PEDF), a potent antiangiogenic factor, can induce THP-1 apoptosis and necrosis by inducing PPARγ protein expression. In their study, PEDF-induced apoptosis was shown to be PPARγ-induction-dependent. Treatment with PPARγ antagonist and PPARγ siRNA attenuated PEDF-induced apoptosis. Transient expression of PPARγ using a PPARγ expression plasmid reproduced the PEDF-effects. Importantly, the PPARγ induced by PEDF was transcriptionally active. These results suggest a PPARγ-dependent induction of apoptosis in THP-1 cells [173].

5.1.2.   Chronic myeloid leukemia (CML)

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder that affects all hematopoietic cell types. It constitutes 15 to 20% of adult leukemias [174]. The American Cancer Society anticipated diagnosis of about 4570 new cases of CML in 2007 [174]. CML is characterized by a genetic abnormality known as Philadelphia (Ph) chromosome, resulting from a translocation between chromosomes 9 and 22, t(9;22)(q34;q11). This translocation generates a fusion protein called BCR-ABL which is a constitutively active tyrosine kinase responsible for uncontrolled cell proliferation and enhanced cell survival [175]. Treatments for this disease include splenic irradiation, stem cell transplantation, and interferon alpha (IFNα) administration with combination chemotherapy. A specific tyrosine kinase inhibitor, Imatinib, was introduced in the late 1990s and is a standard treatment for CML. However, clinical resistance to imatinib has been described in CML patients, where BCR-ABL gene mutations or amplifications have occurred [176,

177]. Therefore, development of new therapeutic strategies to overcome imatinib resistance is needed. Dual PPARα and γ ligands have been tested, either alone or in combination with Imatinib, to overcome drug resistance. A characteristic cell line used to study CML is K562, which was established from a patient with CML in the acute phase [178]. Recently, a study was performed using a synthetic dual PPARα/PPARγ agonist, TZD18, in human CML myeloid blast crisis cell lines [63]. In this study, treatment with TZD18, both alone and in combination with Imatinib, inhibited CML proliferation and induced apoptosis. These effects were PPARα and PPARγ-independent, as neither PPARα nor PPARγ antagonists were able to rescue cell proliferation and survival. These results were reported previously by the same group in Ph-positive lymphocytic leukemia cell lines, where TZD18 promoted cell death and acted synergistically to enhance the effect of Imatinib [179]. Hirase et al. tested the effects of TZDs in K562 cells, which have an erythroid nature and the potential to differentiate into megakaryocytes [180]. TZD inhibited both cell proliferation and the erythroid phenotype of K562 cells. These results were accompanied by a reduction in erythroid lineage-transcription factor, GATA-1, levels [180]. Therefore, PPARγ ligands may serve a therapeutic use for the treatment of other types of myeloproliferative disorders where there is an overproduction of erythrocytes, such as polycythemia vera (PV).

5.2.   Lymphoid malignancies

5.2.1.   Acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphomas

Acute lymphoblastic leukemia (ALL) is a malignant disorder that arises from uncontrolled proliferation of lymphocytic progenitors. The disease is most commonly diagnosed in children, but can also occur in adults. About 80–90% of ALL patients can achieve complete remission with currently available therapy. Yet, many patients eventually relapse, and only 35% of individuals have a long-term leukemia-free survival (LFS) [181, 182]. Therefore, development of new treatment approaches to improve both the cure rate and the quality of life of patients with ALL is greatly needed. ALL involving hyperproliferation of B lymphocyte progenitors (B-ALL) is frequently associated with a translocation between the c-myc gene on chromosome 8q24 and any of the three immunoglobulin genes located on chromosomes 14q32, 2p11, or 22q11. This translocation results in c-myc overexpression and correlates with poor prognosis [183, 184]. The members of the Myc family, including c-myc, are involved in regulation of proliferation and development of normal and malignant cells [185].

An investigation by Zang et al. revealed that the PPARγ ligands pioglitazone and 15d-PGJ$_2$ suppressed cell growth in G1 phase and induced apoptosis in a dose-dependent manner in B-ALL cell lines. Apoptosis was found to be partly caspase-dependent, as treatment with a pan-caspase inhibitor partially reversed this effect [186]. Similar findings were shown in B-ALL with t(14;18), in which troglitazone not only induced G1 phase growth arrest and apoptosis,

but also downregulated the expression of *c-myc* mRNA and protein [187].

Our group has demonstrated that: (1) both normal and malignant B lineage cells express PPARγ mRNA and protein, and (2) exposure to certain small molecule PPARγ ligands, including 15d-PGJ$_2$, inhibits proliferation and induces apoptosis in these cells [85, 113]. Subsequently, we reported that PPARγ ligand-induced apoptosis was mainly PPARγ-independent, since it was not prevented either by a PPARγ antagonist nor a dominant negative form of PPARγ (PPARγ-DN) [94]. We reported that the apoptotic mechanism regulated by 15d-PGJ$_2$, but not by ciglitazone, was related to the production of ROS and the reduction in intracellular GSH [94]. CD40 signaling through CD40-ligand (CD40L) enhances B cell survival and prevents BCR-induced apoptosis by activating the transcription factor NF-κB [188]. Therefore, we tested whether CD40 ligation could protect normal and malignant B cells from PPARγ ligand-induced apoptosis. CD40L was able to partially rescue normal and malignant B cells from PPARγ ligand-induced apoptosis by activating NF-κB. Similarly, Piva et al. reported 15d-PGJ$_2$-induced apoptosis in human Burkitt's lymphomas and multiple myeloma cell lines through inhibition of NF-κB activity. These effects lead to the downregulation of NF-κB-dependent antiapoptotic protein production and therefore decreased cell survival. The apoptotic effects could also be mimicked by NF-κB p65 subunit knockdown by siRNA [189]. These results suggest a possible mechanism for the proapoptotic action of PPARγ agonists.

We have also demonstrated that PPARγ ligands can induce apoptosis in cells from human T cell leukemias (Jurkat), lymphomas (J-Jahn), and T-ALL cells (CCRF-CEM) by a PPARγ-dependent mechanism [103]. Interestingly, normal T cells were not adversely affected by PPARγ ligands, suggesting the use of PPARγ agonists as selective therapeutic drugs for T-cell malignancies [103]. However, data from Yang et al. raised questions on the antiproliferative effects of PPARγ-ligands in T-lymphoma cells [190]. They demonstrated that low concentrations of PPARγ-ligands promoted T-lymphoma cell survival, while high concentrations promoted cell death. These results suggest that in T-lymphoma cells, PPARγ ligands can have contradictory effects when used at different concentrations and require further examination.

Cutaneous T cell lymphoma (CTCL) is a group of T cell malignancies that accumulate in the skin. The most common CTCLs are (1) the Mycosis fungoides (MF), which develops as patches, plaques, or tumors containing apoptosis-resistant CD4$^+$ CD45RO$^+$ helper/memory T cells; and (2) the Sézary syndrome (SS), which is the leukemic form of CTCL that develops with erythroderma and the appearance of atypical T cells in the peripheral blood [191]. Current therapies for CTCL include the use of bexarotene, an RXR ligand, with good efficacy in the late stages of the disease [191]. Zhang et al. demonstrated the expression of PPARγ in three CTCL lines (MJ, Hut78, and HH) and freshly isolated peripheral blood lymphocytes (PBL) from SS patients with circulating atypical T cells (CD4$^+$CD26$^-$) [192]. CDDO exposure caused a dose-dependent induction of apoptosis

in MF/SS cell lines and SS patients' PBL [192]. These findings suggest that PPARγ ligands may be beneficial for the treatment of CTCL and may have synergistic effects when used in combination with bexarotene.

Mantle cell lymphoma (MCL) is a rare type of non-Hodgkin's lymphoma (NHL), constituting about 6% of NHL [193, 194]. In 85% of MCL cases, a translocation between chromosome 11 and 14, t(11;14), is involved in the pathogenesis. This translocation leads to the overexpression of cyclin D1, a protein that increases cell survival and proliferation by positively regulating cell cycle entry into the S-phase [193]. Despite the success of current therapies, patients with mantle cell lymphoma have a shorter life span compared to patients with other B cell lymphomas [193]. Recently, a study demonstrated that treatment with pioglitazone and rosiglitazone, as well as with 15d-PGJ$_2$ induced MCL cell apoptosis and downregulated cyclin D1 expression without altering cell cycle progression [195].

5.2.2. Chronic lymphoblastic leukemia (CLL) and diffuse large B cell lymphoma (DLBCL)

CLL is a clinically heterogeneous disease originating from B lymphocytes that differ in activation, maturation state, or cellular subtype [196]. CLL is one of the most common forms of leukemia in adults [141]. In B-CLL, resistance to apoptosis has been associated with increased Bcl-2 expression, due to either promoter hypomethylation or to chromosomal deletion of the genes which encode two natural Bcl-2 antisense RNAs [197, 198].

To date, there are few studies that evaluate the use of PPARγ-ligands against these malignancies. The effects of the triterpenoid CDDO were evaluated in refractory B-CLL cells. CDDO induced apoptosis in a dose-dependent manner in both previously untreated and chemoresistant CLL samples [199]. In this study, CDDO induced the activation of caspase-8, but not caspase-9, indicating the involvement of a mitochondrial-independent pathway [199]. CDDO also negatively affected the levels of an endogenous caspase-8 inhibitor, c-FLIP (caspase-8 homolog Fas-ligand interleukin-1-converting enzyme (FLICE)-inhibitory protein). However, downregulation of c-FLIP expression was not the sole pathway activated by CDDO, as c-FLIP antisense oligonucleotides did not induce CLL apoptosis [199]. Subsequently, Inoue et al. further investigated the mechanism of CDDO-induced apoptosis in primary B-CLL and Jurkat cell lines. In contrast to the studies discussed earlier [160, 164, 199], where CDDO activated both the intrinsic and extrinsic apoptotic pathways, Inoue et al. proposed that CDDO induces apoptosis exclusively through the intrinsic pathway [200]. In their study, CDDO exposure induced an initial caspase-independent mitochondrial depolarization, followed by caspase cleavage. Using caspase inhibitors, the authors were able to define caspase 9 as the primary activated caspase. Moreover, CDDO induced cell death in caspase-8 and FADD-deficient but not in Bcl-xL-overexpressing Jurkat T cells. In CLL, CDDO induced an initial release of proapoptotic intermediates, cytochrome c, and Smac/DIABLO from the mitochondria and led to apoptosis [200]. According to these results, CDDO
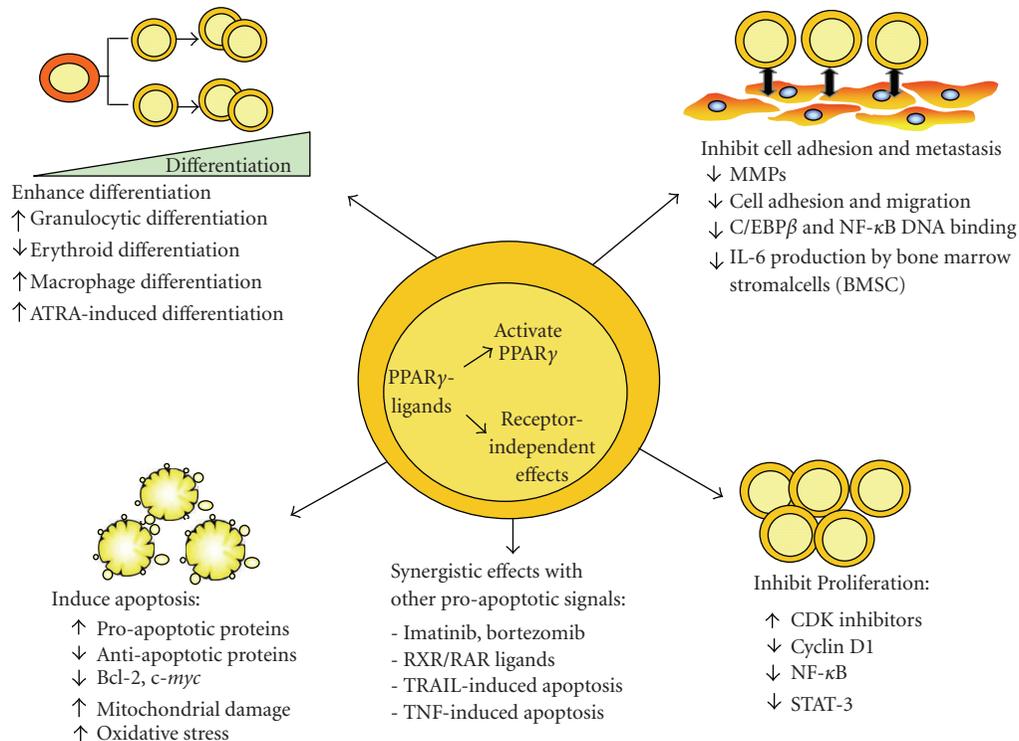
Figure 3: *Mechanisms of action of PPARγ ligands in hematological malignancies.* PPARγ ligands can bind to and activate PPARγ to regulate gene transcription or they can exert PPARγ-independent mechanisms. PPARγ ligands have antiproliferative, prodifferentiation, antimetastatic, and proapoptotic effects on several hematological malignancies making them promising candidates for use in therapeutic regimens.

mainly activates the intrinsic apoptotic pathway in both cell lines [200].

Diffuse large B-cell lymphomas (DLBCLs) are the most common lymphoid neoplasms, composing 30–40% of adult NHL [201]. The gene expression pattern (using DNA microarrays) of DLBCL was compared with that of normal B cells, including those from the germinal center (GC) and in vitro-activated peripheral blood B cells [202]. Based on the results, DLBCL were classified into two groups: those resembling B cells from the GC (GC-DLBCL) and those resembling in vitro-activated B cells (ABC-DLBCL). Patients with cancer of the GC-DLBCL-type have a more favorable prognosis than those with the ABC-DLBCL-type [202]. Although some DLBCL patients are cured with current therapies, most succumb to the disease. In addition, poor prognosis correlates with Bcl-2 overexpression, which may be responsible for the impaired apoptotic response of ABC-DLBCL to chemotherapy [203, 204].

Recently, a study by Ray et al. showed that CDDO induced growth inhibition and apoptosis in human DLBCL and that these effects were PPARγ-independent [205]. Interestingly, CDDO induced NF-κB activation and enhanced DLBCL apoptosis when combined with NF-κB inhibitors. These findings suggest that NF-κB may be activated as a survival pathway to antagonize the apoptotic effects of CDDO [205]. A recent study by Brookes et al. elucidated another mechanism for CDDO-induced cell death [206]. In

this study, CDDO, CDDO-Im, and the dinitril derivative of CDDO, Di-CDDO induced both normal and malignant B cell apoptosis. The CDDO derivatives were more effective than CDDO itself. It was demonstrated that CDDO directly interacted with and modified several mitochondrial protein thiols, resulting in large molecular weight protein aggregates. These aggregates led to a loss in mitochondrial thiol status by constitutively opening cyclosporin A-insensitive permeability transition (PT) pores [206], thereby reducing mitochondrial transmembrane potential and resulting in cell death. These findings suggest a novel mechanism for triterpenoid-induced cell death and predict the development of new therapeutic drugs that can elicit unregulated PT pore formation in cancer cells.

5.3. Multiple myeloma

Multiple myeloma (MM) is a neoplastic disorder characterized by clonal proliferation of differentiated plasma cells in the bone marrow, accompanied by accumulation of monoclonal paraprotein levels in serum and urine. Common clinical symptoms include bone lesions, anemia, immunodeficiency, and renal failure [207]. MM constitutes ~10% of hematological cancers and ranks as the second most frequent hematological malignancy in the United States after NHL [208, 209]. Current therapies for the disease include chemotherapy with or without stem cell

transplantation, glucocorticosteroids, thalidomide, and the proteasome inhibitor Bortezomib (Velcade) and combinations of these agents. However, most of these treatments are not curative, and newer approaches are needed [209]. The therapeutic potential of PPARγ ligands has also been evaluated in MM [13, 210, 211]. PPARγ agonists have been demonstrated to have inhibitory effects in Waldenstrom's macroglobulinemia (WM), a rare plasma cell malignancy [212]. In addition, our laboratory demonstrated that human multiple myeloma cells modestly express PPARγ. Treatment with PPARγ ligands induced MM apoptosis via caspase activation and mitochondrial depolarization. These proapoptotic effects were not reversed by the addition of the MM growth factor IL-6. Moreover, we showed that these cells express RXR and that addition of an RXR ligand (9-*cis*-RA) enhanced PPARγ-ligand-induced apoptosis [210]. Farrar's group found that PPARγ ligands 15d-PGJ$_2$ and troglitazone completely abolished IL-6-dependent MM cell proliferation and induced apoptosis. PPARγ agonists inhibited MM cell survival by specifically blocking the IL-6-dependent transactivation of STAT3 (signal transducer and activator of transcription)-activated genes, including *c-myc* and *mcl-1* [13]. Recently, the same group has revealed that PPARγ ligands inhibit (1) MM cell adhesion to bone marrow stromal cells (BMSC), (2) MM cell expression levels of adhesion molecules, and (3) BMSC secretion of IL-6, which is triggered by MM cell adhesion. The inhibitory effects of PPARγ ligands correlated with PPARγ-dependent transrepression of the transcription factors 5′-CCAAT/enhancer-binding protein β (C/EBP-β) and NF-κB [213]. The PPARγ ligands CDDO and CDDO-Im have also been tested in MM cells, both alone and in combination with the proteasome inhibitor PS-341 (Bortezomib) [214–216]. The mechanisms of CDDO-induced apoptosis include loss of mitochondrial membrane potential, which increases release of ROS and depletes glutathione, as well as activation of caspases and reduction of c-FLIP protein levels [214]. These results correlated with the studies described earlier, using CDDO in CLL [199]. Combination treatments of CDDO-Im with Bortezomib had synergistic apoptotic effects in MM cells [215], abolished NF-κB and Bcl-2-mediated cytoprotective effects and overcame drug resistance to Bortezomib [215]. Overall, these findings suggest the use of CDDO-Im, either alone or in combination with bortezomib, to treat drug-resistant MM and improve patient prognosis.

6. CONCLUSIONS AND FUTURE DIRECTIONS

In summary, although the exact role of PPARγ in controlling malignant cell growth and apoptosis remains unclear, PPARγ has been commonly implicated as a tumor suppressor in hematological cancers (see Figure 3 for overview). Evidently, a better understanding of the mechanism of action of PPARγ is needed. It is important that studies be performed to carefully analyze PPARγ levels, as well as the activation status of PPARγ in hematological cancers. In addition, since many of the existing studies have demonstrated that the proapoptotic and antiproliferative effects of PPARγ ligands are independent of the receptor; additional studies are required to elucidate PPARγ-dependent from independent events by using tissue specific knockouts, siRNA approaches, and overexpression studies. Understanding the mechanisms of action of these agents has become a priority to develop drugs that have beneficial effects on tumor suppression without having major side effects. Certain advances may be possible through the discovery of SPPARγMs that can activate only a subset of desired genes. This will require the identification of PPARγ target genes that mediate the antitumorigenic effects in hematological malignancies.

The fact that PPARγ can be modified by phosphorylation through MAP kinases and that this modification decreases PPARγ transcriptional activity, and the fact that PPARγ activation itself increases PPARγ degradation by the proteasome may be exploited for therapeutic benefit. PPARγ ligands in combination with inhibitors of MAP kinases and/or proteasome inhibitors (e.g., Bortezomib) may be useful in the treatment of malignancy. Therefore, studies should be performed to assess the effectiveness of these combination therapies as well as those combining PPARγ ligands with drugs such as Imatinib or RXR/RAR ligands. Our current knowledge of the anticancer potential of PPARγ ligands predicts that such therapies may prove to be of great benefit for future treatments of hematological cancers.

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Review Article

The Roles of Dietary PPARγ Ligands for Metastasis in Colorectal Cancer

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Dietary peroxisome proliferator-activated receptor (PPAR)γ ligands, linoleic acid (LA) and conjugated linoleic acid (CLA), showed anticancer effects in colorectal carcinoma cells. LA is metabolized by two pathways. Cyclooxygenase (COX)-2 produces procarcinogenic prostaglandin E2, whereas 15-lipoxygenase (LOX)-1 produces PPARγ ligands. The 15LOX-1 pathway, which is dominant in colorectal adenomas, was downregulated and inversely COX-2 was upregulated in colorectal cancer. LA and CLA inhibited peritoneal metastasis of colorectal cancer cells in nude mice. The inhibitory effect was abrogated by PPARγ antisense treatment. A continuous LA treatment provided cancer cells quiescence. These quiescent cells formed dormant nests in nude mice administrated LA. The quiescent and dormant cells showed downregulated PPARγ and upregulated nucleostemin. Thus, short-term exposure to dietary PPARγ ligands inhibits cancer metastasis, whereas consistent exposure to LA provides quiescent/dormant status with possible induction of cancer stem and/or progenitor phenotype. The complicated roles of dietary PPARγ ligands are needed to examine further.

1. INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide and the third leading cause of cancer deaths in Japan [1]. The frequency of colorectal cancer in Japan doubled in the last three decades according to the alteration of life style from Japanese to Western [2]. Especially, the increase in fat intake and decrease in fiber intake have been regarded as the most important nutritional influence on colon cancer development [3, 4]. In this review, we focused on the fatty acids, which possess ligand activity for peroxisome proliferator-activated receptor (PPAR)γ in foods. PPARγ is activated by endogenous secreted prostaglandins and fatty acids. 15-deoxy-δ(12,14)-prostaglandin J2 is a strong endogenous ligand of PPARγ [5]. Linoleic acid (LA) is one of the essential fatty acids, which we must intake from food. Metabolic products of LA, such as 9-hydroxyoctadecadienoic acid (9-HODE), 13-hydroxyoctadecadienoic acid (13-HODE), and 13-oxooctadecadienoic acid (13-OXO), are known as PPARγ ligands [6]. Conjugated linoleic acid is a stereoisomer of LA [7]. CLA is contained in beef, lamb, and also in vegetable oils

[8]. Roles of these dietary PPARγ ligands on PPARγ activation are not still unclear. Colorectal cancer is a good model for influence of nutritional factors to cancer development and progression [9, 10]. In this review, roles of LA and CLA for colorectal cancer progression and therapeutic possibility are discussed as dietary PPARγ ligands.

2. METABOLIC PATHWAYS OF LINOLEIC ACID

Prostaglandins (PGs) are bioactive lipids derived from the metabolites of membrane polyunsaturated fatty acids (PUFAs), and play important roles in a number of biological processes [11]. Cyclooxygenases-2 (COX-2)-dependent overproduction of PGE2 is hypothesized to be an important part of sustained proliferative and chronic inflammatory conditions [12, 13]. Several in vivostudies hypothesize that a high amount of ω-6 PUFA such as LA might enhance colorectal carcinogenesis via stimulation of colonic epithelial cell proliferation [14–17]. In fact, rats treated with a genotoxic agent, azoxymethane (AOM), and fed a diet supplemented with LA develop more tumors than those treated with AOM alone [18].

The oxidative metabolites of LA, in particular, 9-HODE, 13-HODE, and 13-OXO, have biological effects as a PPARγ ligand [6]. CLA, a strong ligand for PPARγ, has a substantial anticarcinogenic effect [8, 19]. Synthesized PPARγ ligands including troglitazon have been shown to be effective chemopreventive agents in a rat model of carcinogenesis and in AOM-induced colon cancer in mice [20]. If LA provides PPARγ ligands, LA might act as an anticarcinogenic agent.

Arachidonic acid is a fatty acid and a component of lipid membranes, and also a major substrate for lipoxygenase enzymes. 15lipooxygenase-1 (15LOX-1) is known for its anti-inflammatory properties and has a profound influence on the development and progression of cancers [21, 22]. Recent studies show that ligand activation of PPARγ in colorectal cancer cells attenuates colonic inflammation and causes a reduction growth via the induction of apoptosis [23, 24]. Furthermore, it has been reported that a number of metabolites generated by 15LOX-1 can function as endogenous activators and ligands for PPARγ [25, 26]. We confirmed the anticarcinogenic effect of LA by in vitro transformation assay using a rat intestinal cell line, IEC6, which expressed 15LOX-1, but not COX2. LA treatment inhibited AOM-induced transformation in IEC6 cells [27]. Many literatures reported that PPARγ possesses an anticarcinogenic effect in colorectal cancer [20]. Moreover, a decrease in PPARγ expression is associated with cancer metastasis [28, 29]. Inhibitory effect of PPARγ to cancer metastasis is reported in several cancers, such as nonsmall cell lung cancer, colon cancer, thyroid cancer, and breast cancer [30–33].

3. SWITCHING OF LA METABOLIC PATHWAYS FROM 15LOX-1 TO COX-2 IN COLORECTAL CANCER DEVELOPMENT

Inhibitory effect of LA on intestinal epithelial cell transformation elucidated above suggests that 15LOX-1 LA metabolism suppresses colon carcinogensis [27]. We next focused on the dual roles of LA in human colon cancer development. Expression of 15LOX-1 and COX-2 was examined in human colon adenoma and carcinoma to elucidate the balance of the two LA metabolic pathways in malignant transformation of human colon epithelium.

We examined the expressions of COX-2 and 15LOX-1 in 54 adenomas, 21 carcinoma-in-adenoma lesions, and 36 serosa-invading advanced carcinomas in the colon [34]. We examined 15LOX-1 mRNA and COX-2 protein by in situ hybridization and immunohistochemistry, respectively. In the nonpathological colon mucosa, which expressed 15LOX-1 but not COX-2, proliferation of colon epithelial cells was controlled at constitutive levels. 15LOX-1 mRNA was found in 96% of adenomas, 43% of adenoma in carcinoma-in-adenoma lesions, and 10% of carcinoma in carcinoma-in-adenoma lesions, but not in advanced carcinoma ($P <$ .0001). In contrast, COX-2 production was found in 11% of adenomas, 52% of adenoma in carcinoma-in-adenoma lesions, 71% of carcinoma in carcinoma-in-adenoma lesions, and 92% of advanced carcinoma ($P <$ .0001). Concurrence of COX-2 induction with 15LOX-1 downregulation was found in 6% adenomas, in 33% adenoma components, and 71% carcinoma components of carcinoma-in-adenoma lesions (all mucosal cancer), in 89% cases in nonmetastatic serosa-invading carcinomas, and in 100% cases of nodal metastasized carcinomas. Our data showed that induction of COX-2 expression and downregulation of 15LOX-1 were sequentially increased from adenomas, adenoma components, and carcinoma components in carcinoma-in-adenoma lesions, to invasive carcinomas. Interestingly, low grade-adenoma components with in carcinoma-in-adenoma lesions showed COX-2 expression and 15LOX-1 downregulation at more frequency than low grade-adenomas, which suggests that the biological property is different in the same histological atypia. In contrast, high grade-adenoma components showed no difference from high grade-adenomas in expressions of COX-2 and 15LOX-1, which suggests that high grade-adenomas might already possess malignant potential as high as adenoma components in carcinoma-in-adenoma lesions. This sequential alteration of concurrence of COX-2 induction with 15LOX-1 downregulation possibly shows close association of the switching of LA-metabolizing pathways with colon cancer development and progression.

15LOX-1 is revealed as an apoptosis inducer in human cancers and inhibits cancer progression. The reduction of 15LOX-1 and the isoform 15LOX-2 is correlated with the disease progression of breast cancer and the poor clinical outcome [35]. Induction of 15LOX-1 provides apoptosis in oral cancer [36]. 15LOX-1 expression is downregulated in colon adenomas, and ectopic expression of 15LOX-1 induces apoptosis in Caco-2 colon cancer cells [37].

In expression of 15LOX-1, inflammatory cytokines play an important role. Interleukin (IL)-4 and IL-13 induce 15LOX-1 expression via Jak2/Tyk2/Stats pathway [36, 38–40]. In prostate cancer, ratio of ω-3/ω-6 fatty acids is associated with expressions of 15LOX-1 and COX-2 [41]. 15LOX-1 induction by nonsteroidal anti-inflammatory drugs (NSAIDs), such as sulindac sulfone, provides apoptosis in SW480 colon cancer cells. Sulindac sulfone inhibits GMP (cGMP)-phosphodiesterases and activates protein kinase G, which enhances 15LOX-1 expression transcriptionally [42]. GATA-6 transcriptional factor is involved in NSAID-induced 15LOX-1 induction in RKO and DLD-1 colon cancer cells [43]. GATA-6 activates 15LOX-1 promoter in Caco-2 colon cancer cells but not in the cells induced differentiation by sodium-butyrate [44]. These are supported by conventional NSAIDs activates PPARγ [45]. COX-2 expression also involves Jak2/Stats pathway and nuclear factor (NFκB) [46–48]. Activation of PPARγ downregulates COX-2 expression by inhibition of NFκB and activator protein (AP)-1 [49]. This negative regulation of COX-2 expression by PPARγ activation might be one of the mechanisms of reversal expressions between 15LOX-1 and COX-2. Furthermore, promoter DNA methylation is responsible for silencing 15LOX-1 expression, which is reversed by 5-aza-2-deoxycytidine treatment [50, 51]. The epigenetic alteration might be a trigger to switch 15LOX-1 repression and COX-2 upregulation along with malignant transformation and disease progression in colorectal cancer. Thus, switching of LA metabolism from 15LOX-1 to COX-2 is thought to be

a common mechanism to escape from antitumoral effect of LA.

4. ANTITUMOR EFFECT OF CLA

We next argue the effect of CLA, which is an isomer of linoleic acid and is established as a PPARγ ligand without metabolization by 15LOX-1. CLA is a natural content of some foods, such as beef, lamb, and also vegetable oils [8]. CLA is one of essential fatty acids, which possesses characteristic bioactive properties [8]. CLA is composed of positional- and stereoisomers of octadecadienoate (18 : 2). Chemoprotective properties of CLA are reported in experimental cancer models and in vitro examinations [8, 19]. Differently from LA, CLA is not a precursor of prostaglandines. CLA activates PPARγ as a ligand [7, 52]. Through activation of PPARγ, CLA might act as an antimetastatic agent as well as an anticarcinogenic agent. We examined the antimetastatic effect of CLA on peritoneal dissemination [53]. Cell growth of MKN28 human gastric cancer cells and Colo320 human colon cancer cells was suppressed by CLA in a dose-dependent manner with an increment in apoptosis. CLA significantly inhibited invasion into type IV collagen-coated membrane of MKN28 and Colo320 cells. CLA-induced growth inhibition was recovered by the exposure to antisense S-oligodeoxynucleotides (ODN) for PPARγ in both cell lines. BALB/c nu-nu mice were inoculated with MKN28 and Colo320 cells into their peritoneal cavity, and administrated with CLA intraperitoneally (weekly, 4 times). CLA treatment did not affect food intake or weight gain of mice. CLA treatment significantly decreased metastatic foci of both cells in the peritoneal cavity. Survival rate in mice inoculated with MKN28 or Colo320 cells was significantly recovered by CLA treatment. PPARγ initiates transcription of genes associated with energy homeostasis, cell growth, and anti-/proinflammatory effect [24, 54–57]. Protein production in MKN28 and Colo320 cells treated with CLA showed a decrease in epidermal growth factor receptor (EGFR) and transforming growth factor (TGF)-α and an increase in Bax. Our results showed that CLA inhibits cell growth and invasion, and induces apoptosis in cancer cells. Our data are supported with the reports, which indicate that PPARγ downregulates EGFR, and upregulates Bax, p21Waf-1, and E-cadherin, which are associated with antiproliferative, proapoptotic, and prodifferentiation effects [33, 58–60].

We also reported the tumor suppressive effect of CLA on established peritoneal tumors using a syngeneic mouse peritoneal metastasis model [61]. C57BL6 mice were inoculated with LL2 cells into their peritoneal cavity. Two weeks after the inoculation, colonized peritoneal cancer foci (2.2 ± 0.4 mm in diameter) were treated with CLA administrated intraperitoneally (600 pmol/mouse, weekly, twice). CLA treatment decreased the number of peritoneal tumors to 26% of that in untreated mice ($P < .0001$). CLA treatment also decreased size of peritoneal tumors to 27% of that in untreated mice ($P < .0001$). In CLA-treated tumors, proliferating cells were decreased ($P < .0001$), whereas apoptotic cells were increased ($P < .0010$). CLA-treated

LL2 tumors showed decrease in PPARγ and EGFR proteins, and increase in Bax protein in comparison with untreated tumors.

5. ANTITUMOR EFFECT OF LINOLEIC ACID

We confirmed antimetastatic effect of PPARγ by CLA in gastric and colon cancer cells. We next examined antimetastatic effect of LA.

The effect of LA on peritoneal metastasis was examined using in vitro treatment of cancer cells and mouse peritoneal metastasis models as well as CLA examination. Firstly, cell growth of MKN28 human gastric cancer cells and Colo320 human colon cancer cells were suppressed by LA in a dose-dependent manner with increment of apoptosis. LA significantly inhibited invasion into type IV collagen-coated membrane of MKN28 and Colo320 cells ($P < .05$). The inhibition of growth and invasion and the induction of apoptosis by LA were abrogated by exposure to antisense S-ODN for PPARγ. Moreover, the decrease in 15LOX-1 expression by exposure to antisense S-ODN for 15LOX-1 suppressed the inhibition of growth and invasion and the induction of apoptosis by LA. LA-induced growth inhibition was recovered by the exposure to antisense S-ODN for PPARγ or 15LOX-1. BALB/c nu-nu mice inoculated with MKN28 and Colo320 cells into their peritoneal cavities were administrated IP with LA (weekly, 4 times). The LA treatment significantly diminished the number of metastatic foci of both cells in the peritoneal cavity ($P < .05$). Protein production in MKN28 and Colo320 cells treated with LA showed a decrease in EGFR and an increase in Bax. PPARγ activation is reported to decrease EGFR expression and increase Bax expression [58, 60]. Our data suggest that LA possesses the same mechanism to CLA of PPARγ ligand; however, its efficacy was 10^3 times weaker than CLA. LA-metabolites thought to be weaker agonists of PPARγ. MKN28 and Colo320 cells expressed both COX-2 and 15LOX-1. At least, relative high concentration of LA might be metabolized dominantly by 15LOX-1, which consequently provides antimetastatic effect in these cells.

Thus, LA and CLA suppress occurrence of cancer metastasis and reduce existing metastatic tumors in animal models. These findings encourage clinical usage of LA and CLA for treatment of cancer metastasis.

6. EFFECT OF LA ON CANCER DORMANCY

A short-term treatment with LA or CLA induced apoptosis in a dose-dependent manner through PPARγ activation as described above. On the contrary, in a long-term continuous treatment with adequate concentrations, LA induced reversible cell growth-arrest in cancer cells that escaped from apoptosis [62].

Cancer cell tumorigenicity in nude mice depends on several factors in cancer cells themselves and their host. To form macroscopical tumors, cancer cells must survive in their host tissue against host immunity, and proliferate with utilization of the host endothelial cells to make tumor
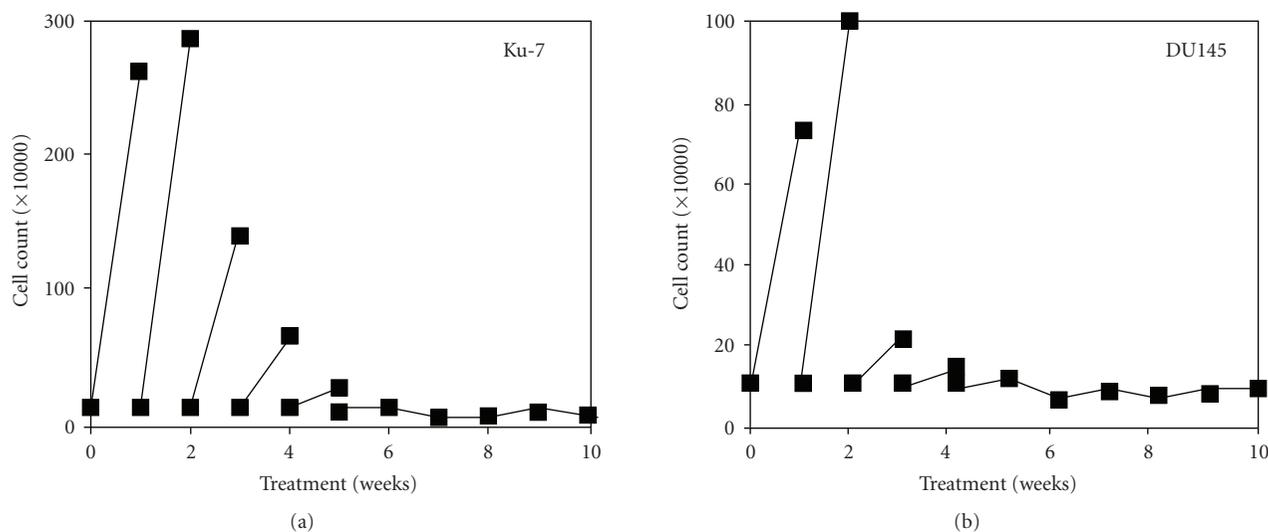
FIGURE 1: Effect of LA on growth inhibition in Ku-1 bladder cancer cells and DU145 prostate cancer cells by long-term treatment. Ku-7 cells and DU145 cells were continuously treated with LA ($100 \, \mu$g/mL) for the indicated period with weekly reseeding by 1×10^5 cells per well. If cells were less than 1×10^5 cells per well, all cells were reseeded. Standard deviation of each cell number was less than 10% of the value.

vasculature. These steps are similar to those in metastasized cancer cells on the metastasis targets [63]. If cancer cells do not endure the attacks by host immunity, they cannot form tumor. When they survive but not proliferate, the condition resembles quiescent or static dormancy. When they survive and proliferate but do not generate tumor vasculature, the cancer cells stay microscopical cell aggregation; the condition is to be an equivalent condition to tumor dormancy [64].

In vitro cell growth was suppressed by 48-hour treatment with LA in a dose-dependent manner in MKN28 and Colo320 cells. Continuous treatment with LA induced quiescence in both cells at 5 to 7 weeks after the LA treatment. The finding was also observed in Ku-7 bladder cancer cells and DU145 prostate cancer cells (Figure 1). In LA-induced quiescent cancer cells, protein production of Bcl-2 was increased, whereas Bak, EGFR, and vascular endothelial growth factor (VEGF) levels were decreased [62]. These alterations might be associated with inhibition of cell growth, angiogenesis, and apoptosis [65, 66], which explained well the characteristics of LA-treated cancer cell aggregation. These alterations of protein levels were the same as those in cells treated with PPARγ ligands, toroglitazon, and CLA [62]. The PPARγ expression was also decreased in quiescent MKN28 and Colo320 cells by continuous treatment with LA. The LA metabolites by 15LOX-1 activate mitogen-activated protein kinase (MAPK) and increase PPARγ phosphorylation, but downregulate PPARγ activity [67]. Continuous PPARγ phosphorylation might decrease PPARγ expression by long-term LA treatment.

When MKN28 and Colo320 cells were inoculated to nude mice subcutaneous tissue, LA-induced quiescent MKN28 and Colo320 cells showed higher tumorigenicity than non-treated cells in nude mice. In the contrary to the tumorigenicity, LA-induced quiescent cancer cells showed 1/10

slower tumor growth than nontreated cells. LA withdrawal after the inoculation provided regrowth in the cancer cells, which subsequently grew into macroscopic tumors. LA-induced quiescent cells were different from growth-arrest cells by aging or senescence, which are irreversible, and refractory to growth factors [68].

In mice treated with LA weekly administration after the inoculation, inoculated quiescent cancer cells did not form macroscopical tumors. Histological examination revealed less than $500 \, \mu$m-sized cancer-cell aggregations in the inoculation site. These cancer cell nests showed no proliferative activity, no vascularity, and no immune cell infiltration [62]. These features of the cancer cell nests were similar to those in tumor dormancy status [64]. The dormant cells expressed undetectable levels of PPARγ protein, which suggests that inactivation of PPARγ might be associated with tumor dormancy formation. In contrast, withdrawal of LA might break the dormancy status in cancer cells. Moreover, PPARγ-negative dormant cells expressed increased levels of nucleostemin. Nucleostemin possesses a role for maintaining stemness [69, 70]. PPARγ inhibits Wnt and LIF (leukemia inhibitory factor) signaling pathways, which maintain pluripotency and self-renewal of stem cells [5, 71]. Downregulation of PPARγ might induce dedifferentiation and stem cell/progenitor phenotype in cancer cells, which might be associated with cancer dormancy and metastasis.

In this story, several possibilities are considered. Firstly, LA-induced PPARγ downregulation provides stemness in cancer cells. Secondly, LA-induced apoptosis abolishes PPARγ-positive cancer cells and PPARγ-negative cancer stem cell/progenitor cells remain. Thirdly, LA possesses direct effect on cancer stem niche. To confirm the mechanism underlying LA-induced cancer dormancy, further examination is requested to focus on the relation of PPARγ with cancer stem cells.

7. CONCLUSION

In this article, we described that the dietary PPARγ ligands, LA and CLA, are deeply involved in colorectal cancer development and progression through PPARγ activation. LA and CLA provide remarkable anticancer effects by short-term treatment. In contrast, long-term continuous treatment with LA induces quiescent and dormancy in cancer cells with PPARγ downregulation. These conditions might be associated with phenotypes of cancer stem cells/progenitor cells. LA is a dietary factor to be taken from food everyday. Cancer cells might be continuously exposed to various concentrations of LA in human body. Taken together, short-term administration of LA and CLA is an effective therapeutic tool for cancer metastasis. We are requested to evaluate the effect of dietary LA on cancer metastasis for prevention of cancer metastasis and cancer dormancy.

NOMENCLATURE

PPAR: Peroxisome proliferator-activated receptor
LA: Linoleic acid
CLA: Conjugated linoleic acid
COX: Cyclooxygenases
LOX: Lipoxygenase
HODE: Hydroxyoctadecadienoic acid
OXO: Oxooctadecadienoic acid
PG: Prostaglandin
AOM: Azoxymethane
IL: Interleukin
NSAID: Nonsteroidal anti-inflammatory drug
NF: Nuclear factor
AP: Activator protein
ODN: Oligodeoxynucleotides
EGFR: Epidermal growth factor receptor
TGF: Transforming growth factor
VEGF: Vascular endothelial growth factor
MAPK: Mitogen-activated protein kinase
LIF: Leukemia inhibitory factor.

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Review Article

Do PPARγ Ligands Suppress the Growth of Cholangiocarcinoma or the Cholangiohepatitis Induced by the Tumor?

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Cholangiocarcinoma is a predominantly fatal cancer, which can be difficult to treat. It has been reported that the administration of pioglitazone temporarily improved not only diabetic control, but also bile duct carcinoma-induced cholangiohepatitis. Pioglitazone is considered to have both direct and indirect mechanisms of action on the tumor-related hepatitis. Several molecules induced by thiazolidinedione, including Smad pathway-related molecules, adipokines, and other lipid metabolism-related proteins, may directly or indirectly suppress tumor development and/or tumor-induced cholangiohepatitis. Although the most frequent and critical side effect of thiazolidinedione is drug-induced hepatitis, it can probably be avoided by careful monitoring of serum hepatic enzyme levels. Thiazolidinedione should be considered for management of tumor-induced hepatitis in the presence of diabetes unless severe side effects occur.

1. INTRODUCTION

The primary effects of thiazolidinedione, a peroxisome proliferator-activated receptor γ (PPARγ) agonist, are the reduction of insulin resistance and improvement of insulin sensitivity, resulting in reduction of fasting plasma glucose, insulin, and free fatty acid levels [1].

Cholangiocarcinoma is a predominantly fatal cancer, which can be difficult to treat. We reported previously that administration of the thiazolidinedione, pioglitazone, in a 73-year-old male patient who developed cholangiocarcinoma with cholangiohepatitis and diabetes improved not only diabetic control, but also the tumor-induced cholangiohepatitis, and improved the patient's quality of life [2]. One and half years after treatment, the patient again showed deterioration of cholangiohepatitis and diabetic control. He finally died of obstructive jaundice.

There are two possible mechanisms to explain the initial improvement of hepatitis in our case: the PPARγ ligand may have directly suppressed the abnormal cell growth, or the PPARγ ligand may have indirectly suppressed tumor growth after the ligand improved hepatitis and/or diabetes.

In this review, we discuss the mechanisms of the temporary beneficial effects of the agent, especially the above two possibilities, with regard to the literature concerning PPARγ and cholangiohepatitis. In addition, we also discuss the positive choice of thiazolidinedione, despite elevated serum concentrations of hepatic enzymes.

2. DIRECT EFFECTS ON THE DEVELOPMENT OF CHOLANGIOCARCINOMA

These mechanisms were supported by the results of basic experiments using various cholangiocarcinoma cell lines [3–6]. PPARγ ligand mediates the inhibition of cholangiocarcinoma cell growth through p53-dependent mechanisms [3]. The PPARγ ligand, 15-deoxy-delta 12,14-PGJ2, induces apoptosis in cholangiocarcinoma cell lines although regulation of apoptosis-related protein expression varies [4, 5], while artificial regulation of PPARγ expression in cholangiocarcinoma cell lines suggests that PPARγ may actually promote tumor cell growth viathe Smad pathway [6]. It has been reported that PPARγ ligands can suppress proliferation and induce apoptosis although PPARγ itself may have

divergent effects on cellular growth in cholangiocarcinoma cell lines [7].

3. INDIRECT EFFECTS ON THE DEVELOPMENT OF CHOLANGIOCARCINOMA

Thiazolidinedione seems not to improve insulin sensitivity and glucose disposal by direct effects on either the liver or muscle. PPARγ is expressed preferentially in adipose tissue, and its levels of expression in the liver and skeletal muscle are low [8]. Thus, it is more likely that the primary effects of these drugs are on adipose tissue, followed by secondary benefits on other target tissues of insulin [9]. In our case, there was no evidence that pioglitazone directly reduced the tumor size. In contrast, cholangiohepatitis was improved by administration of this agent. In addition, the progressive cholangiohepatitis was probably related to the cholangiocarcinoma. In general, cholangiocarcinoma development is based possibly upon the cytotoxicity of bile constituents, that is, cytotoxic bile acids and lysolecithins. These humoral factors may affect tumor progression. Thus, it was suggested that pioglitazone indirectly improves cancer-mediated inflammation, such as cholangiohepatitis, rather than directly suppressing tumor growth.

As mentioned above, it is now generally accepted that adipose cells send molecular signals, including cytokines, to other tissues. Thus, it is possible that PPARγ activation controls one or more genes that regulate systemic tumor promotion (see Figure 1). The interesting candidate genes in this regard are TNF-α, adiponectin, and leptin. Other lipid-related genes regulated by PPARγ ligands, such as lipoprotein lipase and fatty acid binding protein, may also control tumor development [10].

3.1. TNF-α

Thiazolidinedione reduces TNF-α expression in human and rodent adipocytes [11]. A series of studies using cholangiocarcinoma cell lines demonstrated that TNF-α itself attenuates the growth of cholangiocarcinoma cells and induces apoptosis [11–13]. However, several recent studies have demonstrated that TNF-α promotes invasiveness and accelerates migration of cholangiocarcinoma cells [14–16]. These observations imply that the suppression of TNF-α production may attenuate the progressive invasion of tumor cells into healthy hepatobiliary cells.

3.2. Adiponectin and leptin

PPARγ agonists have been reported to increase the expression and circulating levels of adiponectin, an adipocyte-derived protein with insulin-sensitizing activity [17], in diabetic rodents and in patients with type 2 diabetes [18]. There have been many reports, especially in breast cancer, that adiponectin plays roles in the inhibition of tumor cell growth [19]. The expression of leptin, a suppressor of feeding behavior, is negatively regulated by thiazolidinediones [20]. Leptin has been reported to induce tumor development
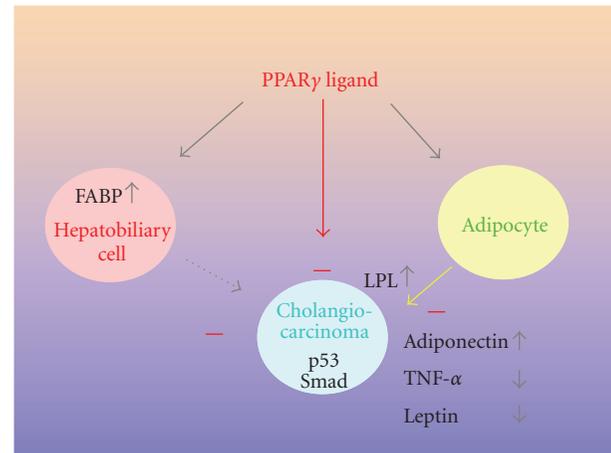

Figure 1: Inhibitory effects of PPARγ ligand on the development of cholangiocarcinoma. PPARγ ligand directly suppresses tumor progression through p53 and Smad pathways (red arrow) and also stimulates adipocyte and hepatobiliary cells (gray arrow). Secretion of adipokines (TNF-α, adiponectin, and leptin) and production of lipid-related proteins (FABP and LPL) are regulated by PPARγ ligand. Up- and downregulation of various gene signals from adipocytes (yellow line) and hepatobiliary cells (broken line) promoted suppression of tumor growth. As a result, PPARγ indirectly suppressed tumor growth of cholangiocarcinoma through adipocytes and hepatobiliary cells. Currently, evidence of suppressive signals from hepatobiliary cells to cholangiocarcinoma is unavailable (broken line). FABP: fatty acid binding protein, LPL: lipoprotein lipase, TNF-α: tumor necrosis factor-α.

in breast cancer [21], suggesting that suppression of leptin secretion may reduce tumor progression.

3.3. Other lipid-related proteins

Other lipid-related proteins, such as lipoprotein lipase (LPL) and fatty acid binding proteins (FABPs), are positively regulated by the PPARγ ligand GW1929 [22]. Although there have been no studies related to cholangiocarcinoma and these lipid-associated proteins, there is a great deal of evidence that the proteins promote reduction of tumor growth. Intestinal polyp formation was suppressed by increasing LPL activity [23]. As FABPs play roles not only as lipid chaperones but also as free radical scavengers, the molecules may affect tumor progression through the oxidative stress pathways. The protein expression of liver FABP was reduced in neoplastic lesions of CuZn superoxide dismutase-deficient mice [24]. It has been reported that FABP reduces cellular damage from hypoxia/reoxygeneration [25]. These lipid-related proteins may also play roles in the reduction of tumor growth and/or suppression of tumor-mediated liver damage.

4. HEPATIC SIDE EFFECTS

The most frequent and critical side effect of thiazolidinedione that must be taken into consideration before starting thiazolidinedione administration in cases of cholangiocarcinoma is drug-induced hepatitis. Although some data

are available from animal studies suggesting that hepatic toxicity may be a characteristic of the thiazolidinedione class [26], current clinical evidence indicates that pioglitazone treatment does not result in liver toxicity [27]. However, this agent causes mild transient increases in serum ALT levels. The FDA recommends monitoring ALT levels and not using these drugs in patients with liver disease [28]. Moreover, it was reported that patients receiving pioglitazone may develop serious liver injury and should be monitored for evidence of hepatitis [29].

Unlike other existing antidiabetic medications that show a very rapid onset of activity, pioglitazone and rosiglitazone exhibit a characteristic delay of 4–12 weeks in the onset of their therapeutic effects. It has been suggested that thiazolidinedione should be continued for at least one month to obtain results. In our case, initial improvement of the elevated hepatic enzymes was observed two weeks after starting administration of this agent. These data indicate that the effectiveness in cases of tumor-related hepatitis could be assessed within two weeks rather than 4–12 weeks when diabetic control is obtained.

5. PERSPECTIVES

Taken together with these considerations, as PPARγ ligands are probably effective in the suppression of tumor development, especially on the reduction of tumor invasiveness through molecular signals from adipocytes, thiazolidinedione should be chosen not only for diabetic control, but also as an attenuator of tumor progression in patients with diabetes. Drug-induced hepatitis can be avoided by meticulous monitoring of serum hepatic enzyme levels.

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Review Article

The Role of PPARγ Receptors and Leukotriene B₄ Receptors in Mediating the Effects of LY293111 in Pancreatic Cancer

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Pancreatic cancer is a devastating disease in which current therapies are inadequate. Separate lines of research have identified the 5-lipoxygenase/leukotriene B₄ receptor pathway and the PPARγ pathway as potential targets for prevention or treatment of this disease. LY293111 was originally designed as a potent leukotriene B₄ receptor antagonist for treatment of inflammatory conditions. LY293111 was also known to have inhibitory effects on 5-lipoxygenase, which is upstream of the production of leukotrienes. LY293111 was shown to have potent anticancer effects in pancreatic cancer and several other solid malignancies, where it caused cell cycle arrest and marked apoptosis. Subsequently, it came to light that LY293111 exhibited PPARγ agonist activity in addition to its effects on the 5-lipoxygenase pathway. This raises the question of which of the two targets is of greatest importance with regard to the anticancer effects of this agent. The evidence to date is not conclusive, but suggests that the effects of LY293111 may be mediated by both LTB₄ receptors and PPARγ.

1. INTRODUCTION

Pancreatic Cancer is a devastating disease with more than 80% of all patients presenting with surgically inoperable tumors. It remains the fourth leading cause of cancer death in both men and women in the USA. The median survival is usually less than six months even with the addition of chemotherapy [1–3]. Surgical resection is the only effective treatment option, but there are few long-term survivors even after apparent curative resection [1–3]. Alternative effective treatment strategies are desperately needed for this disease.

1.1. *Fatty acids and human cancer*

Epidemiological and animal studies show that a high fat consumption is associated with a higher incidence and growth of tumors at several specific organ sites including breast, pancreas, and prostate [4–11]. Recent studies indicate that diets containing a high proportion of polyunsaturated omega-6 fatty acids (n-6 FA), such as linoleic acid (the precursor of arachidonic acid) are associated with a more advanced disease stage at the time of diagnosis of several kinds of cancer [4–6, 8, 10, 11]. In contrast, long-chain n-3 fatty acids, such as docosahexaenoic acid and eicosapentaenoic acid (EPA) inhibit the growth and metastasis of several cancers including pancreatic cancer [7, 9]. Omega 3 fatty acids inhibit tumor growth by a number of mechanisms including suppression of COX-2 expression and, for EPA at least, the alternative substrate produces different cyclooxygenase (PGE_3) and lipoxygenase (LTB_5) products that have anti-inflammatory and anticancer effects.

1.2. *Eicosanoid pathways*

Arachidonic acid is a substrate for three distinctively different enzymatic pathways. Among them, prostaglandin endoperoxide synthases (cyclooxygenases) catalyze the committed step that leads to prostaglandin biosynthesis [12–14]. The second pathway is the epoxygenase pathway that appears to have no role in cancer. The third pathway for metabolizing arachidonic acid, the lipoxygenase pathway catalyzes

Normal pancreas

Duct

Duct

Islet

Pancreatic cancer

PanIN

Cancer

Islet
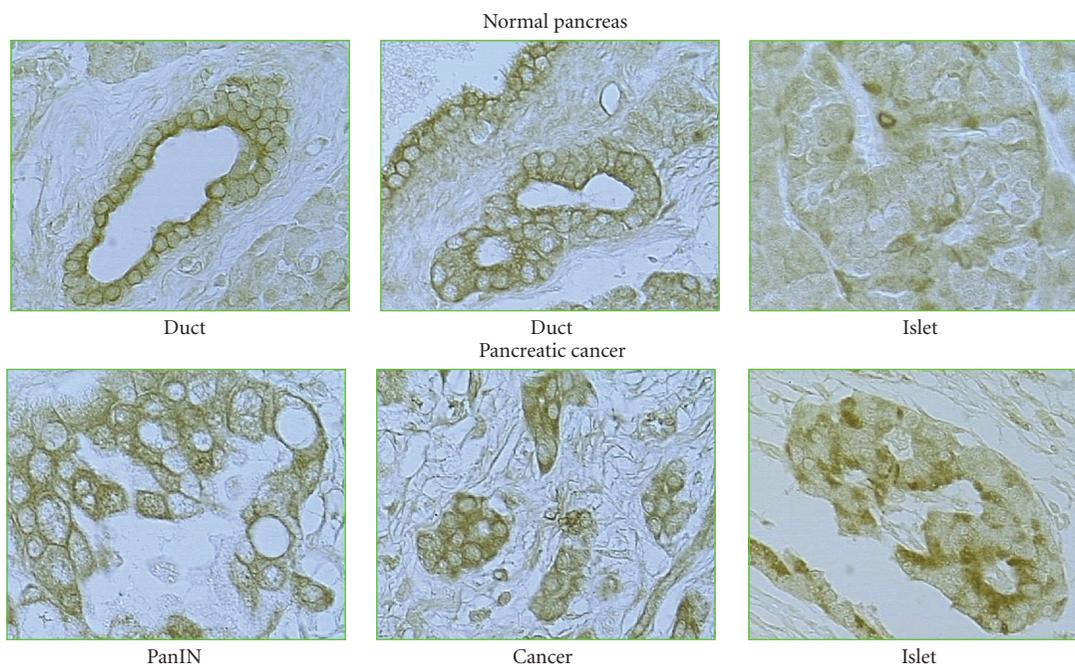
FIGURE 1: Immunocytochemistry of PPARγ receptor in normal human pancreas and in a PanIN lesion and a pancreatic cancer. The PPARγ receptor antibody shows a similar intensity of nuclear staining in normal ducts and in pancreatic cancer cells as well as cells in the PanIN lesion. In contrast, no staining is seen in normal islets, but nuclear staining is seen in islets from tissue adjacent to a cancer. These pictures are representative of eight samples of each tissue type. Staining was similar using antibodies from two different commercial sources.

the incorporation of one oxygen molecule into polyunsaturated fatty acids to yield a 1-hydroperoxy-2, 4-trans, cis-pentadiene product [14–16]. Mammalian lipoxygenases possess regiospecificity during interaction with substrate, and on this basis have been designated as arachidonate 5-, 12-, and 15-lipoxygenase (5-LOX, 12-LOX, and 15-LOX) [14–16]. The three distinct enzymes insert oxygen at carbon 5, 12 or, 15 of arachidonic acid, and the primary product is 5S-, 12S-, or 15S-hydroperoxyeicosatetraenoic acid (5-, 12-, or 15-HPETE), which can be further reduced by glutathione peroxidase to hydroxy forms (5-, 12-, 15-HETE), respectively [14–16]. 5-LOX is noteworthy because it is the only pathway that can turn arachidonic acid into leukotrienes [15, 17]. The activity of 5-LOX is dependent upon a second factor termed 5-LOX-activating protein (FLAP) [15, 17]. Considerable effort has been expended by the pharmaceutical industry to produce inhibitors of FLAP, 5-LOX as well as leukotriene antagonists, because the 5-LOX products, leukotrienes (LTB$_4$, LTC$_4$, LTD$_4$, and LTE$_4$) have been implicated as mediators of inflammation and immediate hypersensitivity reactions, in particular, human bronchial asthma [18, 19].

1.3. Leukotriene receptor antagonists and the development of LY293111

The pharmaceutical industry has focused on several targets to suppress leukotriene activity in inflammatory conditions such as bronchial asthma [18, 19]. One approach is to directly inhibit 5-lipoxygenase activity, thereby blocking

secretion of all leukotrienes. The most widely studied clinical inhibitor of 5-lipoxygenase is zileuton, which inhibits the active site of 5-lipoxygenase atconcentrations that do not inhibit cyclooxygenase, 12-lipoxygenase, or 15-lipoxygenase [18–22]. Another avenue to inhibit leukotriene formation is via blocking FLAP activity, thus preventing cytoplasmic to membrane translocation and activation of 5-lipoxygenase [16–18]. MK-0591 is a widely used 5-lipoxygenase-activating protein inhibitor for biomedical research [16–18]. Even though it strongly inhibits 5-lipoxygenase activity and blocks leukotriene generation, its use in clinic is limited by marked side effects. The final pharmacological approach to block leukotriene activity is to selectively block the actionsof LTB$_4$ or the sulfidopeptide leukotrienes using specific receptor antagonists.

Several synthetic LTB$_4$ receptor antagonists have been developed. Early compounds included SC-41930; ONO-4057, which was orally active; LY223982, a benzophenone dicarboxylic acid; and LY255283 a hydroxyacetophenone [23–27]. These latter two compounds from the Lilly Research Laboratories potently block LTB$_4$ binding to its receptors within the nM range and inhibit the biological functions of LTB$_4$ in vitro [28, 29]. Unfortunately, they showed poor oral bioavailability [28]. In 1995, investigators at the Lilly Research Laboratories reported a new LTB$_4$ antagonist, LY293111. This compound is a novel derivative of LY255283, but is orally stable and more potent as an LTB$_4$ receptor antagonist [28, 29]. Compared with other LTB$_4$ receptor antagonists, LY293111 is superior at blocking the cellular functions induced by LTB$_4$ [28, 30].
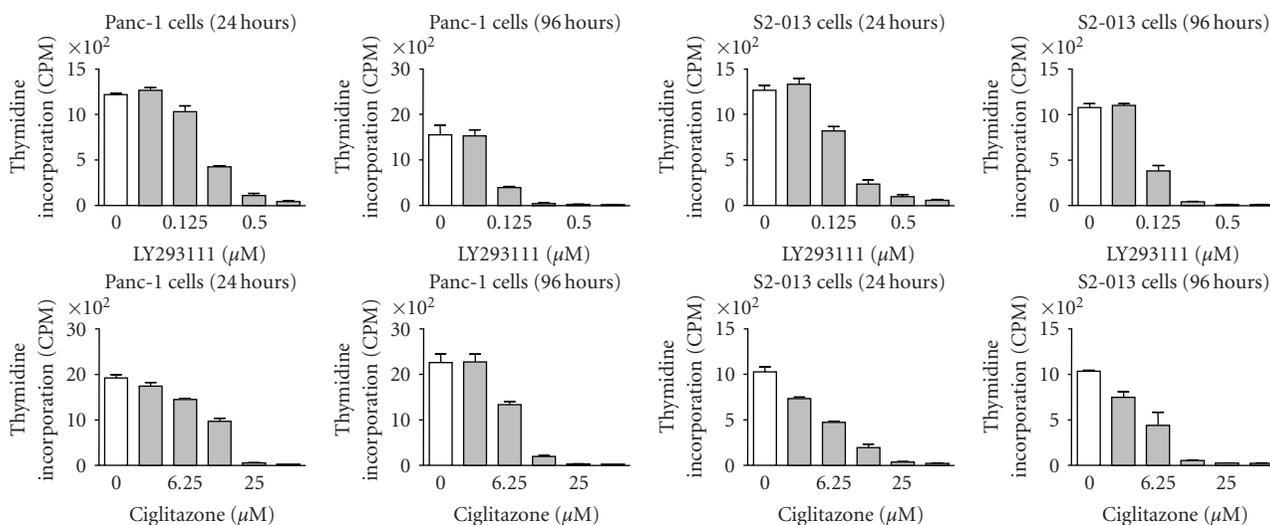
FIGURE 2: Comparison of the effects of different concentrations of LY293111 and ciglitazone on proliferation of two human pancreatic cancer cell lines (Panc-1 and S2-013) after 24 and 96 hours of treatment, measured by thymidine incorporation. LY293111 was approximately 50 times more potent than ciglitazone at inhibiting proliferation of both cell lines. Data shown is mean±SEM from four separate experiments.

1.4. Inflammation, Cyclooxygenases, Lipoxygenases, and cancer

The epidemiological data show a clear and strong association between chronic inflammatory conditions and cancer development, even though the conditions causing inflammation may vary [31–36]. It can be due to chronic infection caused by a virus, bacteria, or parasite or it may be due to noninfective, physical, or chemical irritant [31–36]. For example, chronic infection with the bacterium *Helicobacter pylori* causes atrophic gastritis, which can lead to dysplasia and adenocarcinoma [37]. Hepatitis B and C viruses account for more than 80% of cases of hepatocellular carcinoma worldwide [34]. The inflammatory bowel diseases, ulcerative colitis and Crohn's disease, predispose to the development of cancers of the large bowel and/or terminal ileum, although a causative infectious agent has never been conclusively identified [38]. For noninfectious inflammation, chronic reflux of gastric acid and bile into the distal esophagus causes chemical injury and on the long-term can lead to Barrett's esophagus and eventually to esophageal adenocarcinoma [35]. Thus it is apparent that chronic inflammation is a common underlying theme in the development of many different malignancies.

Although the mechanisms for the association between inflammation and cancer are not fully understood, growth factors, cytokines, and chemokines released into inflammatory environment are associated with tumor development and progression [32, 36]. High concentrations of free radicals and nitric oxide can induce DNA damage and promote cancer development [32, 36]. Over the past decade, much attention has been paid on the role of cyclooxygenases in cancer development, specifically its inducible isoform, the cyclooxygenase 2 (COX-2) [39–41]. COX-2 is active within both inflamed and malignant tissues [37–39]. The expression of COX-2 and COX-2 metabolites increases during the multistage progression of tumors [39–41]. By metabolizing arachidonic acid to prostaglandins, COX-2 induces cellular resistance to apoptosis, modulation of cellular adhesion and motility, promotion of angiogenesis, and immunosuppression [42–47]. Epidemiological data has implicated COX-2 in the pathogenesis of a number of epithelial malignancies, especially colorectal cancer [48, 49]. Inhibition of the enzyme with COX inhibitors is associated with a dramatic reduction in the incidence, morbidity and mortality of colorectal cancer [48–51]. Recent attention has also been focused on the role of 5-LOX, 12-LOX, and 15-LOX in cancer [52–60]. In pancreatic cancer, activation of the 5-LOX and 12-LOX pathways enhances cancer cell proliferation, while the 15-LOX pathway is protective against cancer development [61–64].

1.5. The 5-lipoxygenase/leukotriene B₄ pathway and cancer

Accumulating evidence suggests that the 5-LOX pathway has profound influence on the development and progression of human cancers [61–64]. 5-LOX is overexpressed in pancreatic cancer tissues but is not expressed in normal pancreatic ductal cells [65]. Furthermore, this pathway is already up-regulated in pancreatic intraepithelial neoplasias (PanINs), which are the precursor lesions of pancreatic adenocarcinoma [66]. Blockade of 5-LOX activity inhibits proliferation and induces apoptosis in pancreatic cancer cells both in vitro and in vivo [67–69]. Pancreatic cancer cells secrete LTB₄ and LTB₄ induces proliferation in these cells [62]. Two G-protein-coupled LTB₄ receptors (BLT1 and BLT2) have been cloned and characterized. BLT1 and BLT2 are high- and low-affinity LTB₄ receptors, respectively, and form a gene cluster in humans. Both BLT1 and BLT2 are up-regulated in pancreatic cancer tissues, and expression was
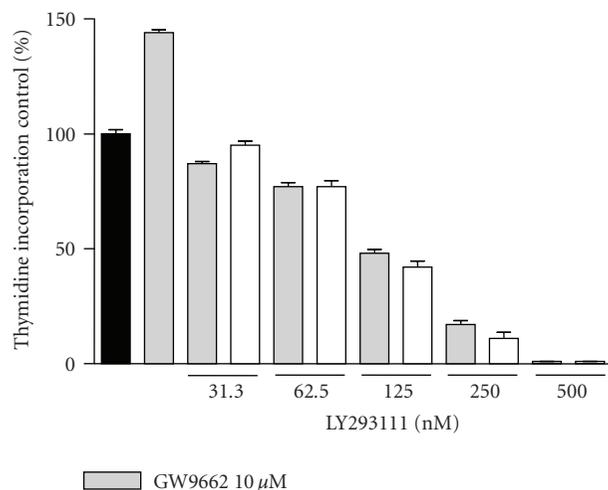
FIGURE 3: Effect of a PPARγ receptor antagonist, GW9662 on the inhibition of proliferation induced by LY293111 in AsPC-1 human pancreatic cancer cells after 24 hours of treatment. These cells express both the PPARγ receptor and LTB$_4$ (BLT1 and BLT2) receptors. While GW9662 alone was able to significantly increase thymidine incorporation, it was not able to block the inhibitory effect of different concentrations of LY293111.

seen in all of the tested pancreatic cancer cell lines [65, 70]. As with other proteins in the 5-LOX/LTB$_4$ pathway, BLT1 and BLT2 are already up-regulated in pancreatic intraepithelial neoplasias (PanIN lesions) which are the precursors of pancreatic adenocarcinomas [70]. This suggests that they may be valuable targets for chemoprevention.

1.6. PPARγ and pancreatic cancer

Peroxisome proliferator activated receptor-γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. PPARγ is expressed at high levels in adipose tissue and plays a central role in adipocyte differentiation and energy homeostasis. Recent studies have implicated PPARγ in the pathogenesis of several human malignancies [71–74]. Previous studies have suggested that PPARγ is up-regulated in pancreatic cancer [75]. Our own studies, employing two separate commercially available antibodies, show that PPARγ is expressed in pancreatic cancer, but that expression in the cancer cells does not appear to be different from that in normal pancreatic ductal cells (Figure 1). In contrast, PPARγ staining was seen in the islets surrounding cancers, but not in islet cells from normal pancreatic tissues obtained from multiorgan donors (Figure 1). In animal models, PPARγ ligands have preventive effects against chemical carcinogenesis [76]. Several studies have shown that PPARγ agonists, including the natural ligand 15-deoxy-Δ12,14-prostaglandin J2, and thiazolidinedione antidiabetic agents, such as citiglitizone and rosiglitizone, inhibit growth and induce apoptosis in pancreatic cancer [75, 77–81]. In contrast, one paper suggests induction of differentiation without apoptosis [82]. The apoptosis appears to be preceded by a morphological change to a more differentiated cell type which perhaps undergoes apoptosis when DNA repair turns out to be not possible [80]. In some studies, PPARγ agonists also block invasion and angiogenesis [83, 84]. However, this is controversial since PPARγ agonists induce secretion of vascular endothelial growth factor, which would have a promoting effect on metastatic tumor growth [85].

1.7. LY293111 and cancer

As might be expected from the growth-stimulatory effects of LTB$_4$ in pancreatic cancer, the LTB$_4$ receptor antagonist, LY293111 inhibits cancer growth and induces apoptosis both in vitro and in vivo [86–89]. LY293111 inhibits proliferation and induces apoptosis in a wide range of pancreatic cancer cell lines as well as cells of other tumor types, such as breast, prostate, and colon cancer cells [86–89]. These effects on growth and apoptosis are both time and concentration dependent, with effects seen at 100–500 nM in vitro [86, 87]. To confirm the involvement of LTB$_4$ receptors in mediating the effect of LY293111 on human pancreatic cancer cell proliferation, another selective LTB$_4$ receptor antagonist, U75302 was used in comparison with a selective LTD$_4$ antagonist, LY171883 [86]. U75302 inhibits the proliferation of pancreatic cancer cells; but it is less potent than LY293111 as expected from the lower receptor affinity of this drug [28, 29, 90]. In contrast, the selective LTD$_4$ antagonist, LY171883 had no significant effect on pancreatic cancer cell growth. LY293111 causes cell cycle arrest in the S phase of the cell cycle with suppression of expression of cyclin A, cyclin E, and cdk2. In parallel with growth inhibition, LY293111 induced apoptosis in all cancer cell lines tested [86, 87]. LY293111 induced dramatic morphological changes in human pancreatic cancer cells following a short period of treatment [86, 87]. The treated cells became rounded and exhibited membrane blebbing, chromatin condensation, and nuclear fragmentation, finally they were detached from the microplate. Induction of DNA fragmentation by LY293111 was confirmed by TUNEL assay (terminal deoxynucleotidyl transferase-mediated nick end labeling) and apoptosis was also established by annexin V binding [86, 87]. Apoptosis is triggered through the mitochondrial pathway, with a change in the ratio of proapoptotic proteins, such as Bax to antiapoptotic proteins, such as Bcl-2 and Mcl-1, release of cytochrome C, activation of caspase (but not caspase 8), and subsequent activation of the downstream caspase cascade with activation of caspase 3 and caspase 7 and cleavage of the caspase 3 substrate, poly ADP-ribose polymerase (PARP) [87].

LY293111 markedly slows down the growth of subcutaneous xenografts of human pancreatic cancer in athymic mice at a dose of 250 mg/kg/day [86]. To confirm the antipancreatic cancer effect of LY293111, pancreatic cancer cells with stable expression of enhanced green fluorescent protein (GFP) were orthotopically implanted into the duodenal lobe of the pancreas of athymic mice. Our data show that LY293111 significantly inhibits the growth of the orthotopically implanted pancreatic cancer cells in concert with blocking metastatic spread to the liver and other organs

[91]. LY293111 also dramatically increased the number of TUNEL positive cells in pancreatic tumors harvested from the subcutaneous transplant experiments in athymic mice [86, 91].

1.8. LY293111 as a PPARγ agonist

Following the disclosure of anticancer effects of LY293111, researchers at the Lilly Research Laboratories found that LY293111 is also a PPARγ agonist [92, 93]. This finding was initially based on structural analysis and was supported by functional studies. The PPARγ agonist activity of LY293111 is evidenced by its ability to induce adipogenic differentiation in vitro [92]. Normalization of circulating glucose levels by LY293111 in the ZDF rat diabetes model further suggests that LY293111 is an antidiabetic, PPARγ agonist [92]. Further studies suggested that the anticancer effect of LY293111 might be mediated, at least in part, by PPARγ [92, 93]. More extensive studies have subsequently shown that LY293111 is also an inhibitor of 5-lipoxygenase, although this effect is less potent than the LTB_4 and PPARγ targets.

1.9. Mechanisms by which LY293111 functions in cancer

Since our findings suggest that all pancreatic cancer cells express both PPARγ and BLT1, it is possible that the anticancer effects of LY293111 could be mediated by either receptor or both receptors. It has been reported that PPARγ negative-expressing cancer cells are less responsive to LY293111-induced growth inhibition [92, 93]. However, there is also evidence in favor of BLT1 being the major target. Firstly, the effects of LY293111 on proliferation and apoptosis are extremely potent. A comparison between the effects of LY293111 and the PPARγ agonist, ciglitazone is shown in Figure 2. As this figure shows, LY293111 is approximately 50 times more potent than ciglitazone in inhibiting the proliferation of Panc-1 and S2-013 human pancreatic cancer cells. LY293111 was also more potent than another PPARγ agonist, rosiglitazone and the PPARα agonist, WY-14643. The antiproliferative effects and induction of apoptosis are seen at 250 nM LY293111, which is much lower than the IC_{50} of the drug for PPARγ receptors ($\sim 4 \mu M$) [92, 93]. Indeed, its effects on cancer cells are more potent than several PPARγ agonists, including ciglitazone and rosiglitazone. Secondly, LY293111 is able to completely inhibit the effects of LTB_4 on proliferation and MAP kinase activation in pancreatic cancer cells [62]. However, preliminary studies have shown that the antiproliferative effects of LY293111 in pancreatic cancer are not inhibited by the PPARγ antagonist, GW9662 in vitro (Figure 3). Finally, data from our own studies and those of others show that PPARγ agonists induce cell cycle arrest in the G0/G1 phase, whereas LY293111 induces S phase cell cycle arrest [82, 87]. It is even possible that anticancer effects of LY293111 might also be partially mediated by other unknown mechanisms. However, based on the current data, both the leukotriene B_4 receptor and PPARγ are likely to be involved in the antitumor activity of LY293111.

2. EFFECT OF LY293111 IN COMBINATION WITH OTHER AGENTS IN CANCER

Several studies have demonstrated that LY293111 enhances anticancer effects of gemcitabine, which is widely used as the standard therapy in pancreatic cancer patients in adjuvant and palliative treatment settings [89, 91, 94]. Gemcitabine only improves survival by a few weeks, but clinical data show improvement in the quality of life for pancreatic cancer patients. The effects of LY293111 in combination with gemcitabine were investigated in an orthotopic model of pancreatic cancer in athymic mice [91]. This model is superior to subcutaneous transplantation since it is less likely to modify the biological characteristic of pancreatic cancer cells, providing a favourable growth environment for them. It also allows easy monitoring of hepatic and lymph node metastasis with GFP stable expressing cells. In this model, animals without any treatment following implantation of GFP-expressing, S2-O13 pancreatic cancer cells developed end-stage disease with invasive cancer obstructing the duodenum and bile duct [91]. The animals develop liver, lung, and lymph node metastases and eventually peritoneal carcinomatosis with malignant ascites and cachexia [91]. Either gemcitabine or LY293111 alone significantly inhibited tumor growth and reduced the incidence of liver metastasis. However the combination of LY293111 and gemcitabine was significantly more effective than either treatment alone in blocking tumor growth [91]. Combined treatment also significantly relieved tumor-induced cachexia and maintained stable body weights compared with either drug alone, and also significantly decreased the incidence of biliary obstruction and metastasis [91]. These experimental results show that combined therapy of gemcitabine and LY293111 potently inhibits the growth and metastases of the very rapidly growing and aggressive pancreatic adenocarcinoma and suggest that it might be a valuable way for treatment of pancreatic cancer patients. LY293111 has also been shown to increase the effectiveness of gemcitabine in a colon cancer model [89]. The in vitro effects of LY293111 have been tested with other classical chemotherapeutic agents. The effects of the active metabolite of irinotecan, SN-38 or the active metabolite of capecitabine, 5′-DFUR were enhanced by LY293111 in multiple cell lines, including breast, bladder, and sarcoma cells [94].

3. CLINICAL TRIALS WITH LY293111

Three phase I clinical trials with LY293111 alone or in combination with gemcitabine or irinotecan have been reported. LY293111 was generally well tolerated [95–97]. The side effects were mild to moderate; the major ones gastrointestinal with diarrhea and pain. These initial phase I trials looked promising and LY293111 could be safely administered orally [95–97]. For example, in combination with gemcitabine, three patients had partial responses [96]. One had pancreatic cancer previously treated with gemcitabine, one with pancreatic cancer previously treated with 5-flurouracil and radiation, and one with non-small-cell lung cancer treated with one prior regimen. Two phase II

trials have been completed and preliminary data reported in abstract form [98, 99]. One of these compared the combination of LY293111 with gemcitabine compared with gemcitabine with placebo in pancreatic cancer [98]. The second compared LY293111 with cisplatin and gemcitabine versus the placebo combined with the latter two drugs in patients with non-small-cell lung cancer [99]. Unfortunately, LY293111 did not improve progression-free survival in either of these two trials [98, 99].

4. CONCLUSIONS

LY293111 is an interesting compound that has biological effects on several different targets. It acts as an antagonist on LTB_4 receptors, as a PPARγ agonist and as a 5-lipoxygenase inhibitor. Indeed, some investigators have referred to LY293111 as a multiple eicosanoid pathway inhibitor [94, 99]. Since LY293111 has anticancer effects on multiple tumor types, the target involved in mediating the effects of the drug is clearly of interest. The evidence to date is not conclusive, but suggests that effects may be mediated by both LTB_4 receptors and PPARγ. Preliminary reports regarding the phase II clinical trials have unfortunately been disappointing. It remains unknown whether this compound will eventually find a use in the clinic for cancer therapy; however this recent clinical experience perhaps makes this unlikely now.

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Review Article

PPAR-γ Thiazolidinedione Agonists and Immunotherapy in the Treatment of Brain Tumors

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Thiazolidinediones (TZDs) are selective agonists of the peroxisome proliferator-activated receptor (PPAR) gamma, a transcription factor belonging to the superfamily of nuclear hormone receptors. Although activation of PPARγ by TZDs has been best characterized by its ability to regulate expression of genes associated with lipid metabolism, PPARγ agonists have other physiological effects including modulating pro- and anti-inflammatory gene expression and inducing apoptosis in several cell types including glioma cells and cell lines. Immunotherapeutic approaches to reducing brain tumors are focused on means to reduce the immunosuppressive responses of tumors which dampen the ability of cytotoxic T-lymphocytes to kill tumors. Initial studies from our lab show that combination of an immunotherapeutic strategy with TZD treatment provides synergistic benefit in animals with implanted tumors. The potential of this combined approach for treatment of brain tumors is reviewed in this report.

Thiazolidinediones (TZDs) are synthetic compounds originally designed as oral antidiabetic drugs [1, 2], but several reports have indicated other potent biological effects as anti-inflammatory agents [3, 4] and regulators of cell survival [5]. It has been shown that TZDs induce growth arrest and cell death in a broad spectrum of tumor cells [6–10] and hematopoietic cells in vitro and in vivo [11]. At the molecular level, TZDs are specific ligands of the peroxisome proliferator-activated receptor gamma (PPAR-γ), a member of the nuclear hormone receptor family. PPAR-γ is expressed in many normal tissues, with the highest levels in adipocytes, consistent with its role in lipid metabolism and adipocyte differentiation [2]. Although the ability of TZDs to induce PPAR-γ mediated cell differentiation has been clearly demonstrated [12–14], the molecular mechanisms responsible for the growth inhibitory effects of these PPAR-γ ligands have not been established [15]. TZDs also bind to a receptor present on the outer mitochondrial membrane termed "mitoNEET" [16] which may mediate some of their metabolic effects and also contribute to induction of apoptosis in tumor cells [17].

Considerable interest has been focused on PPAR-γ ligands as potential therapeutic agents in the treatment of gliomas. It has been shown that PPAR-γ ligands can induce death in both rodent and human glioma cell lines [18–28]. The antineoplastic effects of TZDs have been related to the ability of these drugs to activate apoptotic pathways [29, 30] or to interfere with the cell cycle through downregulation of cyclin D1 [31] and the upregulation of CDK inhibitors [32, pages (21, 27)]. Interestingly, some studies [24, 27, 33] have directly compared the effects of TZDs on primary astrocytes versus transformed cells, with contrasting results. Two studies [20, 25] showed that ciglitazone, a TZD PPAR-γ agonist, was toxic to glioma cells as well as to primary astrocytes, whereas in a third study [27] no toxicity was induced by ciglitazone in normal astrocytes after eight days of incubation. The basis for differential sensitivity of transformed versus nontransformed cells to TZDs is not well understood but may involve differences in metabolic responses [33].

There is some evidence suggesting that PPAR-γ also has an immunomodulatory role. In particular, it has been

reported that TZDs mediate significant inhibition of proliferative responses of both T cell clones and splenocytes [34]. This inhibition occurs in part because the ligands for PPAR-γ mediate inhibition of interleukin-2 (IL-2) secretion by T cell clones while not inhibiting IL-2 induced proliferation of such clones. It has also recently been demonstrated that PPAR-γ is a negative regulator of dendritic cell maturation and function [35]. Sustained PPAR-γ activation in murine dendritic cell reduced maturation-induced expression of costimulatory molecules and IL-12 and profoundly inhibited their capacity to prime naïve CD4$^+$ T cells. Finally, there is some evidence to suggest that TZDs are potent inhibitors of glioma cell migration and brain invasion largely by transcriptional repression of TGF-β [36]. This is particularly important because TGF-β is an immunosuppressive cytokine that has been shown to have a major role in the malignant phenotype of gliomas [37]. Furthermore, inhibition of TGF-β signaling restores immune surveillance and is associated with improved survival in a glioma model [37].

We previously reported the immunotherapeutic properties of interleukin-2 secreting syngeneic/allogeneic cells in the treatment of brain tumors in mice [38]. Mice with an intracerebral (i.c.) glioma treated solely by intratumor injections with allogeneic cells genetically modified to secrete IL-2 survived significantly longer than mice in various control groups. The antitumor response was mediated predominantly by CD8$^+$ T cells and NK/LAK cells [39]. Intratumoral injections of the cytokine-secreting cells resulted in the killing of only the neoplastic cells; nonneoplastic cells were unaffected. Of special interest, mice injected intracerebrally with the cytokine-secreting allogeneic cells alone exhibited no neurologic deficit and there was no adverse effects on survival. The injection of IL-2 secreting allogeneic cells into the microenvironment of an i.c. tumor induced an antitumor immune response capable of prolonging survival.

In another study, the possible benefits of combining administration of the chemotherapeutic agent paclitaxel with immunotherapy in the treatment of C3H/He mice bearing an established highly aggressive intracerebral breast cancer was explored [40]. Paclitaxel is a widely-used chemotherapeutic agent which is known to induce apoptosis, although the mechanism of action is poorly understood [41]. The mice were treated by injection into the tumor bed with the DNA-based vaccine, with paclitaxel administered intraperitoneally or by paclitaxel followed by immunization with the DNA-based vaccine. The results indicated that the survival of mice with an established intracerebral breast cancer was prolonged by treatment with either paclitaxel or the DNA-transfected fibroblasts ($P < .025$), but survival of mice receiving the combined therapy did not exceed that of tumor-bearing mice receiving either form of treatment alone. The suppression of the peripheral white blood cell count attributed to paclitaxel, although relatively brief, makes paclitaxel along with most chemotherapeutic agents somewhat antagonistic when administered with immunotherapeutic treatment strategies. Nevertheless, the combination of systemic chemotherapy along with immunotherapy has been used to treat patients with advanced-stage carcinoma [42]. It has been proposed that dying tumor cells, particularly those killed by chemotherapy, engage with antitumor immune responses [43].

Since the tumor cell population is known to be heterogeneous and includes cells that are resistant to cellular immune mechanisms, it is likely that there is a subpopulation of tumor cells that are resistant to host immune mechanisms. In order to control tumor growth, a combination of therapeutic strategies will be required. Although thiazolidinediones reduce the antigen presenting capacity of dendritic cells along with reducing T cell proliferation and cytokine secretion, TZDs do not suppress the immune system and bone marrow in the same fashion as typical chemotherapeutic agents such that these agents should not compete with immune therapeutic strategies in the treatment of various tumors. In addition, their metabolic effects would be expected to occur independent of cell origin.

To test this, we carried out studies in C57Bl/6 mice with an established glioma [44]. The results of that study showed that oral pioglitazone was not effective in prolonging survival in mice bearing a highly malignant glioma (Gl261) grown intracerebrally in syngeneic C57Bl/6 mice. Intracerebral injection of pioglitazone was effective in prolonging survival in mice with an intracerebral glioma. Furthermore, intracerebral injection of fibroblasts genetically engineered to secrete IL-2 into an established intracerebral glioma was effective both in prolonging survival and stimulating a systemic antitumor immune response as measured in the spleen cells using an IFN-γ ELISPOT assay. In previous studies, it was confirmed that the antitumor immune responses in the tumor bearing mice were mediated predominantly by CD8$^+$ and NK/LAK cells [39]. However, there was a synergistic response in prolonging survival of the animals with an established intracerebral glioma treated with both IL-2 secreting fibroblasts and pioglitazone. It should be noted nevertheless that splenic T cells isolated from mice treated with both IL-2 secreting cells and pioglitazone showed no increase in response as compared with the spleen cells isolated from animals treated with IL-2 secreting fibroblasts alone as measured by the ELISPOT IFN-γ assay. These results suggest that the tendency of pioglitatzone to increase the mean survival time in mice bearing a glioma is not due to an increase in systemically driven T cell immunity against the Gl261 cells.

The above results suggest that treatment with TZDs can synergize with immunotherapeutic approaches to increase glioma cell death. While the mechanisms involved remain to be elucidated, it is likely to be a combination of events including a reduction in the immunosuppressive responses of the tumor cells leading to an increase in cytotoxic T cell numbers or activity as well as a decrease in tumor cell mitochondrial function making the cells more susceptible to induction of apoptosis by cytokines.

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Review Article

Synergistic Effects of PPARγ Ligands and Retinoids in Cancer Treatment

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Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily. The activation of PPARs by their specific ligands is regarded as one of the promising strategies to inhibit cancer cell growth. However, recent clinical trials targeting several common cancers showed no beneficial effect when PPAR ligands are used as a monotherapy. Retinoid X receptors (RXRs), which play a critical role in normal cell proliferation as a master regulator for nuclear receptors, preferentially form heterodimers with PPARs. A malfunction of RXRα due to phosphorylation by the Ras/MAPK signaling pathway is associated with the development of certain types of human malignancies. The activation of PPARγ/RXR heterodimer by their respective ligands synergistically inhibits cell growth, while inducing apoptosis in human colon cancer cells when the phosphorylation of RXRα was inhibited. We herein review the synergistic antitumor effects produced by the combination of the PPAR, especially PPARγ, ligands plus other agents, especially retinoids, in a variety of human cancers. We also focus on the phosphorylation of RXRα because the inhibition of RXRα phosphorylation and the restoration of its physiological function may activate PPAR/RXR heterodimer and, therefore, be a potentially effective and critical strategy for the inhibition of cancer cell growth.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are members of a superfamily of nuclear hormone receptors comprising three isoforms, PPARα, PPARβ/δ, and PPARγ, which act as ligand-activated transcription factors. PPARs play key roles in energy homeostasis by modulating glucose and lipid metabolism and transport. Through these metabolic actions, PPARs can regulate cell proliferation, differentiation and survival [1, 2]. PPARs also control immune and inflammatory responses [3]. Because these physiological activities of PPARs are closely associated with normal cell homeostasis, the aberrant expression and function of PPARs have been observed in a variety of human malignancies. Moreover, these reports also suggest the possibility that targeting PPARs might be a critical strategy for inhibiting the development and growth of cancers. Indeed, numerous in vivo and in vitro studies have demonstrated that PPAR agonists, especially, PPARγ ligands can inhibit cell growth, cause apoptosis, and thus exert antitumor effects in various types of human malignancies [4–6]. Based on the antigrowth and prodifferentiation action of PPARs, several clinical studies have been conducted using the PPAR ligands in human cancers. However, with the exception of a small trial on liposarcomas, the clinical trials have so far indicated that the PPAR agonists may not be useful as a monotherapy for advanced malignancies [7–10].

On the other hand, recent preclinical studies show absorbing evidence that the combined treatment with PPAR ligands plus a variety of other agents can cause a synergistic effect to inhibit growth in cancer cells. For instance, we recently found that the activation of PPARγ/RXR heterodimer by their respective ligands synergistically inhibited cell growth and induced apoptosis in human colon cancer cells [11]. Therefore, the aim of this paper is to review the possibility that the combined usage of the PPAR ligands with other agents may therefore be a critical strategy for the treatment of certain types of human cancers. We also review the significance of the aberrant phosphorylation of
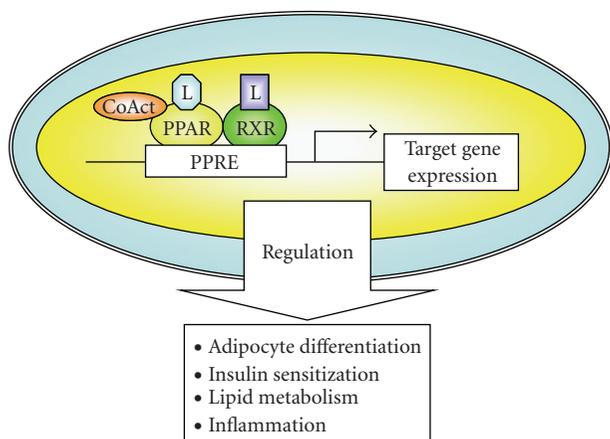
FIGURE 1: PPAR activation pathway and transcriptional regulation of target genes. After ligand binding, PPARs form heterodimers with RXR in the nucleus. The PPAR/RXR heterodimers interact with transcriptional coactivators (CoActs) and bind to sequence specific PPRE located in the target genes that control glucose and insulin homeostasis, lipid metabolism, inflammation, and cellular differentiation. L: ligand.

retinoid X receptor (RXR), which is a heterodimeric partner for PPARs, as described in the next section.

2. RXRs AND PPARs

RXRs and retinoic acid receptors (RARs), both of which are composed of three subtypes (α, β, and γ), are also members of the nuclear hormone receptor superfamily. The ligands for RXRs and RARs are the retinoids, a group of structural and functional analogues of vitamin A, and the retinoids have a profound effect on such cellular activities as growth, differentiation, apoptosis, and morphogenesis primarily through binding to RXRs and/or RARs. A small portion of dietary retinoids is converted to retinoic acid (RA), which is an active metabolite of the retinoids. RXR is specific for the 9-cis RA, while RAR binds both 9-cis RA and all-trans RA (ATRA). The nuclear retinoid receptors are ligand-dependent transcription factors that bind to the retinoic acid receptor responsive element (RARE) and retinoid X receptor responsive element (RXRE), which are present in the promoter regions of retinoid responsive target genes, thereby modulating the gene expression [12, 13]. Other nuclear receptors, including PPARs, also require RXR as a heterodimeric partner in order to exert their function. After ligand binding, PPARs can regulate target gene expression by binding to the peroxisome proliferator responsive element (PPRE) in target genes as a heterodimer with RXRs (see Figure 1) [14, 15]. Therefore, RXRs play a fundamental role in controlling normal cell proliferation and metabolism and act as a master regulator of nuclear receptors. Among the retinoid receptors, RXRα is thought to be one of the most important receptors with respect to the regulation of the essential effects of cell activities.

3. STRUCTURE OF RXRα AND SIGNIFICANCE OF RXRα PHOSPHORYLATION

RXRs have a variable N-terminal domain (A/B domain; AF-1), a highly conserved DNA-binding domain (DBD), a nonconserved hinge, and a moderately conserved C-terminus including the ligand-binding domain (LBD). *Transcriptional activation is mediated by LBD, which contains four more-or-less overlapping surfaces: a ligand-binding pocket for the binding of small, lipophilic molecules, a transactivation domain (AF-2 or helix 12), a cofactor binding surface, and a dimerization surface [16].* Recent studies revealed that phosphorylation processes are critical for the transcriptional activity of RAR/RXR heterodimers. Bruck et al. [17] reported that the activation of c-Jun N-terminal kinases (JNKs) induces phosphorylation of both at three residues (serine 61, serine 75, and threonine 87) located in the N-terminal AF-1 domain and one residue (serine 265) in the Omega loop in LBD (AF-2 domain) of RXRα. The RA-induced phosphorylation of the same three residues in the AF-1 domain is required for the cooperation of RXRα with RARγ for maximal transcriptional activity [18]. The phosphorylation of RXRα in its N-terminal domain plays a role to activate a subset of RA-responsive genes and for the antiproliferative effect of RA [19]. These findings suggest that RXRα "positively" regulates the transactivation of target genes through phosphorylation [20].

On the other hand, there are some contrary reports which show the phosphorylation of RXRα to "negatively" modulate the function of its heterodimeric binding partners. Indeed, MAPK-mediated phosphorylation of the RXRα LBD impairs the transcriptional activity of RXR/RAR [21, 22] and RXR/vitamin D$_3$ receptor (VDR) [23]. These "negative" effects of RXRα via its phosphorylation might be associated with certain types of human diseases, including cancer [20]. In the next section, we review the specific roles of the aberrant phosphorylation of RXRα in carcinogenesis, especially focusing on the development of hepatocellular carcinoma (HCC).

4. RXRα PHOSPHORYLATION AND CANCER

Abnormalities in the expression and function of retinoids and their receptors play an important role in influencing the development of various human malignancies and, therefore, might be critical targets for cancer chemoprevention and/or chemotherapy [24]. Specifically, we previously reported that hepatocarcinogenesis is accompanied by an accumulation of the phosphorylated (i.e., inactivated) form of RXRα and the inhibition of RXRα phosphorylation may thus be an effective strategy for preventing the development of HCC. Initially, we showed that the RXRα protein is anomalously phosphorylated at a specific site of the serine/threonine residues and is accumulated both in human HCC tissue as well as in HCC cell lines [22]. Phosphorylation at serine 260 of RXRα, a consensus site of mitogen-activated protein kinase (MAPK), is closely linked to its retarded degradation, low transcriptional activity, and the promotion of cancer cell growth, and the abrogation of phosphorylation by
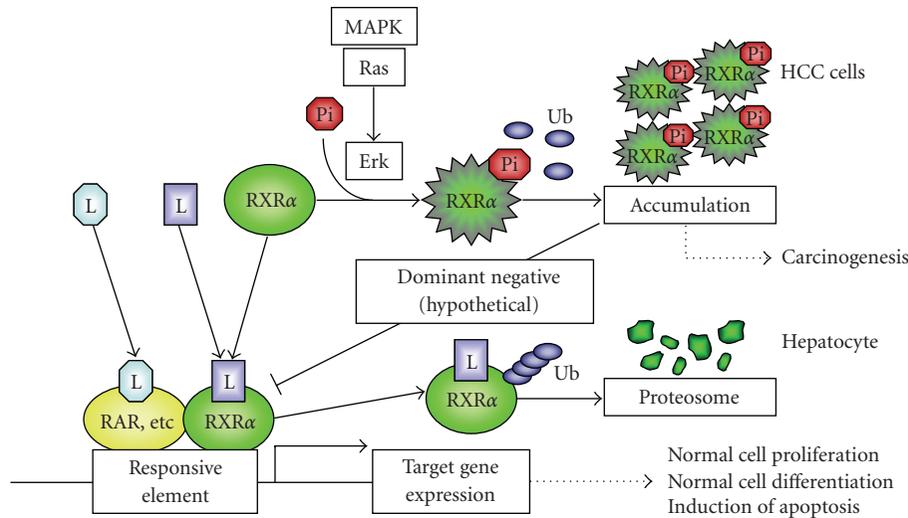
Figure 2: A schematic representation of RXRα phosphorylation in HCC cells. In normal hepatocytes, when the ligand (retinoid) binds to and activates RXRα, the receptor becomes able to heterodimerize with other nuclear receptors, including RAR, and then activates the expression of the target genes, which may regulate normal cell proliferation and differentiation, by binding to the specific responsive element. In HCC cells, the Ras/MAPK pathway is highly activated and phosphorylates RXRα at serine residues, thus impairing the functions of the receptor. Therefore, the accumulated p-RXRα interferes with the remaining normal RXRα, presumably, in a dominant negative manner, thereby playing a critical role in the development of HCC. L: ligand. Ub: ubiquitin.

MAPK-specific inhibitors restores the degradation of RXRα in a ligand-dependent manner [22, 25]. In addition, the phosphorylated form of RXRα (p-RXRα) is also resistant to ubiquitination and subsequent proteasome-mediated breakdown in both human HCC tissues and a human HCC cell line, whereas RXRα protein is unphosphorylated and highly ubiquitinated in the normal liver and in nonproliferating hepatocyte cultures [26]. The phosphorylation of RXRα abolishes its ability to form heterodimers with RARβ, thus resulting in the loss of cell growth control, resistance to retinoids, and the acceleration of cancer development [27]. These findings suggest that the accumulation of p-RXRα (i.e., nonfunctional RXRα), which can escape from the proteasome-mediated degradation system, may interfere with the function of normal RXRα in a dominant-negative manner, thereby playing a critical role in the development of HCC (see Figure 2) [28].

In addition to HCC, a malfunction of RXRα due to a posttranslational modification by phosphorylation is also associated with the development of other types of human malignancies. We recently reported that RXRα protein is highly phosphorylated and also accumulated in human colon cancer tissue samples as well as human colon cancer cell lines, while the levels of expression of p-RXRα do not increase in normal colonic epithelial cells; RXRα protein is phosphorylated in 75% of colorectal cancer tissues when compared with corresponding normal colon epithelial tissues [11]. Similar results have also been observed in human pancreatic cancer (manuscript in preparation). Moreover, Kanemura et al. [29] reported the abnormal phosphorylation of RXRα protein to play a role in the enhancement of cell proliferation, while producing an antiapoptotic effect, and also presumably

acquiring RA-resistance in HL-60R human leukemia cells. In addition to these malignancies, full-length RXRα is anomalously phosphorylated and accumulated in leiomyoma when compared to myometrial cells and this is associated with a resistance to ligand-mediated ubiquitination and a delay in the receptor proteolytic degradation [30].

What are the precise mechanisms by which phosphorylation of RXRα loses its transcriptional activity? Recent studies indicate that the phosphorylation of RXRα can regulate the function of its heterodimeric binding partners. For instance, Solomon et al. [23] reported that phosphorylation of RXRα at serine 260, which is located in the Omega loop of the LBD, results in the attenuation of ligand-dependent transactivation by RXR/VDR complex in human keratinocytes, thus resulting in the induction of malignant transformation. The residues located in the AF-2 domain are also phosphorylated in response to stress agents, including JNKs and MKK4/SEK1 [21, 31], among which serine 265 located in the Omega loop [31], and these phosphorylations are closely linked to inhibit the transcription of RA target genes. The phosphorylation of RXRα at serine 260 is also associated with retinoid resistance [22, 27]. Therefore, these findings indicate that RXRα phosphorylation, which occurs at specific residues located in the Omega loop of the LBD, is apparently associated with a malfunction in the retinoid-dependent signaling pathway. The Omega loop, located between helices H1 and H3, is a very flexible and dynamic region that moves substantially during the conformational rearrangement that accompanies ligand binding to the LBD [32]. It has therefore been proposed that phosphorylation of the residues in this loop might alter the dynamics of this region and create conformational changes within the

LBD, thus disrupting the interactions with coactivators and therefore inhibiting the activation of RA-responsive genes [17, 33].

5. PHOSPHORYLATED RXRα IS A CRITICAL TARGET FOR CANCER TREATMENT

The above findings support the possibility that the inhibition of RXRα phosphorylation and the restoration of its physiological function as a master regulator of nuclear receptors must be an effective strategy for controlling cell growth in various types of human cancers. It has been shown that the new synthetic retinoid, acyclic retinoid (ACR, NIK-333: Kowa Pharmaceutical Company Ltd., Tokyo, Japan), which was originally developed as an agonist for both RXR and RAR [34, 35], can restore the function of RXRα by inactivating the Ras-Erk signaling system and thereby inhibiting RXRα phosphorylation [25]. Practically, this agent has demonstrated several beneficial effects in experimental studies both in vivo and in vitro. For instance, ACR inhibited chemically induced hepatocarcinogenesis in rats as well as spontaneously occurring hepatoma in mice [36]. This agent also inhibited growth and induced apoptosis in human HCCderived cells [37–42]. Similar growth inhibitory effects are also observed in other types of human cancer cells, such as squamous cell carcinoma or leukemia cells [43, 44].

In addition, we also confirmed the chemopreventive effect of ACR on recurrent and second primary HCCs in patients who received anticancer treatment for an initial HCC in a double-blind and placebocontrolled clinical study. Namely, the oral administration of ACR for 12 months significantly reducedthe incidence of posttherapeutic recurrence of HCC and improved the survival rate in patients who underwent potentially curative treatments, without causing any severe adverse effects [45–47]. These findings suggest that ACR is a promising agent for the chemoprevention of HCC and that p-RXRα may be a critical target for the chemoprevention and/or treatment of some types of human cancers, including HCC, which show the accumulation of p-RXRα protein.

6. SYNERGY BETWEEN PPARγ LIGANDS AND RETINOIDS IN CANCER

Since RXR forms a permissive heterodimeric complex with PPAR, and the activation of PPARγ exerts antigrowth effects in cancer cells [4–6], it seems to be reasonable that the combination of RXR and PPARγ agonists may offer new therapeutic strategies for various types of human malignancies. Firstly, Tontonoz et al. [15] reported that the combined use of PPARγ and RXRα specific ligands is able to trigger terminal differentiation of primary human liposarcoma cells in vitro. This result suggests that the combination of these ligands may be a useful therapy for the treatment of liposarcoma [15]. Beneficial effects for the combined treatment with PPAR ligands plus retinoids are extensively reported in preclinical studies of the hematologic malignancies [48–51]. Therefore, the combination of PPARγ

ligand with RXR agonist or RAR agonist can enhance the differentiating and growth-inhibitory effects in human leukemia cells [48]. The combination of PPARγ ligand, ciglitazone, and ATRA synergistically reduces the cell growth rates and cell cycle arrest at the G1 phase in HL-60 human leukemia cells, and this is associated with synergistic upregulation of PTEN expression [49]. The combination of 9-cis RA and PPARγ ligand shows significant synergistic effects for the induction of apoptosis in multiple myeloma cells [50]. These reports suggest that the combination of PPARγ ligands plus retinoids holds promise as a novel therapy for some types of hematologic malignancies by activating the transcription of target genes that control apoptosis and differentiation in these malignant cells.

In addition to the hematologic malignancies, a number of preclinical studies indicate the preferable effects by the combination of PPAR ligands plus retinoids on the inhibition of cell growth in solid malignancies, especially in breast caner [52–55]. For instance, Rubin et al. [55] showed that a combination of ligands for PPARγ and RXR inhibits breast aromatase expression induced by tumor-derived factors. Because aromatase activates estrogen biosynthesis, the combination of these ligands may be able to find utility in thetreatment of estrogen-dependent carcinogenesis, such as breast cancer and endometrial cancer [56]. The combination of RXR ligand with ciglitazone also cooperatively inhibits the growth of breast cancer and lung cancer cells by activating the RARE promoter activity and inducing RARβ, which plays a critical role in mediating the growth-inhibitory effects of retinoids in various cancer cells [57]. In addition, the synergistic or cooperative effect of RXR and PPARγ agonists for growth inhibition and apoptosis induction is found in colon cancer cells [11, 58]. The detailed effects of PPARγ ligands plus retinoids to inhibit growth in colorectal cancer cells are discussed in the next section.

What are the molecular mechanisms by which the combination of PPARγ ligands and retinoids synergistically induce anticancer effects? Yang et al. [59] reported that the PPARγ and RXR ligands have been shown to differentially recruit subsets of transcriptional coactivators (i.e., p160 by RXR and DRIP205 by PPARγ) to the receptor complex, thus leading to an enhanced transcriptional activation and cellular effects. The transcriptional activity of PPRE is additively induced by treatment with a PPARγ activator plus 9-cis RA, and RXRα accumulation, by inhibiting its degradation due to the proteasome system, therefore contributes to the enhancement of PPARγ/RXR activation [60]. The transactivation of the PPRE by PPARγ/RXR heterodimer enhances the expression of the *glutathione S-transferase* gene, which is responsible for the cellular metabolism as well as the detoxification of several xenobiotics and carcinogenic compounds [61]. The findings of these reports suggest that the accumulation of the unphosphorylated form (i.e., functional form) of RXRα activates the transcriptional activity of PPRE and thereby enhances the expression of important target genes. The significance of the restoration of RXRα by inhibiting its aberrant phosphorylation is reported in the studies using the cell lines of HCC [22, 28], leukemia [29], and colon cancer [11], as discussed below.

7. SYNERGY BETWEEN PPARγ LIGANDS AND RETINOIDS IN COLORECTAL CANCER

Among the PPAR targeting therapies, the activation of PPARγ by its ligand is regarded as a potentially useful strategy for the chemoprevention and/or treatment of colorectal cancer because many in vivo and in vitro preclinical studies have demonstrated that PPARγ ligands can inhibit cell growth, cause apoptosis, and thus exert antitumor effects in this malignancy [5, 62–65]. As a result, there has been considerable interest in utilizing the combination of ligands for PPARγ and RXR for the prevention and treatment of colorectal cancer. In fact, it has been reported that in human colon cancer cells the combination of the RXR and PPARγ agonists produces greater efficacy in growth inhibition than either single agent alone, and this is associated with a cooperative reduction in the levels of cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE$_2$) synthesis [58]. The simultaneous exposure of HT-29 human colon cancer cells to ciglitazone and 9-cis RA results in an increased apoptotic effect and greater inhibition of COX-2 expression, in comparison to cells treated with either ciglitazone or 9-cis RA alone [66]. We recently reported that the combination of 9-cis RA and ciglitazone causes a synergistic inhibition in the growth of human colon cancer Caco2 cells, which express high levels of p-RXRα protein, and this is associated with the induction of apoptosis and inhibition in the expression of both COX-2 and c-Jun proteins and mRNAs. The combination of these agents has a synergistic effect in increasing the PPRE activity and decreasing the AP-1 activity. However, we should emphasize that these preferable effects are observed when the phosphorylation of RXRα protein is inhibited [11]. Therefore, the inhibition of the phosphorylation of this protein appears to play a critical role in inducing the synergistic growth inhibitory effect in colon cancer cells.

The above findings indicate that the activation of the RXR/PPARγ heterodimer by their specific ligands can decrease the expression of COX-2, which is one of the main mediators in the inflammatory signaling pathway. This seems to be significant because COX-2 plays a critical role in the development of colorectal cancer and might, therefore, be an important molecular target for colorectal cancer prevention and treatment [67]. Recent studies have revealed 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis to be significantly reduced after the administration of both PPARγ and RXR agonists, and this beneficial effect is reflected by the reduction in the NF-κB DNA binding activity in the colon [68]. The inhibition of the β-catenin mediated pathway, which promotes the development of colon cancer and is stimulated by COX-2 as well as PGE$_2$ [69, 70], by nonsteroidal anti-inflammatory drugs, requires a high-level expression of RXRα and PPARγ [71]. Therefore, the activation of the RXR/PPARγ heterodimer by the coadministration of their ligands is clinically useful for the prevention and/or treatment of colon cancer as well as colonic inflammation [72], due to their synergistic effects on the COX-2/PGE$_2$ axis.

8. SYNERGY BETWEEN PPARs LIGAND AND THE OTHER DRUGS EXCEPT FOR RETINOIDS IN CANCER

In addition to the retinoids, the synergistic effects of PPARγ ligands with other agents have also been reported by many investigators. Girnun et al. [73] found that agonist activation of PPARγ synergistically increases the growth-inhibitory effect of the platinum-based drugs cisplatin and carboplatin in several different types of cancers in both in vivo and in vitro studies. This synergy is associated with the reduction of multiple members of the *metallothionein* gene family expression, which play a role in the resistance of certain cancers to platinum-based drugs [74] by PPARγ [73]. The synergistic or enhancing effects induced by the combination of PPAR ligands plus other conventional chemotherapeutic agents to inhibit cell growth are also reported in several types of cancer cells [75–77]. In addition, it is also reported that the histone deacetylase (HDAC) inhibitors have a synergistic effect with the thiazolidinediones in the activation of PPARγ target genes [78]. In studies using cancer cells, the combination treatment using the PPARγ agonist pioglitazone and the HDAC inhibitor valproic acid has been reported to be more efficient at inhibiting prostate tumor growth than each individual therapy alone [79]. An enhanced growth inhibition is observed when neuroblastoma cells are treated with a PPARγ ligand and a HDAC inhibitor, thus suggesting that a combination therapy to treat neuroblastoma might prove more effective than using either agent alone [80]. These findings suggest that a combination therapy using PPARγ agonists and HDAC inhibitors might therefore be potentially effective for the treatment of some types of human malignancies.

Recently, molecular-targeted therapy is attractive as a new effective strategy to inhibit the growth of cancer cells, and therefore, the combination therapy using such specific molecular-targeting agents plus PPAR ligands may become an important regimen in near future. For instance, the proteasome inhibitor bortezomib, which can inhibit the NF-κB activity, augments the antiproliferative effects of the PPARγ agonist rosiglitazone in human melanoma cells [81]. The dual ligand specific for PPARα/γ synergistically enhanced the antiproliferative and proapoptotic effect of imatinib, a specific inhibitor of BCR-ABL tyrosine kinase, in Philadelphia chromosome-positive lymphocytic leukemia and chronic myelogenous leukemia blast crisis cell lines [82, 83]. The growth inhibitory effects by gefitinib, an EGFR tyrosine kinase inhibitor, on the human lung cancer cell line are potentiated by the treatment with PPARγ ligand, and this is associated with an increase in the expression of PTEN, but a reduction in the expression of p-Akt [84]. It is interesting to note that the activation of PPARγ by its ligand causes a dramatic inhibition of the tyrosine phosphorylation of HER2 and HER3 receptors, the other member for the EGFR family of receptor tyrosine kinases (RTKs), in human breast cancer cells [85]. The PPARγ ligand also blocks phosphorylation of other member of RTKs, such as IGF-1R, thereby suppressing the proliferation of breast cancer cell lines [85]. These findings may explain the mechanisms in regard to precisely how the PPARγ ligands can enhance the effects of specific RTK
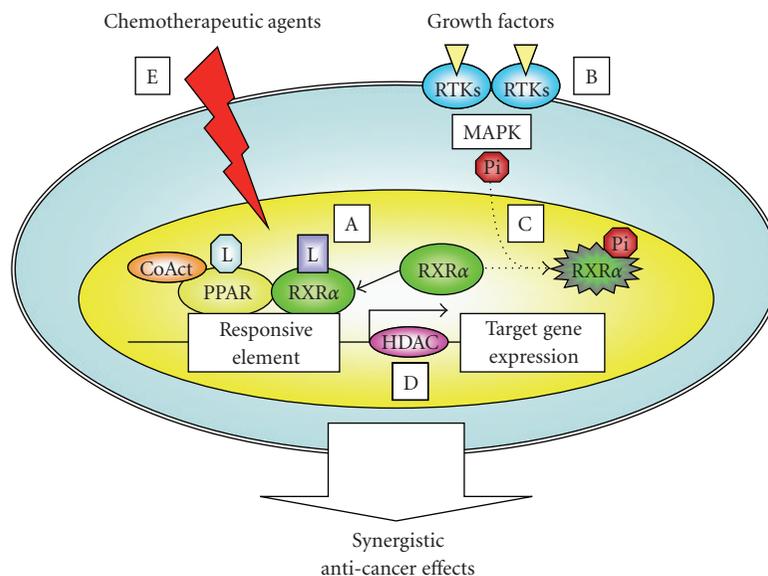
FIGURE 3: A hypothetical schematic representation of the synergistic anticancer effects of the combination of PPAR ligands plus other agents. When PPARs are activated by ligand binding, they are able to heterodimerize with RXR and activate the target gene expression by binding to the PPRE element. Therefore, the retinoids which bind to RXR may be the most preferable partner for the PPAR agonists (A). However, in some types of cancers, the MAPK pathway phosphorylates RXRα, and the accumulated nonfunctional p-RXRα interferes with the function of the remaining normal RXRα, thereby promoting the growth of cancer cells. The activation of RTKs by their specific ligands (growth factors) can play a critical role in the stimulation of the MAPK pathway. Therefore, the agents which target the activation of RTKs (B) and/or the MAPK pathway (C) restore the function of RXRα as a master regulator of nuclear receptors in cancer cells and this will support the synergistic growth inhibition by PPAR and RXR ligands in cancer cells. The HDACs enforce a tight chromatin structure and thereby repress the transcription of target genes controlled by PPAR/RXR. Therefore, the combination of a PPAR agonist plus an HDAC inhibitor is more efficient to inhibit the growth of cancer cells (D). Finally, the conventional chemotherapeutic agents also cause synergistic or enhancing effects to inhibit cancer cell growth by the combination of PPAR ligands (E). L: ligand.

inhibitors, although some other molecules may also play a role.

9. IS PPAR PHOSPHORYLATION ASSOCIATED WITH CANCER?

As mentioned above, a malfunction of RXRα due to phosphorylation is associated with cancer cell growth and retinoid resistance [11, 22, 27, 29]. However, a question which arises here is whether the phosphorylation of PPARs also plays a role in carcinogenesis and/or resistance to the PPAR ligands. Recent studies have shown that PPARs are phosphoproteins, and their transcriptional activity is affected by several kinases, including ERK/MAPK, both in a ligand-dependent and/or -independent manner [86]. Although the significance of the PPARs phosphorylation in cancer has not been clarified, at least in PPARγ, the transcriptional activity of this receptor is inhibited by phosphorylation [87–89]. Extracellular signals that activate intracellular phosphorylation pathways can influence the degradation process of PPARγ [89, 90]. These reports may suggest that as well as RXRs [22, 27], the phosphorylation-mediated inhibition of transcriptional activity of PPARs is associated with cancer [20]. Hedvat et al. [91] conducted an interesting study, reporting that the activation of PPARγ is sustained by the presence of HER-kinase inhibitor, suggesting that HER-

kinase and its downstream ERK/MAPK pathway phosphorylate PPARγ and, therefore, abrogate the effects of PPARγ activity through degradation of this nuclear receptor. In this study, the inhibition of HER-kinase activity was sufficient to inhibit PPARγ protein degradation [91]. These findings suggest that, in future studies, the combination of PPARγ/RXR ligands plus a specific agent which targets the RTKs and/or Ras/MAPK signaling pathway may therefore become a promising strategy to inhibit the growth of cancer cells by inhibiting the phosphorylation of PPARs/RXRs.

10. CONCLUSION

The combined use of two or more agents is often advantageous since it may permit to lower the clinical dosages, thereby decreasing the overall toxicity, and thus providing the potential for synergistic effects between agents. In this review, we made an attempt to show the synergism between PPARγ agonists and other agents (see Figure 3). Among such preferable candidates, retinoids seem to be the best partner of PPARγ ligands in order to exert synergistic antitumor effects. However, phosphorylation of RXRα represses the PPARγ/RXR-dependent anticancer effects. In some cases, the inhibition of PPAR phosphorylation may also support the antitumor function of these nuclear receptors. In addition, we should keep in mind the fact that the encouraging results

obtained from the combined use of PPARγ agonists plus other agents have been exclusively reported in preclinical studies, and PPAR agonist monotherapy did not achieve a significant result for advanced malignancies in clinical trials, except for a small trial on liposarcomas [7–10]. Therefore, it might be expected that some combination with other agents may lead to breakthrough in the clinical application of PPARγ agonists for chemoprevention and/or treatment of malignancies.

In conclusion, the combination treatment using the PPARγ agonists and other agents might be an effective and promising strategy for chemoprevention and/or treatment of various types of cancers. Future studies will be necessary to improve the anticancer efficacy of PPARγ agonist plus retinoids by combining with appropriate specific kinase inhibitors.

ABBREVIATIONS

PPAR:	Peroxisome proliferator-activated receptor
PPRE:	Peroxisome proliferator responsive element
RXR:	Retinoid X receptor
RAR:	Retinoic acid receptor
p-RXRα:	Phosphorylated RXRα
RXRE:	Retinoid X receptor responsive element
RARE:	Retinoic acid receptor responsive element
DBD:	DNA-binding domain
LBD:	Ligand-binding domain
VDR:	Vitamin D_3 receptor
RA:	Retinoic acid
ACR:	Acyclic retinoid
ATRA:	All-trans retinoic acid
MAPK:	Mitogen-activated protein kinase
ERK:	Extracellular signal-related kinase
JNK:	c-Jun N-terminal kinase
HCC:	Hepatocellular carcinoma
COX-2:	Cyclooxygenase-2
Akt:	Protein kinase B
AP-1:	Activator protein-1
NF-κB:	Nuclear factor-κB
PGE$_2$:	Prostaglandin E$_2$
RTK:	Receptor tyrosine kinase
HDAC:	Histone deacetylase
HER:	Human epidermal growth factor receptor.

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Review Article

Combination Therapy of PPARγ Ligands and Inhibitors of Arachidonic Acid in Lung Cancer

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Lung cancer is the leading cause of cancer death in the United States and five-year survival remains low. Numerous studies have shown that chronic inflammation may lead to progression of carcinogenesis. As a result of inflammatory stimulation, arachidonic acid (AA) metabolism produces proliferation mediators through complex and dynamic interactions of the products of the LOX/COX enzymes. One important mediator in the activation of the AA pathways is the nuclear protein PPARγ. Targeting LOX/COX enzymes and inducing activation of PPARγ have resulted in significant reduction of cell growth in lung cancer cell lines. However, specific COX-inhibitors have been correlated with an increased cardiovascular risk. Clinical applications are still being explored with a novel generation of dual LOX/COX inhibitors. PPARγ activation through synthetic ligands (TZDs) has revealed a great mechanistic complexity since effects are produced through PPARγ-dependent and -independent mechanisms. Furthermore, PPARγ could also be involved in regulation of COX-2. Overexpression of PPARγ has reported to play a role in control of invasion and differentiation. Exploring the function of PPARγ, in this new context, may provide a better mechanistic model of its role in cancer and give an opportunity to design a more efficient therapeutic approach in combination with LOX/COX inhibitors.

1. INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States. Despite increasing amount of effort on lung cancer research, five-year survival is still around 15% [1].

Growing evidence suggests that molecular pathways involved in chronic inflammation may contribute the progression of carcinogenesis [2, 3]. Arachidonic Acid (AA) metabolism is intimately involved in the inflammatory response. Its deregulation in epithelial cancers has been regarded as an early step in the transformation process [4, 5]. AA is released from the membrane by phospholipidic enzymes, mainly through cPLA2α activity [6]. AA could be metabolized by two major pathways: lipoxygenase (LOX) pathway producing hydroxy derivatives and leukotrienes and the cyclooxygenase (COX) pathway producing various prostaglandins. Overexpression of LOX and COX enzymes has long been associated with tumor progression [6–8], and targets for those pathways have been a primary interest in research for therapeutics agents [9–11]. However, specific COX-2 inhibitors have been associated with cardiovascular

toxicity [12–15]. It has also been reported that products of the LOX pathway and inhibitors of this pathway may induce activity of peroxisome-proliferator-activated receptor gamma (PPARγ) [9, 16, 17].

PPARγ is a member of the nuclear-hormone-receptor superfamily characterized as having a role in lipid metabolism and adipose differentiation [18]. Several synthetic ligands activate PPARγ and reduce cell growth by inducing apoptosis in lung cancer [19, 20]. Combination therapy of 5-LOX inhibitor, PPARγ ligand, and PPARγ binding partner has resulted in an additive effect on cell growth decrease and induction of apoptosis [21]. However, synthetic PPARγ ligands such as thiazolidinedione derivatives (TZDs), a class of antidiabetic drugs, are also responsible of PPARγ-independent effects [22, 23]. TZDs compounds (pioglitazone, rosiglitazone, trosiglitazone, ciglitazone) have shown interesting clinical activity in diabetes and metabolic syndrome but also have been associated with rare but significant clinical toxicities [24, 25].

New COX/LOX inhibitors have been recently reported and initially these drugs showing a more favorable GI and cardiovascular tolerability [26]. Exploring the potential of these new agents, together with a more comprehensive mechanistic model of the PPARγ function, may provide a solid foundation for a better design of novel combination therapies for lung cancer.

2. INHIBITION OF LOX/COX PATHWAYS

Two isoforms of (COX) enzymes have been identified and targeted for their clinical and pharmacological interest [27, 28]. Characterization of COX-1 and COX-2 enzymes led to a proposed model where COX-1 was constitutively expressed and COX-2 was an inducible enzyme activated in inflammatory response [29, 30]. Overexpression of inducible COX-2 has also been reported in malignant conditions associated to cell growth, protection against apoptosis, and induction of angiogenesis in lung cancer [31–34]. Selective COX-2 inhibitors have reduced cell growth and increased apoptosis in lung cancer cell lines [32, 35, 36]. However, increased cardiovascular risk has been associated with selective inhibition of COX-2 [12–15].

LOX pathway is more complex since at least six different enzymes have been identified in humans, and it has not been as extensively developed for clinical applications [37]. Studies of LOX expression and activities in normal and cancerous lesions have shown that 15-LOX-1 and 15-LOX-2 are usually expressed in normal tissues and benign lesions, whereas 5-LOX and 12-LOX are absent in normal epithelia and constitutively expressed in epithelial cancers such as lung, colon, skin, esophageal, pancreatic, and prostate cancers [38]. Targeting 5-LOX with specific inhibitors or by inhibition of 5-lipoxygenase activating protein (FLAP) has resulted in decreased cell growth and increased apoptosis in lung and breast cancer cell lines [39]. In that report, 5-LOX downstream metabolites were reduced due to a diversion of the metabolic products from 5-LOX to other LOX (12-LOX and 15-LOX) and COX pathways. Substrate of inhibited 5-LOX is metabolized by the other available enzymes of both LOX and COX pathways. This result has been described as endoperoxide shunting [39]. This property of the AA pathways adds another layer of complexity to this mechanism. To address this complexity, Hong et al. [40] analyzed, in epithelial cancer cell lines, the correlation between expression of AA metabolizing enzymes and effect on cell growth of specific enzyme inhibitors. No correlation was observed for inducible enzymes (LOX-12, LOX-15, and COX-2). However, LOX inhibitors have a more potent effect on cell growth in vitro than COX inhibitors on constitutively expressed enzymes, LOX-5, and COX-1 [40]. Of interest, pan-COX inhibitor ketorolac did not inhibited oral cancer growth in vitro, but it was associated with significant reduction of heterotransplant growth in vivo [11]. Cytokine-producing inflammatory cells are present in the in vivo assay. Stimulated macrophages and other inflammatory cells are able to produce a variety of cytokines which could promote growth differentially on clonal populations of epithelial cells. Hong et al. [11] have suggested that IL-6 plays, through

STAT3 signaling, an important role in oral cancer regulation in a paracrine and autocrine way. This report suggests a potential role for inflammatory cells stimulating cancer cell growth by COX-driven cytokine production.

Recent animal studies have shown COX-2 constitutive expression in normal tissues, where it plays a role in gastric mucosal protection, renal homeostasis, and endothelial PGI_2 production [41, 42]. This result, along with the previously described risk of thrombotic complication after selective inhibition of COX-2, motivated the search for an alternative strategy [43]. Since inhibition of one pathway of the AA metabolism might induce the activity of the alternate pathway, a dual inhibition of both LOX and COX pathways has been proposed as a new approach to improve clinical utility [44]. Moreover, inhibition of COX-2 increases production of leukotrienes (LTs), especially in the gastric mucosa. Given the proinflammatory effects of LTs and their deleterious effects on gastric mucosa, dual inhibition of LOX and COX pathways might improve gastric tolerability [45]. On the other hand, free unmetabolized AA may induce a concentration-dependent apoptosis on cancer cells [46]. Therefore, blocking LOX/COX pathways simultaneously may prevent recruitment of alternate pathways within the AA pathway and may lead to an accumulation of AA that could increase apoptosis induction.

The use of combination of LOX and COX specific inhibitors has been described in colon and pancreatic cancer models [47, 48]. Recently, Schroeder et al. [49] have reported that treatment of A549 lung cancer cell line and transformed cell 1198, derived from BEAS-2B, with a triple combination of clinical relevant concentrations of celecoxib (COX inhibitor), MK886, and REV 5901 (both LOX inhibitors) resulted in significant suppression of growth and cell death induction in both cell lines. Interestingly, premalignant cells, derived from BEAS-2B, revealed a greater sensitivity to this LOX/COX inhibitors combination than malignant cells A549. This result raises the possibility that combination of AA metabolism inhibitors might be more effective in precancerous states than in lung cancer therapy.

However, designing a single compound that might target both LOX and COX pathways is a strategy that offers several benefits in terms of cost, risk, and adverse effects [50]. By using different approaches, a number of new compounds able to target LOX-5 and COX-2 have been designed but to date limited data is available concerning their potential as antitumorigenic agents [51]. First generation of compounds showing dual inhibition of LOX-5 and COX-2 such as Benoxaprofen is not longer in use due to their liver toxicity [52]. A new generation of compounds has been developed offering a more balanced inhibition of LOX-5 and COX-2 enzymes by acting as a substrate competitor. Licofelone is one of the most promising candidates and is currently on phase-III clinical trials for treatment of osteoarthritis as anti-inflammatory drug [26]. Licofelone inhibits LOX-5, COX-1, and COX-2, decreases production of PGs and LTs [53, 54], and presents lower GI toxicity compared to nonsteroidal anti-inflammatory drugs (NSAIDs) naproxen and rofecoxib [55, 56]. Interestingly, it has been reported

recently that Licofelone inhibits LOX/COX pathways and induces apoptosis in HCA-7 colon cancer cells [57].

3. PPARγ ACTIVATION

Active PPARγ forms a heterodimer with retinoid X receptor RXR [22, 58, 59]. Coactivators and corepressors interact with the PPARγ-RXR heterodimer which binds specific regions known as PPRE (PPARγ response elements) within promoter of target genes. Different interactions of coactivators and corepressors with PPARγ are responsible for important changes on the transcription pattern of target genes. Some natural ligands of PPARγ have been identified such as leukotrienes, prostaglandin D_2, prostaglandin J_2 (15d-PGJ$_2$), and some polyunsaturated fatty acids. In addition, antidiabetic drugs, such as rosiglitazone, ciglitazone, pioglitazone, and trosiglitazone, included in the group of thiazolidinediones (TZDs), are also ligands of PPARγ. Activation of PPARγ in NSCLC by TZDs has induced cell growth arrest and apoptosis [60, 61] and affects expression of genes such as PTEN, fibronectin, and integrin alpha 5 [62–64]. However, PPARγ ligands have shown effects on NSCLC cell lines that remain elusive to understand with our current notions of PPARγ mechanism of action. For instance, rosiglitazone inhibits cell growth by increasing phosphorylation of AMPKα and reducing phosphorylation of p70S6K. Treatment with PPARγ antagonist, GW9662, has no effect on AMPKα and p70S6K phosphorylation status [65].

PPARγ could also be regulated from upstream elements of the AA pathway; interestingly, it has been reported that cPLA2α, responsible of AA release from the membrane, affects PPARγ activity and modulates expression of COX-2 and IL-8 through PPARγ response elements [66, 67].

4. LOX/COX PATHWAYS AND PPARγ CROSS-TALK

Deregulation of important elements of the AA pathway has been observed in tumor progression in several reports as shown in Figure 1 [4, 5]. Targeting overexpression of inducible enzymes, 5-LOX and COX-2 enzymes, as a way to control cell proliferation was a first logical step. However, as we have previously discussed, downregulation of one pathway of the AA metabolism may induce, through endoperoxide shunting, the other AA pathways, balancing the initial effect [68–72].

Affecting the LOX/COX pathway has also an effect on the activity of PPARγ. Inhibition of 5-LOX by MK886 results in activation of PPARγ in breast and lung cancer cell lines [9, 21]. Inhibition of COX-2 by celecoxib reduces PGE2 production, downregulates cPLA2α expression in lung cancer cell lines, but also induces PPARγ expression and activity [73, 74]. On the other hand, PPARγ ligand ciglitazone may modulate COX-2 expression and PGE2 production through a PPARγ-independent mechanism. Thus, it has been speculated that ciglitazone could suppress transcriptional factors involved in COX-2 mRNA production. It has also been suggested that ciglitazone could downregulate COX-2 through a histone deacetylase mechanism [75]. Furthermore,
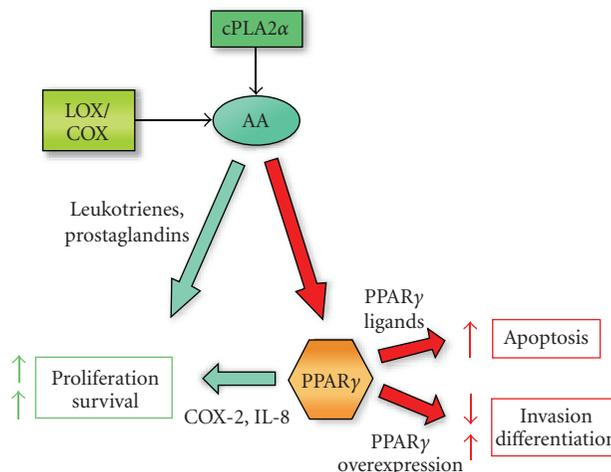

FIGURE 1: Inflammatory products stimulate cPLA2α which increases levels of AA available to be metabolized by the LOX/COX pathways. LOX/COX enzymes' products, leukotrienes and prostaglandins, induce cell proliferation. Natural ligands for PPARγ are also produced by the LOX/COX pathways. Activation of PPARγ balances the effect of leukotrienes and prostaglandins inducing apoptosis through various mechanisms. However, activation of PPARγ may also induce COX-2 and IL-8 expressions. Furthermore, overexpression of PPARγ may decrease invasion and induce differentiation from a mesenchymal to an epithelial-like phenotype.

PPARγ ligands rosiglitazone and pioglitazone decrease PGE2 by upregulating 15-hydroxyprostaglandin dehydrogenase independently of PPARγ and COX-2 [76].

MK886, inhibitor of FLAP (5-lipoxygenase activating protein), results in inhibition of 5-LOX and induction of PPARγ activity [9]. Induction of PPARγ might be a direct effect of MK886 [77] or an indirect effect as a result of changes in the equilibrium of AA available for each different LOX enzymes after inhibition of 5-LOX. Thus, production of 15-HETE might increase which could induce PPARγ expression [16, 17]. An interesting example of the double effect, inhibition of 5-LOX and activation of PPARγ, has been provided by Avis et al. [21] showing that a combination of low-dose MK886, ciglitazone (PPARγ ligand), and retinoic X receptor (RXRα; transcriptional partner of PPARγ), interacting in a superadditive manner, causes an inhibition of cell growth in lung cancer cell lines A549 and H1299. Moreover, a novel compound, LY293111, an LTB$_4$ receptor antagonist and inhibitor of 5-LOX, is able to induce PPARγ as well [78]. LY293111 has proved to be effective in reducing cell growth in different types of cancer such as pancreatic, colon, and lymphoma [79–83]. However, no results have been published so far in lung cancer.

Induction of PPARγ by 15-LOX metabolites and by COX-2 inhibitors and PPARγ effects on COX-2 activity, among other results, are pointing out a cross-talk between effectors of the AA pathway (LOX and COX products) and PPARγ activity. Deregulation of this cross-talk is thought to allow tumor progression.

5. OPTIMIZING THERAPEUTIC EFFECTS OF THE LOX/COX/PPARγ CROSS-TALK

Despite the great effort in research in lung cancer basic biology and cancer early detection and prevention, there has been no significant improvement in five-year survival. As we have been previously described, several reports are showing that combination therapy against enzymes of the AA metabolism has a dramatic potential in chemoprevention and chemotherapy [21, 49, 51, 57]. Moreover, new drugs, such as Licofelone, designed to aim at a dual inhibition of the LOX and COX pathways, have proved to be effective in reducing growth in cancer cell lines. Interestingly, a recently published report, that uses a mathematical model to study the interactions of the AA metabolic network, has revealed that a dual inhibitor against LOX/COX is more effective than a combination of single COX and LOX inhibitors [84]. A successful attempt to reduce cell growth in cancer, through the AA metabolic pathway, may have great potency if involves inhibition of both the LOX and COX pathways and activation of PPARγ. More research is needed in this subject to confirm the safety of dual inhibitors regarding GI toxicity and cardiovascular tolerability. In addition, efficiency of dual inhibitors in preventing cancer growth and inducing cell death has to be proved in other cancers especially in lung cancer.

To manage the systemic toxicity for lung cancer therapy, we have used a regional delivery strategy [85, 86]. With aerosolized drug delivery, there is direct delivery of the therapeutic agent to the target transforming cell population injured by chronic exposure tobacco smoke. Despite attractive results with relevant animal models and early clinical experience [87], this approach has not received serious commercial attention. However, aerosolized drug delivery has the appeal of avoiding certain important complications of PPARγ activation, related to the complicated enterohepatic metabolism of these drugs [88].

Increasing evidence suggests that PPARγ acts as a key control element of the AA pathway. Pawliczak et al. [66, 67] have shown that cPLA2α, responsible of the activation of AA, regulates PPARγ expression and COX-2 and IL-8 expressions through PPARγ in lung cancer cell lines. This last result is especially intriguing since it suggests that PPARγ can also stimulate growth in addition of its role as suppressor of cell growth and inductor of apoptosis. Despite several evidence of the mainstream activity of PPARγ, always in the context of an increased expression of the LOX pathway and/or COX-2, we should consider a more general role for PPARγ as feedback regulator of the AA pathway.

In this context, using PPARγ agonists, such as the TZDs, has provided a considerable amount of information about the PPARγ function. But it has also revealed the great complexity of their indirect effects not related to PPARγ [22, 23]. One approach to study PPARγ function, without the interference of TZDs, would be to overexpress PPARγ in NSCLC. Overexpression of PPARγ has no effect on proliferation but it affects anchorage-independent growth, invasiveness and induces a differentiation from a mesenchymal to an epithelial-like phenotype [89]. In the same vein, some recent studies are suggesting a role for PPARγ in control of anoikis by interacting with focal adhesion proteins [90, 91]. To better understand the PPARγ function, a more direct approach might be to study the effect of overexpression and silencing of PPARγ on mechanisms of cell proliferation and apoptosis. Generating such data could enable the development of a mechanistic model that could explain the inefficient response of PPARγ, in the context of overexpression of the LOX/COX pathways, in lung cancer. This model may also allow a better understanding of LOX/COX interaction and PPARγ function. As a result of this approach, a combination therapy, based on dual inhibitors of the LOX/COX pathways, could be developed in a more rationale fashion and provide a better result in chemoprevention and chemotherapy.

6. CONCLUSION

With considering the clinical significance of AA metabolism, it may be useful to partition certain aspects of this complex biology. For example, it is known that COX products are constitutively required for the maintenance of normal gastric epithelium, and from a clinical tolerance perspective, it would be good not to interfere with this function.

As we have discussed, chronic injury initiates an excessive release of cytokines and other inflammatory mediators such as arachidonic acid metabolism products which could trigger carcinogenesis. In devising therapeutic strategies, with the arachidonic acid products, greater attention to the impact of levels of mediators may be rewarding. For beneficial effects with carcinogenesis, it would be of interest to evaluate if reduction, rather than complete elimination, in levels of arachidonic acid products would be efficacious without incurring clinical toxicity. Moreover, exploring the effects of PPARγ activity in different contexts, such as anoikis, could provide relevant mechanistic information about PPARγ function that might allow an improved design of combination therapies.

Important opportunities exist to reduce the occurrence of low frequency but significant hepatic toxicity by considering the use of aerosolized drug delivery strategies. This would potentially greatly enable the use of combination approaches, as discussed in this review, for early lung cancer management applications.

Development of robust clinical pharmacology tools, such as the assay for urinary PGM, could allow for a precise and adaptive method to define optimal dosing for LOX/COX inhibitors. This approach may provide an important new opportunity in learning how to more effectively exploit the effects of inhibiting LOX and COX pathways, in combination with PPARγ activation, on control of proliferation and apoptosis in lung cancer.

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Review Article

Potential Therapeutic Use of PPARγ-Programed Human Monocyte-Derived Dendritic Cells in Cancer Vaccination Therapy

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Dendritic cells (DCs) can regulate all elements of the immune system, and therefore are an ideal target for vaccination. During the last two decades, as a result of extensive research, DCs became the primary target of antitumor vaccination as well. A critical issue of antitumor vaccination is the phenotype of the dendritic cell used. It has been recently shown that several nuclear hormone receptors, and amongst them the lipid-activated nuclear receptor and peroxisome proliferator-activated receptor gamma (PPARγ), have important roles in effecting the immunophenotype of human dendritic cells. It regulates primarily lipid metabolism and via this it influences the immunophenotype of DCs by altering lipid antigen uptake, presentation, and also other immune functions. In this review, we summarize the principles of antitumor vaccination strategies and present our hypothesis on how PPARγ-regulated processes might be involved and could be exploited in the design of vaccination strategies.

1. DENDRITIC CELLS IN TUMORS

Dendritic cells (DCs) were discovered in mouse spleen by Ralph Steinman and Zanvil Cohn in 1973 [1]. Immature dendritic cells (IDCs) are sentinels of the immune system, continuously monitoring peripheral tissues for invaders, capture and process antigens, and migrate to the draining lymph nodes where they present peptides to naive Treg cells (T cells) and activate them [2]. The full activation of T cells requires special peptide-MHCI or peptide-MHCII complexes and additional signals from DCs in the form of various costimulatory molecules and cytokines. Furthermore, activated CD4$^+$ T cells could be polarized to T helper 1 (Th1) and T helper 2 (Th2) subtypes. These processes are dependent on the cytokines interleukin-12 (IL-12), IL-4, and IL-10 secreted by mature DCs (MDCs). In response to IL-12, T cells polarize to Th1 and enhance CD8$^+$ cytotoxic T-cell response against tumor cells or pathogens, while IL-4- and IL-10-activated Th2 cells promote humoral immune response and/or tolerance. Another important point is that

DCs can induce T-cell tolerance to self-antigens and via this prevent and reduce autoimmune diseases.

Importantly, it appears that tumor tissues have characteristic immune environments with distinct DC subset distributions. Different DC subset localization within the compartments of tumor has been reported in colorectal cancer and oral squamous cell carcinoma patients. Dadabayev et al. investigated the infiltration pattern of DCs in human colorectal tumor samples analyzed with S100 and HLA class II DC markers. S100$^+$ and CD1a$^+$ DCs were found in tumor epithelium, in parallel with intraepithelial CD4$^+$ or CD8$^+$ T-cell infiltration and suggested increased disease-free survival, while HLA class II$^+$ cells were observed in the stromal compartment, correlated with adversed outcome of the tumor [3]. Later they utilized CD208 (DC-LAMP) marker for marking MDCs and proved that CD208$^+$ DCs were detectable in the peritumoral area, and infiltration of MDCs into tumor epithelium was correlated also with decreased patient survival [4]. In primary squamous cell carcinoma patients, IDCs and MDCs were characterized with

distinct tissue localization patterns. Immature Langerhans cells (LCs) and DC-SIGN^{+} interstitial DCs were found inside the tumor tissue while the number of mature CD208^{+} DCs was limited. Moreover, CD123^{+} plasmacytoid DC representation in the tumor area was correlated with poor survival [5]. Importantly, DCs interact with tumor cells and cytokines produced by tumor cells or immune cells influence DC function and maturation. The tumor microenvironment affects DC differentiation from CD14^{+} monocytes and haematopoietic precursors promoting an early and dysfunctional maturation of DCs. Several reports described reduced the number of DC in peripheral blood, tumor tissues, and draining lymph nodes in cancer patients. Partial maturation of DCs by tumor-derived factors like IL-10, vascular endothelial growth factor (VEGF), and TGF-β induces self-tolarence and promotes conversion of naive T cells to regulator T cells, favoring development of suppressive T cells. Presence of tolerogenic T cells in tumor beds induces local immune suppression and alters the function of anticancer effector T cells. These cells, isolated from draining lymph nodes of patients with pancreas or breast cancer secrete IL-10 and TGF-β, prevent activated CD4^{+} CD25^{−} and CD8^{+} effector T cells, and suppress tumor-specific immune response [6]. Apart from the fact that DCs are involved in activation of Treg, there is an increasing amount of evidence about T cells, which can be recruited into tumors and affect DC development. Decreased CD80 and CD86 cell surface markers by T cells lead to reduced T-cell stimulatory ability of DCs [7, 8]. Immunosuppressive B7-3/4 molecules are upregulated on DCs upon DC-Treg interaction reserving a possible feedback loop to generate more regulatory T cells [9, 10]. Another immunosuppressive cycle is the conversion of DCs by Treg-secreted INF-γ and CTLA-4 into an indolamine 2, 3-dioxigenase (IDO) expressing cells which induce Treg generation and effector T-cell apoptosis [11, 12].

Classically, CD4^{+} T cells have been categorized into Th1, Th2, Treg, and Th17 subsets. However, TGF-β has a crucial role in Treg and Th17 cell development, the dichotomy of Treg/Th17 is dependent on IL6. Only a few pieces of evidence has been reported on the presence and regulation of Th17 cells by IL-2 in human cancer and experimental tumors. Muranski et al. reported that tumor-specific Th17 polarized cells mediated successful treatment of large established tumor in cutaneous melanoma-bearing mice. The therapeutic effect of the cells was dependent on their INFγ production [13].

Modulating factors released by the tumor environment cause defective functional maturation of DCs and affect the differentiation of immature myeloid-derived suppressing cells (MDSCs). The portion of MDSC is significantly increased in spleen, peripheral blood, and bone marrow of tumor-bearing patients and correlates with tumor progression [14–16].

In conclusion, DCs are one of the potent regulator cells in tumor development. The effects of DCs in cancer patients are contraversial: several reports demonstrated that myeloid-derived MDCs induce effective antitumor immune response and tumor regression. In spite of this, the suppressive tumor environment can alter the properties of DCs. The functional defects of DCs have an essentional role in cancer patient to impede succesful antitumor immune response. These tolerogenic DCs function as tumor-promoting cells. The future challenge of anticancer-based therapies is to overcome DC tolerogenecity and to reduce their negative effects in tumor progression.

2. DENDRITIC CELL-BASED CANCER THERAPY

It is clear that however in low number DCs are present in tumors and can be used to elicit antitumor immune response. The challenge and goal of anticancer therapy is to elicit an effective cellular immune response against tumor cells and evoke clinical response in treated patients with negligible side effects. The discovery of isolation techniques and methods for differentiating DCs in vitro gave us the possibility to generate DCs that could be loaded by tumor-specific antigens or peptides. In this therapeutic approach, one can define DC-vaccine as a DC loaded with tumor-specific antigen. The first DC-based clinical trial against B-cell lymphoma was reported by Hsu et al. [17]. One important question in DC vaccination is to decide whether to use an ex vivo or in vivo vaccination strategy. The ex vivoapproaches (see Figure 1) allow to monitor the quality of the cells during the differentiation procedure, analyze cell surface markers, the proper maturation state, cell viability, or subtype specificity of DCs by FACS analysis. It is also possible to evaluate the effective tumor antigen-specific T-cell response by ELISpot, mixed leukocyte reaction (MLR) before targeted DCs are introduced back to the patient. The possible sources of human DCs are CD34^{+} precursors, hematopoetic progenitors, and monocytes, isolated from blood by cytopheresis, adherent techniques or magnetic-based immunoselection, or immunodepletion [18–20]. IDCs can be differentiated from peripheral blood-derived mono-cytes in vitro in the presence of GM-CSF and IL-4 [18, 20]. Alternatively, DC precursors can be isolated from human peripheral blood, but for effective anticancer therapy one has to obtain high amount of targetable DCs. In a clinical trial, FLT-3 ligand-expanded DCs were prepared from the blood of colon and nonsmall cells of lung cancer patients. Because of the limited blood DC number, patients underwent FLT-3 treatment before DC isolation. As a result, three times more PBMC was obtained from these patients after standardized leukopheresis as compared to control patients. The isolated patient-derived DCs showed immature CD83^{−}/CD40^{low}/CD80^{low}/CD86^{low} phenotype, but after two days in culture, cells started to express CD83, elevated levels of CD86 and CCR7 proteins, which reflect MDC phenotype and migration capacity [21]. DC-vaccine studies utilize DCs loaded with peptide fragments or whole proteins providing an opportunity to present all potential peptide sequences of the antigen to recognize even more specific T-cell clones and tumor lysates exogenously [22]. Alternatively, one can target DCs endogenously with antigen-coded mRNA or cDNA [23]. After loading IDCs with tumor-specific antigens, it is very important to add adequate maturation agents (e.g., proinflammatory cytokines, LPS, CD40L) to ensure that DCs achieve their maximum migratory capacity to the lymph
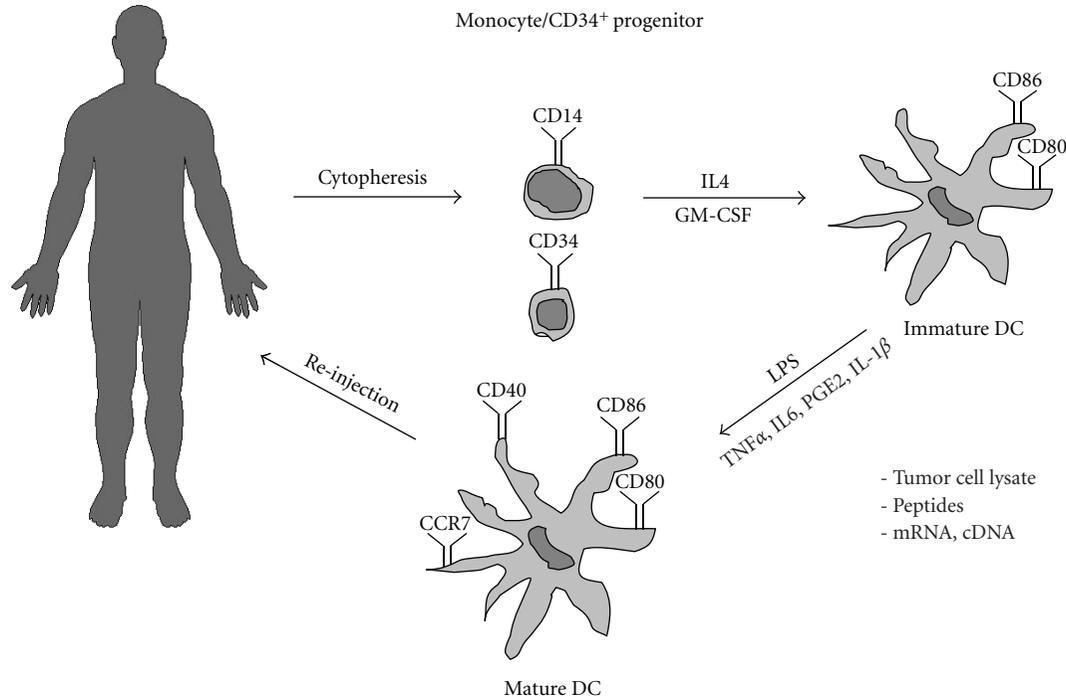
FIGURE 1: *General scheme of anticancer vaccination*. Dendritic cell progenitors (either CD34^{+} or CD14^{+} cells) are obtained using cytopheresis. Cells are differentiated using cytokines GM-CSF and IL-4. Immature dendritic cells are loaded with tumor lysate, peptides, or expression vector. DC maturation is induced and DCs are reinjected to patient.

nodes, otherwise only a small portion of antigen-loaded DCs could migrate to the site of the naive T-cell activation. Following quality control steps, the generated DC vaccines have to be reinjected into the patients. An important issue is also the DC injection site. DC vaccines could be reinjected to patients by intravenous, subcutaneous, intradermal, or intralymphatic injections. In a clinical study, the efficiency of different injection sites was compared by Fong et al. [24]. According to their results, the intradermal or intralymphatic administration was more effective compared to intravenous injection. In general, DC delivery via the skin is preferable to intravenous injection. Combining the different routes of reinjections may be beneficial, depending on the tumor localization. Most of the early phase I clinical trials, using the ex vivo approach, have not shown long-term tumor regression or improved survival. Probably it is mostly due to the fact that in these studies the researchers selected only advanced-stage cancer patients who were immonosuppressed by recurrent tumors or by chemotherapy. However, an in vitro approach could provoke Th1 cell response in metastatic malignant melanoma [25].

As far as the migratory capacity of DCs is concerned, less than 5% of the MDCs reach the lymph nodes after intradermic injection [26]. Therefore, it would be more beneficial to activate and target DCs within the host. In this case, we are not concerned with cell isolation or differentiation protocols, but rather DC-initiated tumor-specific immune responses have to be monitored inside the body. Monoclonal antibodies and fusion construct can be used for more productive tumor antigen delivery directly into the DCs

and probably the cells do not need to be cultured in vitro. Many vaccination studies target DC-specific c-type lectin receptors for efficient targeting of tumor antigens into the cells. These receptors bind to particular self- or nonself-sugar patterns by means of their carbohydrate recognition domain (CRD) and have roles in endocytotic antigen uptake [27]. One of them, DEC205/DC205, is expressed at high levels by MDC in mice, but human B cells, NK cells, monocytes, and macrophages also express this receptor [28]. Bozzacco et al. designed a fusion monoclonal antibody construct by taking the light and heavy chain coding cDNA sequence of an anti-DEC205 antibody and by inserting different gag p24 peptides at the carboxy terminus of the heavy chain. According to their results, these antibodies increased antigen presentation in the treated HIV-infected patients. They could further demonstrate DC-primed cross-presentation of internalized, nonreplicating proteins to MHCI complexes inducing CD8^{+} T-cell activity [29]. These results support the feasibility of engineering tumor-specific peptide fragment into DC-targeted antibodies against various types of cancer in vivo.

3. THE ROLE OF THE PPARγ RECEPTOR IN DENDRITIC CELLS

3.1. PPARγ-altered phenotype of DCs

Nuclear hormone receptors are ligand-activated transcription factors. There are three different PPAR isoformes in the human body and these show distinct tissue-specific distribution with different physiological functions. PPARα is

most highly expressed in the liver, skeletal muscle, kidney, and heart, and it regulates fatty acid oxidation [30]. PPARδ shows a ubiquiter distribution while PPARγ expression can be detected in various cell types like adipocytes, macrophages, and DCs. The receptor was initially described in mouse adipose tissue [31] and its role in myeloid development was shown by Nagy and Tontonoz in 1998 [32, 33]. PPARγknockout mice are lipodystrophic and die of placental defect, showing the essential regulatory role for the receptors in embryonic differentiation [34]. Moreover, high level of PPARγ expression can be detected in monocyte-derived macrophages in atheroscleric lesions [33]. PPAR receptors heterodimerize with retinoid X receptors (RXRs) in the nucleus and bind to certain receptor-specific response elements (PPREs) in the promoter or enhancer regions of their target genes [35]. The PPRE contains direct repeat sequences separated by one base pair (DR1). Endogenous or exogenous ligands bind into the ligand-binding domain (LBD) of PPARγ and modulate PPARγ-mediated gene expression. PPARγ can be activated by components of the oxidized low-density lipoprotein (oxLDL) and prostaglandin derivate (e.g.,15d-PGJ2) [32, 36]. Ligand activation of the receptor induces the expression of CD36 scavenger receptor which in turn leads to oxLDL uptake of macrophages and this metabolic process can lead to foam cell formation [32]. From these studies, we know that PPARγ regulates fatty acid uptake into the cell by induced cell surface receptors and it also promotes lipid storage and accumulation. Beside this fundamental regulatory role in metabolism, the receptor also functions as a key modulatory factor in macrophage immune function [37]. Earlier microarray data suggested that the PPARγ gene is upregulated during monocyte-to-DC differentiation [38]. According to our experiments and those of others, the receptor is immediately upregulated in cultured DCs, while it is barely detectable in monocytes [39]. We have shown that the transcription factor in this system is active, because synthetic agonists induce dose-dependent gene expression of the bone fide PPARγ target gene FABP4 in IDCs [39, 40]. Through global gene expression analysis, we found that PPARγ-activated genes involved primarily in the first 6 hours are involved primarily in lipid metabolism and transport (CD36, LXRα, and PGAR). Genes, coupled to the immune regulatory role of human DCs, were upregulated only for 24 and 120 hours after ligand treatment. It is possible that immunophenotype of DCs could be altered by PPARγ activation indirectly through activation of lipid metabolism and signaling pathways [41].

In terms of DC-based vaccination therapy, the most important question is how PPARγ activation might effect the DC-initiated immune responses and DC phenotype. PPARγ expression was first detected in murine DCs by Faveeuw et al. and they reported that there is a PPARγ receptor-dependent inhibition of IL-12 secretion of IDCs and MDCs [42]. Furthermore, it was also shown that PPARγ ligand activation caused anti-inflammatory cytokine production in macrophages [37]. These findings support the idea that PPARγ might have an essential role in the APC-based DC-vaccine therapies. The DC-secreted IL-12 is indispensable for Th1 cell promotion and CD8+ T-cell activation. Earlier

publications by Gosett et al. and Nencioni et al. assessed that PPARγ ligand activation alters the immunogenicity of human monocyte-derived DCs [43, 44]. During DC maturation, costimulatory molecules (CD40, CD80, and CD86) are upregulated on the surface of DCs [2]. Some bacterial products, such as LPS, are able to induce signals via TLR receptors or CD40 molecules induce IL-12 secretion of DCs. They have also found that upon ligand activation of PPARγ, the phenotype and cytokine expression patterns of the cells were changed [43]. PPARγ ligands altered iDC-specific surface markers involved in APC function. The CD83 activation marker expression in treated MDCs was uneffected, which means that PPARγ ligand-activated cells showed mature phenotype. After ligand activation, elevated CD86 protein level was detected on the surface of MDCs. They also showed that activation of PPARγ inhibits the secretion of IL-12p70 active form into the supernatant by MDCs while the levels of IL-6 and IL-10 were unchanged. Furthermore, chemokines involved in Th1 cell recruitment (IP-10, RANTES, and MIP1α), were also decreased after ligand treatment in the same study. Nencioni et al. later characterized the effects of PPARγ on DC maturation and found that ligand activation reduced the surface expression of CD1a molecule in a concentration-dependent manner, resulting in an unusual phenotype of differentiated IDCs [44]. Lower levels of IL-10, IL-6, and TNFα cytokines were measured upon ligand treatment. PPARγ agonist impaired the allogenic T-cell stimulating capacity in MLR assays and the secreted INFγ concentration was also reduced. T-cell activation capacity could not be restored by IL-12 administration suggesting that the impaired T-cell activation of MDCs was not only due to lack of IL-12 expression but also to other effects that modulate DC maturation process were involved.

In conclusion, the PPARγ ligand-activated cells not only impede the naive T cell to Th1 cell differentiation, but these cells also showed decreased antigen-specific T-cell response. Appel et al. reported that important anti-inflammatory effects of the receptor as ligand activation of the PPARγ receptors inhibited the LPS-activated MAP kinase and NF-κB proinflammatory signaling pathways, probably due to transrepression mechanism in DCs that subvert IL-12 expression [45].

Flow cytometry measurements performed by some of us largely supported the phenotypic results reviewed above [39]. Furthermore, when treated DCs with PPARγ, we detected that enhanced endocytosis in the form of enhanced latex bead uptake and ligand-treated cells were CD1a−. We could not detect any differences in case of HLA-ABC molecule expression, suggesting that the MHCI-mediated peptide antigen presentation capacity of the cell is probably not affected [46]. As reported by Angeli, PPARγ inhibits the expression of CCR7 on the surface of MDCs and this decreased the migration of DCs in mice. In this model, TNFα-induced epidermal LC motility from epidermis to dermal lymph nodes was reduced by PPARγ ligand treatment. They also found that ligand-activated PPARγ impaired the steady-state migration of DCs from the mucosal to the thoracic lymph nodes, but the maximal inhibitory

effect was detected at a considerably high concentration of the PPARγ agonist rosiglitazone (10 μM) that suggested receptor-independent effects [47].

Summarizing these data, PPARγ activation in DCs prevented IL-12 secretion, lowered CD80/CD86 ratio, and probably shifted naive T-cell differentiation toward Th2 cells. According to our own experiments, PPARγ agonist rosiglitazone at 2.5 μM concentration did not decrease the activation of allogeneic T cell and INFγ production [39]. So far, no one was able to detect Th2 response in MLR in response to PPARγ ligand activation.

3.2. The role of PPARγ in CD1d-mediated lipid antigen presentation

Szatmari et al. provided evidence that PPARγ activation could effect the lipid antigen presentation capacity of monocyte-derived DCs through upregulated expression of CD1d molecule on the surface of DCs [39]. This finding links PPARγ to invariant natural killer T (iNKT) cells. After isolation, monocytes fail to express CD1 group I molecules (CD1a, b, c) but the CD1a protein is upregulated during monocyte-to-IDC differentiation [20]. Inversely, the CD1 group II molecule CD1d is expressed at high levels on monocytes and downregulated on the surface of DCs [39]. Induced signaling pathways are able to regulate CD1d gene expression and lipid metabolism upon PPARγ ligand treatment [41]. Utilizing PPARγ agonist treatment, Gogolak et al. could induce the expression of CD1d molecules along with downregulation of CD1a at both mRNA and protein levels [48]. Later we established that PPARγ ligand activation enhanced indirectly the CD1d expression by turning on endogenous lipophilic ligand synthesis in the DCs through activation of the expression of retinol dehydrogenase 10 (RDH10) and retinaldehyde dehydrogenase type 2 (RALDH2) enzymes, which are involved in retinol and retinal metabolism and endogenous all-trans retinoic acid (ATRA) production from retinol [46]. The intracellularly synthesized ATRA induced CD1d and other retinoic acid receptor alpha (RARα) target genes in DCs. We looked at the functional role of PPARγ-induced retinoid-regulated CD1d expression on DC surface. DCs pulsed with synthetic alpha-galactosilceramide (αGalCer) ligand for 24 hours elevated iNKT cell expansion and INFγ secretion [39, 41]. As CD1d-mediated lipid antigen presentation is essential for iNKT cell activation, we could conclude that PPARγ-induced CD1d expression can be translated to the increased activation and proliferation of iNKT cells under these in vitro conditions [39]. Our results suggest that combination of PPARγ activator ligands along with αGalCer during the differentiation of DCs might be beneficial in iNKT-based adoptive transfer therapy (see Figure 2).

4. CD1d-RESTRICTED iNKT CELLS IN CANCER THERAPY

4.1. iNKT cell-based anticancer effects in animal models

Besides DCs and T cells, there are other important cell types contributing to antitumor immunity. iNKT cells are a unique T-lymphocyte population. These cells share both NK (CD161) and T-cell-specific markers (TCRs) on their surfaces. iNKT cells have restricted T-cell receptors (TCRs): in mice, the most frequently expressed α-chain rearrangement is Vα14-Jα18 while human NKT cells express Vα24-Jα18/Vβ11 TCRs (reviewed by Godfrey and Kronenberg [49]). For iNKT activation, it is essential to interact with cells displaying the evolutionarily conserved CD1d, nonclassical antigen-presenting molecules that present glicolipids in the context of hydrophobic antigen binding to these cells [50, 51]. αGalCer is the most frequently used lipid ligand for iNKT activation. It is derived from a marine sponge.

αGalCer has shown antimetastatic activity in various experimental tumor models (e.g., B16 melanoma, Lewis lung carcinoma, FBL-3 erytroleukemia, Colon26, and RMA-S 3LL tumor cells) in vivo [52–54]. This effect of the compound was tested in CD1d$^{-/-}$, Ja281$^{-/-}$ RAG$^{-/-}$ NKT mice, which have no iNKT cells and in NK-depleted wild-type mice. The results indicated that the antitumor effect of the glycolipid was abolished on all of the three tested genetic backgrounds in mice and the αGalCer-mediated antimetastatic function likely acts through iNKT cell activation and NK-like effector function [52]. Adoptive transfer experiments provided further proof for the key role of iNKT cell-secreted INFγ in the antimetastatic role of αGalCer in mice. Furthermore, activation and proliferation of NK cells downstream to iNKT activation, and subsequent INFγ production was also required to be essential for antimetastatic cytotoxic activity in vitro and in vivo [55, 56]. αGalCer activates iNKT cells, which produce INFγ, and secondary activates NK cells. These activated NK cells have been implicated also in the regulation of angiogenesis during tumor development. The αGalCer treatment inhibits the subcutaneous tumor growth, tumor-induced angiogenesis, and epithelial cell proliferation, which are required for tumor vessel formation [57]. Later it has been established that αGalCer treatment, in combination with IL-21, prolonged and elevated the NK cell cytotoxicity, maturing NK cells into perforine-expressing cells by IL-21. Moreover, this combination inhibited spontaneous tumor metastases. Presentation of αGalCer by DC to iNKT cells in contrast to soluble compound injection was even more effective in the suppression of metastasis formation [58]. Similar successful antitumor effects of αGalCer-pulsed DC have been published by Toura et al. using B16 melanoma liver metastasis and lung metastasis of LLC model in vivo. Beside the inhibition of metastatic nodule formation in these tissues, αGalCer-DC administration also has a significant beneficial effect in the regression of established nodules [59, 60].

4.2. iNKT cells in human cancer therapy

Human Vα24$^+$ iNKT cells also mediate αGalCer-dependent antitumor activity by perforin-dependent cytotoxic lysis against Daudi lymphoma and other various cell lines [53, 61]. Others demonstrated effective direct iNKT-mediated cytotoxicity only against CD1d$^+$ cell lines such as U937, while CD1d$^-$ cell lines were killed only after CD1d transfection
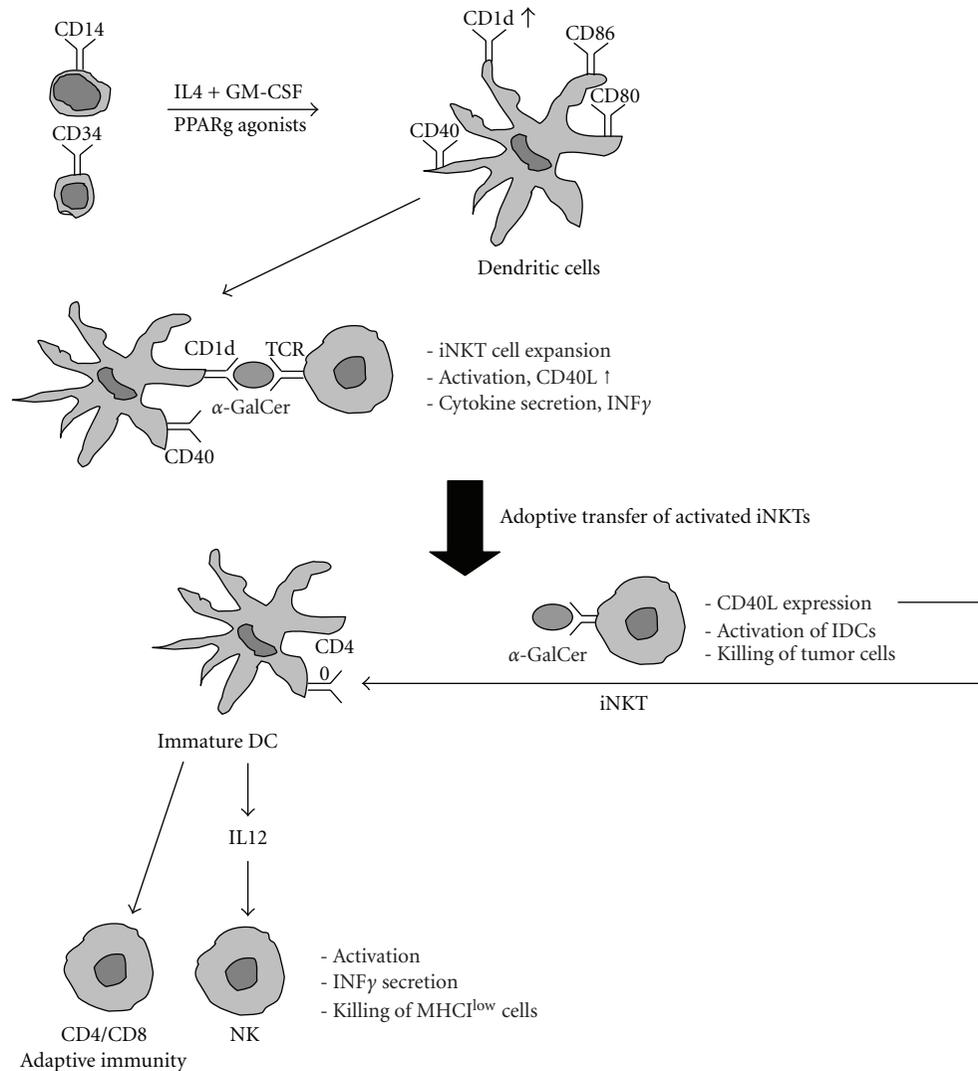
Figure 2: *The molecular basis for the potential use of PPARγ-programed dendritic cells during tumor vaccination.* DC progenitors are differentiated in the presence of PPARγ agonists. A PPARγ-programed DC showed increased CD1d expression. In the presence of αGalCer, the treated DC is capable of inducing iNKT cell expansion. The adoptively transfered iNKTs can induce activation of iDCs and IL-12 secretion in cancer patients. This can lead to improved ability to kill tumor cells.

into the cells. NKT cells provoked NK cell-induced cytotoxicity by IL-2 and INFγ secretion [62].

The crosstalk between innate and adaptive immunity was established in several anticancer studies. This linkage could be mediated via reciprocal interaction between iNKT cells and iDCs. Upon αGalCer activation, iNKT cells express CD40L [63]. The CD40 ligand binds to the CD40 molecules of DCs and triggers IL-12 expression and secretion by the DCs. The produced IL-12 generates a positive feedback and induces INFγ secretion by the iNKTs [63, 64]. The secondary activation of DCs leads to NK, CD4+, and CD8+ T-cell activation by standard activation of memory T cells and adaptive immune response against peptides presented by DCs [65–67].

The fact that αGalCer-loaded DC could trigger iNKT expansion and mediate antitumor immune response in sev-

eral in vitro experiments and in vivo antimetastatic models in mice supported the notion that using αGalCer-pulsed DCs for iNKT activation in cancer patients in situ might induce an effective anticancer therapy. Other alternative approach could be the adoptive transfer of in vitro expanded and activated iNKT cells to patients.

The number of NKT cells in cancer patients is significantly lower compared to healthy volunteers. Giaccone et al. showed the disappearance of iNKT cells after 24 hours of αGalCer administration from the peripheral blood of patients and only transient iNKT activation was registered in some individuals [68]. Others found quantitative defects in iNKT cell-derived INFγ production among patients with advanced prostate cancer [69]. Showing that NK cells were able to respond to IL-12, those cells could secrete increased levels of INFγ demonstrating the selective loss of

INFγ-secreting capacity of iNKT cells in patients [69]. As it was expected from mouse experiments, Chang et al. were able to expand the number of iNKT cells for more than a month in all treated patients, proving that αGalCer-pulsed MDCs could be more effective than using αGalCer-pulsed IDC or the soluble compound [70]. However, the levels of IL-12p40 and IL-10 in the serum were elevated after the treatment and iNKT cells showed reduced-INFγ secretion. The future challenge of this type of tumor therapy is to induce extended iNKT number and activity. One possible approach is to use additional pharmacological ligands upon cancer therapies. It has been shown that the thalidomide analogue, lenalidomide (LEN), enhanced the predominant iNKT cell expansion in vitro and in vivoin response to αGalCer-loaded DC. LEN elicits higher level of INFγ secretion in response to αGalCer-loaded DC, suggesting that LEN might be restoring the INFγ-producing activity of iNKT cells in cancer patients [71]. Alternative possibility is the adoptive transfer method: in phase I clinical trial, adoptive transferred iNKTs were used in patients with malignancy to increase the number of iNKT cells [72]. They expanded iNKT cells in vitro in the presence of IL-2 and αGalCer. The activated iNKT cells showed cytotoxic activity against PC-13 and Daudi human cancer cell lines. Reinjection of activated iNKT cell into the patients enhanced the level of INFγ secreting iNKT cells in the peripheral blood from day one up to two weeks [72].

5. DENDRITIC CELL PPARγ IN ANTICANCER THERAPY

Despite the enormous research effort, cancer is still a significant clinical problem as well as basic science problem. However, immunotherapy opened up some possibility in the fight against cancer. General APC features of DCs are capturing antigens in the periphery, processing, and enhancing MHC-peptide complex presentation capacity to naive T cells. These phenomena highlighted possible roles for DCs in anticancer therapy. However, DC-based vaccination often does not elicit clinical responses and fails to ensure long-time tumor regression in patients with malignant tumors. One reason for this failure could be the immunosuppressed state of the patient, for example, due to chemotherapy in the therapeutical history. Recently, we have identified a new target gene, ABCG2, which is transcriptionally regulated by PPARγ in DCs. ABCG2 transporters modify the drug resistance against anticancer agent of PPARγ agonist-treated DCs. PPARγ has a protective function in these cells, and using PPARγ-specific ligands during in vitro differentiation could revert the xenobiotics-induced toxicity in DCs [73].

Due to the fact that we did not find reduced capacity of DCs to activate T cell in MLR assays, we concluded that ligand activation does not suppress DC-mediated peptide antigen presentation. For effective anticancer therapy, one should provoke adaptive immune response against tumor-specific peptides presented by DCs. In mouse experiments, simultaneously added αGalCer and peptide-loaded DCs induced CD4/CD8 T-cell-specific anticancer immune response mediated by iNKT cell. In case of human patients, αGalCer-loaded DCs could not induce adaptive immunity, partly because of the ineffective INFγ secretion by iNKT cells.

Pharmacological approaches like LEN may solve this problem. The ability of PPARγ to upregulate CD1d expression on DCs raises the possibility to use receptor agonists in iNKT-based adoptive transfer treatments.

Many features of DCs, which are critical during DC-vaccination design, are affected by PPARγ. Reduced migratory capacity, inhibited IL-12 cytokine production, inadequate Th1 and CD8$^+$ T-cell response, and presumed generation of IL-10-producing tolerogenic DC could influence the outcome of DC-based vaccination therapies against cancer. Based mainly on these in vitro results, activation of the PPARγ receptor in tumor peptide-pulsed DCs could be less beneficial in terms of in vivo vaccination strategies. At the same time, increased phagocytic capacity, increased CD1d expression, and iNKT activation potential are useful features of PPARγ-programed DCs. In spite of the vast amount of in vitro obtained results on the potential role of PPARγ in DCs, the most controversial issue remains open: whether synthetic PPARγagonists have significant modifying effects on antitumor immune response in vivo or not. Further in vivo studies are needed to clarify the receptor-specific immunomodulatory effects of PPARγ ligands (agonists or antagonists) in cancer patients. For that, the use of siRNA-based gene-silencing techniques or DC-specific PPARγ, KO animal models would probably be useful.

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Review Article

Pathophysiological Roles of PPARγ in Gastrointestinal Epithelial Cells

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Although the highest levels of PPARγ expression in the body have been reported in the gastrointestinal epithelium, little is known about the physiological functions of that receptor in the gut. Moreover, there is considerable controversy concerning the effects of thiazolidinedione PPARγ agonists on the two major diseases of the gastrointestinal track: colorectal cancer and inflammatory bowel disease. We will undertake to review both historical and recently published data with a view toward summarizing what is presently known about the roles of PPARγ in both physiological and pathological processes in the gastrointestinal epithelium.

1. INTRODUCTION

Peroxisome proliferator-activated receptor-gamma (PPARγ, NR1C3) has been described as a master regulator of adipocytes differentiation [1]; and since the discovery that the insulin-sensitizing thiazolidinedione drugs are PPARγ agonists [2], the role of this receptor in adipogenesis has been studied in detail. PPARγ is also abundant in the gastrointestinal tract, where it is highly expressed in epithelial cells [3, 4]. High-level expression of PPARγ in the gut epithelium suggests some important physiological role in the gut, although this role is not well understood. From the standpoint of pathology, PPARγ has been implicated in both transformation and inflammation in the gut. However, there are conflicting data concerning the efficacy and even the safety of PPARγ agonists in clinical management of gastrointestinal cancer and inflammation. We will undertake in this review to summarize both historical and recent data that relate to three questions: What is the physiological role of PPARγ in gastrointestinal epithelial cells? Is PPARγ a colon cancer suppressor? And what is the role of PPARγ in inflammation?

2. THE PHYSIOLOGICAL ROLE OF PPARγ IN GASTROINTESTINAL EPITHELIAL CELLS

PPARγ is expressed in epithelial cells of both the large and small intestines [3, 4]. Relatively lower levels of expression are observed in the small intestine, which appears to express PPARγ at more or less uniform abundance from the duodenum to the caecum [5]. Very high levels of PPARγ are expressed in the proximal colon, with somewhat lower levels observed in the mid and distal colon [5, 6]. The highest levels of PPARγ in the body are observed in highly differentiated lumenal epithelial cells of the proximal colon. It has also been observed that PPARγ is induced when Caco2 cells undergo differentiation in culture [6], leading to the suggestion that induction of PPARγ is associated with differentiation of colonic epithelial cells. However, a careful analysis of PPARγ expression in the colon suggests that this conclusion is correct only within a limited context [5]. Basal epithelial cells of proximal crypts are highly differentiated, yet express significantly lower levels of PPARγ than lumenal epithelial cells from the same crypts. Furthermore, PPARγ expression in the distal colon is more or less uniform throughout the crypts, with slightly higher levels of expression observed in the less differentiated basal crypt cells, rather than the more highly differentiated lumenal epithelial cells [5, 7]. Thus, it is not generally the case that induction of PPARγ is associated with differentiation of colonic epithelial cells. It is, however, the case that all colonic epithelial cells express significant levels of PPARγ. This is an important point, since some of the effects of PPARγ are manifest in the transit amplifying cells that support renewal of the colonic epithelium. Whether

PPARγ is expressed in colonic stem cells remains an open question.

A significantly different pattern of PPARγ expression is observed in the epithelium of the small intestine. Transit amplifying cells within the crypts of Lieberkühn express little or no PPARγ [8, 9]. Instead, this receptor is induced at the crypt/villus junction, where small intestinal epithelial cells undergo differentiation into mature villus epithelial cells. Thus, in the small intestine it is unambiguously the case that induction of PPARγ is associated with differentiated function, and it has been reported that PPARγ collaborates with Hic5 to promote differentiation of embryonic small intestinal epithelial cells [10].

Efforts to understand the role of PPARγ in differentiated function of gut epithelial cells have been hindered by the lack of good cellular models to study the mechanisms of action of PPARγ in culture. There are no nontransformed colonic epithelial cell lines, and primary colonic epithelial cells have proved to be difficult, if not impossible, to maintain in culture for even a few hours. There are several nontransformed epithelial cell lines derived from the rat embryonic small bowel (e.g., RIE1 and IEC6), but these cells are derived from proliferative crypt cells and, like the transit amplifying cells from which they were derived, these cells express no PPARγ. It has been possible to engineer RIE1 cells to express PPARγ, thereby modeling the transition that occurs at the crypt/villus junction [8, 11]. Activation of PPARγ in such cells results in irreversible withdrawal form the cell cycle, promotes motility, and reduces cellular adhesion.

Genomic profiling indicates that PPARγ targets in such cells fall into four major functional cohorts [8]. The largest of these cohorts consists of genes that are involved in metabolism, and a large proportion of lipid transport and metabolism genes are evidenced within this group. This observation is consistent with our understanding of the metabolic role of PPARγ in other tissues [12–16]. A second cohort of PPARγ target genes was ontologically linked to signal transduction. The data suggest that there is extensive crosstalk between PPARγ and other signaling pathways within intestinal epithelial cells. A third cohort of genes was linked to proliferation, consistent with the observation that activation of PPARγ within these cells results in inhibition of culture growth and irreversible withdrawal from the cell cycle. Somewhat surprisingly, the forth functional cohort of PPARγ target genes was ontologically linked to cellular motility and adhesion. Such processes have not been generally thought of as linked to activation of nuclear receptors. Nevertheless, activation of PPARγ in intestinal epithelial cells potently induces cellular motility, through a mechanism that involves Rho family GTPases and MAPK activation [11]. Renewal of the intestinal epithelium is tightly coupled to migration of differentiated epithelial cells from the crypts to the villus tips, and the observation that PPARγ regulates intestinal epithelial cell motility provides a very important clue into the potential physiological role of this receptor in the gastrointestinal epithelium.

Genomic analysis of PPARγ targets in colonic epithelial cells isolated from thiazolidinedione-treated mice indicates that, in general, similar processes are regulated in epithelial cells from the colon and small intestine [5]. Major ontological cohorts were identified that link PPARγ activation to metabolism, signal transduction, and migration/motility. In contrast to the results obtained with intestinal epithelial cells in culture, no proliferative cohort of PPARγ target genes was identified in colonic epithelial cells isolated from thiazolidinedione-treated mice. This result was unanticipated since such drugs significantly inhibit BrdU incorporation into both proximal and distal colonic epithelial cells. Failure to detect a cohort of proliferation-related PPARγ target genes in vivo is probably attributable to the fact that only a small subpopulation of cells is involved in proliferation in the colonic epithelium, such that the contribution of RNA from such cells is diluted by the much larger postmitotic population. Genomic studies of this sort are, therefore, useful for analysis of PPARγ effect on the differentiated, postmitotic epithelial cells; but such studies are unlikely to reveal much information about the effects of PPARγ on proliferative colonic epithelial cells, which are presumably the targets for transformation.

PPARγ expression and distribution are very different in the proximal and distal colonic epithelium, implying that this receptor may have different functions in the proximal and distal colon. Genomic analysis indicates that the majority of PPARγ target genes are expressed in both proximal and distal epithelial cells [5], suggesting that there is substantial overlap between the physiological functions of PPARγ in these tissues. However, a subset of PPARγ target genes is restricted to the proximal colon, and a second subset is expressed predominantly in the distal colon. Intriguingly, the proximal PPARγ target genes are all induced by thiazolidinediones, whereas the distal target genes are all repressed. The significance of this observation is unknown at this time. However, the observation that PPARγ represses genes that are differentially expressed in the distal colon is consistent with the hypothesis that PPARγ may suppress differentiated function in that tissue. This hypothesis tends to contradict a large number of observations to the effect that PPARγ promotes differentiated function, and additional studies are needed to confirm this unanticipated observation.

In summary, the lack of appropriate cellular models to study the mechanism of action of PPARγ in nontransformed gut epithelial cells has significantly impaired our ability to understand the role of this receptor in gut physiology. Nevertheless, genomic analyses of genetically engineered intestinal epithelial cells and colonic epithelial cells isolated from thiazolidinedione-treated mice have provided important clues. The data suggest that PPARγ is a potent metabolic regulator in gut epithelial cells. This suggestion is obviously consistent with what we know about PPARγ in mesenchymal cells. There is a strong suggestion that PPARγ is involved in extensive crosstalk with other signal transduction pathways, suggesting that this receptor plays an important role in integrating the physiological response to a wide variety of extracellular signals in vivo. Finally, both genomic and cellular data indicate that PPARγ plays a very important role in regulating cellular motility, which is one of the major differentiated functions of intestinal epithelial

cells. The challenge at this time is to put the information we have into a physiological context and to use these data to understand the role of PPARγ in malignant transformation of gastrointestinal epithelial cells.

3. PPARγ AS A COLON CANCER SUPPRESSOR

The evidence in support of PPARγ as a colon cancer suppressor is based upon a relatively small number of observations, and not all of these observations are consistent with each other. The most compelling data come from studies using the azoxymethane (AOM)-treated rodent model, which has been widely studied as a model of sporadic colon carcinogenesis. Two initial studies in AOM-treated rats used troglitazone, a relatively weak PPARγ agonist [17, 18]. The endpoint in these experiments was aberrant crypt foci (ACF), rather than colon tumors. Nevertheless, both of these studies indicated that troglitazone potently inhibits ACF formation, thereby presumably reducing the risk of subsequent tumor formation in rats. It was subsequently shown that troglitazone, pioglitazone, and rosiglitazone inhibit ACF formation in AOM-treated Balb/c mice [19]. This was the first study that demonstrated that thiazolidinediones inhibit colon tumor formation, in addition to ACF formation, in the AOM model of colon carcinogenesis. This observation was consistent with the report that whole animal hemizygous knockout of PPARγ suppressed AOM-mediated colon tumor formation in mice [7]. It has subsequently been reported that RS5444, a very high-affinity third generation thiazolidinedione, inhibits ACF formation and blocks tumor formation in AOM-treated C57BL/6 mice [5, 20]. Overall, these data unambiguously indicate that PPARγ inhibits some very early step in transformation of colonic epithelial cells in AOM-treated rodents.

In contrast to the data cited above, two reports have concluded that thiazolidinediones induce caecal tumors in mice [21, 22]. These reports have not been independently confirmed, and other investigators have not observed such an effect. However, this may reflect the fact that caecal tumors were observed only in mice that had received very high concentrations of thiazolidinediones for very long periods of time [21, 22]. The pathological significance of these observations is unclear at this time. Caecal tumors are very rare in both mice and humans, and the concentrations of thiazolidinediones that were used in these experiments were very likely far beyond any dose that would be tolerated in humans, in which peripheral edema is the dose limiting response to such drugs. Nevertheless, the potential significance of these disturbing observations warrants additional consideration.

The major controversy in the PPARγ field has revolved around two high-profile papers that reported increased colon tumor formation in APC$^{+/Min}$ mice treated with thiazolidinediones [23, 24]. One of these papers reported a significant increase in colon tumor size in APC$^{+/Min}$ mice treated with either troglitazone or rosiglitazone. The companion paper reported a slight, but significant, increase in the number of colon tumors in APC$^{+/Min}$ mice treated with troglitazone. No increase in tumor size was observed in the second report,

which was, therefore, not entirely consistent with the first. Moreover, two subsequent reports failed to reproduce the effect of thiazolidinediones in APC$^{+/\Delta1309}$ or APC$^{+/Min}$ mice [25, 26]. It was also reported that whole animal hemizygous knockout of PPARγ had no affect on tumor number or size in APC$^{+/Min}$ mice [7], an observation that is at variance with the notion that PPARγ promotes tumor formation in mice that contain activating germ line mutations in the Wnt/APC/β-catenin pathway. It has recently been reported that biallelic knockout of PPARγ in colonic epithelial cells promotes tumor formation in APC$^{+/Min}$ mice [27], indicating that PPARγ is, in fact, acting to suppress tumor formation in the Min mouse. On the whole, the evidence no longer supports the hypothesis that activation of PPARγ promotes tumor formation in mice with germ line APC mutations.

A recent report describes the effects of PPARγ agonists in pre-established tumors in AOM-treated mice [20]. Such tumors invariably contain somatic mutations that activate the Wnt/APC/β-catenin signaling pathway [28]. Thiazolidinediones had no effect on growth or incidence of colon tumors when the drug was given after tumor formation had occurred [20]. However, activation of PPARγ under these circumstances had a profound inhibitory effect on tumor progression. This effect was most strikingly apparent in the development of carcinoma in situ, which was detected in about 1/3 of the control tumors but was never observed in thiazolidinedione-treated tumors. Since formation of carcinoma in situ involves invasion of the surrounding stoma, it is tempting to speculate that this observation indicates that PPARγ inhibits invasion in vivo, consistent with several reports that indicate that invasion by human colon cancer cell lines in culture is inhibited by PPARγ [9, 29]. Notably, activation of PPARγ in pre-established tumors had no significant effect on BrdU incorporation, consistent with the lack of any significant effect on tumor size. This observation is at variance with several reports that thiazolidinediones inhibit proliferation of human colon cancer cell lines in culture and in xenografts [9, 29–35]. The significance of this discrepancy requires additional investigation, but the data are consistent with the hypothesis that the suppressive effects of PPARγ on established tumors may be due to inhibition of tumor progression, rather than inhibition of tumor growth.

On balance, the data seem unambiguously clear in one respect: PPARγ suppresses colon carcinogenesis in mice. The primary effect appears to be inhibition of some early stage in transformation. The ability of PPARγ to block early stage transformation is presumably due to some effect on normal colonic epithelial cells, which emphasizes the importance of understanding the functions of this receptor in the normal colonic epithelium. It is also clear that whereas PPARγ inhibits proliferation of normal intestinal epithelial cells, this response is attenuated early in transformation. Although the antiproliferative effects of PPARγ appear to be lost in cells that have either germ line or somatic mutations in β-catenin signaling, the data indicate that this receptor still retains the ability to inhibit tumor progression, at least in AOM-induced tumors. Finally, we submit that there is very little solid evidence that PPARγ promotes colon carcinogenesis under any pharmacologically relevant conditions.

The role of PPARγ as a suppressor of colon carcinogenesis in rodents is beyond question, but the evidence that PPARγ is a colon cancer suppressor in humans is not so compelling. Although an early report indicated that loss-of-function mutations in PPARγ were common in colon cancer [36], this claim has not subsequently been confirmed [37]. It has been reported that a polymorphism in codon 12 of PPARγ is associated with colon cancer risk [38]. However, this polymorphism is manifest in PPARγ2, which is expressed at relatively low levels in the colonic epithelium [3, 4], and not in the major colonic PPARγ isoform, PPARγ1 which differs in N-terminal sequence from PPARγ2. PPARγ expression is reduced in ulcerative colitis [39] and acromegaly [40], two conditions that predispose to colon cancer; and one might extrapolate from data with hemizygous knockout mice [41] to postulate that a reduction in PPARγ expression increases the likelihood of transformation in the human colon. However, the evidence in support of such a conclusion is not very strong. Finally, a small phase II trial in which troglitazone was used to treat patients with late stage metastatic colon cancer produced no objective response [42]. One might argue that lack of response in this case was due to the rather low potency of the agonist or the very advanced stage of cancer in these patients. Alternatively, one might point to the data in mice, which indicate that PPARγ has little or no effect on growth of established colon tumors in AOM-treated mice [20].

The best evidence for a tumor suppressive role of PPARγ in humans comes from studies of established human colon cancer cell lines [9, 29–31, 33–35, 43]. Some of these cells exhibit growth arrest in culture when treated with thiazolidinediones, and growth of colon cancer xenografts has also been observed in thiazolidinedione-treated mice. However, many (in our experience most) human colon cancer cell lines are resistant to growth inhibition by concentrations of thiazolidinediones that are sufficient to maximally activate PPARγ. Such observations raise two important questions: why are some colon cancer cell lines resistant to PPARγ agonists? And to what extent are the effects of thiazolidinediones dependent upon PPARγ expression and/or activity? Both of these questions are significant in terms of the use of thiazolidinediones as chemotherapeutic agents in colon cancer.

The most compelling case for pharmacological application of PPARγ agonists in colon cancer is as a preventive agent. Clearly, PPARγ is preventive in mouse models of sporadic colon cancer. However, the legal climate in USA at this time does not favor the development of chemopreventive drugs, particularly those that may have cardiovascular side effects [44]. These considerations do not preclude a prophylactic use for PPARγ agonists. Patients with large numbers of ACF are at increased risk for development of subsequent colon cancers [45–47], and it is plausible that short-term treatment with PPARγ agonists might result in a decrease in ACFs and a subsequent reduction in colon cancer risk. From a clinical standpoint, it is therefore important to ascertain if activation of PPARγ in humans suppresses ACF formation, as has been observed in AOM-treated mice.

Finally, it is the case that several million individuals are taking thiazolidinediones for management of type II diabetes. It would seem that an epidemiological analysis of these individuals might, once and for all, determine if PPARγ agonists have chemopreventive efficacy. Our initial attempts to collect data on time to formation of a second polyp in patients who are taking thiazolidinediones indicates that there are great many confounding variables, which limit the power of such an analysis and may account for the fact that few comprehensive anaylses of the effects of PPARγ agonists on colon cancer incidence have been reported [48].

In summary, we conclude that both genetic and pharmacological data indicate that PPARγ suppresses some early stage in transformation of colonic epithelial cells. We submit that the balance of evidence is inconsistent with the hypothesis that PPARγ promotes colon carcinogenesis under any circumstances. Although it is unlikely that PPARγ agonists will ever be developed as chemopreventive agents, it is plausible that we may, by studying the mechanism of action of PPARγ, elucidate downstream effectors that may be chemopreventive targets. Furthermore, it is possible that PPARγ agonists may have clinical applicability in prophylactic management of individuals who are at increased risk of colon cancer due to large numbers of ACFs.

4. PPARγ AND INFLAMMATORY BOWEL DISEASE

PPARγ has become a potential pharmacological target for treatment of inflammatory diseases of the colon, particularly ulcerative colitis (UC). PPARγ is highly expressed in both colonic epithelial cells and macrophages critical for innate immunity and gut homeostasis. Over the past decade, in vitroand in vivo studies have defined an anti-inflammatory role for macrophage and epithelial PPARγ in regulating colonic inflammation. Studies utilizing in vitro cell culture models have established that thiazolidinedione PPARγ agonists can reduce NFκB activation and inflammatory gene expression in colonic epithelial cells [49, 50], macrophages [51–54], dendritic cells [55–58], and T-cells [59, 60]. However, the magnitude by which thiazolidinediones reduce inflammatory gene expression and act directly through PPARγ has been confusing. The anti-inflammatory effects of thiazolidinediones vary among studies due to differences in cell models, the concentration, duration, and type of thiazolidinedione (rosiglitazone, pioglitazone) used, as well as the context of inflammation studied (i.e., inducing agent-LPS, TNF-α, etc. [61]). More importantly, thiazolidinediones can reduce inflammation in both a PPARγ dependent and independent manner, with the latter resulting from the use of high concentrations [62, 63]. Despite confusion, it has been generally accepted that thiazolidinediones can reduce inflammatory gene expression via PPARγ in epithelial and immune cells when used at appropriate concentrations.

Perhaps the strongest evidence for an anti-inflammatory role of PPARγ comes from landmark studies indicating that heterozygous PPARγ deficient mice were more susceptible to DSS- and TNBS-induced colitis [64, 65]. DSS-induced colitis, in particular, is an acute inflammation model primarily driven by epithelial disruption and macrophage infiltration. This data indicates that PPARγ expression in certain cell types of the colon plays an anti-inflammatory

role. Recent studies elaborated on these findings by showing that mice deficient in PPARγ expression in epithelial cells and macrophages displayed increased proinflammatory gene expression and susceptibility to DSS colitis [66, 67]. These findings suggest that PPARγ expression in at least two cell types, epithelial and macrophage, can protect against at least one model of acute colitis (DSS). Questions remain regarding the importance of macrophage and epithelial PPARγ in other models of acute and chronic colitis. For example, what is the role of macrophage or epithelial PPARγ in a chronic colitis model driven by T-cells? Likewise, little is known about the role of dendritic and T-cell specific expression of PPARγ in colitis, as PPARγ knockout animals currently do not exist for these cell types. Experimental models of colitis can be initiated by distinct mechanisms and driven by infiltration of different cell types, both epithelial and immune [68–70]. Given that no single model accurately mimics human colitis, much remains to be understood regarding the tissue specific importance of PPARγ in controlling gut inflammation. Furthermore, the importance of tissue specific PPARγ expression may depend largely on the model of colitis examined, emphasizing the need to utilize multiple models to accurately represent manifestations of human colitis. Collectively, knockout animals have confirmed a potential protective role of PPARγ in colitis but additional research is still required to understand fully how tissue specific PPARγ expression influences different manifestations of colonic inflammation.

Based on the evidence that (a) thiazolidinediones can suppress inflammatory gene expression in vitro and (b) PPARγ expression protects against the development of colitis in several animal models, it seems logical that PPARγ might be a good target for treatment of gut inflammation. In reality, the preventative and therapeutic efficacy of targeting PPARγ with thiazolidinediones for treatment of colitis is debatable. It remains unclear what phase, if any, would be best for targeting PPARγ with thiazolidinediones for treatment of colitis: during the initiation, low grade, moderate, high grade, or remission phases of colitis. Our knowledge is largely based on animal studies in which acute preventive doses of thiazolidinediones were administered before the initiation of colitis. When given acutely (0–3 days) before inducing stimuli (i.e., DSS or TNBS) [50, 64–67, 71–73] or in the early life stages of animals that develop spontaneous cancer (IL-10$^{-/-}$ mice) [74], thiazolidinediones provide beneficial effects in the amelioration of inflammation. These data indicate that at least one mode of PPARγ action is to suppress the initiation of colitis, and suggest that thiazolidinediones may be a useful chemopreventative agent for the treatment of colitis. However, given their potential cardiovascular side effects, it is unlikely that thiazolidinediones would be approved as a chronic preventive agent for the management of gut inflammation. Moreover, the effectiveness of long term-preventative thiazolidinedione treatment on suppressing gut inflammation remains to be fully established. A recent report indicates that long-term treatment of mice with rosiglitazone exacerbated DSS-induced colitis [75], raising concerns about the preventative use of thiazolidinediones in gut inflammation.

The alternative scenario is that thiazolidinediones alone or in combination with anti-inflammatory/immunosuppressive drugs may be used to therapeutically target active inflammation. However, data from human and animal studies regarding the usefulness of therapeutic doses of thiazolidinediones in the treatment of colitis are inconsistent. When given after the initiation of DSS colitis, several studies found that thiazolidinediones had little or no value in improving colitis symptoms [50, 65, 73]. Likewise, therapeutic doses of thiazolidinediones given after established inflammation in the IL-10$^{-/-}$ model of colitis provided no value [74]. Similar results were observed in a small open end clinical trial in which patients with moderate colitis receiving rosiglitazone experienced only modest improvement [76], however interpretation of these results are difficult as the trial lacked a proper control group. In contrast, a few studies have reported that therapeutic doses of rosiglitazone improved colitis symptoms in DSS and TNBS colitis [64, 77, 78]. Recently, a multicenter, randomized, double blind placebo-controlled trial for treatment of mild to moderately active UC with rosiglitazone showed clinical response in 44% of patients [79]. However, endoscopic remission rates were not significantly different. The discrepancies in thiazolidinedione effectiveness may reside with the doses of thiazolidinediones administered, with the magnitude of inflammation at the time of thiazolidinedione administration, or differences in scoring. Alternatively, the anti-inflammatory actions of thiazolidinediones may be inhibited or not strong enough to suppress inflammation during active colitis. Most likely, however, the lack of therapeutic efficacy of thiazolidinediones during active colitis can be explained by a loss of PPARγ expression and/or activity that coincides with the level of inflammation. Indeed, PPARγ levels have been shown to be downregulated in epithelial cells [39] and macrophages [73] during colitis. Moreover, Katayama et al. showed that the lack of therapeutic responsiveness to thiazolidinediones during colitis could be restored by adenoviral-mediated reexpression of PPARγ in the colon [73]. In addition, Necela et al. showed that NFκB drives down PPARγ expression in response to lipopolysaccharide, thereby obviating the actions of PPARγ and promoting an inflammatory state in macrophages [80].

In summary, although the current data supports a role for PPARγ expression and activation in epithelial and immune cell types in the control of colonic inflammation, it remains unclear whether targeting PPARγ with thiazolidinediones will be an effective strategy for treating gut inflammation. While animal models suggest a favorable role of thiazolidinediones in chemoprevention, the efficacy and safety of long-term use of thiazolidinediones as preventative agents or maintenance therapy in UC patients remains to be assessed. Likewise, the use of thiazolidinediones as therapeutic agents for treatment of colitis is controversial. It remains unclear during what clinical phase of inflammation thiazolidinediones would be most effective for treating UC patients or which patients would be most likely to benefit from treatment. Moreover, there is considerable concern regarding whether the adverse effects of thiazolidinediones would outweigh the potential benefit for patients with UC.

In general, the majority of studies are in agreement that thiazolidinediones may be better exploited for therapeutic treatment of mild-moderate active colitis rather than during severe inflammation. Our understanding of the preventative and therapeutic potential of targeting PPARγ with thiazolidinediones is largely limited by several factors, including the lack of (a) experimental usage of distinct models of colitis, (b) understanding of tissue specific roles of PPARγ in different models of colonic inflammation, (c) understanding of factors affecting thiazolidinedione efficacy (PPARγ levels, etc.), (d) understanding of the mechanism of the anti-inflammatory actions of PPARγ, and (e) understanding of the adverse effects of short- and long-term use of thiazolidinediones during inflammation. Additional animal and clinical studies should resolve these discrepancies and provide further insight into the appropriate use, preventative or therapeutic, of thiazolidinediones for treatment of gut inflammation.

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Review Article

The Role of PPARγ in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide. This cancer develops mainly in cirrhotic patients. The cirrhotic liver is considered to be a preneoplastic organ, suggesting the rationale for cancer prevention. PPARγ is a nuclear transcription factor whose activation leads to interaction in the metabolism of lipids, insulin sensitization of peripheral cells, anti-inflammatory action. It can also induce differentiation and inhibits proliferation of cancer cells. Until now, data using PPARγ ligands in HCC have demonstrated mainly in in vitro models that its activation could be due to an antiproliferative effect. PPARγ ligand administration has also been associated with a diminution of liver fibrosis in animal models, and potentially also on tumoral cell death. Soma data show that the favorable effect of natural and synthetized PPARγ agonists could also be independent of PPARγ activation. Furthermore, in some situations, PPARγ antagonists have also an anticancer effect. Therefore, we can conclude that the link between activation of the PPARγ pathway and an anticancer activity is suggested but until now not firmly established in HCC.

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Its prognosis is poor, with up to 40% of patients diagnosed at an advanced stage, when only palliative treatments can be offered. Most patients that develop HCC have cirrhosis, considered as a preneoplastic organ. Strategies to prevent the development of HCC have been implemented, by preventing the appearance of cirrhosis (e.g., HBV vaccination and treatment) [2] or the reappearance of HCC in cirrhotic patients (e.g., interferon in HCV-related cirrhotic patients) [3].

PPARγ is a nuclear transcription factor, member of the nuclear hormone receptor superfamily, being activated by binding its ligand, then heterodimerizing with the Retinoid X receptor, to finally bind with specific response elements in the nucleus, called peroxisome proliferating response elements. Activation of PPARγ by agonists such as thiazolidinediones (TZDs) has been shown to have anticancer effect in vitro and in vivo in many cancer types [4]. However, as emphasized by articles in this special issue of PPAR research, in some situations, like in colon cancer models, PPARγ agonists can have tumor promoting effects [5].

Effect of PPARγ agonists on HCC has been studied in the last years both in cell cultures and in animal models. Here, we summarize the main findings of these works and attempt to define the role of PPARγ and its modulation in HCC.

2. EXPRESSION OF PPARγ IN RODENT AND HUMAN LIVER

PPARγ RNA and protein expression has been demonstrated in vitro in several cell lines, including Hep3B, HepG2, Huh7, and others [6–11]. Its expression was generally higher than positive control used but variable among cell lines, with mRNA levels not necessarily correlating protein expression.

In human HCC, reports from 3 papers show conflicting results: in the first paper, PPARγ protein expression was assessed by western-blot (WB) in 5 cirrhotic patients and showed no difference between HCC and nontumoral cirrhotic liver [12]. The second paper showed a constant overexpression of PPARγ protein in 20 HCCs and no expression in surrounding liver, assessed by immunohistochemistry (IHC). In this paper, no information was given regarding the presence of cirrhosis [13]. Finally, a recent report, analyzing

mRNA and protein expression (by WB) in 20 patients, with cirrhosis ($n = 12$) and chronic hepatitis ($n = 8$), showed a statistically significant decrease in PPARγ expression in HCC compared to adjacent liver [14]. Furthermore, PPARγ mRNA was less expressed in poorly differentiated HCC compared to well-differentiated ones. The latter [2] results, using 2 different methods (IHC versus WB) in different populations, can therefore not be compared, and certainly other analyses will be needed before we can draw any conclusions on PPARγ expression in HCC and cirrhosis compared to normal liver, where it is known to be low [15].

3. EFFECT OF PPARγ AGONISTS ON CELL PROLIFERATION

The effect of the natural PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2, and the synthetic agonists thiazolidine-diones has been tested in many human HCC cell cultures [6, 7, 9, 11, 12, 14, 16, 17]. Overall, cell growth was inhibited at variable concentrations. Troglitazone, the most studied TZD, was shown to inhibit cell growth already at 5–10 μMol concentrations, although some contradictory reports have been published [10]. Pioglitazone and ciglitazone, the other main TZDs tested, were less active, with concentrations from 50 to 100 μMol needed to have significant growth inhibitory effect. The antiproliferative effect was shown to be due to a cell cycle arrest, with cells accumulating at the G1 phase. Koga et al. have performed the most thorough mechanistic investigations in the field [7, 12]. Using Troglitazone at a dose of 50 μMol, they could show on several cell lines that the inhibitory effect on cell cycle was linked to an increase in the mRNA and protein expression of the Cyclin-dependent kinase inhibitor (Cki) p21$^{waf1/cip1}$ and also an increase in protein expression only of p27^{kip1}, another CKi. These proteins were shown to bind to the Cyclin-dependent kinase CDK2, thus inhibiting its activation. Because the intracellular concentration of p27^{kip1} is tightly regulated by its degradation through the ubiquitin-proteasome pathway, the authors have analyzed and could show that indeed, troglitazone had an inhibitory effect on p27^{kip1} degradation via inhibition of Skp2, an F-box protein of ubiquitin-ligase complex. These results were reproduced by others [11, 14, 18].

In vivo, four papers showed an inhibition of the development of preoplastic and neoplastic liver nodules by PPARγ agonists. Kawaguchi et al. [19], using pioglitazone in male Wistar rats fed with a choline-deficient L-amino acid defined diet, which is an animal model of nonalcoholic steatohepatitis (NASH) model, showed that pioglitazone, at a concentration of 0.01% wt/wt, inhibited the formation of GSTp positive foci by a factor 2. GSTp, the placental form of Glutathione S-transferase, is an early marker of malignant transformation, widely used in carcinogenic models [20]. Guo et al. [21], using three TZDs, namely, troglitazone, rosiglitazone, and pioglitazone, in male Wistar rats showed that all could suppress the formation of GSTp foci that were induced by the strong mutagen diethylnitrosamine (DEN). In a longer experiment aiming at tumor formation, they found that pioglitazone at a 200 ppm concentration,

equivalent to 0.05% wt/wt, prevented the appearance of macroscopic tumors. However, in this paper, no histologic proof of hepatocellular carcinoma was demonstrated, and the quantitation of tumors is subject to criticism, with a "stereomicroscopical" method used that is not described. We recently published our results [22], using pioglitazone at a dose of 0.01% wt/wt, in a carcinogenic 2-stage rat model, using DEN and acetylaminofluorene. We could show that given early in the carcinogenic process, pioglitazone inhibited the formation of preoplastic foci to 50% of the control group. This was accompanied by a significant decrease of proliferation in the transformed foci, and an overexpression of the CKi p27^{kip1} in the pioglitazone-treated group compared to control, thus reproducing some of the results obtained in vitro. Finally, Yu et al. published their results on the effect of troglitazone, in vitro and in vivo in a nude mice model, injected subcutaneously with the human Huh7 HCC cell line [14]. They could show quite convincingly that troglitazone, at the 200 ppm concentration, either inhibited the appearance of HCC, or decreased the growth of existing ones. The authors demonstrated that troglitazone induced a decrease in proliferation, assessed by PCNA, an increase in p27^{kip1} and a decrease of COX-2 expression in tumors compared to controls.

It is important to mention that none of these studies, using TZDs, has clearly demonstrated the antiproliferative effect of these drugs to be due to activation of PPARγ pathway. In other cancer types, authors have demonstrated that troglitazone and ciglitazone could inhibit cell proliferation in PPAR$\gamma^{-/-}$ cell lines, by inhibition of translation initiation [23].

4. ROLE OF PPARγ IN THE PROGRESSION OF LIVER FIBROSIS

An important paper published in 2002 by Galli et al. [24] demonstrated that, given early, TZDs could significantly reduce the development of liver fibrosis induced in rats, either by chronic administration of dimethylnitrosamine, carbon tetrachloride, or bile duct ligation. The authors could show that this antifibrotic effect was driven through a PPARγ-dependant decreased activation of hepatic stellate cells. These early results showing the importance of PPARγ activation in the pathogenesis of fibrosis were confirmed by others [25, 26]. Though, we could show that the protective effect was very dependant on the rodent type [27] and the severity of fibrosis at the time of initiation of treatment [28]. These data are thus to be interpreted with caution before going to clinical application. In humans, one randomised double-blind placebo-controlled study assessed the effect of pioglitazone versus placebo in patients with NASH [29]. One of the aims of the study was to assess fibrosis on liver biopsy before and after 6 months of a calorie-restricted diet with or without pioglitazone. The study failed to show differences in terms of fibrosis regression, assessed by histology, between groups ($P = 0.08$), although patients in the pioglitazone group showed significantly less fibrosis after the 6-month regimen compared to before treatment ($P = 0.002$).

5. PPARγ MODULATION IN ASSOCIATION WITH CELL DEATH

The role of PPARγ in apoptosis and anoikis is less clear. Several in vitro works report induction of apoptosis in HCC cell lines, constantly by troglitazone and not by others TZDs [9, 14, 30–32]. The main apoptotic mechanisms remain poorly understood, although some publications report an effect of troglitazone on the bax/bcl-2 balance [32], suggesting a role for the mitochondrial pathway. In another work, authors have analyzed the effect of the natural PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (15D-JG2) on apoptosis of SK-Hep1 and hepG2 cells. They indeed showed that given at a dose of $50\,\mu$Mol, 15D-JG2 could induce apoptosis by caspase 3 activation. Using PPARγ antisense oligodeoxynucleotides, they demonstrated that apoptosis was induced independently of PPARγ expression. They also showed that 15d-PGJ2 inhibited NF-kB activation induced by TNFα when PPARγ was normally expressed or downregulated.

6. IS THERE A ROLE FOR PPARγ INHIBITION IN CANCER?

Although, as discussed earlier in the text, the vast majority of available data deals with PPARγ agonists, some authors analyzed the effect of PPARγ inhibitors on cancer cell growth. One publication reported the effect of PPARγ inhibitors on cell adhesion and anoikis [13]. On the contrary to the paper by Yu et al. [14], authors found overexpression of PPARγ in human HCC. They could show that these PPARγ inhibitors increased loss of adherence in vitrofollowed by caspase-dependent apoptosis, a finding reproduced using PPARγ siRNAs, reflecting the specificity of the PPARγ-dependent activation driving cell death. They also showed that inhibitors were at least 5-fold more potent in reducing cell number than troglitazone and rosiglitazone. Another study evaluated the effect of PPARγ agonists and antagonists on cell growth, migration, and invasion in four different HCC cell lines [33]. Authors could show that antagonists inhibited cell growth and migration more efficiently than PPAR agonists. They suggest that this effect could be due to Vimentin cleavage, thus interacting negatively with the cellular cytoskeleton.

7. CONCLUSION

Several in vitro and in vivo papers suggest a role of the PPARγ pathway in the prevention and treatment of HCC. PPARγ mRNA and protein expression are constantly found in cell lines. In normal liver, it is known to be expressed at low levels, ten times less than in adipose tissue. We do not know its actual level of expression, neither in cirrhotic nontumoral liver, nor in HCC, with conflicting results reported. Thiazolidinediones, which are active and specific PPARγ agonists, have shown antitumoral activity in cell lines and in animal models. This anticancer activity is due to inhibition of cell proliferation, by interfering with important cell cycle cyclins and cyclin-dependent kinase inhibitors, such as p27^{kip1}, but also by a proapoptotic action. The link between activation of the PPARγ pathway and the anticancer activity of TZDs is, however, not established. Finally, PPARγ antagonists have shown antitumor activity, by different mechanisms, mainly involving loss of cell adherence, migration, and invasion.

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Review Article

Peroxisome Proliferator-Activated Receptors (PPARs) as Potential Inducers of Antineoplastic Effects in CNS Tumors

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The peroxisome proliferator-activated receptors (PPARs) are ligand-inducible transcription factors which belong to the superfamily of nuclear hormone receptors. In recent years it turned out that natural as well as synthetic PPAR agonists exhibit profound antineoplastic as well as redifferentiation effects in tumors of the central nervous system (CNS). The molecular understanding of the underlying mechanisms is still emerging, with partially controverse findings reported by a number of studies dealing with the influence of PPARs on treatment of tumor cells in vitro. Remarkably, studies examining the effects of these drugs in vivo are just beginning to emerge. However, the agonists of PPARs, in particular the thiazolidinediones, seem to be promising candidates for new approaches in human CNS tumor therapy.

1. REVIEW CRITERIA

For this review we searched NCBI PubMed articles including early-release publications. Search terms included peroxisome proliferator-activated receptor (PPAR) in conjunction with "glioma" or "glioblastoma" or "astrocytoma" or "neuroblastoma." The abstracts of retrieved citations were reviewed and prioritized by relevant content. Full articles were obtained and references were checked for additional material when appropriate. Only papers published in English between 1995 and 2008 were included.

2. PPARs

The peroxisome proliferators-activated receptors (PPARs) are ligand-inducible transcription factors which belong to the superfamily of phylogenetically related proteins termed nuclear hormone receptors (NHRs). Three different PPAR isotypes (PPARα, PPARβ, also called δ, and PPARγ) have been identified in various species and show structural homology [1, 2]. PPARγ is found in two different isoforms, PPARγ1 and PPARγ2 [3].

PPARα, PPARβ/δ and PPARγ show unique spatio-temporal tissue-dependent patterns of expression during fetal development in a broad range of cell types with ectodermal, mesodermal, or endodermal origin. PPARs are involved in several aspects of tissue differentiation and development, such as the differentiation of the adipose tissue, brain, placenta, and skin [4]. Therefore, it appears that the PPAR isoforms developed from a common PPAR gene with broad ligand-binding specificity, itself derived from the ancestral orphan receptor [5].

PPARs regulate gene expression via multiple mechanisms, thereby functioning as obligate heterodimers with retinoid-X-receptors (RXRs). Like the other members of the NHR superfamily, PPARs are composed of four domains. The highly conserved DNA-binding domain together with its zinc finger domain is a common attribute of all family members. The DNA binding domain is linked to the C-terminal ligand binding domain by the hinge region. The E/F domain is responsible for the dimerization of PPARs with RXRs and the ligand-dependent transactivation function of the receptor. The N-terminal domain finally is involved in the ligand-independent regulation of the receptor activity (reviewed in [6]).

PPARs stimulate gene expression through binding to conserved DNA sequences, termed peroxisome-proliferator response elements (PPREs), present in the promoter region of their target genes. In the absence of ligands, these heterodimers are physically associated with corepressor complexes which suppress gene transcription [4]. However, upon binding of a ligand to the receptor, the NCor-containing corepressor complexes are dismissed and replaced with coactivator complexes. These coactivators are then linked to the basal transcriptional apparatus, thereby activating gene transcription [7].

PPARs act prinicipally as lipid sensors and regulate whole body metabolism in response to dietary lipid intake and direct their subsequent metabolism and storage [8]. The prototypic member of the family, PPARα, was initially reported to be induced by peroxisome proliferators, and now denotes the subfamily of three related receptors. The natural ligands of these receptors are dietary lipids and their metabolites. The specific ligands interacting with the individual receptors have been difficult to establish, owing to the relatively low-affinity interactions and broad ligand specificity of the receptors.

PPARα acts primarily to regulate energy homeostasis through its ability to stimulate the breakdown of fatty acids and cholesterol, driving gluconeogenesis and reduction in serum triglyceride levels. This receptor acts as a lipid sensor, binding fatty acids and intiating their subsequent metabolism. PPARα binds a number of lipids including fatty acids, eicosanoids, and other natural lipid ligands. Its dominant action is to stimulate adipocyte differentiation and to direct lipid metabolites to be deposited in this tissue. PPARγ operates at the critical metabolic intersection of lipid and carbohydrate metabolism. PPARγ activation is linked to reduction in serum glucose levels, likely as a secondary effect of its ability to regulate endocrine factors. It is this latter activity that has led to the development of specific PPARγ agonists for the treatment of type-2 diabetes [9]. The PPARβ/δ binds and responds to VLDL-derived fatty acids, eicosanoids including prostaglandin A1 [10] and appears to be primarily involved in fatty acid oxidation, particularly in muscle.

Binding of PPARs to their specific ligands leads to conformational changes which allow co-repressor release and co-activator recruitment. Even though all PPARs can be attributed to a common ancestral nuclear receptor, each PPAR isotype has its own properties with regard to ligand binding. Synthetic thiazolidinediones (TZDs), which are commonly prescribed for the treatment of type-2 diabetes, are selective PPARγ ligands. Naturally occurring PPARγ ligands include eicosanoids and the cyclopentenone prostaglandin 15d-PGJ2. The best characterized PPARγ agonists are the TZDs including pioglitazone (Actos) and rosiglitazone (Avandia), which are Food and Drug Association (FDA) approved for treatment of type-2 diabetes. The TZD troglitazone (Rezulin) was introduced in the late 1990s but turned out to be associated with an idiosyncratic reaction leading to drug-induced hepatitis. It was withdrawn from the US market in 2000, and from other markets soon afterwards. There are a number of non-TZD-based PPARγ agonists, such

as GW78456 and others that have been developed. PPARα ligands include fibrates that are commonly used for the treatment of hypertriglyceridemia and the synthetic agonists WY14,643 and GW7647. PPARβ/δ agonists include the prostacyclin PGI$_2$, and synthetic agents including GW0742, GW501516, and GW7842. All three PPAR isotypes can be activated by polyunsaturated fatty acids with different affinities and efficiencies [8, 11]. An overview addressing the affinity of several natural and synthetic ligands has been summarized recently [12].

All PPARs have been described in the adult and developing brain as well as in the spinal cord. Furthermore, it has been suggested that PPAR activation in neurons may directly influence neuron cell viability and differentiation [13–17]. While PPARβ/δ has been found in neurons of numerous brain areas, PPARα and γ have been localized to more restricted brain areas [18, 19]. The localization of PPARs has also been investigated in purified cultures of neural cells. PPARβ/δ is expressed in immature oligodendrocytes where its activation promotes differentiation, myelin maturation and turnover [20, 21]. The γ isotype is the dominant isoform in microglia. Astrocytes possess all three PPAR isotypes, although to different degrees depending on the brain area and animal age [22, 23].

The role of PPARs in the CNS is mainly related to lipid metabolism; however, these receptors have been implicated in neural cell differentiation and death as well as in inflammation and neurodegeneration. The expression of PPARγ in the brain has been extensively studied in relation to inflammation and neurodegeneration [14]. PPARα has been suggested to be involved in the acetylcholine metabolism [24] and to be related to excitatory amino acid neurotransmission and oxidative stress defense [18]. However, mice lacking PPARα function appear healthy and fertile and do not show neurological phenotypes, suggesting that PPARα is dispensable for brain development [25]. In contrast, loss of PPARγ has been shown to be embyonically lethal [26]. Whereas PPARβ/δ remains highly expressed in the rat CNS, the expression of PPARα and γ decreases postnatally in the brain [27]. In retina, all three receptors are expressed [23, 27, 28]. Even though this pattern of expression, which is isotype-specific and regulated during development, suggests that the PPARs may play a role during the formation of the CNS, their function in this tissue is still poorly understood. Both in vitro and in vivo observations show that PPARβ/δ is the prevalent isoform in the brain beeing found in all cell types, whereas PPARα is expressed at very low levels predominantly in astrocytes [29]. Acyl-CoA synthetase 2, which is crucial in fatty acid utilization, is regulated by PPARβ/δ at the transcriptional level, providing a facile measure of PPARβ/δ action. This observation strongly suggests that PPARβ/δ participates in the regulation of lipid metabolism in the brain. This hypothesis is further supported by the observation that PPARβ/δ null mice exhibit an altered myelination of the corpus callosum. Such a defect was not observed in other regions of the central nervous system, and the expression of mRNA encoding proteins involved in the myelination process remained unchanged in the brain [30].

As mentioned above, PPARs were at first identified as controllers of lipid metabolism. Presently, it turned out that PPARs also play a role in controlling important cellular functions like energy homeostasis, diabetes, cell proliferation and cell death, differentiation, inflammation, and even cancer [6, 31]. Especially PPARγ and its agonists have been demonstrated to induce antineoplastic effects in several types of cancer (reviewed in [7]). In the following we focus on the role of PPARs as potential inducers of antineoplastic effects in highly abundant CNS tumors, namely astroglioma and neuroblastoma.

3. ASTROGLIOMA

Malignant astrocytic gliomas represent the largest proportion of all primary brain tumors in adults [32, 33]. The characteristic feature of glioma cells is a high proliferation rate, accompanied by the ability to invade far into the healthy brain tissue. According to the WHO classification of tumors of the nervous system [32], gliomas are ranked with increasing malignancy in four classes from WHO grade I to WHO grade IV. The vast resistance against irradiation and chemotherapy and the prevalent recurrence after surgical resection are the main reasons for the poor prognosis in treatment of malignant astrocytic gliomas. Despite multimodal therapeutic approaches, the mean survival time of patients with WHO grade IV glioblastoma multiforme, which is also the most frequent brain tumor, is only about one year after diagnosis [33]. Although medical research has been intensified in the past decades, the overall survival of patients with malignant astrocytic gliomas was not essentially improved [34].

All isoforms of PPARs are expressed in the brain [35, 36] as well as in a variety of rat and human astroglial cell lines [7, 37–44]. PPARγ has been shown to be expressed at high levels in human glioblastomas [31, 37, 45, 46]. Based on findings in other neoplastic disease, several natural and synthetic ligands of PPARs have been tested for their efficacy in the treatment of astroglioma. Bezafibrate and gemfibrozil, both PPARα agonists, inhibited the cellular viability of glioblastoma cell lines [47]. A different effect was observed when human T98G glioblastoma cells were treated with other PPARα ligands, clofibrate and Wy-14,643. These ligands strongly downregulated the expression of semaphorin 6B, a member of the semaphorin family of axon guidance molecules [39], suggesting suppression of glioma invasion mechanisms by these PPARα agonists. However, no direct influence of Wy-14,643 on proliferation or induced cell death was observed in either human or rat glioma cells [43].

Treatment with conjugated linoleic acid (CLA) inhibited growth in primary human glioblastoma cells as well as ADF glioblastoma cells [13, 40, 48]. In ADF cells this was associated with an increase of PPARα and a decrease of PPARβ/δ expression, whereas PPARγ levels were unaltered [40]. Cimini et al. found that CLA and the PPARγ-specific agonist GW347845 reduced glioma cell growth and induced apoptosis [13, 48]. The authors suggested that this effect was mediated by PPARγ activation. This conclusion was supported by the finding that the PPARγ antagonist GW259662

completely prevented both the CLA and GW347854X-induced effects on cell growth and apoptosis. Furthermore, PPARγ agonists reduced cell adhesion, cell migration, and tumor invasion which was associated with a decrease in matrix metalloproteinase 2 (MMP2) levels. The authors stated that activation of PPARγ is likely to be responsible for these latter effects, since the PPARγ antagonist GW259662 completely abolished these effects [13]. Furthermore, treatment with CLA and GW347845 significantly decreased VEGF isoforms, indicating that PPARγ may also inhibit angiogenesis in gliomas [48].

Pérez-Ortiz et al. reported that generation of reactive oxygen species (ROSs) was likely to be responsible for glitazone-induced glial cell death [35, 49], which is in line with findings of Kang et al. [50]. Interestingly, in four different glioma cell lines (A172, U87-MG, M059K, M059J) rosiglitazone led to inhibition of proliferation and induction of apoptosis in a PPARγ-dependent way since there the antagonist GW9662 partially reverted this effect [46]. Ciglitazone and the putative natural PPARγ ligand PGJ$_2$ inhibited proliferation and induced apoptotic cell death in human [38] and rat glioma cells, and apoptotic cell death was correlated with the upregulation of Bax and Bad protein levels [43]. Similar effects have been described by Zang et al. [44], who also reported that a combination of pioglitazone with all-trans retinoic acid (ATRA) increased the cytotoxic effect. Tetradecylthioacetic acid (TTA), a saturated fatty acid and PPAR ligand, inhibited growth of BT4Cn rat glioma cells at increased levels as compared to the PPARγ ligand rosiglitazone [37]. Furthermore, TTA reduced tumor growth and led to a longer survival of rats with implanted BT4Cn tumors. The use of the PPARγ antagonist GW9662 reversed the effect of rosiglitazone but not for TTA, indicating that TTA might act both via PPARγ-dependent and PPARγ-independent pathways [37].

Grommes et al. reported that the nonthiazolidinedione tyrosine-based PPARγ ligand GW7845 reduced viability of rat C6 and human glioma cells and induced apoptotic cell death in a PPARγ-dependent mechanism as shown by the inhibition of these effects by the specific antagonist GW9662 [51]. Primary astrocytes were not affected, demonstrating the specificity of the effects of GW7845 on neoplastic cell types. GW7845 also reduced proliferation of rat C6 glioma cells and reduced both the migration and invasion of glioma cells [51]. These investigators have subsequently reported [52] that the PPARγ agonist pioglitazone reduced cellular viability of rat and human glioma cell in vitro. Furthermore proliferation in rat glioma cells was inhibited, as measured by Ki-67 expression. Glioma cells overexpressing PPARγ-cDNA showed reduced cellular viability after pioglitazone treatment, whereas treatment of glioma cells overexpressing a mutant cDNA lacking transcriptional activity, showed no antineoplastic effects [52]. Grommes et al. extended these findings to in vivo studies, using a C6 rat glioma model [52]. In this study, tumor volumes were dramatically reduced following pioglitazone administration intracerebrally, as well as orally, indicating that pioglitazone is able to cross the blood-brain barrier (BBB). It has not been established whether TZDs other than pioglitazone penetrate

Signal mechanisms involved in antineoplastic effects of PPAR ligands
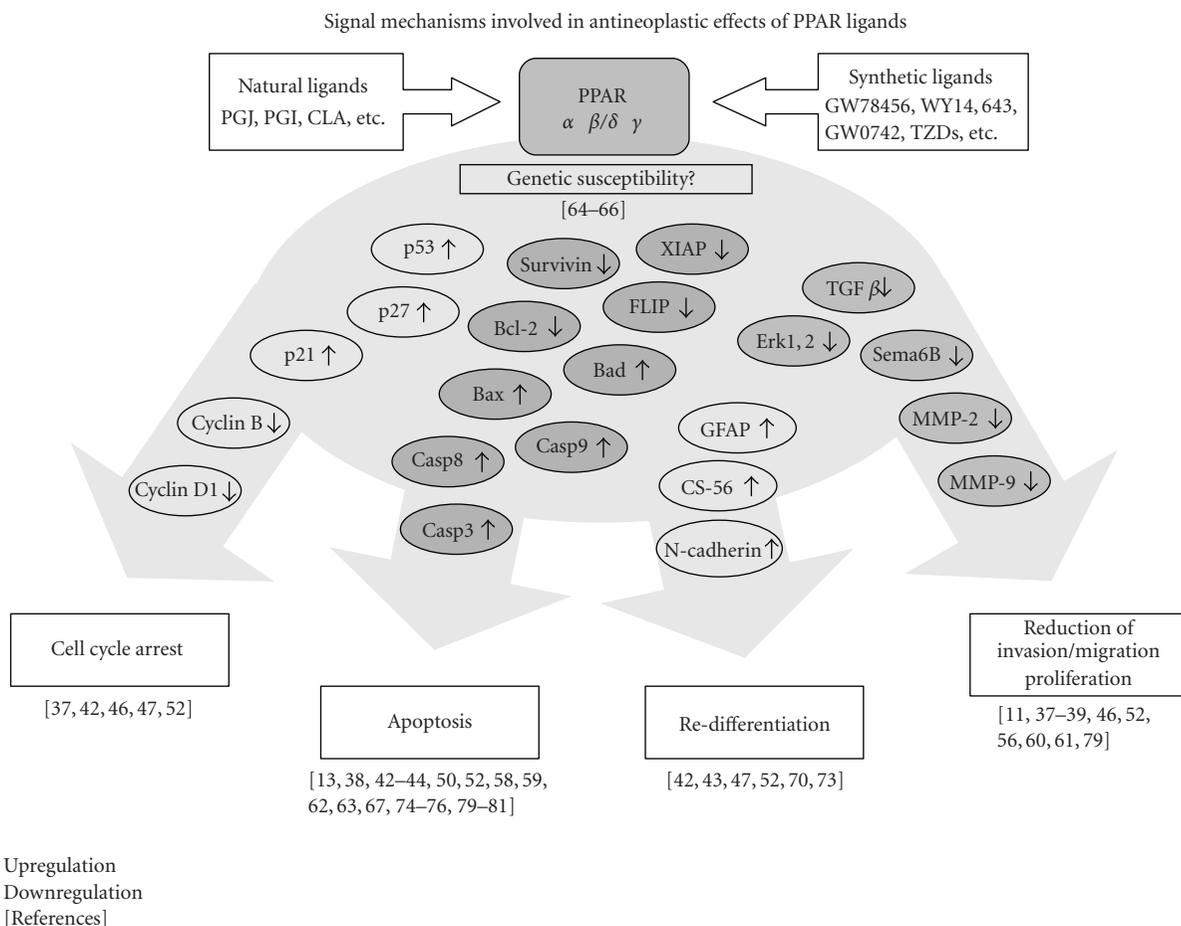
FIGURE 1

the BBB. However, in vitro studies provide evidence that troglitazone is actively incorporated by the bidirectional transporter Oatp14 (Slco1c1) expressed in brain capillary endothelial cells, which is likely to provide homeostasis of troglitazone and may be of other TZDs [53]. Treated animals showed drug-induced apoptosis in the tumors by activation of proapoptotic proteins. Grommes and coworkers also observed decreased tumor invasion in vivo which was correlated with reduced MMP9 levels. Indeed, PPARγ agonists suppressed tumor migration in vitro in a Boyden chamber assay. Finally, they described a pioglitazone-induced upregulation of the astrocytic redifferentiation marker CS-56 in tumor cells both in vivo and in vitro. Primary astrocytes were not affected by pioglitazone, indicating the restriction of these effects to neoplastic cell types [52]. A possible explanation for this neoplastic specificity is given by Spagnolo et al., who showed differences in metabolic responses in GL261 glioma cells as compared to primary astrocytes when treated with the TZD troglitazone [54].

The same authors also presented a study exploring C57/Bl6 mice with an intracerebral glioma derived from GL261 cells [55]. Mice were treated with a combined therapy of interleukin (IL)-2-secreting syngeneic/allogeneic fibroblasts administered into the tumor bed along with the TZD pioglitazone. In contrast to the data of Grommes et al., only intracerebrally administered pioglitazone prolonged the survival of mice harboring an intracerebral glioma, whereas pioglitazone administered orally showed no effect. Finally, combination of pioglitazone and Il-2-secreting fibroblasts significantly prolonged the survival of the treated mice as compared to untreated animals [55].

Using an organotypic glioma invasion model, closely resembling extracellular matrix environment present in the brain, Coras et al. show that micromolar doses of troglitazone blocked glioma progression without neurotoxic damage to the organotypic neuronal environment observed [56]. The authors stated that the intriguing antiglioma property of troglitazone appears to be only partially based on its moderate cytostatic effects. Concordant with the data presented by Grommes et al., the authors showed that troglitazone effectively inhibits glioma cell migration and brain invasion. Interestingly, the antimigratory effects of troglitazone could be mimicked by inhibition of TGF-ß signaling which has shown to be intimately involved in glioma cell migration, suggesting both mechanisms to be interlinked. In this study, the authors identified troglitazone

as a potent inhibitor of TGF-ß release, suggesting that troglitazone reduced glioma cell motility by counteracting TGF-ß signaling [56].

More than 10 years ago, Prasanna et al. [57] reported that treatment with lovastatin (a HMG-CoA reductase inhibitor) led to growth arrest in glioma cells, accompanied with an increased expression of PPAR. A combination therapy of lovastatin and the PPARγ agonist troglitazone reduced cellular viability in the DBTRG-05MG human glioblastoma cell line [58]. Interestingly, the combination of lovastatin with two other PPARγ agonists, rosiglitazone and ciglitazone, did not lead to the same effect. The authors suggested that it may be possible that PPARγ is an essential, but not sufficient, factor in this synergism.

PPAR agonists have also been shown to exhibit effects on tumor biology through PPAR-independent mechanisms. For example, the PPARα/γ dual agonist TZD 18 inhibited growth of T98G human glioblastoma cells and induced apoptosis through PPAR-independent mechanisms, since their respective antagonists MK-886 and GW9662 did not reverse this effect [59]. The TZD-mediated antineoplastic properties from PPARγ was argued to arise from off-target, receptor-independent actions of the drugs as well as those of rosiglitazone and pioglitazone [35, 38, 43, 60]. The glitazones were toxic for the human glioma cell line U251 and rat glioma cell line C6, but not for primary rat astrocytes [43]. Indeed, PPARγ seems not to be involved in these effects of the TZDs, since the inhibitor GW9662 had nearly no effect on attenuation of cytotoxicity. Using PPARγ positive and PPARγ deficient mouse embryonic stem (ES) cells, it has been demonstrated that the TZD troglitazone inhibited the growth of tumors formed by injection of PPARγ+ and PPARγ− cells to the same extent, indicating that PPARγ is not essential for the antiproliferative effects of troglitazone [60]. Moreover, troglitazone derivatives which are unable to activate PPARγ suppress cancer cell proliferation similar to troglitazone, giving further evidence that the antiproliferative effects of troglitazone are at least in part PPARγ-independent [61]. Furthermore, troglitazone sensitized human glioma cells to TRAIL-induced apoptosis in a process independent of PPARγ [62, 63]. Troglitazone treatment led to a marked downregulation of the antiapoptotic proteins FLIP and survivin [63] as well as Bcl-2 [62] and so could possibly counteract the capability of tumor cells to become resistant to apoptosis. Hence a combination therapy of troglitazone and TRAIL might be a promising experimental approach. Conversely, in A172 human glioma cells Kang and colleagues showed that the TZD ciglitazone induced cell death dependent of PPARγ, but independent of caspase and AIF. Furthermore, the authors demonstrated that downregulation of XIAP and survivin is involved in the cell death mechanism [50]. A possible explanation for the differentiative effects of PPARγ agonists was supposed to rely on PPARγ dysfunction. Single strand conformational polymorphism (SSCP) analysis was carried out in different tumor and nontumor tissues, showing somatic loss-of-function mutations in different carcinomas [64, 65]. Genetic analysis of American patients with glioblastoma multiforme revealed an overrepresentation of the H449H polymor-

phism in the PPARγ gene, possibly being an important low penetrance susceptibility locus for glioneural tumors [66].

4. NEUROBLASTOMA

Neuroblastoma is a phenotypically heterogeneous tumor, containing cells of neuronal, melanocytic or glial/Schwann cell lineage. Regardless of the phenotype, PPARγ is expressed in neuroblastoma cell lines [67], in primary neuroblastoma cells [7] as well as in samples of patients harbouring neurblastoma [68]. Data about the expression of PPARβ/δ in neuroblastomas are scarce [69–71], and only a few studies report the expression of PPARα at mRNA or protein level in human neuroblastoma cell lines [71–74]. Therefore, most studies that assess the influence of PPARs on treatment of neuroblastoma evaluate the impact of its natural or synthetic ligands.

The putative natural PPARγ agonist 15d-PGJ$_2$ inhibits cellular growth, decreases cellular viability and induces apoptosis in human neuroblastoma cells in vitro [67, 69, 74–76]. Rodway et al. [74] show that the PPARα agonist WY-14643 has no effect on the growth of the IMR32 neuroblastoma cell line, whereas PGJ$_2$ induces growth inhibition in the same neuroblastoma cells. This occurs through programmed cell death type II or autophagy, and the serum lysolipid LPA is responsible for modulating this cellular response. In the neuroblastoma cell line ND-7, the same group shows that the degree of PPARγ activation induced by PGJ$_2$ is modulated through an interaction with retinoblastoma protein (Rb) and the class I histone deacetylase 3 (HDAC3) [75]. A combination therapy consisting of PGJ$_2$ and the histone deacetylase inhibitor trichostatin A (TSA) enhanced the growth inhibition effects and is therefore proposed as a promising new strategy in the treatment of neuroblastoma. It should be noted that the effects of 15d-PGJ2 can also arise from its actions on the NFκB pathway [77]. Di Loreto et al. report that a specific PPARβ agonist as well as oleic acid induced redifferentiation in SH-SY5Y neuroblastoma cells [70].

The best studied synthetic PPARγ agonists are the TZD class of antidiabetic drugs, also referred to as glitazones [7]. Valentiner et al. [78] tested four glitazones (ciglitazone, pioglitazone, troglitazone, rosiglitazone) and reported their in vitro effects on cell growth in seven human neuroblastoma cell lines (Kelly, LAN-1, LAN-5, LS, IMR-32, SK-N-SH, SH-SY5Y). All the glitazones inhibited cell growth and viability of the human neuroblastoma cell lines in a dose-dependent manner, whereas the effectiveness of the single drugs differed strongly between cell lines. Similar results for ciglitazone and rosiglitazone have been reported [75, 79]. Cellai et al. show that high concentrations of rosiglitazone significantly inhibit cell adhesion in vitro, invasiveness and apoptosis in SK-N-AS, but not in SH-SY5Y human neuroblastoma cells [79]. The authors argued that this effect may be related to cellular differences in PPARγ transactivation. Furthermore, Jung et al. report that the TZD rosiglitazone protects SH-SY5Y cells against MPP+ as well as acetaldehyde-induced cytotoxicity, which may be ascribed to the induction of the

expression of antioxidant enzymes and also to the regulation of Bcl-2 and Bax expression by rosiglitazone [80, 81].

5. CONCLUSION

The understanding of the molecular mechanisms underlying the antineoplastic effects mediated by PPAR agonists is still emerging. Over the past years, an increasing number of reports were published, presenting evidence for several involved pathways concerning cell cycle arrest, apoptosis, redifferentiation and inhibition of invasion/migration, that have been found to be affected by PPAR agonist treatment. Figure 1 presents an overview of signal mechanisms involved in the antineoplastic effects of PPAR ligands. Interestingly, there are partially controverse findings regarding the receptor dependency of the observed effects. Besides the number of natural and synthetic ligands, as well as to the number of different tumor cell lines used, a further explanation may be that most studies were performed on long-term cultured cell lines which may have undergone alterations while being in cell culture. Only few studies use primary cell cultures of tumor cells or organotypic models, like Benedetti et al. or Coras et al. [48, 56], trying to resemble natural conditions as close as possible. Remarkably, studies examining the effects of PPAR agonists in vivo are just emerging for gliomas [52, 55], and are still missing for neuroblastomas.

From all natural and synthetic PPAR ligands, the group of thiazolidinediones is the one with the best characterized antineoplastic properties. The fact that TZDs like pioglitazone (Actos) and rosiglitazone (Avandia) are FDA-approved for treatment of type-2 diabetes and therefore readily available for clinical studies may be the main reason for this. Very recently, a phase 2 clinical study was published, presenting for the first time a combination of low-dose chemotherapy with COX-2 inhibitors and PPARγ agonists in high-grade gliomas [82]. Unfortunately, the trial had to be closed prematurely, due to the moderate efficacy as compared to other clinical trials, which however investigated PPARγ agonist treatment of different tumor entities. It is questionable whether the tumor biology of astroglioma, which are extremely heterogeneous and rarely metastasize, can be compared to these different tumors, and thus the degree of response to a PPARγ agonist-based therapy. Of note, depending on the particular astroglioma and region within the tumor, the poor blood brain barrier penetration of the TZDs may also account for limited efficacy. Therefore, further in vivo studies are warranted to unravel the molecular mechanisms underlying the antineoplastic effects of PPAR agonists in malignant astrocytic gliomas.

Nevertheless, agonists of PPARs, in particular the TZDs, seem to be promising candidates for new therapeutic approaches in human CNS tumor therapy due to their profound antiproliferative and anti-invasive effects as well as their positive effects on apoptosis and redifferentiation.

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Review Article

Multiple Interactions between Peroxisome Proliferators-Activated Receptors and the Ubiquitin-Proteasome System and Implications for Cancer Pathogenesis

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The peroxisome proliferator-activated receptors (PPAR) α, β/δ, and γ are ligand-activated nuclear receptors involved in a number of physiological processes, including lipid and glucose homeostasis, inflammation, cell growth, differentiation, and death. PPAR agonists are used in the treatment of human diseases, like type 2 diabetes and dyslipidemia, and PPARs appear as promising therapeutic targets in other conditions, including cancer. A better understanding of the functions and regulation of PPARs in normal and pathological processes is of primary importance to devise appropriate therapeutic strategies. The ubiquitin-proteasome system (UPS) plays an important role in controlling level and activity of many nuclear receptors and transcription factors. PPARs are subjected to UPS-dependent regulation. Interestingly, the three PPAR isotypes are differentially regulated by the UPS in response to ligand-dependent activation, a phenomenon that may be intrinsically connected to their distinct cellular functions and behaviors. In addition to their effects on gene expression, PPARs appear to affect protein levels and downstream pathways also by modulating the activity of the UPS in target-specific manners. Here we review the current knowledge of the interactions between the UPS and PPARs in light of the potential implications for their effects on cell fate and tumorigenesis.

1. INTRODUCTION

Despite the everyday progress in understanding the genetic and molecular bases of cancer, this disease still strikes millions of people worldwide. The quest for new targets and more effective therapeutics is currently a major driving force in cancer research. Multiple mutations that affect critical cellular pathways lead to uncontrolled proliferation, increased survival, and block of differentiation in cancer cells [1]. Several cellular pathways (e.g., cell surface receptors, signal transduction pathways, apoptosis, angiogenesis, transcription, chromatin regulation, and proteasome-mediated degradation) have provided relevant targets and opportunities for development of clinically useful therapeutics [1]. Unfortunately, targeting each of these major pathways individually may not be sufficient. Extensive cross-talks

occur between regulatory pathways and it is not unlikely that the same proteins play multiple roles in different processes. The peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors may represent a prime example of proteins interacting with multiple cellular pathways and exerting diverse and sometime apparently contrasting functions. Here, we review how PPARs interact with the ubiquitin-proteasome system (UPS), which is the major cellular system responsible for protein turnover, and how these two systems might reciprocally affect each other activity and functions.

2. THE UBIQUITIN-PROTEASOME SYSTEM

Ubiquitin is a 76-amino acid polypeptide that is post-transcriptionally linked to proteins via a covalent linkage to

one or multiple lysine residues [2]. Several proteins including cell surface receptors, cell cycle regulators, and transcription factors are ubiquitinated and protein ubiquitination affects many cellular processes including proliferation, cell cycle progression, DNA damage repair, and cell death [2]. Ubiquitination is a regulatory signal that affects the fate and function of proteins. Ubiquitination regulates mainly protein turnover directing ubiquitinated proteins to proteasome-mediated proteolysis. Other nonproteolytic functions, like control of protein-protein interactions, cellular localization, and catalytic activity, are emerging [2]. The proteasome is a multicatalytic complex that comprises a 20S core with proteolytic activity and a 19S subunit that recognizes poly-ubiquitinated proteins, unfolds them, and passes into the 20S catalytic core for degradation. Ubiquitination is catalyzed by three types of enzymes, called E1, E2, and E3 [2, 3]. Ubiquitin is first activated by an E1 ubiquitin-activating enzyme in an ATP-dependent reaction. The activated ubiquitin is then transferred to an E2 ubiquitin-conjugating protein (UBC). Finally, E3 ubiquitin-ligases, which are the most critical enzymes in the process, catalyze the transfer and covalent attachments of the activated ubiquitin to the target protein. In human cells, a single E1 and about 60 E2 enzymes have been identified, while there are approximately a thousand E3 enzymes, which ensure a high degree of substrate specificity to the system [2, 3]. E3 enzymes are split in two major subfamilies: the Ring-H2 and the HECT domain proteins. The human genome contains also more than 70 deubiquitinating enzymes (DUBs) that remove ubiquitin chains from ubiquitinated proteins and can rescue them from proteasomal degradation [4].

Protein ubiquitination is a highly dynamic process and ubiquitination-deubiquitination cycles can serve to rapidly modulate protein level and function [4]. Ubiquitin and proteasomal components play an important role in transcription [5, 6]. Ubiquitin ligases and proteasomal subunits are present as integral components of transcription regulatory complexes [5, 6]. Histones, the main component of chromatin, are ubiquitinated and the process affects chromatin remodeling and transcription [6, 7]. RNA polymerase II is also directly regulated by ubiquitination [6, 8]. Moreover, the UPS regulates the abundance, activity, and subcellular localization of many transcription factors [5, 6]. Transcription factors are ubiquitinated and degraded by the proteasome and, paradoxically, the process is often essential for their transactivating ability [6]. In fact, transcription activation and degradation domains of transcription factors often overlap [6]. In addition, mono-ubiquitination (i.e., addition of single ubiquitin tag to a protein) can act as a post-translational modification that modulates activity of transcription factors and regulates transcription efficiency by nonproteolytic mechanisms [6]. Degradation of inhibitors of transcription factors is also often required to release active transcription factors. For example, activation of the transcription factor NF-κB is controlled by a signaling cascade based on multiple ubiquitination and proteasome-dependent events [6].

Alterations of the UPS are frequent in cancer. They are mainly due to loss or gain of function of specific components of the UPS and alterations of UPS substrates, like oncogene and tumor suppressor gene products, which become less or more susceptible to proteasomal-dependent degradation [9]. Tumor suppressor proteins are often the targets of UPS alterations. The human papillomavirus (HPV), a cause of cervical cancer, encodes two oncogenic proteins, E6 and E7. These viral proteins promote degradation of the tumor suppressor p53 via ubiquitination by the E6-associated protein (E6-AP) E3 ubiquitin ligase [10]. HDM2 is another E3 ubiquitin ligase that targets p53 to proteosomal degradation [11]. Aberrant expression of HDM2 is found in many human cancers [12]. Single nucleotide polymorphism in the HDM2 promoter leading to HDM2 overexpression has been recently associated to the development of sporadic and hereditary cancers [13]. The E3 ubiquitin ligase Skp2 is responsible for ubiquitination of the cell cycle inhibitor and tumor suppressor p27 [14]. Skp2 overexpression is observed in cancer cells leading to degradation and inactivation of this tumor suppressor protein [15]. Oncogenic proteins are also affected by alterations of UPS components. The E3 ubiquitin ligase encoded by the von Hippel-Lindau gene (pVHL) mediates the ubiquitination and degradation of the hypoxia-inducible transcription factor HIF-1α [16, 17]. Mutations in pVHL gene predispose patients to renal cell carcinoma and other cancers. In these tumors, the level of HIF-1α is increased resulting in a potent oncogenic and angiogenic stimulus.

Due to the unique mechanism of cleavage at the proteolytic active sites, selective proteasome inhibitors have been synthesized and some, like bortezomib (Velcalde, PS341), have undergone clinical evaluation as anticancer agents [18]. Bortezomib is a peptide boronate proteasome inhibitor that blocks the chymotryptic activity of the 26S proteasome [18]. The anticancer effect of bortezomib is likely to be achieved through its inhibitory effects on protein degradation and modulation of important cellular pathways, including inhibition of the NF-κB pathway [18]. Bortezomib is currently approved for clinical use for treatment of multiple myeloma. Clinical trials with bortezomib and second generation proteasome inhibitors as single agents or in combination with other chemotherapeutic agents are ongoing in various tumor types [18].

3. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

PPARs emerged in the nineties as nuclear receptors regulating transcription of genes involved in metabolic processes like lipid and glucose homeostasis [19, 20]. Later, PPARs have found to be implicated in many physiological and pathological processes [20]. PPARs belong to the nuclear hormone receptor super-family, which is one of the largest families of transcriptional regulators in the human genome with more than 40 distinct nuclear receptors [21]. Nuclear receptors bind small lipophilic molecules, such as steroid hormones, vitamins, and fatty acid derivatives, and function as ligand-activated transcription factors, interacting with specific DNA sequences (i.e., hormone response elements, HRE) in target genes and stimulating their transcription
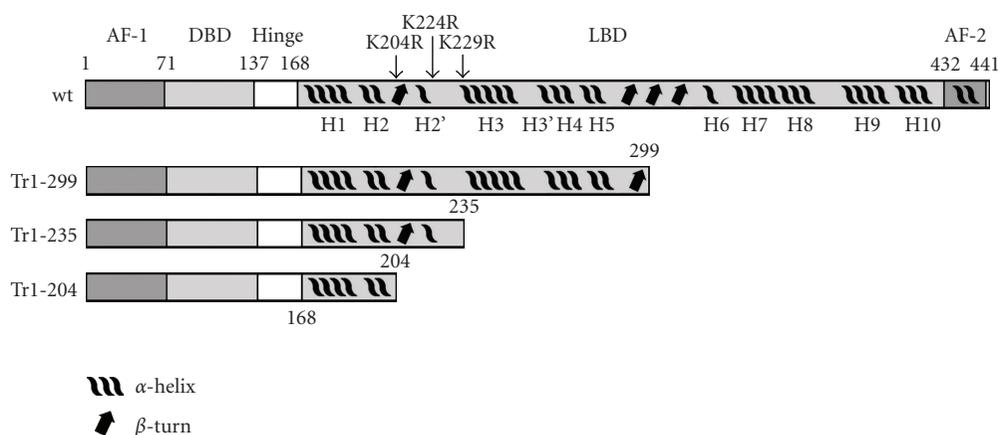
Figure 1: Domain structure of PPARδ and truncated forms of the receptor. AF-1, N-terminal ligand-independent activation function 1 (aa 1–70). DBD, DNA binding domain (aa 71–136). Hinge, flexible hinge region (aa 137–167). LBD, ligand-binding domain (aa 168–431). AF-2, C-terminal ligand-dependent activation function-2 (aa 432–441). The position of the mutations (K204R, K224R, and K229R) introduced in the region 204–235 is shown.

[21]. Thus, nuclear receptors provide a direct link between small lipophilic signaling molecules present in the cells or their environment and the cellular transcriptional machinery, turning on specific subsets of genes containing the appropriate HRE and inducing complex cellular responses [21, 22]. The nuclear receptor super-family includes the steroid hormone receptors (i.e., estrogen, progesterone, androgen, and glucocorticoid receptors) and receptors for nonsteroidal hormones [21–23]. The latter include the PPARs, vitamin D (VDR), and retinoic acid (RAR) receptors [21, 23]. The ligands of most nonsteroidal receptors are dietary fatty acids or generated locally by lipid metabolism within the target cell or tissue, while steroid and thyroid hormones are produced by distant endocrine organs and released in the blood [21, 22].

Nuclear receptors exhibit a characteristic modular structure comprising an N-terminal domain with the ligand-independent activation function domain (AF-1), a DNA binding domain (DBD), and a C-terminal domain containing the ligand binding (LBD), and the ligand-dependent transactivation (AF-2) domain [23]. The DBD contains two zinc finger modules and determines the DNA binding specificity of the receptors. The LBD is involved in homo- and heterodimerization and interaction with cofactors [23, 24]. The structure of the LBD is highly conserved among nuclear receptors, comprising a large hydrophobic cavity that accommodates the ligand. Variations of the size and shape of the ligand binding pocket ensure ligand specificity among receptors [23, 24]. Ligand-binding induces a conformational remodeling of the LBD that exposes surfaces required for interaction with coactivators and affects the affinity of the receptors for corepressors [23, 24]. The nonsteroidal receptors are found primarily in the cell nucleus and are bound to HRE as heterodimers with the retinoic X receptor (RXR) [19, 23]. These receptors can affect both positively and negatively transcription of target genes with the LBD mediating alternatively transcriptional activation or repression, although the mechanisms of transrepression by PPARs

are still poorly understood [25]. Transcriptional repression is due to recruitment of corepressors, like NCoR/SMART, by the unliganded and DNA-bound receptor and formation of multiprotein complexes containing histone deacetylases and other chromatin remodeling enzymes [23, 25]. In the presence of ligands, corepressor complexes are released and replaced by coactivators, like SRC1 and CBP-p300, thus switching on transcription [23, 25]. Transcriptional activation is associated with histone modifications, chromatin remodeling, and assembly of the transcription initiation complex. Thus, transcriptional activation and repression by nuclear receptors are very dynamic processes involving the formation of protein complexes in which multiple coactivators and corepressors need to be rapidly exchanged [25–27]. The UPS is perhaps the major system controlling the assembly and turnover of these regulatory complexes ensuring their timely interaction with the transcriptional machinery [26]. Ubiquitin and proteasome components are associated with corepressor and coactivator complexes recruited by nuclear receptors [25, 26]. Most nuclear receptors, including thyroid hormone, estrogen, glucocorticoids receptor, RAR, and RXR receptors, as well as coactivators, corepressors, and general components of the transcription machinery are ubiquitinated and degraded by the proteasome [26, 28].

PPARs have the typical modular structure of the nuclear hormone receptors with a poorly characterized N-terminal domain with putative ligand-independent AF-1 function, a central DNA-binding domain (DBD), and a C-terminal ligand binding (LBD) and ligand-dependent AF-2 domain (Figure 1) [19, 23]. However, despite the high sequence and structural homology, the three PPAR isotypes have distinct ligand specificity, functions, and behaviors [19, 20]. PPARα is a key regulator of energy homeostasis and plays a major role in lipid metabolism and glucogenesis. PPARα is expressed in tissues with significant fatty acid and cholesterol catabolism, like brown adipose tissue, liver, kidney, intestine, heart, and skeletal muscle [29]. PPARγ exists in two isoforms (γ1 and γ2) that differ only at the N-terminus. PPARγ2 is present at

high levels in adipose tissue, whereas PPARγ1 expression is broader and is present in gut, brain, vascular cells, immune cells, and retina [30]. PPARγ plays a role in adipocyte differentiation, glucose metabolism, and lipid homeostasis, and participates in monocyte/macrophage differentiation [30]. Moreover, PPARγ influences fatty acid storage in the adipose tissue and is implicated in insulin resistance and atherosclerosis [30]. PPARδ is ubiquitously expressed with high levels in colon, skin, and brain [20]. PPARδ also functions in processes linked to lipid metabolism, like fatty acid catabolism, cholesterol efflux, lipid uptake in macrophages, and preadipocyte differentiation [31]. This nuclear receptor plays also a role in placental and gut development, embryo implantation, tissue injury, and wound healing [20, 32].

PPARs possess a broad ligand-binding cavity that allows binding of a wide range of synthetic and natural lipophilic compounds [19]. Medium- and long-chain unsaturated fatty acids (e.g., linoleic acid), conjugated and oxidized fatty acids (e.g., phytanic acid), and eicosanoids bind to PPARα [19]. Fibrates, like bezafibrate, fenofibrate, and clofibrate, which are used for the treatment of dislipidemias and cardiovascular diseases, are selective PPARα agonists [29]. PPARγ binds to long-chain fatty acids, prostaglandin J$_2$ (PG J$_2$), and other eicosanoids [19]. Synthetic PPARγ agonists, such as pioglitazone and rosiglitazone, are insulin sensitizers used to treat type 2 diabetes [30]. PPARδ has high affinity for prostaglandin I$_2$ (PGI$_2$), fatty acids, and synthetic compounds [19, 31].

Beside their metabolic functions, PPARs have an important role in inflammation. PPARα and PPARγ agonists can ameliorate chronic inflammatory conditions, such as athrosclerosis, arthritis, and inflammatory bowel disease [20, 29, 30]. PPARs repress genes of the inflammatory response pathway, such as cytokines (TNFα, IL-1β, IL-6), cell adhesion molecules (MMPs), and other proinflammatory molecules (iNOS) [25]. These effects are mediated in large part by the ability of PPARs to antagonize other transcription factors, like AP-1, STAT1, and NF-κB, which have proinflammatory functions [25]. Different mechanisms have been proposed to explain the phenomenon of transrepression by PPARs, including sequestration of limiting cofactors, direct physical interaction, and antagonism between PPARs and other transcription factors, and promoter-specific block of corepressor/coactivator exchange by PPARs in selected target genes [24, 25]. The latter involves a block of the ubiquitin and proteasome-dependent processing of corepressor complexes as in the case of PPARγ-mediated repression of proinflammatory NF-κB target genes [25]. PPARγ and PPARα can also interact physically with NF-κB and c-Jun blocking transcriptional activation [33, 34]. Reciprocally, NF-κB and c-Jun can repress PPARγ and PPARα-induced transcription, respectively, by inhibiting the binding to PPRE in target genes [33–35]. Also PPARδ has a role in inflammation controlling expression of proinflammatory genes in macrophages in a ligand-dependent manner [31]. Unliganded PPARδ binds to corepressor molecules including Bcl-6, which is a repressor of inflammatory gene expression [31, 36]. Ligand binding re-leases the corepressor complexes resulting in transcription

of PPARδ target genes. At the same time, PPARδ-bound Bcl-6 is also released and is free to repress its own target genes suppressing the inflammatory response [31, 36]. Paradoxically, PPARδ knockout has the same effects of the agonists on the expression of Bcl-6 target genes since it also leads to release the transcriptional repressor [36].

The involvement of PPARs in carcinogenesis has been widely discussed, although it is still controversial whether the different isotypes either favor or inhibit tumorigenesis [37, 38]. This may still represent a major concern for developing PPAR-targeted therapeutics for clinical applications because of the potential risk of promoting tumorigenesis as indicated by studies in rodents [39]. PPARs are expressed in several human cancers and PPAR ligands have been shown to modulate tumor growth [37, 38]. Inactivating mutations, deletions and chromosomal translocations of PPARγ have been found in various cancers pointing to a tumor suppressor role of this nuclear receptor [40–42]. PPARγ ligands promote differentiation, growth arrest, and death of cancer cells in vitro [38]. PPARγ ligands reduce growth of human tumor xenografts and spontaneous and carcinogen-induced tumors in rodents [38]. PPARα is also expressed in various tumors and cancer cell lines [43, 44]. Activation of PPARα in cancer cells inhibits proliferation and suppresses metastatic potential [45–47]. PPARα ligands have shown antitumor activity also in murine models [46, 48, 49]. PPARδ participates in a number of important pathways controlling adhesion, proliferation, differentiation, and survival [37]. Unlike the other isotypes, PPARδ has been shown to prevent apoptosis and induce cell growth in normal cell types, like primary mouse keratinocytes, preadipocytes, vascular smooth muscle cells, hepatic stellate cells [37]. Consistent with an antiapoptotic role, PPARδ increases the expression of antiapoptotic genes and activates prosurvival signaling pathways in keratinocytes [50]. PPARδ agonists stimulate proliferation and survival of cancer cells in vitro and promote tumor growth in mice [51–57]. PPARδ is a downstream target of β-catenin/T cell factor-4, which is central in colon cancer pathogenesis and regulates other cancer-promoting genes like c-myc and cyclin D1 [58]. Cyclooxygenase-2 (Cox-2) modulates PPARδ activity and nonsteroidal anti-inflammatory drugs that have chemopreventive effects in colon cancer inhibit PPARδ activity and expression [58–60]. Cox-2 is frequently upregulated in cancer and preneoplastic lesions, and Cox-2 products like PGI$_2$ act as selective agonists of PPARδ [58–60]. To further support a protumorigenic role of PPARδ, PPARδ expression is elevated in cancers, like colorectal, endometrial, and head and neck cancers [58, 59, 61]. Additional evidence pointing to a tumor promoting function of PPARδ comes from experiments in mice where disruption of PPARδ decreased tumorigenicity of cancer cells in nude mice and PPARδ activation increased tumor growth [55, 57, 62].

Despite this large body of evidence, some controversial results in animal experiments cast doubts both on the anti- and protumorigenic activities of PPARs [37, 38]. Experiments in rodents have shown increased frequency and enhanced tumor growth by PPARγ agonists [38, 63, 64]. Similar contradictory data have been reported for PPARα,

whereby prolonged administration of PPARα agonists caused hepatocarcinogenesis in rats and mice [65]. The frequency of intestinal tumors also increased in PPARδ knockout mice [66, 67] or decreased upon treatment of the animals with PPARδ ligands [68]. These contradictory results between cellular and animal models and different animal models suggest that the function of these nuclear receptors is more complex than that has been assumed so far and may depend heavily on cell and tissue context, cross-talks with multiple signaling pathways and noncell autonomous mechanisms. A hint to this complexity is given by recent studies of the role of PPARs in tumor angiogenesis. In addition to cancer cell-autonomous effects, PPARs affect strongly tumor angiogenesis and inflammation, two processes that have a critical role in tumor pathogenesis and progression. PPARγ and PPARα agonists have anti-inflammatory properties, which may contribute greatly to their in vivo antitumor activity under certain circumstances. PPARγ ligands are also potent angiogenic inhibitors [69, 70] and PPARα agonists suppress VEGF production, endothelial cell proliferation, and tumor growth in mice [48, 49]. PPARδ activation stimulates VEGF production in mice, which at least in part had an autocrine prosurvival effect on cancer cells [71]. PPARδ has been recently identified as a critical node in a tumor angiogenic network linking angiogenesis to inflammation and carcinogenesis [72]. Knockout of PPARδ in host tissues but not in tumor cells reduced tumor growth by impairing angiogenesis [72]. Interestingly, the in vivo antitumor activity of PPARα agonists also depended heavily on the effects of host endothelial and stromal cells rather than cancer cells blocking angiogenesis and inflammation [48, 49]. Paradoxically, PPARα knockout impaired tumor growth in mice, because it resulted in a strong inflammatory response and production of anti-angiogenic factors, like TSP-1 and endostatin [73]. This paradoxical response is similar to the effects of PPARδ on inflammatory gene expression in macrophages, where both receptor activation and knockout suppressed expression of a subset of target genes [31, 36]. This dual mode of regulation of gene expression, whereby ligand- dependent and independent mechanisms lead to transrepression, derepression, or trans-activation of distinct subsets of genes, seems a common theme for these nuclear receptors and needs to be taken into account when examining their functions in physiological and pathological processes.

4. THE UBIQUITIN-PROTEASOME SYSTEM AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

4.1. *UPS and control of PPAR turnover*

Important factors to consider when studying the multiple and complex functions of PPARs are their connections with other cellular systems and how these interactions reciprocally impact on each system activity. Recent reports suggest that the activity of PPARs is linked in many ways to the UPS [28]. All three PPARs are short-lived proteins that undergo ubiquitination and proteosomal degradation and

the UPS is mainly responsible for the turnover of these nuclear receptors [28]. However, the three PPAR isotypes have different behaviors with respect to ligand-dependent receptor turnover. PPARγ undergoes negative autoregulation upon agonist binding. PPARγ is ubiquitinated and degraded by the proteasome in a negative feedback loop that probably serves to attenuate receptor-mediated gene transactivation [74]. PPARα turnover is controlled by ligands in a slightly different manner. Instead of enhancing ubiquitination and degradation, PPARα ligands prevent ubiquitination and lead to increased stability of the receptor [75]. The protective effect of the ligand, however, is maximal during the first 3 hours of exposure to the ligand and the receptor is then rapidly degraded [75].

We have recently examined the ligand-dependent turnover of PPARδ and the role of the UPS in this process [76]. Our study revealed interesting differences between PPARδ and other PPAR isotypes with respect to ligand-dependent receptor turnover and interaction with the UPS. We found that PPARδ, like other nuclear receptors, is ubiquitinated and rapidly degraded by the proteasome [76]. Brief incubation of cells expressing both endogenous and recombinant PPARδ with proteasome inhibitors led to rapid accumulation of the receptor in cell nuclei. Interestingly, in the presence of proteasome inhibitors, PPARδ was transcriptionally competent as shown by luciferase reporter assays and assessment of endogenous target genes by RT-PCR [76]. Thus, PPARδ was different from other nuclear receptors, including the estrogen, androgen, thyroid hormone, and retinoic acid receptors, whose transcriptional activity is reduced by proteasome inhibitors [26]. Furthermore, while in the absence of ligands PPARδ had a very short half life (~30 minutes), the addition of ligand increased considerably the receptor half life [76]. The effects of the synthetic and natural ligands were rapid with an increase of PPARδ protein level within 4 hours upon addition to the cell culture medium. The receptor level remained high as long as the ligands were present [76]. Removal of the ligands was followed by rapid reversal with return to the baseline level within few hours. Once again, PPARδ behavior was unique among nuclear receptors, whose turnover is generally accelerated by their own ligands [26, 77]. The progesterone receptor, thyroid receptor, estrogen receptor, RAR, and RXR all show ligand-dependent increase of degradation associated with transcriptional activation [26, 77]. The direct consequence of these events is a rapid decrease of the receptor half life and switching-off the transcriptional response. Only vitamin D3 receptor is known to be stabilized by the ligand with a similar kinetics [78]. As mentioned above, PPARγ is also rapidly degraded upon exposure to ligands [74] and PPARα is stabilized only transiently by ligands [75]. Further work demonstrated that ligand-induced stabilization of PPARδ was due to a selective block of receptor ubiquitination [76]. This ubiquitination block depended on the continuous presence of the ligand, was rapidly reversed after removal of the ligand, and was due to the direct interaction of the ligand with the receptor [76]. Disruption of the LBD in PPARδ/Tr1-299 abolished the effect of the ligand on ubiquitination and proteolysis, although the truncated form of the receptor
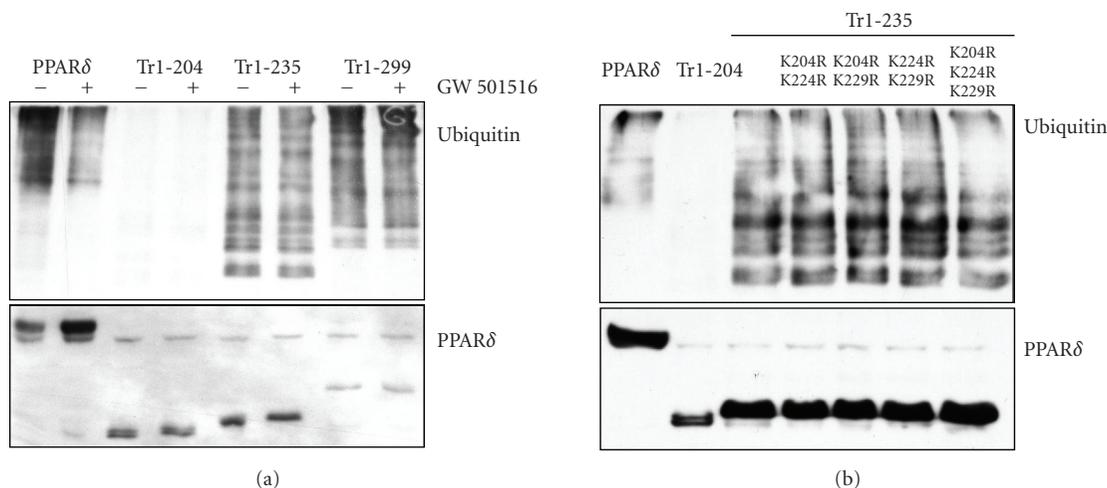
Figure 2: Ubiquitination of truncated and mutated forms of PPARδ. (a) U2OS cells were transfected with HA-ubiquitin expressing vector along with wild type His-PPARδ or truncated forms of the receptor (PPARδ/Tr1-204, Tr1-235 and Tr1-299). After 24 hours, cells were incubated overnight with vehicle or the PPARδ ligand GW501516 ($5\,\mu$M) and subsequently all samples were incubated with $10\,\mu$M the proteasome inhibitor PS341 for 4 hours. His-tagged wild type and truncated PPARδ were pulled-down with nickel affinity gel under denaturing conditions. PPARδ was detected in pull-down fractions using an anti-His antibody and ubiquitinated proteins with an anti-HA antibody. (b) U2OS cells were transfected with HA-ubiquitin vector along with the indicated PPARδ expressing vectors. PPARδ/Tr1-235 had wild type sequence or the indicated double or triple mutations (K204R, K224R and K229R). Cells were treated and analyzed as above.

was still ubiquitinated and degraded by the proteasome [76]. Thus, binding of the ligand to the LBD induced a conformational change that, in addition to allowing receptor trans-activation, blocked the interaction of PPARδ with an ubiquitin ligase or, alternatively, promoted binding of a deubiquitinating enzyme.

Using site-directed mutagenesis, we investigated further the role of distinct PPARδ domains in the ligand-dependent regulation of receptor turnover [76]. This analysis revealed additional differences between PPARδ and other PPAR isotypes. Mutations in the DBD of PPARδ reduced the effect of ligands on receptor ubiquitination [76]. This suggested that the ligand acted preferentially on the DNA-bound receptor preventing its ubiquitination. Interestingly, mutations in DBD of PPARγ did not affect ligand-dependent turnover, indicating that DNA binding was not a prerequisite for ligand-induced degradation of this receptor [74]. On the other hand, we showed that the AF-2 domain of PPARδ was not required for ligand-induced block of ubiquitination, indicating that the effect was independent of coactivator binding [76]. For most nuclear receptors, the transactivating function is linked to proteolytic degradation and mutations in the transactivating domain affect also receptor ubiquitination and proteolysis [77]. The AF-2 domain of PPARγ has a similar role and mediates ligand-induced degradation of the receptor [74]. For PPARγ and other nuclear receptors, conformational changes induced by the ligands may favor the concomitant interaction with coactivators and components of the UPS. Overexpression of transcriptional coactivators led also to a decrease of PPARα level in the presence of ligand, showing that the interaction with coactivators via the AF-2 domain promoted proteolysis of the α isotype [79]. Thus, for PPARα the initial stabilization is probably followed by

the recruitment of coactivators along with other factors that trigger proteolysis of the receptor. In contrast, in the case of PPARδ we showed that transactivation and receptor ubiquitination are functionally separated [76]. The absence of a link between these two processes allows independent control of receptor transactivation and ubiquitination upon ligand binding and may be a prerequisite to avoid rapid degradation and sustain its transcriptional activity once it is engaged in transcriptional activation complexes. Further analysis of PPARδ mutants indicates that the region between amino acid 204 and 235 may play a role in controlling ubiquitination and proteolytic degradation of the receptor (**Figure 1**). This region has a poor secondary structure, forms a loop exposed to the surface, and may be in an environment prone to ubiquitination [80, 81]. In addition, the region is quite diverse between the PPAR isotypes, possibly explaining the divergent responses in terms of ligand-dependent turnover. Pull-down experiments showed that the truncated PPARδ/Tr1-235 was ubiquitinated, while the shorter PPARδ/Tr1-204 was not (**Figure 2(a)**). Different scenarios can explain these results and are under consideration. The region between amino acid 204 and 235 may contain lysine residues that are the major sites of ubiquitination of PPARδ. However, mutations of the three lysines present in this region (K204R, K224R and K229R) did not affect ubiquitination of the PPARδ/Tr1-235 (**Figure 2(b)**). Thus, alternatively the region 204–235 may be needed for the binding of an ubiquitin ligase or cofactors that mediate the interaction of the receptor with the UPS.

Thus, even if the PPAR isotypes are structurally very similar, binding to specific ligands induces divergent responses as far as receptor turnover. PPARγ upon ligand binding becomes ubiquitinated and prone to degradation, whereas ligands prevent or delay ubiquitination and degradation of

PPARδ and PPARα. Most nuclear receptors exhibit negative autoregulation upon interaction with the respective ligands [26, 77]. Ligand-induced stabilization is a less common and has been observed only for very few nuclear receptors. The system in place for PPARδ may be geared to prevent both accumulation of high levels of the receptor and its prolonged activation [76]. Overactivity of PPARδ may be detrimental to cells, perhaps due to its antiapoptotic and potentially tumorigenic activity [32, 37]. The level of PPARδ is low and constantly controlled via UPS-dependent proteolysis, which may affect greatly the ligand-independent functions of the receptor like transrepression of other transcription factor target genes. Under physiological conditions, the low abundance and short half life of natural PPARδ ligands, like PGI$_2$, would contribute to keep the receptor in the unbound state [32]. In the presence of high concentrations of ligands, the DNA-bound and liganded PPARδ is protected from proteasomal degradation by the inhibition of its ubiquitination [76]. The stabilized DNA-bound receptor would be able to transactivate target genes as long as enough ligand is present. This would be consistent with the fact that in processes, such as wound healing, inflammation, and cancer, PPARδ levels seem to increase concomitantly with upregulation of cyclooxygenase-2 and other enzymes for the production of lipid metabolites capable of stabilizing and activating PPARδ [32, 37, 55, 58, 59]. In the absence of this coordinated increase of ligand and receptor levels, PPARδ might not be able to act as antiapoptotic and growth-promoting factor. How ligand-induced stabilization of PPARδ affects ligand-dependent interactions with other transcription factors leading to transrepression or derepression of gene expression is still unknown.

4.2. UPS, PPARs, and interactions with other signaling pathways

In addition to ligand-dependent receptor turnover, the UPS is an important way to control PPAR activity in response to upstream signal transduction pathways (Figure 3). Receptor phosphorylation by cellular kinases can regulate both basal and ligand-induced activity of PPARs as well as modulate their protein level by indirectly controlling proteasome-dependent degradation [82]. In colorectal cancer cells, the polypeptide hormone gastrin promotes cell proliferation and the effect is associated with decreased PPARγ level. This was mediated by phosphorylation of PPARγ involving the epidermal growth factor receptor and ERK1/2 kinase leading to increased PPARγ proteasome-mediated degradation [83]. In fat cells IFN-γ treatment induces a rapid reduction of PPARγ protein level, which is blocked by proteasome inhibitors [84]. On the other hand, there are instances in which PPARs enhance stabilization or degradation of proteins by affecting their susceptibility to UPS-mediated degradation. Perhaps the best example of a signaling pathway in which both PPARs and the UPS are implicated is the Wnt pathway. Suppression of the canonical Wnt signaling is required for differentiation of preadipocytes into adipocytes. The process is in part mediated by PPARγ-induced degradation of β-catenin, which is a central element in the Wnt pathway. Activation of PPARγ
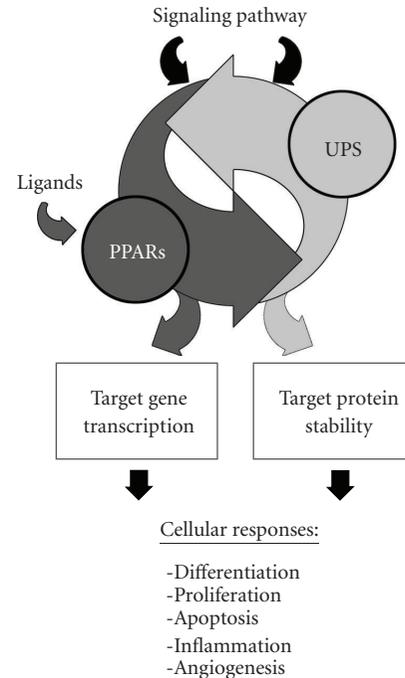

Figure 3: *Interactions between PPARs and the ubiquitin-proteasome system (UPS) affect multiple cellular pathways.* The UPS regulates activity of PPARs by controlling receptor turnover in ligand dependent and independent manners and affecting the ability of PPARs to regulate target gene transcription. Signaling pathways can modulate PPAR activity by affecting UPS-mediated turnover (e.g., increased PPARγ degradation in response to growth factors or hormones). PPAR can also affect biological pathways and cellular responses by increasing or decreasing susceptibility of proteins to proteasomal degradation (e.g., enhanced degradation of β-catenin and suppression of the Wnt pathway by PPARγ).

promotes degradation of β-catenin in glycogen synthase kinase 3β (GSK3B)-dependent or independent manner [85, 86]. β-catenin mutations that inhibit degradation block expression of a subset of adipogenic and PPARγ target genes [85]. PPARγ-dependent degradation of β-catenin requires an active APC-containing destruction-complex. Mutations of the T cell factor/lymphocyte enhancer factor (TCF/LEF) binding domain of β-catenin or of a catenin-binding domain (CBD) within PPARγ block proteasomal degradation of β-catenin [87]. The interaction between β-catenin and PPARγ affect their respective oncogenic and tumor suppressor function [87]. A functional APC was found to be required also for PPARγ-mediated suppression of colon carcinogenesis [88]. Activation of PPARγ induces degradation of cyclin D1, which has a critical role in cell cycle regulation, along with β-catenin in hepatocytes [89]. Reduced cyclin D1 protein level was observed also in breast cancer cells upon PPARγ activation by selective ligands and cyclin D1 downregulation was blocked by inhibition of the proteasome [90]. However, the ability of thiazolidinedione ligands to reduce β-catenin and cyclin D1 levels might be in part PPARγ-independent and determined by direct effects of these compounds on protein degradation [91, 92]. Beside the induction of

proteosomal degradation, activation of PPARγ has been shown to increase the level of proteins by blocking their proteolysis. Activation of PPARγ in human hepatocarcinoma cells inhibits proteosomal degradation of p27, a cyclin-dependent kinase inhibitor, with consequent inhibition of cell proliferation [93]. Similarly, PPARγ inhibits claudin 4 degradation resulting in urothelial cell differentiation [94]. In both cases, the increased protein level is probably due to reduced ubiquitination. Interestingly, transcriptome analysis of ovarian cancer cells exposed to a PPARγ agonist revealed that PPARγ activation resulted in upregulation of several genes involved in protein modification and ubiquitination, including many ubiquitin ligases and ubiquitin-conjugating enzymes [95]. This finding may provide a plausible explanation for the broad effects that PPAR-γ agonists have on protein ubiquitination and turnover and clearly deserves further investigation [95].

PPARα agonists also enhance protein degradation. In LPS-treated macrophages PPARα agonists enhance degradation of inducible nitric oxide synthase (iNOS), reducing nitric oxide (NO) production, which is an important mediator in inflammatory processes. PPARα agonists did not affect iNOS expression and proteasome inhibitors reversed the effect on iNOS protein levels, indicating that PPARα agonists enhanced degradation of this protein by the proteasome [96]. PPARδ has been found to regulate ubiquitin C expression and this has been linked to the modulation of protein kinase Cα (PKCα) and attenuation of cell proliferation in the skin. The level of PKCα was lower in the skin of PPARδ wild-type mice treated with TPA compared to the skin of PPARδ-null mice [97]. On the other hand, the amount of ubiquitinated PKCα was lower in skin of TPA-treated PPARδ-null mice compared to wild-type mice and inhibition of the proteasome prevented TPA-induced downregulation of PKCα. Thus, the effects of PPARδ on cell proliferation in the skin could be due to ubiquitin-dependent turnover of PKCα that in turn modulated the activity of the PKCα-dependent pathways [97].

Finally, the UPS is involved in the reciprocal regulation of PPARs and other transcription factors. Activation of NF-κB is achieved when the inhibitor IκB, which normally holds NF-κB in the cytoplasm, is phosphorylated and recognized by the E3-β-transducin repeat containing protein (β-TRCP). Ubiquitinated IκB is degraded by the proteasome, allowing NF-κB to translocate to the nucleus and induce gene transcription [98]. NF-κB has a critical role in inflammation. In experimental rat models of autoimmune myocarditis stabilization and translocation of NF-κB were inhibited by PPARγ-dependent expression of IκB [99]. Likewise, PPARα activation induced IκB in aortic smooth muscle cells and in human hepatocytes [100]. The transcription factor AP-1, which is another key player in inflammation, interacts with the PPARs and may be regulated in a similar combinatorial manner by PPARs and the UPS [33, 34].

5. CONCLUSIONS

Here, we have presented the current evidence linking PPARs and the UPS. Ubiquitination and proteasomal degradation control the level and modulate the activity of PPARs in many ways. Ligand binding and proteolytic degradation affect turnover and transcriptional activity of the PPAR isotypes in distinct ways. PPARδ ubiquitination is selectively blocked by agonist ligands ensuring the accumulation of DNA-bound receptor engaged in transcriptional activation complexes. The opposite is true for the other PPAR isotypes. Distinct cellular pathways can exploit the UPS to modulate PPAR turnover and activity affecting their multiple functions. Furthermore, PPARs can control the level of specific proteins by modulating the activity of the UPS. This could be mediated by their ability to control the expression of components of the UPS, like ubiquitin ligases, or via protein-protein interactions. Controlling turnover of the receptors, the UPS can affect also the ligand-independent functions of PPARs. In this context, the control operated by the UPS on nuclear receptor levels might affect their ability to modulate activity of other transcriptional regulators. Increased proteolysis might reduce PPAR levels and produce apparently paradoxical responses with derepression or transrepression of distinct subsets of genes as seen in certain PPAR knockout experiments. The contribution of the multiple interactions between PPARs and the UPS need to be taken in consideration when examining the effects of PPAR overexpression, knock down or ligand-dependent activation on complex biological processes, like inflammation, angiogenesis, and tumorigenesis.

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Review Article

Activation and Molecular Targets of Peroxisome Proliferator-Activated Receptor-γ Ligands in Lung Cancer

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Lung cancer is the leading cause of cancer death, and five-year survival remains poor, raising the urgency for new treatment strategies. Activation of PPARγ represents a potential target for both the treatment and prevention of lung cancer. Numerous studies have examined the effect of thiazolidinediones such as rosiglitazone and pioglitazone on lung cancer cells in vitro and in xenograft models. These studies indicate that activation of PPARγ inhibits cancer cell proliferation as well as invasiveness and metastasis. While activation of PPARγ can occur by direct binding of pharmacological ligands to the molecule, emerging data indicate that PPARγ activation can occur through engagement of other signal transduction pathways, including Wnt signaling and prostaglandin production. Data, both from preclinical models and retrospective clinical studies, indicate that activation of PPARγ may represent an attractive chemopreventive strategy. This article reviews the existing biological and mechanistic experiments focusing on the role of PPARγ in lung cancer, focusing specifically on nonsmall cell lung cancer.

1. INTRODUCTION

Lung cancer is the leading cause of cancer death for both men and women in the USA. In fact, more deaths will occur this year due to lung cancer than breast, prostate, and colorectal cancers combined [1]. In spite of intensive research, 5-year survival in patients with lung cancer remains dismally low, with overall survival at 15% [2]. A major reason for this problem is the presence of metastasis at the time of diagnosis. While smoking cessation will clearly reduce the risk of lung cancer, a majority of diagnosed cases are being detected in exsmokers [3]. Therefore, in addition to new chemotherapeutic approaches, there appears to be a critical need for chemopreventive strategies which can be administered to patients at risk for developing lung cancer. In this article, we will review recent data, both from basic sciences experiments and from clinical studies indicating that activation of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) may represent a novel strategy for the treatment and prevention of lung cancer.

2. BIOLOGY OF LUNG CANCER

Lung cancers are categorized as small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC). As a group, the NSCLC constitute the bulk of lung cancers and are subdivided into squamous, adenocarcinoma, and large cell carcinoma phenotypes. Selective changes in specific oncogenes can be used to distinguish the two types of cancer. Activating mutations in ras are associated with NSCLC, with a mutation at codon 12 of the Ki-Ras gene observed in approximately 30% of adenocarcinomas, and just under 10% of other NSCLC types [4]. These mutations appear to be virtually absent from SCLC [5]. In mice, Ki-ras mutations are found in over 90% of spontaneous and chemically induced lung tumors [6]. Overexpression of the c-myc gene is also frequently observed in NSCLC, but appears to be more prevalent in SCLC [7]. Elevated expression of the HER-2/neu gene, a member of the epidermal growth factor receptor family has also been observed in 35% of adenocarcinomas and a slightly lower percentage of

squamous carcinomas [8]. Alterations in tumor suppressor genes have also been reported. Mutations in p53 have been detected in 90% of SCLC and 50% of NSCLC [7]. Mutations in the retinoblastoma gene are more specific for SCLC, occurring in more than 90%, while only a small fraction of NSCLC have mutations in this gene. Recently, mRNA expression profiling has been used to define subclasses of lung adenocarcinoma, which can be defined by distinct patterns of gene expression [9, 10]. These studies suggest that NSCLC may in fact represent multiple diseases characterized by distinct molecular pathways. In contrast to most NSCLC, SCLC displays neuroendocrine features exemplified by the presence of cytoplasmic neurosecretory granules containing a wide variety of mitogenic neuropeptides including gastrin-releasing peptide, arginine vasopressin, neurotensin, cholecystokinin, and many others [11, 12]. Significantly, SCLC also expresses G protein-coupled receptors (GPCR) for these neuropeptides, thereby establishing autocrine-stimulated cell growth. Therapeutic strategies have targeted these neuropeptides using inhibitors of GPCRs. However, the existence of potentially redundant loops mediated by multiple neuropeptides has limited the usefulness of this strategy.

Recently, a great deal of attention has been focused on the EGF receptor, and the use of selective inhibitors of the EGF receptor tyrosine kinase (EGFR-TKI). These agents (gefitinib and erlotinib) have shown therapeutic efficacy in a subset of NSCLC patients which have somatic mutations in this receptor [13, 14]. However, responses have also been observed in patients with wild-type EGFR. Identifying strategies which would sensitize patients to EGFR-TKI therapy is under active investigation (see [15] for review).

3. PPARγ ACTIVATION

PPARγ is a member of nuclear receptor superfamily. Two major isoforms have been described, PPARγ1 and PPARγ2 (see [16] for review). These are splice variants, with PPARγ2 being expressed predominantly in adipose tissue, whereas PPARγ1 has a more widespread distribution, and is expressed in cancer cells, including lung cancer [16]. More recently a number of additional splice variants have been identified [17]. The role of these forms of PPARγ remains to be established. The structure of PPARγ is similar to that of most nuclear receptors; the core of the molecule consists of a DNA-binding region (DBD) and a ligand-binding region (LBD), separated by a hinge region. There are two activation domains, AF-1 at the amino terminal and AF-2 at the carboxyl terminal. The classic pathway of PPARγ activation involves binding as a heterodimer with the retinoic acid X receptor to specific DNA sequences (PPAR-RE). The consensus PPAR site consists of a direct repeat of the sequence AGGTCA, separated by a single nucleotide, designated a DR-1 site. Ligand binding to the LBD causes a conformational change, which results in the release of corepressors and the binding of coactivators, resulting in increased transcription of target genes.

PPARγ is activated by polyunsaturated fatty acids and eicosanoids. In particular, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (dPGJ$_2$) has been shown to specifically activate PPARγ with micromolar affinity [18]. Lipoxygenase products of linoleic acid, 9- and 13-HODE have micromolar affinities for PPARγ [19]. It is not clear whether any of these agents are actual physiologic regulators of PPARγ, and a recent study has found that endogenous levels of dPGJ$_2$ do not change during adipocyte differentiation [20]. Synthetic activators of PPARγ include the thiazolidinediones, such as rosiglitazone and pioglitazone [21]. These compounds have insulin-sensitizing and antidiabetic activity, which is likely mediated at least in part through PPARγ activation. Finally, NSAIDs, which inhibit eicosanoid production, activate PPARγ albeit at higher concentrations than required for COX inhibition [22]. While all of these agents can activate PPARγ, it is clear that they also stimulate "off-target" pathways which may impact their therapeutic potency [23]. Finally, it should be noted that PPARγ can directly bind to other transcription factors, including NF-κB and Sp1 [24]. This mechanism of action complicates the spectrum of genes that could be regulated by PPARγ by engaging regulatory elements distinct from classic PPAR-RE sites [25].

4. CLINICAL ASSOCIATIONS WITH PPARγ IN LUNG CANCER

Analysis of human lung tumors has reported that decreased expression of PPARγ is correlated with a poor prognosis [26]. Further work indicated that expression of PPARγ as detected by immunohistochemistry was more frequently detected in well-differentiated adenocarcinomas, compared to poorly differentiated ones. Recently, a retrospective study demonstrated a 33% reduction in lung cancer risk in diabetic patients using the TZD rosiglitazone [27]. An even more dramatic reduction was observed in African-American patients (75%). This decreased risk appeared to be specific for lung cancer, and no protective effect was observed for prostate or colon cancer. Genetic variants in the PPARγ gene have been identified which are associated with a decreased risk for lung cancer [28]. These findings suggest that chemoprevention strategies using PPARγ activators may be an attractive approach in patients at risk for lung cancer, and that polymorphisms in the PPARγ gene may be a way to screen those patients. There are several chemoprevention trials being initiated using TZDs. However, a concern in these studies is the association of higher rates of adverse cardiac events with chronic TZD treatment, especially with rosiglitazone [29]. As discussed below, agents which target PPARγ through alternative pathways may therefore represent novel therapeutic targets.

5. BIOLOGICAL EFFECTS OF PPARγ IN LUNG CANCER CELLS

A number of studies have examined the effects of TZDs on the growth of lung cancer cells. The majority of these studies have focused on NSCLC. Administration of TZDs has been shown to inhibit growth and induce apoptosis in numerous NSCLC cell lines [30–34]. While the mechanisms for these effects are not completely understood, they appear to be
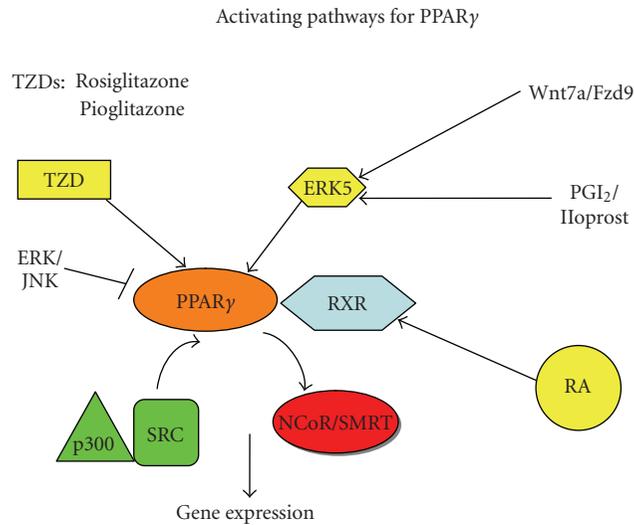
FIGURE 1: *Activation pathways for PPARγ*. PPARγ forms a heterodimer with the retinoic acid X receptor (RXR). Activation can occur by thiazolidinidiones (TZD) such as rosiglitazone or pioglitazone directly binding to the ligand-binding domain. This results in the dissociation of corepressors such as NCor and SMRT, and the binding of coactivators such as p300 and Src, mediating activation of transcription. In lung cancer cells, binding of Wnt7a to its cognate receptor Fzd9 leads to activation of ERK5, which presumably directly binds to the hinge region of PPARγ mediating activation. Prostacyclin (PGI) and analogs such as iloprost can also lead to PPARγ activation, and this may involve ERK5 activation. Conversely, activation of the ERK or JNK family of MAP kinases can inhibit PPARγ activation; this is mediated through direct phosphorylation of the molecule which alters the ligand binding affinity. Finally, activation of PPARγ/RXR heterodimers may be activated through retinoic acid (RA) binding to RXR.

mediated through both PPARγ-dependent and independent effects. Induction of apoptosis may involve the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in some cancer cell lines [35]; these effects appear to be mediated through PPARγ-independent pathways. Recent studies have also demonstrated that PPARγ activation induces proline oxidase, which will result in increased production of cytotoxic reactive oxygen species (ROS) [36]. Growth arrest may be mediated through induction of the cyclin kinase inhibitor p21 [37]. In this case, the mechanism of action involves PPARγ-dependent induction of p21 through interactions with other transcription factors. Several studies, including work from our own laboratory have demonstrated that activation of PPARγ leads to promotion of a more highly differentiated phenotype in NSCLC [32, 38]. This can be assessed by growing cells in 3-dimensional tissue culture, which has been shown to reveal epithelial features. E-cadherin is perhaps to most widely studied marker of epithelial differentiation, and both pharmacological PPARγ activators and molecular overexpression of PPARγ had shown increased protein and mRNA for E-cadherin. Epithelial mesenchymal transition has been associated with cancer progression and metastasis [39]. While this is still somewhat of a controversial area [40], activation of PPARγ in lung cancer cells appears to inhibit invasiveness, at least in part through inhibiting or reversing EMT.

It has become evident during the past several years, that while genetic changes in cancer cells are critical for tumor initiation, progression and metastasis entail a critical contribution from the tumor microenvironment [41]. Specifically, interactions of tumor cells with vascular cells, innate immune cells, and fibroblasts control tumor angiogenesis and promote a more aggressive phenotype. These cell-cell interactions are mediated through cytokines and growth factors initially produced by the tumor cells which recruit stromal cells. Among these cytokines are factors such as MCP-1 and CCL5, critical for macrophage recruitment, and VEGF and other proangiogenic cytokines such as IL-8 which recruit vascular cells [42]. Transcriptional control of these factors is mediated by multiple transcription factors, but specifically, it has been shown that two specific factors, NF-κB and HIF-1, are critical for many of these molecules. Several studies have demonstrated that PPARγ activation can inhibit activation of NF-κB in NSCLC [43, 44]. While effects on HIF-1 have not been documented in lung cancer cells, PPARγ has been shown to inhibit HIF-1 in other systems [45]. These data indicate that activation of PPARγ may disrupt communication between cancer cells and the surrounding tumor microenvironment, thus blocking progression and metastasis, distinct from antiproliferative effects on the tumor cells. In lung cancer, where metastasis has often occurred at the time of diagnosis, agents, which specifically target tumor-stromal interactions, represent a novel therapeutic approach.

6. UPSTREAM ACTIVATION OF PPARγ

While TZDs have received most of the attention as PPARγ activators, it is becoming apparent that activation of PPARγ can occur as a consequence of activation of other signaling pathways (see Figure 1). Phosphorylation by the ERK members of the MAP kinase family has been shown to decrease

PPARγ activity, likely through altering the affinity for ligand binding [46]. Work in endothelial cells has demonstrated that flow-mediated activation of ERK5, a member of the MAP kinase family, results in activation of PPARγ [47], which may mediate anti-inflammatory effects associated with laminar flow. In this case, the mechanism of activation involves direct binding of ERK5 to the hinge region of PPARγ. In lung cancer, our studies have focused on the role of the Wnt signaling pathway. While canonical Wnt signaling has been implicated as promoting colon carcinogenesis, the role of the Wnt pathway in nonsmall cell lung cancer appears to be more complex. Our studies have demonstrated that Wnt7a signaling through its receptor Fzd9 inhibits transformed growth of NSCLC cell lines [48]. Further studies indicated that this pathway leads to increased PPARγ activity through activation of ERK5, and that this increase in PPARγ activity mediated the antitumorigenic effects of Wnt7a/Fzd9 signaling [49].

A connection has also been made between prostacyclin and activation of PPARγ. Prostaglandin I_2 (PGI$_2$, prostacyclin), produced through the cyclooxygenase pathway via prostacyclin synthase (PGIS), is a bioactive lipid with anti-inflammatory, antiproliferative, and potent antimetastatic properties [50, 51]. Our laboratory has shown that transgenic mice with selective pulmonary PGI$_2$ synthase (PGIS) overexpression exhibited significantly reduced lung tumor multiplicity and incidence in response to either chemical carcinogens or exposure to tobacco smoke [52, 53], suggesting that manipulation of the arachidonic acid pathway downstream from COX is a target for lung cancer prevention. IIoprost, a long-lasting prostacyclin analog, also inhibits lung tumorigenesis in wild-type mice. PGI$_2$ can signal through a specific cell surface receptor, designated IP, which is a member of the G-protein coupled receptor family, and signals through increases in cAMP [54]. However, PGI$_2$ has been shown to signal through activation of PPARs, with reports of both PPARγ [55] and PPARδ activation [56, 57]. To define the downstream effector of PGI$_2$ in the chemoprevention of lung cancer, studies were performed in which mice overexpressing PGIS were crossed with mice deficient in IP (A. M. Meyer et al., unpublished observations). In a chemical carcinogenesis model, lack of IP did not affect protection against lung tumorigenesis mediated by PGIS overexpression, suggesting IP-independent pathways. Further study is required to whether prostacyclin can activate PPARγ in vivo, and whether this effect is mediated through IP or represents a direct, IP-independent activation.

To test the role of PPARγ in chemoprevention of lung cancer, we have developed transgenic mice overexpressing PPARγ under the control of the surfactant protein C promoter, which targets expression to the distal lung epithelium. In a chemical carcinogenesis model, these mice showed a marked protection against developing lung tumors [44]. While the connection between prostacyclin analogs and PPARγ activation needs to be more precisely defined, from a therapeutic standpoint, the ability to activate PPARγ through non-TZD mechanisms represents an attractive strategy that may avoid some of the deleterious effects seen with TZD administration.

7. MECHANISMS OF PPARγ ACTION IN LUNG CANCER CELLS

In spite of intensive study examining the biological effects of PPARγ activation in lung cancer, much less is know regarding the direct targets of PPARγ (see Figure 2). As a member of the nuclear receptor superfamily, PPARγ is a ligand-activated transcription factor. Thus, one assumes that there are direct transcriptional targets, where PPARγ, in combination with the RXR receptor, binds to regulatory elements and induced transcription. These targets have been difficult to identify in cancer cells. In fact, most of the responses that have been demonstrated involve suppression of target genes (e.g., cytokines). While PPARγ has been shown to upregulate E-cadherin in NSCLC, there are no studies demonstrating direct binding of PPARγ to the E-cadherin promoter. A family of transcription factors have been identified which act as suppressors of E-cadherin expression. Members of this family include Snail1, Snail2 (Slug), ZEB1, and Twist [58, 59] are potent inducers of EMT. Both Snail and Twist appear to play critical roles in breast cancer metastasis [60, 61]. Overexpression of ZEB-1 has been implicated in mediating EMT in NSCLC cells [62].

Several studies have reported increased expression of the protein and lipid phosphatase PTEN in response to PPARγ activation [63, 64]. Increased expression/activity of PTEN would be anticipated to inhibit signaling through PI-3 kinase/Akt, and downstream effectors such as mTOR. Decreased activation of Akt could lead to inhibition of NF-κB signaling [65–67], although the molecular mechanisms are not well defined.

Elevated expression of cyclooxygenase-2 (COX-2) is common in NSCLC, and mediates increased production of PGE$_2$ [68]. Activation of PPARγ has been shown in inhibit COX-2 expression and decrease PGE$_2$ production in NSCLC [44, 69]. While the mechanisms whereby PGE$_2$ contributes to growth and progression of NSCLC are not completely understood, recent data in colon cancer have shown that PGE$_2$ acting through its cell surface receptor can engage β-catenin signaling, leading to proliferation [70]. Consistent with such a model, TZDs also inhibit expression of the EP2 receptor, which couples to β-catenin signaling [71]. Regulation of PGE$_2$ production by TZDs can also occur through PPARγ-independent pathways. Both rosiglitazone and pioglitazone can directly activate 15 hydroxyprostaglandin dehydrogenase, promoting breakdown of PGE$_2$.

8. CONCLUSIONS AND FUTURE DIRECTIONS

Activation of PPARγ appears to inhibit lung tumorigenesis at several different stages. Animal studies indicate that increased PPARγ may be chemopreventive against developing lung tumors, suggesting that it can block the early stages of epithelial transformation. In established lung cancer, activation of PPARγ can inhibit proliferation, induce apoptosis, and promote a less invasive phenotype through promoting epithelial differentiation, and perhaps blocking EMT. Finally, through disruption of tumor-stromal communication via inhibition of chemokine production, PPARγ can negatively
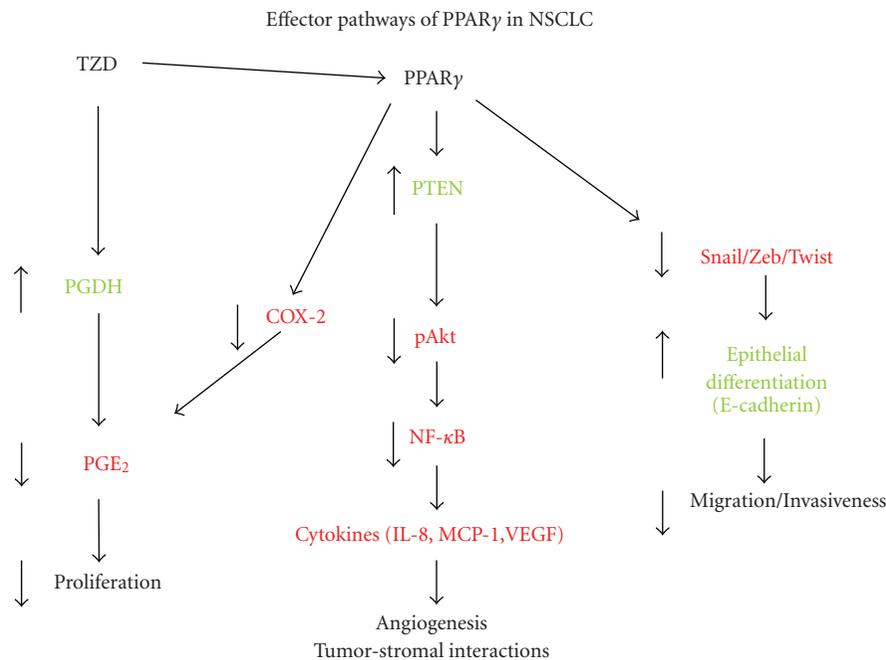
FIGURE 2: *Effector pathways for PPARγ in NSCLC*. PPARγ can increase either expression of enzymatic activity of PTEN. This results in inhibition of Akt activation (pAkt), which may be involved in the growth inhibitory responses seen with PPARγ activation. Decreased Akt activity also can lead to decreased activity of the transcription factor NF-κB. NF-κB is a critical transcription factor in the production of proangiogenic and proinflammatory cytokines such as VEGF, IL-8. Decreased production of these factors would be expected to inhibit recruitment of inflammatory cells such as macrophages, and block tumor angiogenesis. PPARγ-mediated suppression of members of the Snail family of transcription factors, such as Snail, Zeb, or Twist, would lead to derepression of E-cadherin expression and promote the epithelial phenotype, leading to decreased migration and invasiveness. PPARγ-mediated suppression of COX-2 expression in NSCLC has been shown by several investigators. This would result in decreased PGE$_2$ production, which will impact growth. TZDs can inhibit PGE$_2$ production through a PPARγ-independent pathway involving induction of 15-hydroxyprostaglandin dehydrogenase (PGDH). Pathways indicated in green are increased or activated by PPAR, while those in red represent pathways that are inhibited or repressed.

impact tumor progression and metastasis. These data make PPARγ activators attractive agents for the treatment and prevention of lung cancer.

However, a number of significant issues remain to be resolved. In many of the studies described in this article, it is not clear if the biological responses are mediated through "on-target" activation of PPARγ, or through other "off-target" effects. A strategy to address this issue is the use of molecular approaches, either overexpressing or silencing PPARγ in cancer cells to complement studies with pharmacological agents. Genetic mouse models using targeted knockouts of PPARγ in either cancer cells or stromal compartments will also be informative. This strategy also applies to defining the mechanisms mediating the adverse cardiovascular events reported in patients taking TZDs. Defining the molecular targets of TZDs mediating a specific response will be critical in the further development of second-generation PPARγ drugs. If adverse cardiac events are mediated through "off-target" effects, then a more selective PPARγ activator would be therapeutically effective, without leading to adverse cardiac events. Alternatively, if the antitumorigenic effects of TZDs are mediated through "off-target" effectors, then identifying these pathways would lead to novel therapeutic targets. Finally, the majority of studies have focused on NSCLC. Studies defining mechanisms of

activation and downstream targets in SCLC are needed to determine if PPARγ represents a therapeutic target for treating these forms of lung cancer.

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Review Article

The Role of the PAX8/PPARγ Fusion Oncogene in Thyroid Cancer

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Thyroid cancer is uncommon and exhibits relatively low mortality rates. However, a subset of patients experience inexorable growth, metastatic spread, and mortality. Unfortunately, for these patients, there have been few significant advances in treatment during the last 50 years. While substantial advances have been made in recent years about the molecular genetic events underlying papillary thyroid cancer, the more aggressive follicular thyroid cancer remains poorly understood. The recent discovery of the PAX8/PPARγ translocation in follicular thyroid carcinoma has promoted progress in the role of PPARγ as a tumor suppressor and potential therapeutic target. The PAX8/PPARγ fusion gene appears to be an oncogene. It is most often expressed in follicular carcinomas and exerts a dominant-negative effect on wild-type PPARγ, and stimulates transcription of PAX8-responsive promoters. PPARγ agonists have shown promising results in vitro, although very few studies have been conducted to assess the clinical impact of these agents.

1. INTRODUCTION

Thyroid cancer is the most frequent endocrine malignancy. While relatively uncommon, the American Cancer Society estimates that there will be over 37 000 new cases in 2008 in the United States [1]. The majority of thyroid cancers are well-differentiated malignancies originating from the thyroid follicular cells. Papillary thyroid carcinomas (PTCs) are the most frequent histotype, particularly in iodine-sufficient areas, while follicular thyroid carcinoma (FTC) and Hürthle cell carcinomas (HCCs) together represent around 20% of thyroid cancer. However, these latter thyroid cancer types are often more aggressive, more advanced at the time of diagnosis, less responsive to traditional therapy, and more likely to cause both morbidity and mortality. FTC share similar cytologic features with follicular adenomas (FAs) and are distinguished only by the presence of invasion beyond the tumor capsule or into blood vessels on pathology. This common histology suggests an adenoma to carcinoma sequence, with adenomas representing a premalignant lesion, though this remains unproven.

However, the peroxisome proliferator-activated receptors (PPARs), including α, β, δ, and γ subtypes, are part of the ubiquitous nuclear hormone receptor superfamily that has been the focus of considerable research over the last two decades. PPARγ is best known in the endocrine world for its role in adipogenesis and insulin sensitization. However, it also plays a role in cell cycle control, inflammation, atherosclerosis, apoptosis, and carcinogenesis through its influence on gene expression involving multiple cell signaling pathways [2]. Research on PPARγ as a potential thyroid proto-oncogene was accelerated by the discovery of a chromosomal translocation involving the PPARγ-1 gene in a subset of follicular carcinomas [3]. Since that time, considerable effort has gone into further clarifying the possible effect of PPARγ in several cancers, including FTC.

2. PPARγ AND THYROID CANCER

Chromosome analysis and microsatellite mapping techniques have revealed a number of chromosomal changes associated with thyroid carcinogenesis, and pointed to specific loci that might harbor oncogenes or tumor suppressor genes, including a region spanning the fragile site on chromosome 3p14, through 3pter that is often disrupted in follicular thyroid cancers [4]. Jenkins et al. had already described a somatic mutation involving 3p25-p21 region in three cases of follicular thyroid cancer [5, 6], and there followed a number of reports of balanced translocations or aneusomy involving this chromosomal region in FTC, HCC, PTC, and anaplastic thyroid cancers (ATCs) [7–11]. A specific translocation, t(2;3)(q13;p25), was described in a case of aggressive FTC associated with bone metastases [11], though it was also seen in some FAs [10]. The importance of this chromosomal rearrangement and the mechanism of its oncogenic activity remained unclear until Kroll et al. mapped the involved 3p25 locus in a number of FTC to the PPARγ gene in 2000 [3]. Thus these data were the first to convincingly link the PPARγ gene to thyroid cancer. Altered PPARγ activity has subsequently been shown to have a potential role in several types of thyroid cancer.

Several reports of ATC, FTC, and some PTC cell lines have demonstrated PPARγ mRNA expression by reverse-transcription polymerase chain reaction (RT-PCR), without identifiable PPARγ mutations or translocations [12–15]. In these PPARγ-positive cell lines, PPARγ ligands (troglitazone [15, 16], ciglitazone [12, 14, 17], rosiglitazone [12], prostaglandin J2 [13, 15, 17], and RS1303 [13]) inhibit growth of cells, while no change in growth is seen in PPARγ-negative cell lines. Growth suppression was dose-dependent [13, 16, 17], and one study found a correlation between PPARγ expression and response to PPARγ ligands [13], though another did not [16]. Levels of Bax protein and c-myc, both apoptosis-related proteins, were increased in a dose-dependent fashion by treatment with PPARγ ligand [13, 15], although one study found increased levels of a different apoptotic protein, Bcl-2, rather than of Bax [12]. Inhibition of cell invasion [13], attachment [12, 17], and anchorage-independent growth on soft agar [12], all features of malignancy, are also seen with PPARγ ligand treatment in thyroid cancer cell lines. Finally, evidence of cell death, with decreased viable cell numbers and increased rates of necrosis and apoptosis, have been reported by some groups following PPARγ agonist treatment [12, 17]. This reduction in cell viability may be inhibited by coincubation with GW9662, a selective PPARγ antagonist, suggesting that the described changes in cell growth and survival were truly PPARγ-dependent [12].

Addition of PPARγ agonist to PPARγ-positive cells lines led to an increased portion of cells in G_0/G_1 with a reduction of cells in G_2/M and S phase, consistent with decreased cell proliferation [12]. DNA synthesis appeared to be slowed with decreased ^{3}H-thymidine incorporation in these cells, while expression of the cell-cycle progression inhibitors p21^{cip1} and p27^{kip1} were increased [12]. Overexpression of the PPARγ gene by transfection into PPARγ-positive or

-negative cell lines similarly decreased colony formation and triggered nuclear condensation, fragmentation of chromatin and apoptosis, with G_0/G_1 cell cycle arrest [12, 14–16]. Together, these data provide strong support that PPARγ has a tumor suppressive effect in thyroid follicular cells, which is consistent with results in other nonthyroid cell lines [18–23].

One of the few available animal models of follicular thyroid carcinoma was created by generating homozygous mutations in the thyroid receptor gene TRβ, a mutation initially described in a patient with thyroid hormone resistance syndrome [24] and referred to as the PV/PV mutant. Homozygous TR$\beta^{PV/PV}$ mice develop follicular thyroid cancer with predictable progression from thyroid hyperplasia to capsular and vascular invasion, more extensive soft tissue invasion and ultimately lung metastasis at an early age [25]. Analysis of gene activity by cDNA microarray analysis in TR$\beta^{PV/PV}$ mice demonstrates altered regulation of several genes compared to wild-type siblings. Many of these genes are implicated in tumor-formation, metastasis, invasion, cell cycle control and apoptosis. PPARγ-mediated pathways, however, are downregulated in these mice, hinting at a role for PPARγ inhibition in this thyroid cancer model [26].

Early in the histologic progression of thyroid disease in this TR$\beta^{PV/PV}$ mouse model expression of PPARγ mRNA, assessed by Northern blot analysis, was diminished approximately 50% compared to wild-type siblings. Furthermore, not only was PPARγ activity suppressed, but the mRNA activity in mutant mice did not increase with age as was seen in their wild-type counterparts [27]. Data from humans has also implicated a functional downregulation of PPARγ expression in human thyroid tumors that did not carry PPARγ translocations, in studies using semiquantitative PCR, real-time RT-PCR, and microarray analyses [28, 29]. Furthermore, tumors with reduced PPARγ expression showed an increased incidence of distant metastases, local invasion, and areas of poor differentiation [28]. These findings suggest that downregulation of wild-type PPARγ may be a key event in thyroid carcinogenesis.

Homozygous PPAR$\gamma^{+/-}$ mice, incorporating a loss of function mutation in one allele of the PPARγ gene, have been used as an animal model to evaluate the molecular genetic events ultimately leading to carcinogenesis in colon, breast, and ovarian tumors [30, 31]. Deletion of both alleles of the PPARγ gene, however, is universally lethal to embryos. In thyroid cancer, the TR$\beta^{PV/PV}$ mouse model was used to further elucidate the mechanism of PPARγ tumorigenesis. TR$\beta^{PV/PV}$ mice were crossed with PPAR$\gamma^{+/-}$ mice to obtain TR$\beta^{PV/PV}$PPAR$\gamma^{+/-}$ offspring. In these mice, PPARγ mRNA and lipoprotein lipase (LpL) expression were reduced compared to wild-type mice. LpL is a downstream target gene for PPARγ, thereby confirming diminished PPARγ action. Furthermore, LpL mRNA and protein expression were further reduced in the mutant TR$\beta^{PV/PV}$PPAR$\gamma^{+/-}$ mice compared to both wild-type and TR$\beta^{+/+}$PPAR$\gamma^{+/-}$ mice [32].

Western blot analysis demonstrated activation of the NFκB pathway in these TR$\beta^{PV/PV}$PPAR$\gamma^{+/-}$ mice [32], which is consistent with data in the PPAR$\gamma^{+/-}$ mouse model [33]. Expression of cyclin D1, a cell cycle regulator important in the progression from G_1 to S phase and a downstream target
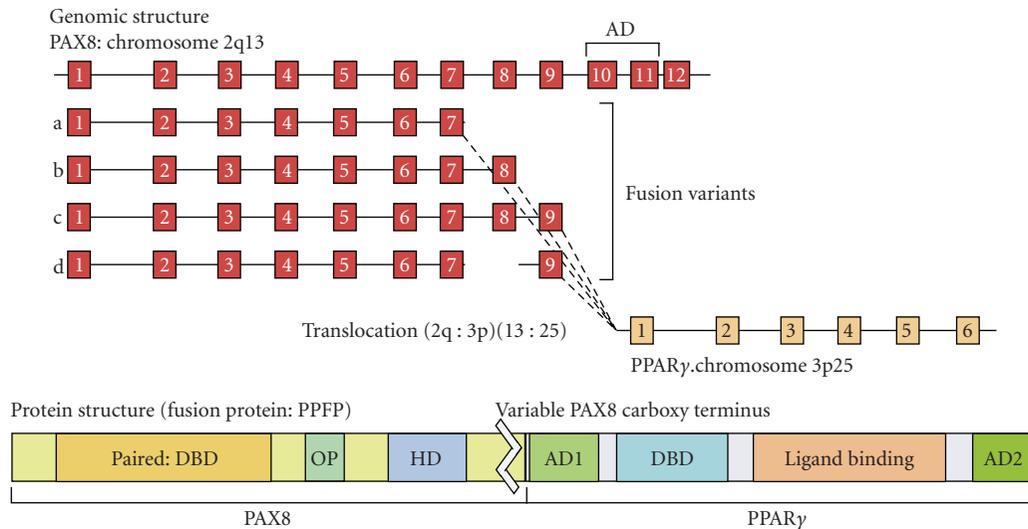
FIGURE 1: PAX8/PPARγ rearrangement illustrating the genomic structure with exon arrangement and sites of fusion. The PAX8 activation domain (AD) is eliminated in all fusion events. The protein structure of the predicted fusion protein is shown and contains the PAX8-paired domain, containing the DNA binding domain (DBD), the octapeptide motif (OP), and the truncated homeodomain (HD). All of the functional domains of PPARγ gene, including activation domains 1 and 2 (AD1 and AD2), DBD, and ligand binding domain are retained in the fusion protein.

of NFκB, was significantly increased. Also, cell cycle analysis in TRβ$^{PV/PV}$PPARγ$^{+/-}$ mice showed a shortened G_0/G_1 phase and decreased apoptosis compared to TRβ$^{PV/PV}$PPARγ$^{+/+}$ mice [32]. Cyclin D1 is known to be overexpressed in human thyroid carcinomas [34] and in vitro addition of a PPARγ agonist to human ATC cell lines suppresses cyclin D1 levels [12], confirming that this effect is related to alterations in PPARγ rather than TRβ. Caspase-3, a gene critical in the apoptotic signal cascade, was significantly reduced in TRβ$^{PV/PV}$PPARγ$^{+/-}$ mice, a finding consistent with previous data on the downstream effects of NFκB activation in prostate cancer cells [35]. Conversely, increased levels of caspase-3 activation were seen in vitro with PPARγ agonist therapy of FTC [17] and ATC cell lines [12]. Importantly, rosiglitazone therapy slowed tumor growth and reduced capsular invasion in TRβ$^{PV/PV}$ mice, suggesting that activation of the PPARγ pathway delays disease progression [32].

In summary, several lines of evidence, in human tumors, cell lines, and animal models strongly support the hypothesis that PPARγ inhibition, downregulation, or insufficiency appears to be tumorigenic in the thyroid. This appears to be mediated through PPARγ effects on cell cycle progression and inhibition of apoptosis, thereby contributing to tumor development or progression.

3. PAX8/PPARγ GENE TRANSLOCATION AND THYROID CANCER

Following the identification of a frequent translocation in follicular thyroid cancer, involving 3p25 and 2q13, Kroll confirmed not only that the 3p25 breakpoint lies within the PPARγ gene, but also that the 2q13 breakpoint lies within the PAX8 gene. Indeed, the translocation brings together these two genes to form a neogene, which expresses a fusion protein (PAX8/PPARγ fusion protein, designated PPFP) [3].

Native PAX8 is a transcription factor important in thyroid follicular cell differentiation and in the regulation of a number of thyroid-specific genes [36]. The chromosomal translocation described by Kroll fuses the promoter and proximal 5' coding sequence of PAX8 inframe with a nearly full-length PPARγ, resulting in the production of the fusion protein PPFP, whose expression is under the transcriptional regulation of the PAX8 promoter [3]. Several splice variants have been identified for PPFP, which appear to be frequently coexpressed [3, 36, 37]. Variants described to date are shown in Figure 1. In each case these PAX8, fragments are fused to PPARγ exons 1 to 6 [3, 36, 37]. All of the known variants include the paired and partial homeobox DNA binding domains of PAX8, as well as the DNA binding, ligand binding, RXR dimerization and transactivation domains of PPARγ-1 [3]. These attributes make it likely that PPFP will retain at least some of the DNA and ligand binding properties of each of the native transcription factors, with the potential for significant impact on either the PAX8- or PPARγ-mediated pathways, or both.

A number of studies, using RT-PCR, nested PCR, FISH, or Western analysis, have confirmed the relatively high prevalence of the PAX8/PPARγ rearrangement or expression of PPFP in follicular thyroid lesions, though the precise incidence varies by cell type and method of detection (Table 1). FTCs have the highest incidence at 36% (range 0–63%) with FAs exhibiting lower rates of around 11% (range 0–55%). Only one case of HCC has shown the PAX8/PPARγ rearrangement. PPFP does not appear to be expressed in classical PTC, but karyotyping has shown at (2;3)(q13;p25)

TABLE 1: Occurrence of PAX8/PPARγ rearrangements in differentiated thyroid carcinomas.

Author	Method	Benign nodular hyperplasia	Follicular adenoma	Follicular carcinoma	Hürthle cell carcinoma	Papillary carcinoma	Follicular variant of papillary	Anaplastic carcinoma
Kroll [3]	RT-PCR	0/10	0/20	5/8 (63%)	—	0/10	—	—
Martelli [14]	RT-PCR	—	—	0/5	—	0/41	—	0/5
Zhu [38]	RT-PCR	—	—	—	—	0/46	0/30	—
Nikiforova [39]	RT-PCR and nested PCR	0/16	2/25 (8%)	8/15 (53%)	0/12	0/23	0/12	0/2
Marques [36]	RT-PCR and nested PCR	0/2	2/16 (13%)	5/9 (56%)	—		0/9	0/4
Aldred [29]	RT-PCR	—	—	2/19 (11%)	—	—	—	—
Lacroix [40]	RT-PCR	—	1/16 (6%)	4/26 (15%)	0/4	—	—	0/5
Hibi [41]	RT-PCR	0/12	0/12	0/6	—	0/12	—	—
Marques [28]	RT-PCR and FISH	0/28	6/36 (17%)	6/24 (25%)	—	0/38	—	—
Sahin [42]	RT-PCR	—	4/31 (13%)	31/54 (57%)	1/23 (4%)	—	—	—
Nikiforova [43]	RT-PCR and FISH	—	1/23 (4%)	13/33 (36%)	0/19	—	—	—
Castro [44]	RT-PCR and FISH	—	—	—	—	—	4/8 (50%)	—
Cheung [37]	RT-PCR	—	6/11 (55%)	6/17 (35%)	—	—	—	—
Foukakis [45]	RT-PCR	—	1/8 (13%)	5/25 (20%)	—	—	—	—
Dwight [46]	RT-PCR, FISH and Western	—	1/40 (3%)	10/34 (29%)	—	—	—	0/13
Castro [47]	RT-PCR and FISH	—	3/9 (33.3%)	10/22 (45.5%)	—	0/2	9/24 (37.5%)	—
Giordano [48]	RT-PCR	—	—	7/13 (54%)	—	—	—	—
TOTAL		0/68	27/247 (11%)	112/310 (36%)	1/58 (2%)	0/172	13/83 (16%)	0/29

in one case of follicular variant of papillary thyroid cancer (FVP) [49] and intense PPARγ immunostaining was seen in three PPFP-negative FVP [50], perhaps arguing for an alternative mechanism of PPARγ overexpression in these tumors. Although Castro et al. [44, 47] found that 37–50% of FVP were positive for PPFP by RT-PCR and FISH, three other studies have found no such rearrangements [36, 38, 39]. It is, nevertheless, tempting to speculate whether those few cases of FVP that develop lung metastasis [51] or display an encapsulated growth pattern [52] might behave with a phenotype more reminiscent of a follicular carcinoma than papillary because of PPFP expression. Larger studies are needed to determine the frequency of expression of PPFP in this tumor variant, and to assess the possible coexpression

of genes known to be associated with PTC, such as RAS and BRAF mutations and RET/PTC rearrangements.

4. PAX8/PPARγ AND PPARγ FUNCTION

To further investigate the function of PAX8/PPARγ−1, Kroll evaluated PPARγ response element (PPRE) activity in the presence of the fusion gene and wild-type PPARγ in a PPARγ-null osteosarcoma U2OS cell line [3]. In contrast to the wild-type PPARγ gene, the fusion gene was ineffective in stimulating ligand-induced gene expression. Furthermore, the coexpression of the fusion gene with wild-type PPARγ abrogated the PPARγ-mediated gene expression, in an apparently dominant negative fashion [3].

In vitro studies using an immortalized human thyroid cell line have shown accelerated growth in cells transiently transfected with PPFP compared to wild-type PPARγ or vector only [53]. Increased proliferation was confirmed with cell cycle transit studies, which showed a lower proportion of PPFP-transfected cells in the resting-phase (G_0/G_1) compared to vector and diminished rates of apoptosis in the PPFP-positive cells. Similar results were seen with stable transfection experiments, in which cells stably expressing PPFP demonstrated a growth advantage over vector-transformed cells. These data are consistent with the hypothesis that PPFP, acting as a dominant negative inhibitor of wild-type PPARγ, inhibits the normal tumor suppressor mechanism of PPARγ, and consequently acts as an oncogene. Furthermore, PPFP stable cell lines showed improved colony-formation on soft agar, a characteristic associated with malignant transformation [53].

Consistent with Kroll's results, cotransfection of PPFP and wild-type PPARγ in immortalized human thyroid cells led to a significant decline in PPARγ transactivation [53]. In these studies, GW9662, a potent PPARγ inhibitor, demonstrated a dose-dependent increase in cell growth of vector-transfected cells, but did not increase growth further in the PPFP-transfected cells. Similar effects were observed upon expression of a dominant negative PPARγ mutant in these cells. Loss of contact inhibition and anchorage dependence, which also correlate with malignant transformation, were also observed upon overexpression of PPFP. The studies of Powell et al. [53] provided the first direct evidence for the oncogenic potential of the PAX8/PPARγ fusion gene, confirming increased proliferation, decreased apoptosis, and a dominant negative effect of PPFP on wild-type PPARγ.

The influence of PPFP on PPRE-dependent transcription appears to be cell line-dependent. Au et al. [54] demonstrated that the fusion gene not only had a dominant negative effect on PPRE expression in HeLa cells, but also stimulated the expression of the PPRE-dependent promoter in a PPARγ ligand-dependent manner in FRTL-5 and Nthy-ori cells, immortalized rat and human thyroid cell lines, respectively. These differences might be related to differences in the ways these cells have been immortalized. Thus HeLa cells utilized the HPV E6 gene and Nthy-ori cells were immortalized with the SV40 large T antigen, whereas FRTL5 cells are a continuous line of functional, nontransformed rat thyroid cells that depend on thyroid-stimulating hormone (TSH) for sustained growth. Whatever the mechanism, the results of Au et al. [54] are in direct contradiction to Powell's findings, in which PPARγ agonists did not augment the PPFP response [53]. The reasons for this discrepancy are not known. Nevertheless, PPFP expression in FRTL-5 cells showed increased proliferation by ^{3}H-thymidine incorporation and soft agar assays [54], findings that are fully consistent with Powell's data [53]. Although further study is clearly warranted to clarify the mechanism of PPFP action in the cell lines, the data indicate that PPFP can act as a dominant negative inhibitor as well as an independent ligand-responsive transcription factor in a promoter-dependent manner.

It is still not clear whether PPFP alone is sufficient to promote tumorigenesis, or whether additional genetic events are a prerequisite for this fusion gene to exhibit an oncogenic impact. One strong candidate, RAS gene mutations, which are seen in up to 50% of follicular tumors, rarely occur within the same tumor as PAX8/PPARγ rearrangements, suggesting that these putative oncogenes form two distinct pathways of carcinogenesis [43]. For each of these pathways, an additional step or series of steps may be required before the development of the full malignant phenotype.

5. PAX8/PPARγ AND PAX8 FUNCTION

Relatively few studies have assessed the impact of PAX8/PPARγ rearrangements on wild-type PAX8 function. Au et al. [54] and Espadinha et al. [55] evaluated the impact of PAX8/PPARγ on genes containing PAX8 response elements: sodium-iodine symporter (NIS), thyroid peroxidase (TPO), thyroid stimulating hormone receptor (TSHR), and thyroglobulin (Tg). Each of these promoters is regulated by PAX8, while the Tg promoter is regulated by both PAX8 and thyroid transcription factor-1 (TTF1), which exhibit a synergistic effect when both promoters are combined in vitro. In human thyroid cancer cell lines, PPFP expression resulted in a complex mixture of stimulatory and inhibitory effects on PAX8-responsive genes, including in PPARγ ligand-dependent and -independent effects. NIS gene expression was stimulated in response to PPFP expression alone in one study [54], although this apparently stimulatory effect required cotransfection of PPFP with wild-type PAX8 in another study [55]. TPO transcription was also increased by PPFP [54], while TSHr expression was inhibited [55]. Repression of the Tg promoter was also seen in response to PPFP [54], but again one study found that cotransfection with PPFP and PAX8 was necessary for this inhibitory effect to be seen [55]. However, in both studies, the fusion gene inhibited PAX8-mediated transcription of Tg in a dominant-negative fashion [54, 55], while the addition of ciglitazone did not reverse this dominant negative effect [54].

Consequently, the effects of the PAX8-PPARγ gene translocation on PAX8 function seem to be complex. TSHr and Tg expressions, both of which are associated with highly differentiated thyroid tissue, are downregulated by PPFP, though it is not clear whether the reduced expression of these genes truly alters cell differentiation status in vivo. In contrast, NIS and TPO expression are enhanced by the expression of PPFP, although once again the impact on cell function is not known. Whether any of these findings relates directly to the oncogenic actions of PPFP, and consequently the impact on the behavior and biology of human FTC, remains to be determined.

6. PAX8/PPARγ REARRANGEMENTS: BENCH TO BEDSIDE

On the basis of the data discussed above, PPFP appears to be important in the development of at least a subset of thyroid follicular neoplasms, and has, therefore, been proposed as

a possible oncogene in follicular thyroid carcinoma. The most obvious direct clinical utility of this discovery is the possibility that PPFP status could provide a presurgical test of malignancy within the troublesome group of biopsy specimens currently described as "suspicious for follicular neoplasm." These lesions represent approximately 20% of all fine needles aspiration biopsies of thyroid nodules, and create a diagnostic challenge because the minority (10–15%) that prove ultimately to be malignant cannot currently be distinguished by cytological criteria from those that prove to be benign. Consequently, the recommendation for all such patients would be to undergo surgery [56], something that might be avoided with a preoperative test of sufficient accuracy. Unfortunately, the finding of a subset of PPFP-positive adenomas reduces the negative predictive value of a preoperative PPFP biopsy finding to 47.4%, while the presence of PPFP in a minority of FTC reduces the positive predictive value to 80.6% (Table 1).

RT-PCR, real-time RT-PCR, and FISH have all been used experimentally to detect translocations, though none is yet proven in a prospective, clinical setting [3, 36, 39, 42]. PCR, probably the technique most easily adapted to a rapid turnover, clinical setting, is concordant with PPARγ immunohistochemistry in up to 80% of cases [36]. When the definition of "positive" PPFP is restricted to only strong, diffuse staining, immunohistochemistry concordance improves to 100% in some studies [7, 39], and it may be that RT-PCR techniques will actually prove superior to the immunohistochemistry "gold standard" for the detection of the fusion event (Algeciras-Schimnich, A. and Grebe, S.K.G., unpublished data). The possibility of false-positive immunostaining is real since normal thyroid tissue, chronic lymphocytic thyoiditis, or benign tissue adjacent to malignancies may show moderate to strong nuclear staining for PPARγ expression. [39, 57], so the RT-PCR-based approach may prove to have a better negative predictive value. There are a number of possible explanations for this apparently "false-positive" staining, including alternate PAX8/PPARγ breakpoints, 3p25 aneusomy, overexpression of wild-type PPARγ, or rearrangements involving PPARγ and a non-PAX8 partner [57].

Despite these challenges, Sahin et al. demonstrated that a reliable preoperative assay for PPFP might improve the accuracy of intraoperative frozen section by significantly reducing the false-negative rate of this technique, and therefore reducing the need for second (completion) surgeries for patients with follicular carcinoma who undergo primary thyroid lobectomy [42]. Immunohistochemistry formed the basis for this retrospective study, so clinical implementation would require confirmation with a more practical preoperative or intraoperative technique (most likely RT-PCR). In this archival tissue analysis, however, the sensitivity was improved from 85% with frozen section alone to 97% with the combination of frozen section and PPARγ status. Several false-positive PPARγ staining results led to a positive predictive value of only 72%, but the overall negative predictive value of frozen section plus immunostaining at this institution was 99%, meaning that five additional cases of carcinoma in this series of 39 cancers could have been

identified intraoperatively, reducing the need for completion thyroidectomy to a single patient (3%).

Currently, there is no evidence that PPFP status predicts outcome in follicular thyroid cancer, with no correlation with proven predictive factors of gender, age, regional nodal spread, or tumor size [28, 39, 42]. The same is true for PPARγ aneusomy or other PPARγ rearrangements found in follicular cancers, which is not correlated with TNM stage [7]. However, patients with PPARγ rearrangements may have a higher prevalence of previous nonthyroid cancers [7] and PPFP rearrangements may be associated with an increased incidence of multifocal capsular invasion or vascular invasion [39], although all of these findings remain in dispute [28, 42]. Larger and more comprehensive outcome analysis will be necessary to resolve these differences in the findings of multiple small studies.

7. PPARγ AGONIST THERAPY IN THYROID CANCER

Follicular cell-derived thyroid cancers carry a generally good prognosis. Standard therapy involves near-total resection of the thyroid with adjuvant ^{131}I radioablation of remnant thyroid tissue in most cases. A subset of tumors exhibits a more aggressive clinical course, and may show features of dedifferentiation, which has been associated with decreased expression of thyroid-specific genes such as Tg, TPO, NIS, and TSHR [15, 58]. These dedifferentiated thyroid cancers may consequently lose their ability to accumulate and concentrate radioiodine, making these tumors unresponsive to further ^{131}I therapy. Traditionally, patients with iodine-insensitive tumors have had few therapeutic options and further basic and applied research is needed to identify suitable therapeutic targets for treatment of these patients; PPARγ provides one such target.

Thiazolidinediones, including troglitazone, rosiglitazone, and pioglitazone, are PPARγ agonists used in the treatment of type 2 diabetes. These and other PPARγ agonists have been investigated in vitro in various cancer cell lines with evidence of growth inhibition and tumor cell apoptosis [18–23, 59]. Small clinical trials in liposarcoma and prostate cancer, which exhibit PPARγ expression, have also been promising in these malignancies, which exhibit PPARγ expression [60, 61]. Recent in vitro evaluations in thyroid cancer cell lines have hinted at a possible role for PPARγ agonist therapy in redifferentiating neoplastic tissue, potentially enhancing the response to currently available therapies [62–67]. Expression of CD97, a marker of cell dedifferentiation, decreased in one follicular carcinoma cell line after therapy with troglitazone [16]. In the same experiment, expression of NIS, a gene associated with well-differentiated thyroid tissue, whose protein product is responsible for iodine concentration within thyroid cells, increased with agonist treatment compared to control in both a papillary and follicular carcinoma cell lines. Such an approach of "redifferentiation" might open up the possibility to restore radioactive iodine sensitivity in some tumors. Anaplastic cancer cell lines have been studied in similar experiments, and expressions of Tg, TSHR, NIS, and TPO were all increased after treatment with rosiglitazone [12].

These findings imply that PPARγ agonists may prove to be an effective therapy for improving response to ^{131}I radiotherapy, even in patients without known PAX8/PPARγ rearrangements.

To date, three reports have been published assessing rosiglitazone therapy in patients with recurrent thyroid cancer as indicated by elevated Tg levels, but negative pretreatment whole body iodine scans (Tg-positive, scan-negative thyroid cancer). Philips et al. [68] treated 2 follicular cancer and 3 papillary cancer patients with rosiglitazone 4 mg daily for one month and then 8 mg daily for three months. Whole body ^{131}I scanning (WBS) using recombinant human TSH (rh-TSH) was negative for all 5 patients at the onset of the study. Basal Tg levels rose in 3 patients while the rhTSH-stimulated Tg increased in two of the 3 patients after rosiglitazone therapy. Posttreatment rhTSH-stimulated WBS was faintly positive in one patient. Elias and Lizotte [69] reported a single case of papillary thyroid cancer in which rosiglitazone caused a marked increase in ^{131}I uptake on WBS. Following treatment with 250 mCi ^{131}I, the WBS became negative, suggesting that the cancer had been effectively treated. Kebebew et al. [70] have reported the largest number of patients to date treated with a thiazolidenedione: 8 PTC, 1 FVP, and 1 FTC. These patients received rosiglitazone 4 mg daily for 7 days, then 8 mg daily for 49 days. Four patients (40%) had conversion from negative to positive WBS after rosiglitazone, suggesting a possible redifferentiation effect. At 6 months of followup, 2 patients had improved Tg levels (one follicular and one FVP patient), 3 had stable levels (all papillary cancer patients), and 5 patients had increased Tg levels compared to baseline. After 11 months of followup, 4 patients had a partial response to rosiglitazone, exhibiting decreased Tg levels or increased ^{131}I uptake, 4 had stable disease, and 2 had progression of disease as indicated by increased Tg levels. In all cases, rosiglitazone was apparently well tolerated with no significant adverse events. Overall, 6 of 16 patients (5 papillary, 1 FVP) showed uptake on WBS after rosiglitazone therapy, indicating that a subset of patients may have experienced redifferentiation of their cancers. Whole body iodine scanning did not always correlate with Tg levels and caution should be exercised in interpreting Tg levels in these studies because an increase in Tg could indicate improved differentiation, rather than being a sign of increasing tumor mass. Only one patient in this study received additional ^{131}I radiotherapy, although therapy was apparently successful, with a low Tg post-radiotherapy. Larger studies with longer followup are needed to see if thyroid cancers treated with PPARγ agonists show improved response to ^{131}I radioablation or decreased mortality, the truly important clinical outcome.

Very little data is currently available on combination therapy with PPARγ and other chemotherapeutic agents. Aiello et al. [12] evaluated the in vitro response of rosiglitazone plus doxyrubicin, a standard agent used in anaplastic thyroid cancer, in ATC cell lines. They found a markedly increased effect of doxyrubicin in PPARγ-positive cell lines when combined with rosiglitazone, but no effect in a PPARγ-negative cell line. Copland et al. [71] combined paclitaxel with a novel PPARγ agonist, RS5444, in a variety of ATC cell lines. This combination demonstrated synergistic effects on inhibition of proliferation and stimulation of apoptosis in vitro. When athymic nude mice were implanted with the responsive ATC cell lines, tumor growth was inhibited by monotherapy with RS5444. No clinical studies or case reports have yet addressed combination therapy in humans with thyroid cancer, though a clinical trial is currently under development by our group.

8. CONCLUSION

Emerging genetic and molecular information acquired over the last 2 decades has begun to unravel the pathogenesis of thyroid cancer and in the future may open the door to potential novel therapies for patients with previously untreatable disease. Research focusing on PPARγ in a variety of cancer cell lines has implied a tumor suppressor function for wild-type PPARγ, while PPARγ downregulation or inhibition may be one factor in the development of at least some thyroid cancer types.

Chromosomal alterations of PPARγ, resulting in the expression of the fusion protein PPFP, may be an early event in the development or progression of follicular thyroid cancer and perhaps the follicular variant of papillary cancer. The detection of these alterations in FAs may support a stepwise adenoma to carcinoma sequence, or indicate the presence of "carcinoma in situ." However, the PAX8/PPARγ rearrangement in itself may not be sufficient for the development of a malignant phenotype: additional genetic or epigenetic events may be required to enable the full phenotypic expression of follicular thyroid carcinoma.

Several lines of data suggest that PPFP, either through PPARγ inhibition, PAX8-dependent gene expression modulation, or both, leads to downstream effects, which are at least in part mediated by the NFκB pathway. These altered pathways stimulate cell proliferation and inhibit apoptosis, but there may be several other paths through which PPFP modulates the tumor phenotype, including alteration of cell differentiation status and expression of the sodium iodide transporter NIS, which may have an impact on the efficacy of our current therapeutic options.

PPARγ represents an attractive therapeutic target in a variety of thyroid cancers, including anaplastic, follicular, and papillary thyroid cancers. Although in vitro data is promising, early studies using PPARγ agonists to treat iodine-insensitive recurrent thyroid cancer are promising, but inconclusive so far. Larger studies with longer followup will be needed to clarify the potential for PPARγ agonists to act as "redifferentiation" agents, Nevertheless, the availability of a number of approved, orally administered, well-tolerated agents makes this group of drugs an attractive option for study.

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Review Article

Chondrosarcoma and Peroxisome Proliferator-Activated Receptor

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Induction of differentiation and apoptosis in cancer cells by ligands of PPARγ is a novel therapeutic approach to malignant tumors. Chondrosarcoma (malignant cartilage tumor) and OUMS-27 cells (cell line established from grade III human chondrosarcoma) express PPARγ. PPARγ ligands inhibited cell proliferation in a dose-dependent manner, and induced apoptosis of OUMS-27. The higher-grade chondrosarcoma expressed a higher amount of antiapoptotic Bcl-xL in vivo. The treatment of OUMS-27 by 15d-PGJ$_2$, the most potent endogenous ligand for PPARγ, downregulated expression of Bcl-xL and induced transient upregulation of proapoptotic Bax, which could accelerate cytochrome c release from mitochondria to the cytosol, followed by induction of caspase-dependent apoptosis. 15d-PGJ$_2$ induced the expression of CDK inhibitor p21 protein in human chondrosarcoma cells, which appears to be involved in the mechanism of inhibition of cell proliferation. These findings suggest that targeted therapy with PPARγ ligands could be a novel strategy against chondrosarcoma.

1. INTRODUCTION

Cancers are associated with dysregulation of differentiation and apoptotic cell death. Recent investigations have demonstrated that induction of these cellular events by targeted therapy with ligands of nuclear hormone receptors could be a novel strategy against cancers [1]. Peroxisome proliferator-activated receptor (PPAR)γ, a member of the nuclear receptor superfamily, acts as a ligand-activated transcription factor, and is involved in many processes important for homeostasis of cells and tissues, including metabolism, immune and inflammatory controls, cell proliferation and apoptotic cell death [2–6]. Because PPARγ is expressed by many malignant tumors, activation of PPARγ by 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$), the most potent endogenous ligand for PPARγ [7], and the synthetic PPARγ ligands (e.g., rosiglitazone, pioglitazone, troglitazone, and indomethacin) have been regarded as a novel therapeutic approach for certain human malignancies through growth inhibition, induction of apoptosis and terminal differentiation, and inhibition of angiogenesis [8]. This review will outline the inhibitory effects of synthetic and endogenous PPARγ ligands and discuss their potential therapeutic effects on chondrosarcoma.

2. CLINICAL FEATURES OF CHONDROSARCOMA

Chondrosarcoma is a malignant tumor of cartilage; the matrix formed by tumor cells is uniformly and entirely chondroid in nature [9]. Human chondrosarcoma is a rare bone tumor, accounting for <10% of primary malignant bone tumors. Chondrosarcoma also arises in pre-existing benign lesions (e.g., osteochondromatosis, enchondromatosis) and is termed "secondary chondrosarcoma."

Primary (conventional) chondrosarcoma arises centrally in a previously normal bone, and mostly grows slowly through the diaphyseal cortex. Most patients are aged >50 years. Conventional chondrosarcoma is more common in men. The commonest sites are the bones of the pelvis, followed by the femur and the humerus. Recognizable histologic variants are clear cell, mesenchymal, and dedifferentiated chondrosarcomas. On the basis of histologic

features (nuclear atypia and cellularity), conventional chondrosarcoma is further subdivided into three grades: I, II, and III [10]. The histologic grade of chondrosarcoma indicates the differentiation status of tumor cells, and is one of the most important factors for prognosis [11]. Progression of a locally aggressive low-grade chondrosarcoma to a metastasizing high-grade chondrosarcoma is associated with loss of cartilaginous phenotype, genomic instability, and aneuploidy [12]. The grading of chondrosarcoma correlates well with clinical behavior, although chondrosarcoma is one of the most difficult malignant tumors of bone to diagnose [13].

Most conventional chondrosarcomas are grade I or II. For low-grade chondrosarcoma, surgical treatment with adequate marginal resection is reported to be associated with better clinical outcomes [14]. Only 5–10% of conventional chondrosarcomas are grade-III lesions, which have definite metastatic potential. The prognosis for high-grade chondrosarcoma is poor, despite adequate surgery, because they are highly resistant to conventional chemotherapy and radiotherapy [15]. These facts, that the differentiation status of chondrosarcoma is predictive of clinical outcomes, suggest the favorable effects of the modification of the differentiation status on clinical behavior. Recent advances in understanding the progression or development of chondrosarcoma have suggested several molecular targets for future development of new adjuvant therapy [16], such as chondrocyte differentiation factors (PTHrP, CTGF) [17, 18], antiapoptotic gene (*Bcl-2*) [19, 20], tumor suppressor gene (*p16*, *p53*) [21, 22], and others (PDGF-α, VEGF, *MDR-1*) [23–25].

3. OUMS-27, A CHONDROSARCOMA CELL LINE

A cell line derived from chondrosarcoma, particularly from high-grade chondrosarcoma, can provide a useful model for the investigation of cell development and treatment of chondrosarcoma [26–30]. The OUMS-27 cell line has been established from grade III human chondrosarcoma [31]. The cells do not show contact inhibition after reaching confluence, grow rapidly in multiple layers, and express proteoglycan, as well as collagen type I, II, III, IX, and XI after 120 passages, showing stable maintenance of the differentiated chondrocytic properties. The transplantation of OUMS-27 cells into athymic mice resulted in formation of grade II chondrosarcoma at the injection site. There have been many studies on the etiology and treatment of chondrosarcoma using this cell line [32–34].

4. EXPRESSION OF PPARγ IN HUMAN CHONDROSARCOMA AND OUMS-27

Subramanian et al. [35] investigated gene expression profiling of ten extraskeletal myxoid chondrosarcomas (EMCs) using 42000 spot cDNA microarrays. Eighty-six genes that distinguished EMC from the other sarcomas were identified by significance analysis of microarrays with 0.25% likelihood of false significance. Of these, PPARG and PPARGC1A, an interacting protein with PPARG and also a coactivator, were highly expressed in EMCs.

Table 1: Summary of immunohistochemical study for PPARγ in human chondrosarcoma tissues.

Positive cell ratios (%)	Pathological grade of chondrosarcoma (%)		
	I ($n = 20$)	II ($n = 6$)	III ($n = 2$)
<10	35	17	50
10–40	40	33	50
>40	25	50	0

In vivo PPARγ protein content was examined in conventional chondrosarcoma specimens from 28 patients undergoing surgery [36]. Immunohistochemical study revealed that human chondrosarcoma cells frequently express PPARγ protein. The positivity (cutoff positivity of 10%) of chondrosarcoma cells were 65.0% in grade I, 83.3% in grade II, and 50.0% in grade III; overall positivity was 67.9% (see Table 1). Expression of PPARγ in OUMS-27 cells at protein and mRNA levels was confirmed by immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, respectively [36]. These data indicated that PPARγ is frequently expressed in primary chondrosarcomas and chondrosarcoma cell line OUMS-27, and led the authors to test the effect of PPARγ activators on cell proliferation and survival of OUMS-27.

5. EVIDENCE OF APOPTOTIC CELL DEATH OF OUMS-27 CELLS AFTER TREATMENT BY PPARγ LIGANDS

In our previous report [36], OUMS-27 cells were treated with increasing concentrations of pioglitazone (synthetic PPARγ ligand) and 15d-PGJ2 for up to 48 hours. The results of immunostain for Ki-67 (cell proliferation marker) and colorimetric MTT assay showed that treatment with both pioglitazone and 15d-PGJ$_2$ for 24 hours inhibited cell growth and reduced cell viability in a dose-dependent manner, respectively. 15d-PGJ$_2$ had more noticeable effects on OUMS-27 cell growth than pioglitazone. It was unclear whether the effects of ligands on OUMS-27 cells were strictly due to PPARγ activation. When cells were treated with 15d-PGJ$_2$ doses of $\geq 5\,\mu$g/mL, they showed relatively round shapes and some cells no longer adhered to the dish.

Semithin sectioned, LR White-embedded cells stained by toluidine blue revealed that many OUMS-27 cells treated with 15d-PGJ$_2$ show apoptotic appearances with cell shrinkage and nuclear condensation (see Figure 1). DNA fragmentations in OUMS-27 cells treated by 15d-PGJ$_2$ ($10\,\mu$g/mL) for 24 hours were confirmed by DNA ladder formation and TUNEL staining. Transmission electron microscopic study revealed sections of OUMS-27 cells treated with 15d-PGJ$_2$ contained many cells consistent with morphological apoptosis with condensed chromatin, many vacuoles in cytoplasm, and membrane budding. Early apoptotic change and the translocation of phosphatidylserine (PS) on the outer leaflet of the cell membrane were demonstrated by FACS analysis. The population of apoptotic cells with PS at the outer membrane of the cells (annexin-V-positive,

(a)

(b)
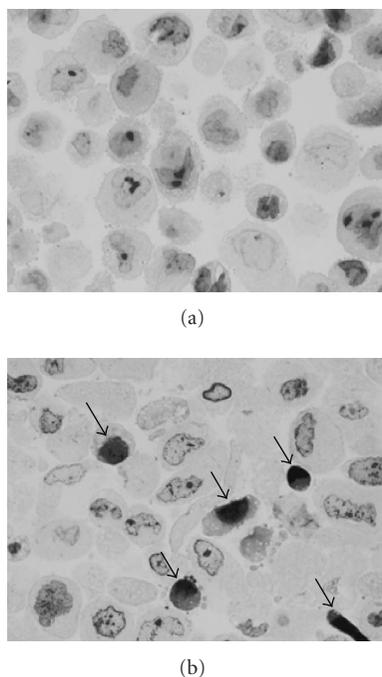
Figure 1: Cell morphology of chondrosarcoma cell line OUMS-27 after incubation with (b) or without (a) 15d-PGJ$_2$. Cells were treated with 10 μg/mL of 15d-PGJ$_2$ for 8 hours, and the cell pellet embedded in hydrophilic resin. Semithin sections stained by toluidine blue show more apoptotic cells with cell shrinkage and nuclear condensation (arrows) after treatment with 15d-PGJ$_2$.

PI-negative) was ~53.9% and 67.6% at 4 hours and 24 hours after coincubation with 15d-PGJ$_2$, respectively.

6. MECHANISM OF APOPTOTIC CELL DEATH OF OUMS-27 CELLS BY PPARγ LIGANDS

cDNA microarray analysis was carried out to comprehensively explore the changes in gene expression pattern during OUMS-27 cell growth inhibition and possible cell cycle arrest caused by treatment with 15d-PGJ$_2$ [37]. Among the 1081 genes analyzed, 52 genes were upregulated and 81 genes were downregulated significantly in OUMS-27 cells after 8-hour treatment with 15d-PGJ$_2$ (10 μg/mL). Microarray analysis is shown in Table 2. Interestingly, the proapoptotic gene Bax was upregulated, and the antiapoptotic gene Bcl-xL was downregulated. The other Bcl-2 members were unchanged. These results were further confirmed by RT-PCR and real-time PCR analysis.

Upregulation of Bax, concurrent with the downregulation of Bcl-xL, can destabilize mitochondria, leading to the release of several mitochondrial intermembrane space proteins such as cytochrome c, AIF, Smac/DIABLO, Endo G, and Omi/HtrA2 into the cytosol, where they are actively involved in apoptotic cell death [38]. This hypothesis is supported by our observations of the release of cytochrome c from mitochondria into the cytosol, and the activation of caspase-3 in 15d-PGJ$_2$-treated OUMS-27 cells. Coincubation

Table 2: Changes in apoptosis-related gene expression in OUMS-27 cells after treatment with 15d-PGJ$_2$.

Gene name	Fold
Clusterin	+3.7
Defender against cell death 1	+3.8
Tumor necrosis factor receptor 1	+3.2
State-induced state inhibitor 2	+2.5
Heat shock protein 60	+2.4
V-akt murine thymoma viral oncogene homolog 1	+2.3
Apoptosis regulator bax	+2.3
Interferon-induced RNA-dependent protein kinase	+2.1
Apoptosis regulator bcl-xl	−2.2
Calpain, small subunit 1	−2.3
Heat shock 70 KD protein 1	−2.4
Endothelin 2	−2.4
Insulin-like growth factor 1 receptor	−2.5
Death-associated protein 6	−2.8

of cells with the broad-spectrum caspase inhibitor Z-VAD-FMK completely inhibited caspase activity, and prevented the cell death induced by 15d-PGJ$_2$. These results indicate that 15d-PGJ$_2$ induced apoptosis in OUMS-27 cells through a caspase-dependent signal transduction pathway which, at least in part, was triggered by cytosolic release of cytochrome c [37].

The decreased expression of antiapoptotic Bcl-xL in OUMS-27 treated by 15d-PGJ$_2$ led us to examine the expression in the tissue of human chondrosarcoma samples to study the clinical application of differentiation therapy by PPARγ activation. The result of immunohistochemical study demonstrated that Bcl-xL was expressed in all three grades of chondrosarcoma; the expression was strongest in grade III. These results indicated that higher-grade chondrosarcoma cells may be resistant to apoptosis by overexpression of Bcl-xL, and 15d-PGJ$_2$ might induce apoptotic cell death by downregulation of Bcl-xL and transient upregulation of Bax [37]. Similar results were reported in renal cell carcinoma cells (786-O and A498 cells) showing the thiazolidinedione (TZD) induction of apoptosis with increased Bax expression and decreased Bcl-2 expression [39].

6.1. Genetic and epigenetic alterations in chondrosarcoma

Little is known about the role of genetic or epigenetic alterations in tumor progression from low-malignant chondroblastic to highly malignant anaplastic chondrosarcoma. The appearance of de novo aberrant DNA methylation is the commonest molecular change in the cancer cell, which inactivates many cellular pathways [40]. The most studied change of DNA methylation in neoplasms is the silencing of tumor suppressor genes by deoxy-cytidylatephosphate-deoxy-guanylate (CpG) island promoter hypermethylation, which targets genes and molecules associated in cell differentiation, such as p16(INK4a), BRCA1, and hMLH1

[41–43]. Röpke et al. reported the p16 and E-cadherin promoter methylation in low-grade chondroid compartment of dedifferentiated chondrosarcoma. Van Beerendonk et al. found p16 promoter methylation by methylation-specific PCR in 5 of 30 tumors, but this did not correlate with protein expression, or with loss of heterozygosity (LOH) at 9p21 region, one of the few consistent genetic aberrations found in conventional chondrosarcoma [44]. In OUMS-27, methylation was not detectable in the promoter of p16 gene (unpublished data).

Some reports suggested that p53 mutation and p53 loss of heterozygosity are involved [43, 45]. In OUMS-27, we have previously shown that the p53 gene is mutated [31]. Asp et al. analyzed p16 and p53 in cartilaginous tumor tissues and showed that the p16 gene was found to be partly methylated in 5 high-grade chondrosarcomas and homozygously deleted in 1 chondrosarcoma, whereas the p53 gene revealed an unchanged structure in all 22 chondrosarcoma samples [46].

7. INDUCTION OF CELL CYCLE ARREST BY 15d-PGJ$_2$ IN OUMS-27

Ligands for PPARγ reportedly induce cell cycle arrest in various cancer cells [39, 47–54]. 15d-PGJ$_2$ induces G$_1$ arrest and inhibits cell growth of human anaplastic thyroid carcinoma through a p53-independent, but p21- and p27-dependent, manner [55]. Activation of PPARγ by troglitazone inhibited cell growth and induced G$_1$ arrest through the increase of cycline-dependent kinase (CDK) inhibitor p27 in several cell lines, including human pancreatic carcinoma cells, gastric cancer cells, and hepatocellular carcinoma cells [56–58]. The effect of troglitazone on the proliferation of cancer cells was inhibited by antisense for p27. Yang et al. also showed TZD decreased the protein levels of proliferating cell nuclear antigen, pRb, cyclin D, and Cdk4, but increased the levels of p21 and p27, in RCC cells [39].

In OUMS-27, 15d-PGJ$_2$ induced the expression of the CDK inhibitor p21 protein, and it was increased within 24 hours. Expression of the other CDK inhibitors, p16 and p27 proteins, were detected at time zero, and were not significantly influenced by 15d-PGJ$_2$ treatment [37]. 15d-PGJ$_2$-induced p21 may exert cell cycle arrest in a p53-independent manner.

Whether 15d-PGJ$_2$ induces p21 expression in OUMS-27 cells through a PPARγ-dependent or -independent pathway is unclear. It is possible that p21 expression is directly regulated by PPARγ activation because p21 gene contains a potentially conserved consensus PPARγ response element in the promoter region [59]. Copland et al. reported [60] that RS5444, a novel high-affinity PPARγ agonist, inhibits anaplastic thyroid cartinoma (ATC) tumor growth and angiogenesis in mice. In DRO cells derived from ATC tumor, they demonstrated that upregulation of p21 by RS5444 is PPARγ dependent, and might be the major mechanism by which RS5444 inhibits DRO cell proliferation. Han et al. demonstrated the link of PPARγ activation and p21 signaling to cell growth inhibition in human lung cell cartinoma cells using p21 antisense oligonucleotides [61]. They also indicated the induction of p21 expression by

PPARγ ligands might be mediated through increased Sp-1 and NF-interleukin 6 (IL6) CAAT/enhancer binding protein (C/EBP)-dependent transcriptional activation.

8. CLINICAL APPLICATION OF PPARγ AGONIST FOR CHONDROSARCOMA SUPPRESSION

Accumulating evidence suggests that PPARγ activators might have clinical therapeutic benefit in the treatment of cancers. Although initial clinical trials with troglitazone reported promising results in liposarcomas [62] and prostate cancers [63], recent studies failed to show the expected therapeutic values of rosiglitazone in liposarcomas [64] and early-stage breast cancers [65], and troglitazone in chemotherapy-resistant metastatic colorectal cancers [66]. However, a single study of a phase-I clinical trial of LY293111 in patients with advanced solid tumors reported a potential efficacy of PPARγ agonist for chondrosarcoma [67]. LY293111 is an orally stable leukotriene B$_4$ (LTB$_4$) receptor antagonist, as well as a PPARγ agonist, as demonstrated by activity in the rat ZDF diabetes model, and the induction of adipocyte differentiation. One patient with progressive chondrosarcoma had stable disease lasting ~336 days of LY293111 administration at the dose of 200 mg bd.

9. FUTURE DIRECTION

In chondrosarcoma, whether the cell death and growth inhibitory effects induced by 15d-PGJ$_2$ are PPARγ-dependent or -independent is unknown. As 15d-PGJ$_2$ at high doses is toxic for most of cell types independent of PPARγ activation, we examined the effects of the caspase inhibitor Z-VAD-FMK, and the PPARγ antagonist GW9662, on caspase-3 activation and cell viability of OUMS-27 cells treated by 15d-PGJ$_2$ [37]. 15d-PGJ$_2$ alone clearly increased cell death; the addition of GW9662 partially inhibited cell death. Cell death was inhibited almost to control level when Z-VAD-FMK was added to 15d-PGJ$_2$. The activity of caspase-3 was attenuated, though not completely, by stimulation of 15d-PGJ$_2$ together with GW9662. These data indicate that the greater proapoptotic effects of 15d-PGJ$_2$ on chondrosarcoma cells may result from the cumulative effects of PPARγ-dependent and -independent pathways. Detailed analysis of the effects of ligands on cells transfected with PPARγ siRNA should provide important clues to understanding this phenomenon. Whether endogenous or synthetic PPARγ ligands can also induce tumor cell death in an experimentally transplanted chondrosarcoma model remains to be examined before human trial.

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Review Article

A Novel Mechanism of PPARγ Regulation of TGFβ1: Implication in Cancer Biology

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Peroxisome proliferator-activated receptor-γ (PPARγ) and retinoic acid X-receptor (RXR) heterodimer, which regulates cell growth and differentiation, represses the TGFβ1 gene that encodes for the protein involved in cancer biology. This review will introduce the novel mechanism associated with the inhibition of the TGFβ1 gene by PPARγ activation, which regulates the dephosphorylation of Zf9 transcription factor. Pharmacological manipulation of TGFβ1 by PPARγ activators can be applied for treating TGFβ1-induced pathophysiologic disorders such as cancer metastasis and fibrosis. In this article, we will discuss the opposing effects of TGFβ on tumor growth and metastasis, and address the signaling pathways regulated by PPARγ for tumor progression and suppression.

1. INTRODUCTION

Peroxisome proliferator-activated receptor-γ (PPARγ) as a ligand-activated transcription factor belongs to the members of nuclear hormone receptor superfamily. PPARγ is implicated in a wide variety of cellular functions, regulating the expression of gene networks required for cell proliferation, differentiation, morphogenesis, and metabolic homeostasis. The transforming growth factor isoforms (TGFβ1, β2, and β3) as the members of the TGFβ superfamily are ubiquitously expressed cytokines [1, 2]. TGFβ exerts multiple functions with differential expression pattern in organs: each form of TGFβ has similar biological activities [3]. Among the TGFβ forms, it is recognized that TGFβ1 plays a major role in the regulation of cell proliferation and differentiation. In this review paper, we will discuss the role of PPARγ on TGFβ gene expression.

Accumulating evidences suggest that the interplay of PPARγ and TGFβ contributes to the regulation of cell proliferation, differentiation, and their associated cellular functions. For instance, the interaction of PPARγ signaling with the proteins affected by the activation of TGFβ receptor determines the outcome of the breast tumor progression [4]. Many studies have shown that agonist-induced activation of PPARγ interferes with TGFβ/Smad-dependent or Smad-independent signaling in different cell types [5–12]. The crosstalk between PPARγ and TGFβ can be achieved not only by PPARγ-dependent modulation of the propagation of TGFβ/TGFβ receptor-mediated signaling pathways, but also by the regulation of TGFβ1 expression itself and TGFβ1-inducible target genes. Hence, suppression of TGFβ signaling by PPARγ could be counteracted by the inhibitory action of TGFβ on the PPARγ-mediated signaling [13–15].

The TGFβ1 expression is regulated at multiple levels. Diverse transcription factors are involved in the transcriptional regulation of TGFβ gene expression and post-translational modification makes precursors bound with TGFβ1 binding proteins mature to TGFβ molecule [16, 17]. The role of PPARγ activation in TGFβ1 gene repression has been examined by the experiments using thiazolidinedione PPARγ agonists [18, 19]. These studies on the regulation of the TGFβ1 gene and the molecular interaction of ligand-activated nuclear receptors for the activation of responsible transcription factor(s) brought insights into

the transcriptional control mechanism. The research results showed that PPARγ activation might transrepress the TGFβ gene, interfering with TGFβ signaling and thereby altering the expression of TGFβ-inducible target genes [18], substantiating the fact that ligand activation of PPARγ modulates TGFβ receptor-mediated gene regulation.

2. TGFβ AND CANCER CELL BIOLOGY

TGFβ1 exerts its diverse biological effects by acting on distinct combinations of type I and type II receptors and recruiting downstream signal transducers including Smads, consequently regulating a group of target gene expression responsible for a specific biological activity. Smad proteins are classified into R-Smads (receptor-regulated Smads: Smads 1, 2, 3, 5, and 8), Co-Smads (common mediator Smad: Smad 4), and I-Smads (inhibitory Smads: Smad 6 and 7), and these play roles as the transcriptional regulators for the superfamily of TGFβ1-inducible target genes [1, 2, 20–22]. Smad 2 and Smad 3 are the specific mediators of TGFβ1, whereas Smad 1, Smad 5, and MADH6/Smad 9 are crucial for bone morphogenic protein signaling [22]. In particular, Smad 3 is involved in the TGFβ1 gene regulation, which is crucial for the autocrine function of TGFβ1 [23].

Following the activation of the TGFβ1 receptor by TGFβ1, TGFβ1-induced receptor kinase activation rapidly phosphorylates Smads proteins and initiates formation of functional oligomeric complexes. The resultant oligomeric complex translocates to the nucleus to regulate target gene expression. Briefly, the type I TGFβ1 receptor kinase phosphorylates serine residues at the C-terminal SSXS motif in the MH2 domain of Smad 3 (or Smad 2) [24]. Phosphorylated Smad 3 (or Smad 2) forms an oligomeric complex with Smad 4, which is crucial for the maximal transcription of diverse TGFβ1-inducible target genes [25, 26]. The oligomeric complexes of Smad 3 (or Smad 2) and Smad 4 recognize DNA binding element tetranucleotide (CAGA) or GC-rich sequences, and several copies of which are present in the promoter regions of many TGFβ1-responsive genes such as plasminogen activator inhibitor-1 (PAI-1), α2(I) procollagen, and type VII collagen [25, 27]. It is well known that the protein products encoded from these genes promote the accumulation of extracellular matrix and that abnormal accumulation of the proteins may lead to fibrosis, which represents a form of the epithelial to mesenchymal transition (EMT).

Moreover, TGFβ1-activated kinase-1, a member of MAPK kinase kinase family, activates its MAP kinase pathways [28, 29]. It is accepted that TGFβ1-activated ERK pathway synergistically enhances Smad signaling of the TGFβ1 receptor due to the positive cross talk between the ERK and Smad pathways [22, 30]. Serine phosphorylation of Smad 3/2, but not phosphorylation of the C-terminal motif, was decreased by MEK-ERK inhibitors [31]. Smad 3/2 are differentially activated by TGFβ1 in hepatic stellate cells as a result of the differential phosphorylations of the Smads. Smad 3 plays a key role in TGFβ signaling, which is strengthened by the observation that the loss of Smad 3 interfered with TGFβ1-mediated induction of target genes

[32, 33]. In addition, activation of CCAAT/enhancer binding protein (C/EBP) β is also involved in the inhibition of TGFβ1 expression [34].

During the process of carcinogenesis, TGFβ action can be either tumor suppressive or tumor promoting, depending on the stage of tumor development [35–37]. In an experimental cell model, TGFβ could induce cell growth arrest and promote apoptosis of carcinoma cells [1]. The antiproliferative action of TGFβ in epithelial cells, for example, is essentially attributed to the cell cycle arrest and the apoptosis concomitantly induced. It is well known that cell cycle arrest induced by TGFβ occurs at G1 phase through enhancing transcription of cyclin-dependent kinase inhibitors, p21$^{Cip1/WAF}$ and p15^{Ink4b}, while suppressing the induction of c-Myc, a progrowth transcription factor, and of Id$_{1–3}$, the inhibitors of differentiation [38–43]. In a model of gastric adenocarinoma, TGFβ-mediated apoptosis contributed to tumor suppression, which resulted from TGFβ-induced caspase-8 activation [44]. Moreover, it has been shown that TGFβ reduced the expression of antiapoptotic Bcl-2 family members in prostate cancer cells [45].

By contrast, TGFβ may also lead to tumor cell proliferation as a consequence of EMT process [46–48], which is a cellular phenomenon characterized by a loss of polarized epithelial phenotype with transition to a mesenchymal or more migratory phenotype. Studies have shown that diverse signaling pathways are involved in the TGFβ-dependent EMT process. Initiation of EMT by TGFβ receptor activation is mediated by either Smad-dependent or Smad-independent pathway [1, 49, 50]. Downstream of the TGFβ receptor activation, the Smads activated by the TGFβ receptor kinase promote transcription of the genes, which eventually play crucial roles in the process of EMT. The responsible transcription factors primarily include Snail, Slug, and LEF-1 [1]. In addition, TGFβ also activates the non-Smad pathways, which include Ras, phosphatidylinositol 3-kinase (PI3K), and Par 6. These molecules regulate the expression of Snail and the activities of glycogen synthase kinase 3β (GSK3β) and RhoA, respectively [51, 52], thereby enhancing the process of EMT. It is now accepted that the EMT phenomenon of primary cancer cells promoted by the action of TGFβ may increase cancer metastasis.

TGFβ acts on tumor cells directly, playing a role in cancer cell migration and invasion. Diverse TGFβ-mediated signaling pathways are responsible for this process. In glioblastoma cells, siRNA knockdowns of TGFβ1 and TGFβ2 resulted in the inhibition of cell motility or invasiveness [53]. As a same token, TGFβ released from tumor tissues might facilitate glioma cell migration and invasion via an autocrine signaling [54]. Several lines of evidence also support the concept that TGFβ-induced Smad signaling is responsible for the invasiveness of cancer cells [55–58]. This is explained in part by the TGFβ-dependent induction of matrix metalloproteases, which are known to be responsible for cell migration and invasion [55, 59–62]. Activation of ERK and JNK by TGFβ and their association with focal complexes may also contribute to cell migration, as shown in the case of breast carcinoma [63]. Moreover, it has been shown that the activation of p38 MAPK pathway by TGFβ

facilitated invasion of head and neck squamous epithelial cells [61].

In addition to the double-edged effects of TGFβ on cancer cells, TGFβ may alter cancer growth by suppressing the growth of multiple immune cells, which compromises the overall immune functions. Studies have shown that the proliferation and activity of T cells are suppressed by the TGFβ blockade of IL-2 production and expression of T cell effector molecules [64–68]. Also, TGFβ attenuates the activity of natural killer (NK) cells by inhibiting NK production of interferon-γ (IFN-γ) [69, 70]. Another study showed that TGFβ inhibited the antigen presentation function of dendritic cells through suppressing the expression of MHC class II and costimulatory molecules [71]. All of these results support the alterations by TGFβ in immune functions, which would impair immune surveillance or attack against cancer cells.

In summary, action of TGFβ1 on cancer cells switches from tumor suppression to tumor promotion, depending on the stage of tumor progression. For instance, during the early phase of breast tumorigenesis, the TGFβ signal inhibits primary tumor growth via cell growth arresting and promoting apoptosis. However, at later stage, cancer cells acquire a capacity to escape from the tumor suppressive effects of TGFβ1 via induction of EMT. Interestingly, the aforementioned conflicting functions of TGFβ might go through the same TGFβ receptor complex and the associated signaling pathways involving Smad transcription factors [1]. Probably, there should be certain stage-dependent modifications in cellular signaling system including changes in receptor function and downstream Smad signaling cascades. Taken together, it is concluded that TGFβ may not only induce growth arrest of cancer cells, but also increase cancer dissemination [1], supporting the concept that the cytokine serves a dual function in tumor development and progression (Figure 1).

3. PPARγ AND CANCER BIOLOGY

PPARγ has been extensively studied as an anticancer target in preclinical and clinical settings [72]. The anticancer effects appeared to be cancer cell-specific. A knock-out or loss of function mutation in PPARγ can be an important risk factor for the incidence of cancer [73–75]. In this sense, PPARγ has been considered as a novel target for designing new anticancer drugs for chemotherapy. This is further supported by the finding that PPARγ activators exert a potent tumor-suppressing activity against various human cancer cells [76–78]. As a matter of fact, PPARγ activators such as troglitazone and ciglitazone exert antiproliferative activities in epithelial cancer cell lines or animal models, which presumably results from the activation of PPARγ receptor and the PPARγ receptor-dependent pathways [76, 79–83]. Nevertheless, other anticancer pathways have also been recognized in association with PPARγ, which might be PPARγ receptor-independent [84, 85]. Multiple PPARγ-independent anticancer targets of PPARγ agonists have been suggested in several cancer cell types. The mechanisms may comprise a variety of pathways such as the blockade of
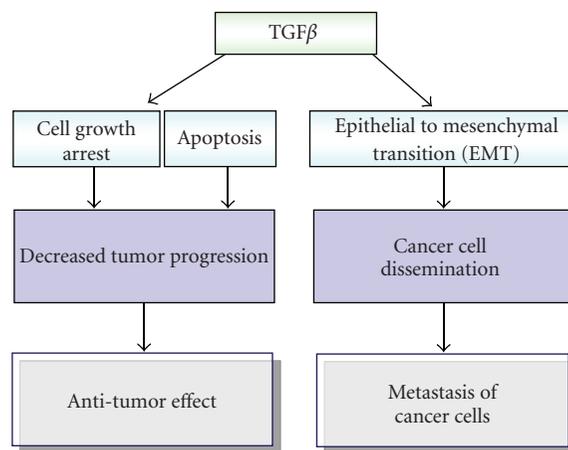

FIGURE 1: A scheme showing the opposing effects of TGFβ on tumor growth and metastasis.

G1-S phase transition by inhibiting translation initiation [86], activation of JNK-dependent cell death pathway [87], induction of the early growth response-1 (Egr-1) gene [88], inhibition of Bcl-xL and Bcl-2 function [85], counteracting TGFβ release by tumor cells [54], and induction of cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ [89]. However, the precise antiproliferative mechanisms of the PPARγ agonists remain to be further studied. On the contrary, there are also other reports available on the opposite effects showing that PPARγ signaling promoted carcinogenesis [90, 91].

It should be noted that the antitumor effects of PPARγ may be explained at least in two different ways. One mechanism involves cell growth regulation [4], which should be further clarified, whereas the other mechanism includes cancer chemopreventive effects mediated by the induction of antioxidant enzymes [92]. It is well recognized that PPARγ affects cell survival, growth, and differentiation by acting on the peroxisomal proliferator-response element (PPRE), thereby modulating an expression of a group of genes controlling cell growth and differentiation pathways [93, 94]. The PPARγ homodimer and PPARγ-retinoic acid X receptor (RXR) α heterodimer have the specificities of DNA-binding with preferential binding of the latter to DR1, which is a PPRE DNA binding site. SRC-1 is a coactivator of PPARγ [95]. Binding of the ligand-activated PPARγ-RXRα heterodimer to its DNA binding sites stimulates the interaction between PPARγ-RXRα and p160/SRC-1 [95].

A number of studies support the concept that cancer chemoprevention is accomplished by the induction of antioxidant enzymes. The results from our laboratories indicated that oltipraz and flavonoids as potential cancer chemopreventive agents activate C/EBPβ in the antioxidant genes such as *glutathione S-transferase (GST) A2* [96, 97]. In addition, treatments of cells with PPARγ activators induced the nuclear translocation of NF-E2-related factor 2 (Nrf2) and C/EBPβ, and activating Nrf2 and C/EBPβ bindings to the antioxidant response element (ARE) and C/EBP response elements, respectively [92]. Moreover, the Nrf2 and C/EBPβ genes contain PPRE sites, which account for the induction

of the target antioxidant proteins by PPARγ activators. Both the ARE and the C/EBP binding site have crucial roles in transactivating the GSTA2 gene by PPARγ and RXR ligands [92]. Therefore, Nrf2 and/or C/EBPβ inductions(s) via the PPARγ and RXRα heterodimer binding to the PPREs in the promoter regions of the target genes contribute(s) to the antioxidant capacity of cells (e.g., GSTA2).

A result of our previous study indicated that specific mutations of these nuclear binding sites in the GSTA2 promoter, which are present as a three-PPRE cluster, caused the complete loss of its responsiveness to PPARγ activators [92]. All of the putative PPRE sites comprising DR1 were functionally active. Therefore, the binding of the activating PPARγ-RXR heterodimer to all of the PPRE sites appeared to be crucial for the inducible gene activation, showing that the PPAR binding site cluster is the functionally active PPRE-responsive enhancer module (PPREM) [92]. This study on the regulation of gene expression by the PPARγ-RXR heterodimer at the promoter containing multiple DR1 elements brought additional insight into the transcriptional control mechanism of the antioxidant enzymes. The identified molecular mechanism would shed light on the contribution of cell viability and cancer chemoprevention as a consequence of the induction of antioxidant targets genes by PPARγ activators.

4. TGFβ REGULATION BY PPARγ-RXR AND CELL SIGNALING

Activation of the PPARγ-RXR heterodimer represses the TGFβ1 gene through dephosphorylation of a transcription factor called zinc finger transcription factor-9 (Zf9), which has been shown to be induced by phosphatase and tensin homolog deleted on chromosome (PTEN)-mediated p70 ribosomal S6 kinase-1 (S6K1) inhibition [18]. Because RXRs are modular proteins with a highly conserved central DNA binding domain and a less conserved ligand binding domain [98], activation of the PPARγ and RXR heterodimer contributes to the gene regulation. The role of PPARγ in repression of the TGFβ1 gene was further evidenced by the effects of thiazolidinediones, and also by the reversal of TGFβ1 repression by the dominant negative mutants, supporting to the novel aspect that PPARγ activation contributes to TGFβ1 gene repression and that RXRα is necessary for the full responsiveness in the gene repression. In fact, the inhibition of TGFβ1 gene by the PPARγ and RXR heterodimer might account for either tumor suppression or tumor promotion [18]. Also, as an effort to identify the molecular basis of TGFβ1 repression by PPARγ activators, the effects of PPARγ and RXR activation on the TGFβ1 gene transactivation, that is regulated by the proximal DNA response elements, have been examined [18]. The potential regulatory sites responsible for the TGFβ1 gene expression have been explored by using the luciferase reporter gene assays, which identified the putative PPREs located at the multiple sites upstream from −453 bp of the promoter region [18]. Promoter deletion analyses indicate that neither the putative PPREs nor the activator protein-1 (AP-1) binding

sites are directly regulated by PPARγ activators forthe gene repression.

S6K1, a ubiquitous serine/threonine kinase, controls the translational efficiency by phosphorylating ribosomal S6 protein [99]. S6K1 functions as a multifunctional kinase for the phosphorylation of ribosomal S6 protein [99], CREM [100], BAD [101], and the eukaryotic elongation factor 2 kinase [102]. Rapamycin, a well-known mammalian target of rapamycin (mTOR) inhibitor, inhibited liver fibrosis and TGFβ1 expression in rats bile duct-ligated or challenged with toxicants [103, 104], with a concomitant decrease in S6K1 activity. It is well recognized that rapamycin inhibits S6K1 activity via mTOR inhibition [105]. Yet, other pharmacological agents that modulate S6K1 activity have not been reported. The mechanism of PPARγ-RXR heterodimer-mediated repression of the TGFβ1 gene has been elucidated in terms of the modulation of S6K1 activity (Figure 2).

The PI3K-mTOR pathway regulates S6K1 for the regulation of transcription factors involved in the TGFβ1 gene transactivation. A study identified the inhibition of S6K1 activity by the PPARγ-RXR, which contributes to TGFβ1 gene repression [18]. Another signaling molecule, PTEN, antagonizes the PI3-kinase-mTOR-S6K1-mediated signaling cascade [106, 107]. Thus, it has been elucidated that PPARγ activators upregulate PTEN, which leads to the S6K1 inhibition, consequently causing TGFβ1 repression [18].

5. TRANSCRIPTION FACTORS RESPONSIBLE FOR TGFβ REPRESSION BY PPARγ-RXR

In the promoter region of the TGFβ1 gene (Figure 3), the putative binding sites for PPARγ-RXR seemed to be neither active nor responsible for the gene repression by the activated PPARγ and RXR heterodimer. It has been claimed that the effects of PPARγ or retinoid ligands on TGFβ1 gene expression might be mediated in part by AP-1 inhibition [108, 109]. Nevertheless, such a result that deletion of the DNA region containing both AP-1 sites still had the capability to repress the gene by PPARγ activator suggests that the AP-1 binding sites might not be a major regulatory target in the TGFβ1 gene repression. Rather, the target molecule altered by PPARγ-RXRα-activated cell signal may be involved in the interaction with the protein recruited on the AP-1 DNA complex. It appeared that the TGFβ1 gene repression may have not resulted from the direct inhibition of AP-1, but other mechanistic basis [18].

Another study showed that the mechanism associated with the inhibition of TGFβ1 by PPARγ activators involves the regulation of c-Fos [108]. In the study, thiazolidinediones inhibit high-glucose-induced TGFβ1 promoter activity. A suggested mechanism was raised based on the observation that treatments of thiazolidinediones reduced high-glucose-induced, activated PKC and c-Fos-mediated TGFβ1 gene expression in mesangial cells [108].

Zf9 as an immediate early gene reduces cell proliferation with the induction of p21^{cip1} and the enhancement of c-Jun degradation [110, 111], thus functioning as a potential tumor suppressor gene. The transcription factors that interact with the known DNA binding sites on the region
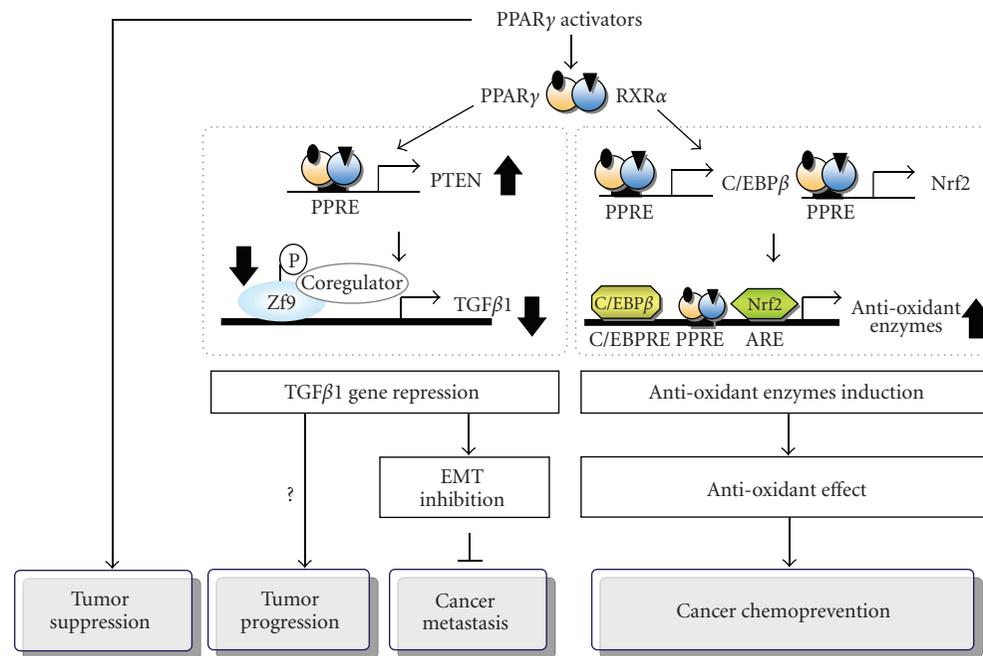
FIGURE 2: A schematic presentation of the multiple pathways regulated by PPARγ for tumor suppression, progression, inhibition of metastasis, and cancer chemoprevention.
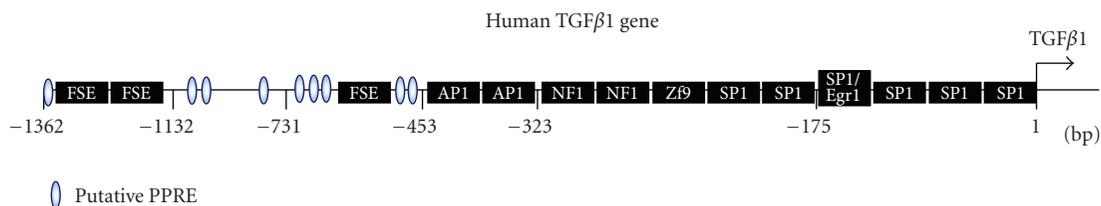

FIGURE 3: The human TGFβ1 promoter region.

downstream within the -323 bp of the TGFβ1 gene include Zf9, NF1, and SP1. It is noteworthy that Zf9 activation induces TGFβ1 during the activation of hepatic stellate cells [112]. Also, Zf9 regulates TGFβ receptors and collagen α1(I), promoting accumulation of extracellular matrix [113]. Studies have shown that Zf9 phosphorylation enhances its nuclear localization and transcriptional activity [111]. Zf9 as a transcription factor plays a crucial role for the induction of TGFβ1 [113]. Thus, phosphorylation status of Zf9 may contribute to the promotion of its target gene expression [114]. Identification of the partners of Zf9 or phosphorylated Zf9 for the TGFβ1 gene regulation and their molecular interactions would be interesting to pursue. The constitutive Zf9 phosphorylation by S6K1 strengthened the important role of S6K1 as a multifunctional kinase for the transcription factor regulation of target genes [100–102].

The TGFβ1 gene contains the DNA response element interacting with Zf9 [16] that regulates multiple genes involved in tissue differentiation. Activation of Zf9 includes its phosphorylation at serine (or tyrosine) residues [114]. Thus, phosphorylation of Zf9 leads to transcription of its target genes [111, 114]. Although the kinase catalyzing Zf9

phosphorylation has not been completely identified, the inhibition of Zf9 phosphorylation by rapamycin that inhibits S6K1 activity via mTOR inhibition supports the role of S6K1 in Zf9 phosphorylation [18]. More importantly, the role of S6K1 in regulating TGFβ1 gene and the associated molecular mechanistic basis have been clarified in terms of Zf9 dephosphorylation [18]. In view of the previous observations that Zf9 is crucial as a transcription factor for TGFβ1 induction in hepatic stellate cells [113] and that a phosphorylated form of Zf9 plays a role in the transactivation of the target gene promoter [114], the potential ability of PPARγ activators to inhibit serine phosphorylation of the transcription factor has also been investigated. Thus, it has been demonstrated that the inhibition of the TGFβ1 gene by the activation of PPARγ-RXR includes Zf9 dephosphorylation [18]. Therefore, TGFβ1 gene repression by PPARγ activators appears to be related with dephosphorylation of Zf9, supporting the conclusion that the PPARγ-RXR heterodimer causes TGFβ1 repression via S6K1 inhibition, and that the inhibition of S6K1 activity provides a central mechanism, by which PPARγ-RXR regulates Zf9-dependent TGFβ1 gene expression (Figure 2).

Moreover, it has been shown that PPARγ activation induces PTEN, which serves as a PI(3,4,5)P$_3$ lipid phosphatase and antagonizes PI3-kinase-mediated cell signaling [106]. Functional PPREs located in the PTEN promoter have been recognized [115]. The induction of PTEN by PPARγ activators may result in TGFβ1 gene repression following S6K1 inhibition. Furthermore, PPARγ activators inhibited phosphorylations of Akt, ERK1/2, p90 ribosomal S6 kinase-1 (RSK1), and mTOR, downstream of PTEN, indicating that PTEN induction by PPARγ activators leads to S6K1 inhibition via the pathways of ERK1/2-RSK1 as well as Akt-mTOR. In conclusion, the result showing that PPARγ activation upregulates PTEN, which has also been implicated in tumor-inhibitory or anti-inflammatory actions of PPARγ [106, 115], gives credence to the concept that PPARγ activators induce PTEN during S6K1 inhibition, and consequently causes TGFβ1 repression. Therefore, the inhibition of tumor proliferation by PPARγ activators may be explained in part by PPARγ-dependent TGFβ1 repression (Figure 2), supporting the concept that the PPARγ activators may be applied for controlling TGFβ1-induced cancer metastasis and fibrosis.

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Review Article
PPARγ, PTEN, and the Fight against Cancer

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Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor, which belongs to the family of nuclear hormone receptors. Recent in vitro studies have shown that PPARγ can regulate the transcription of *phosphatase and tensin homolog on chromosome ten* (*PTEN*), a known tumor suppressor. *PTEN* is a susceptibility gene for a number of disorders, including breast and thyroid cancer. Activation of PPARγ through agonists increases functional PTEN protein levels that subsequently induces apoptosis and inhibits cellular growth, which suggests that PPARγ may be a tumor suppressor. Indeed, several in vivo studies have demonstrated that genetic alterations of PPARγ can promote tumor progression. These results are supported by observations of the beneficial effects of PPARγ agonists in the in vivo cancer setting. These studies signify the importance of PPARγ and *PTEN*'s interaction in cancer prevention.

1. INTRODUCTION

1.1. PPARγ

Peroxisome proliferator-activated receptor gamma (PPARγ) is a ligand-activated transcription factor, belonging to the nuclear hormone receptor family, whose ligand-binding domain is located at the carboxy-terminus. There are several known natural and synthetic PPARγ agonists with 15-deoxy-delta 12, 14-prostaglandin-J2 (15d-PG-J2) being the most notable natural PPARγ agonist. Additionally, linoleic, linolenic, and arachidonic acids are other commonly recognized natural agonists. Synthetic PPARγ agonists, such as the thiazolidinediones (TZDs), are some of the most commonly prescribed medications for the treatment of type II diabetes mellitus. The four commercially recognized TZDs are ciglitazone (Alexis), pioglitazone (Actos), rosiglitazone (Avandia), and troglitazone (Rezulin).

After ligand-activation, PPARγ forms a heterodimer complex with retinoic acid receptor (RXR). This PPARγ/RXR complex subsequently translocates to the nucleus and binds to a peroxisome proliferator response element (PPRE) within a target gene thereby initiating transcription. The primary, and most studied, targets of PPARγ are involved in metabolic pathways and adipocyte differentiation. However, in recent years it has been suggested that PPARγ has a role in cancer development. Indeed, initial studies demonstrated alterations of cellular differentiation, indicative of apoptosis in a breast cancer setting, after PPARγ agonist stimulation. This indicates that PPARγ and its agonists may play an important role in cancer development, prevention, and treatment.

In 1998, Mueller et al. performed one of the first PPARγ agonist studies in a cancer setting [1]. They demonstrated that both 15d-PG-J2 and rosiglitazone (Rosi) could induce changes in epithelial gene expression associated with a more differentiated, less malignant state. Moreover, they described a reduction in the overall growth rate of breast cancer cells when treated with a PPARγ agonist. These data suggest that PPARγ can contribute to the prevention of breast cancer development and its agonists may be a novel therapy for cancer treatment [1]. These results stimulated further studies investigating PPARγ-mediated tumor suppression. One protein, that may play a role in PPARγ-mediated tumor suppression, is phosphatase and tensin homolog on chromosome ten (PTEN), which has an established role in breast cancer development. Interestingly, Mueller et al. characterization of breast cancer cells after PPARγ activation demonstrated a striking resemblance to cells with active PTEN expression [1]. Taken together, these results suggested

that PTEN and PPARγ, together, may modulate breast cancer progression.

1.2. PTEN

In 1995, *PTEN* was identified as the susceptibility gene for Cowden syndrome (CS), which is characterized by breast, thyroid, and endometrial carcinoma as well as macrocephaly [2–8]. Patients diagnosed with CS have a 25–50% lifetime risk of developing female breast cancer, compared to the general population risk of ~13% [9, 10] Additionally, patients have ~10% lifetime risk of developing thyroid cancer, compared to <1% in the general population and have a ~5–10% lifetime risk of endometrial cancer compared to ~2–4% in the general population [9, 11]. Since its identification, research has detected a *PTEN* mutation in 85% of CS patients [11]. Furthermore, somatic alterations in *PTEN*, whether by genetic or epigenetic mechanisms, play some role in the pathogenesis of a broad range of solid tumors, including sporadic carcinomas of the breast, thyroid, endometrium, and colon.

PTEN's protein, PTEN, is a unique phosphatase that has the ability to dephosphorylate both proteins and lipids (Figure 1) [4]. Its lipid phosphatase activity functions as a negative regulator of Akt phosphorylation (P-Akt). PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) at the D3 position generating phosphatidylinositol 4,5-biphosphate (PIP2), decreasing cellular PIP3 levels. Since PIP3 is required for Akt phosphorylation, active PTEN leads to a decrease in the levels of P-Akt and consequently a decrease in Akt -mediated proliferation pathways. PTEN's protein phosphatase activity has been shown to inhibit the SHC/SOS/GRB2 and mitogen-activated protein kinase (MAPK) pathways. The dephosphorylation of SHC by PTEN indirectly decreases the phosphorylated form of MAPK levels, reducing MAPK's activity. Additionally, PTEN's protein phosphatase activity upregulates p27 with a concomitant downregulation of cyclin D1 which coordinates G1 arrest [12]. By regulating these key-signaling pathways, PTEN downregulates cell division and upregulates apoptosis. Additionally, PTEN's protein phosphatase activity has been shown to dephosphorylate focal adhesion kinase (FAK), which inhibits cell spreading and migration [4].

Transcriptional regulation of *PTEN* is only beginning to be elucidated. To date, analysis of *PTEN*'s promoter suggests that there are at least eight regulatory factors that modulate *PTEN*'s transcription (Figure 2). In 2001, Stambolic et al. identified a functional p53 binding site, located at nucleotide positions −1190 to −1157 in *PTEN*'s promoter, which was required for *PTEN's* upregulation [13]. Additionally, early growth response-1 (Egr-1) has been shown to bind to the *PTEN* promoter at −947 to −939 and induce PTEN expression [14]. Recently, our laboratory has identified a USF1 binding site ~2 kb (−2237 and −2058) upstream of the ATG site [15]. CBF-1, Sp1, and c-Jun have also recently been suggested as *PTEN* transcription factors [11, 16–18]. The majority of *PTEN* promoter analyses have been focused on transcription factors that increase PTEN levels. However, recently, suppression of *PTEN* gene expression has been
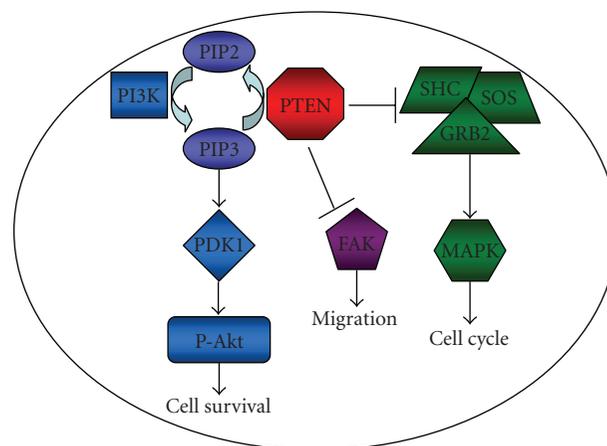

FIGURE 1: PTEN protein signaling pathways. PTEN's lipid phosphatase activity dephosphorylates PIP3 to PIP2 inhibiting PDK1-mediated Akt phosphorylation and downregulating Akt-mediated cell survival. PTEN's protein phosphatase activity inhibits the phosphorylation of FAK to prevent cell migration. PTEN's protein phosphatase activity also dephosphorylates the SHC/SOS/GRB2 complex resulting in the decreased phosphorylation of MAPK and inhibition of the cell cycle.

shown by the tumor necrosis factor-alpha/nuclear factor-kappa B (NF-κB) [19], however the precise mechanism of this inhibition remains unclear.

1.3. PPARγ and PTEN in vitro

In 2001, Patel et al. first showed that PPARγ can be a *PTEN* transcription factor [20]. They observed that Rosi induced PTEN protein expression in both MCF-7 breast and CoCa2 colon cancer cell lines. In addition to the increase in PTEN expression, they observed an inhibition of both Akt phosphorylation and cellular proliferation. They also identified two putative PPREs within the *PTEN* promoter approximately 15 and 13 kb upstream of the ATG site (Figure 2). While this study was significant in demonstrating a potential link between PPARγ and PTEN, it remained correlative.

In 2005, two independent laboratories confirmed Patel's suspicion that PPARγ induces *PTEN* transcription in a breast cancer setting [21, 22]. We demonstrated that of the four TZDs, only Rosi had the ability to induce *PTEN* transcription and subsequently its protein expression in MCF-7 cells [21]. Furthermore, we showed that stimulation with Rosi induces a PTEN protein that is both protein- and lipid-phosphatase active, as evidenced by decreased phosphorylation of Akt and MAPK concomitant with PTEN expression. Additionally, Rosi treatment induced G1 arrest that paralleled with PTEN expression. By using a Rosi analog, Compound 66, that is incapable of activating PPARγ, we confirmed that Rosi induced PTEN expression via a PPARγ-dependent mechanism in several reporter assays [21].

Additionally, in 2005, Bonofiglio et al. also demonstrated that PPARγ could upregulate *PTEN*'s transcription in a breast cancer setting [22]. After cells were stimulated with
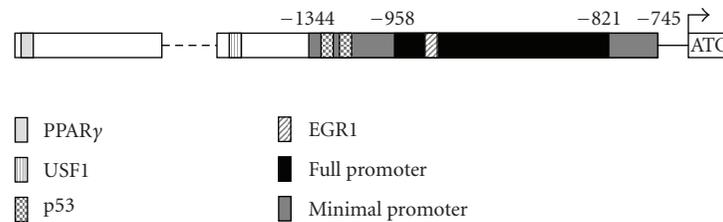
FIGURE 2: *PTEN* promoter and its transcription factors. *PTEN*'s full-length promoter lies between -1344 and -745 (gray bar), while the minimal promoter lies between -958 and -821 (black bar). Four transcription factors are known to directly bind upstream of PTEN: PPARγ (solid gray bar), USF1 (stripped gray bar), p53 (dotted gray bar), and EGR1 (dashed gray bar).

Rosi, an increase in PTEN protein was observed as well as an inhibition of Akt phosphorylation and cellular growth. More importantly, they were able to observe for the first time the specific binding of PPARγ to the *PTEN* promoter (-15376 to -15364; Figure 2). Interestingly, this interaction was enhanced by Rosi treatment. Further analysis indicated that PPARγ and estrogen receptor (ER) could bind to the PPRE both independently and simultaneously. The ER's association with the PPRE inhibited PPARγ's ability to induce transcription as demonstrated by cotreatment of MCF-7 breast cancer cells with both Rosi and 17ß-estradiol. This cotreatment inhibited the induction of PTEN protein that was observed by Rosi stimulation alone [22]. This is an important observation as it is appealing to postulate that this crosstalk, between PPARγ and ER, may significantly affect breast cancer therapeutics as well as lead the way to the discovery of future novel treatment therapies.

In 2006, Zhang et al. showed that Rosi stimulation of hepatocarcinoma cells results in the upregulation of PTEN and PTEN-dependent inhibition of cell migration [23]. This is significant because PTEN expression is decreased or absent in approximately half of all primary hepatocarcinoma patients. As similarly demonstrated by others, Rosi treatment of hepatocarcinoma cells resulted in an increase in *PTEN* mRNA. They further speculated that there may be three other potential PPREs within the *PTEN* promoter, located at -2874 to -2854, -1615 to -1596, and -1594 to -1574, however, it has not yet been determined if these are functional PPREs. Interestingly, Zhang et al. do not observe an increase in transcriptional activity of the *PTEN* promoter in response to Rosi treatment [23]. We observed similar results when examining the full-length *PTEN* promoter, using a luciferase reporter assay and Rosi stimulation (Teresi, Waite, and Eng; unpublished observations). This may suggest that elements beyond the full-length *PTEN* promoter are required for Rosi-mediated *PTEN* transcription.

These initial studies concretely demonstrated that PPARγ acts as a tumor suppressor in a cancer setting by upregulating *PTEN* transcription. However, these studies were performed solely in breast cancer cell lines, leaving the speculation that these observations are cancer-type dependent. To this end, several groups have studied PPARγ's ability to regulate PTEN levels in other cancer backgrounds. Lee et al. observed an inhibition of cellular proliferation and Akt phosphorylation in accord with an increase in G1 arrest and PTEN protein expression in A549 lung cancer cells [24].

Subsequently, PPARγ has been shown to upregulate PTEN expression in nonsmall cell lung cancer, neuroblastoma, adrenocortical, pancreatic, heptocarcinoma, and thyroid cell lines [23, 25, 26].

Interestingly, the majority of these studies utilized Rosi as the PPARγ agonist. This may be due to the combination of our initial study, which demonstrated that of the TZDs only Rosi was capable of inducing PTEN expression, and the fact that natural ligands can be difficult to work with in vitro [21]. Despite this, Chen et al. demonstrated that both ciglitazone and 15d-PG-J2 could upregulate PTEN expression in W-2 thyroid cells [27], which raises the possibility that of the TZDs, Rosi stimulation is limited to breast cancer. This remains to be determined.

1.4. PPARγ and PTEN in vivo

Despite the growing amount of in vitro data supporting the role of PPARγ as a tumor suppressor, only a small number of cancers have had their PPARγ status characterized in vivo and there are very few studies of clinical PPARγ agonist treatment. Nonetheless, current studies provide some essential and encouraging information. One of the first studies to analyze *PPARγ* status in an in vivo cancer setting examined 55 unrelated sporadic colon cancer samples and revealed 4 *PPARγ* mutations [28]. Moreover, these mutations produced an inactive PPARγ protein. This study demonstrated that PPARγ can act as a tumor suppressor in vivo and when its normal activity is altered it can lead to cancer development [29]. Subsequent studies have confirmed these results showing the reduction of PPARγ expression in both acrometaly [30] and ulcerative colitis [31], two predisposing conditions of colon cancer. In contrast to these studies, Ikezoe et al. did not observe any *PPARγ* alterations in their colon cancer study; however they limited their study to only exons 3 and 5 of *PPARγ* [32]. These studies indicate that PPARγ is indeed a tumor suppressor in the colon cancer setting; however none of these studies tested if the TZDs could effect the cancer's progression.

To date, the majority of studies correlating PPARγ with PTEN have been performed in vitro and these studies suggest that PPARγ agonists may be beneficial to PTEN in vivo. Moreover, in vitro data suggest that PPARγ agonists have the potential to be highly effective *PTEN* transcriptional inducers for patients who have one of the following: a hemizygous deletion, a germline nucleotide alteration within

the promoter, and potentially in the circumstance, where a *PTEN* mutation is not identified but a decrease in protein expression is observed.

Despite the potential beneficial effects of TZD treatment, in particular Rosi, one must be aware that the use of these medications may lead to more harm than good. For example, treatment of patients with germline intragenic *PTEN* mutations or those with neoplasias containing somatic intragenic mutations may see a raise of mutant, inactive protein. Recently, PTEN has been shown to induce gain-of-function p53 protein suggesting that TZD treatment in this setting may subsequently induce mutant, nonbeneficial p53 protein. Additionally, our work and others have suggested that not all of TZDs signal through the same pathways, at least in cell culture conditions [21]. Rosi is the only TZD that is known to increase PTEN in breast cancer lines, which indicates that each TZD may lead to its own individual side effects. Indeed in 2000, troglitazone (Rezulin) was pulled off of the market due to liver toxicity. Interestingly, to date, this has not been observed with other TZDs [33]. A recent study demonstrated that Rosi (Avandia) increases the risk of heart complications, specifically heart attacks; however these results have yet to be replicated [34]. This indicates that the significance of Rosi treatment on cardiac function needs to be examined further. Indeed, in this first study, important results, which came to the opposite conclusions, were not included in the meta-analysis. In spite of this, a deeper understanding of the signaling mechanisms behind these side effects should open the door to both new avenues of cancer treatment and personalized health care, allowing physicians to properly weigh the benefits against the known side effects prior to prescribing such a treatment.

Drug-drug interactions are another aspect that physicians will need to be aware of. Bonofiglio's PPARγ-ER-PTEN results are significant in the context of breast cancer and hormone therapies [22]. Their data suggest that women treated with hormones, either through birth control or hormonal therapies, may not benefit from cotreatment with a PPARγ agonist. This further suggests that hormone treatment may actually be detrimental by inhibiting naturally occurring *PTEN* transcription.

1.5. The translation of PPARγ and PTEN into the clinic

A recent study has suggested that Rosi treatment could be beneficial to patients with Gefitinib-resistant lung cancer [24], a cancer which is typically correlated with the loss of PTEN protein. Lee et al. have shown that in the human lung cancer cell line, A549, the combined treatment of Rosi and Gefitinib was more beneficial than Gefitinib treatment alone [24]. Taken together, these data provide support that the upregulation of PTEN levels with Rosi treatment may reverse the Gefitinib resistance in these patients. Such a treatment could have the potential to be advantageous to patients with both sporadic and familial cancer.

PPARγ status is only now beginning to be examined in the in vivo cancer setting, however the TZDs have been used in a variety of clinical trials, although not directly related to PPARγ activation. Seemingly, out of the ordinary, polycystic ovary syndrome (PCOS) is the most commonly studied syndrome with regards to the effects of TZD treatment [35]. While there is still much debate on what treatment is best for these patients, the majority believe that Rosi treatment is beneficial. Studies have demonstrated that Rosi treatment raises insulin and androgen levels in the obese PCOS population, thereby inhibiting tumor progression. Furthermore, Yee et al. recently performed a pilot study in women with breast cancer to determine if Rosi treatment would be beneficial. Thirty-eight women with early stage breast cancer were treated with Rosi for 2–6 weeks with tumor growth inhibition or progression as an end point [36]. The data indicate that short-term Rosi therapy in early-stage breast cancer patients has both local and systemic effects on PPARγ signaling. Both of these studies suggest that Rosi may be used clinically to benefit cancer patients.

1.6. PPARγ and PTEN's future

The culmination of these data strongly suggests that Rosi stimulation may be advantageous to the cancer patient. However, lacking in many of these studies is the role of PTEN. To date, in vitro data has demonstrated a connection between PPARγ and PTEN, yet no in vivo study has concretely confirmed these results. The results obtained from these studies would concretely determine if Rosi treatment is advantageous for cancer patients by upregulating PTEN expression through PPARγ.

While clinical trials are necessary to determine if Rosi treatment is truly beneficial for cancer patients and which patients it is most advantageous for, much remains to be learned at the molecular level. The relevance of the putative PPRE in the *PTEN* promoter identified by Bonofiglio et al. remains to be determined (Figure 2) [22]. This PPRE is located a long distance from the ATG site, thus making it unclear if this site is functional in regulating PTEN expression. It will be interesting to find out the role of this unique site.

While evidence suggests that TZDs induce PTEN expression through PPARγ, further studies are warranted to determine the exact mechanism of action. Evidence by our group suggests that PPARγ may regulate PTEN expression through both transcriptional-dependent and -independent mechanisms [37]. While this may add to the complexity of the role of PPARγ, with regards to PTEN, it may also provide other areas for therapeutic advances. Interestingly, while studying the ability of statins to induce PTEN expression, we observed that statins increase *PTEN* transcription via an unknown PPARγ-mediated mechanism [37]. Retrospectively, we observed a similar response with Rosi stimulation indicating that PPARγ is necessary; however its transcriptional activity is not. These results suggest that PPARγ may induce *PTEN* transcription through an unknown mechanism and an unrecognized transcription factor; however this remains to be determined.

2. CONCLUSION

In recent years, there has been a growing accumulation of data implicating the importance of both PPARγ and PTEN in

cancer prevention, development, and treatment. In vitro data has demonstrated that PPARγ agonists can induce functional PTEN protein that controls cellular growth. In vivo data has suggested that *PPARγ* genetic alteration can lead to cancer development, while its agonists can inhibit tumor progression. Despite this progress, we are only beginning to determine the roles of these two proteins and their complex interactions. Undoubtedly, future studies will clarify the PPARγ-PTEN connection providing a variety of targets that may lead to novel therapeutic treatments for cancer patients.

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Research Article

Rosiglitazone Inhibits Adrenocortical Cancer Cell Proliferation by Interfering with the IGF-IR Intracellular Signaling

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Rosiglitazone (RGZ), a thiazolidinedione ligand of the peroxisome proliferator-activated receptor (PPAR)-γ, has been recently described as possessing antitumoral properties. We investigated RGZ effect on cell proliferation in two cell line models (SW13 and H295R) of human adrenocortical carcinoma (ACC) and its interaction with the signaling pathways of the activated IGF-I receptor (IGF-IR). We demonstrate a high expression of IGF-IR in the two cell lines and in ACC. Cell proliferation is stimulated by IGF-I in a dose- and time-dependent manner and is inhibited by RGZ. The analysis of the main intracellular signaling pathways downstream of the activated IGF-IR, phosphatidyl inositol 3-kinase (PI3K)-Akt, and extracellular signal-regulated kinase (ERK1/2) cascades reveals that RGZ rapidly interferes with the Akt and ERK1/2 phosphorylation/activation which mediates IGF-I stimulated proliferation. In conclusion, our results suggest that RGZ exerts an inhibitory effect on human ACC cell proliferation by interfering with the PI3K/Akt and ERK1/2 signaling pathways downstream of the activated IGF-IR.

1. INTRODUCTION

Adrenocorticalcarcinoma(ACC) is a rare tumor with an incidence of approximately 1-2 per million population per year. ACC is a very aggressive tumor, characterized by a poor-prognosis: approximately 50% of patients do not survive beyond 2 years from the diagnosis and the 5-year mortality rate is between 20% and 60% [1]. Its poor prognosis depends mainly upon the limited therapeutic resources. At present, a complete surgical removal following an early diagnosis is the only valuable option for the tumor's cure. Moreover, other than improved surgical management, the prognosis for ACC has not changed significantly over the past three decades [2]. The tumor is, in fact, resistant to radio- and chemotherapy and medical treatment very rarely leads to a complete remission in the case of recurrences or metastatic spread. Although several new medical therapeutic options have been recently proposed [3], at present the medical treatment of advanced ACC is far from being satisfactory, due to our poor knowledge of the molecular mechanisms leading to malignant transformation of adrenocortical cells. In fact, although some intracellular signaling pathways have been shown to be altered in ACC cells [4], efforts to identify the events leading to neoplastic transformation and tumor invasiveness have met with limited success. The role of IGF-I system in mediating proliferation and progression has been well documented in several cancers, including adrenocortical carcinoma [5]. In particular, ACC, as well as the H295R cell line [6], overexpress both IGF-II [7] and its promiscuous receptor IGF-IR [8] in comparison to adrenal adenomas and normal adrenal tissue. The overexpressed IGF-II is thought to act in a paracrine fashion through the IGF-IR to sustain tumor and cell proliferation [6, 9, 10].

Peroxisome proliferator activated receptor (PPAR)-γ is a ligand-activated transcriptor factor and a member of the nuclear hormone receptors superfamily. Thiazolidinediones

(TZDs), which are a family of PPAR-γ ligands, have been introduced in the therapy of type 2 diabetes mellitus (T2D) because of their ability to reduce insulin resistance. In the last few years, an increasing amount of experimental data showing the ability of these drugs to exert additional pleiotropic actions such as regulation of inflammatory processes and of cancer cell growth has been published [11].

TZD effects seem to be mainly due to their ability to bind and activate PPARγ differentially expressed in adipose and other tissues. Upon ligand binding, PPARγ heterodimerizes with the 9-cis retinoic acid receptor (RXR) on specific responsive elements in the promoters of genes involved in glucose and insulin homeostasis, lipid metabolism, and cellular differentiation. Besides this transactivating activity, a ligand-dependent transcriptional transrepression mechanism involving PPARγ/RXR complex has been described. According to such a model, the heterodimerized receptor represses gene transcription in a DNA-binding independent way by physically sequestering activated transcriptional factors or their coactivators [12]. More recently, an increasing number of TZD effects, and in particular the antineoplastic ones, have been shown to be independent of PPARγ activity [13]. Finally, rapid nongenomic activities of TZDs, not resulting in modulation of gene transcription but affecting posttranslational modifications involved in cell signaling, have been reported [14].

In addition to their action as insulin sensitizers, TZDs inhibit cell growth in breast, colon, prostate, lung, pancreas, stomach, thyroid, liver and adrenal cancers [15]. However, the molecular mechanisms underlying such pharmacological activities remain to be elucidated.

Rosiglitazone (RGZ) and pioglitazone (PGZ), the two most widely used PPARγ agonists, have been shown to inhibit growth and invasiveness of the human adrenal cancer cell line H295R [16] as well as to induce cell differentiation and apoptosis [10]. Moreover, H295R cells and both normal and tumoral adrenal tissue express PPARγ, with no differences in the level of expression between tumoral and normal tissue and no correlation with clinical parameters such as tumor size, hormonal profile, and so forth [10, 16].

In this study, we investigate two different cell models of ACC, namely, SW13 and H295R lines, whether the TZD RGZ may exert its antiproliferative action on human ACC cell lines by interfering with the intracellular pathways activated by IGF-IR.

2. MATERIALS AND METHODS

2.1. Reagents

Anti-phospho [Akt (Ser473), ERK1/2 (T202/Y204)] and anti-Akt antibodies were from Cell Signaling Technology, Inc. (Danvers, Mass, USA). Anti-ERK1/2, anti-IGF-IRβ, and anti-actin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif, USA). Anti-PI3K p85 regulatory subunit antibody was from Upstate Biotechnology (Lake Placid, NY, USA). Rosiglitazone was from Alexis Biochemicals (San Diego, Calif, USA). MTS solution was purchased from Promega (Madison, Wis, USA). IGF-I was from

Sigma Aldrich (San Louis, Mo, USA). [Methyl-^{3}H]thymidine ([^{3}H]TdR) was purchased from NEN Life Science Products (Boston, Mass, USA). NVP-AEW541 was provided by Novartis (Basel, Switzerland).

2.2. Tissue specimens and cell cultures

A total of three normal human adrenal glands, three adrenal carcinomas, and three adenomas were used in this study. Normal adrenal glands were removed during an expanded nephrectomy due to renal carcinoma or from organ donors (age 32–72 years). Approval for the use of human material was given by the Local Ethical Committee. Informed consent was obtained from each patient. Adrenocortical fragments, collected immediately after surgery, were snap frozen in liquid nitrogen and stored at $-80°$C.

The human ACC cell lines H295R and SW13 were obtained from the American Type Culture Collection (Manassas, Va, USA). SW13 were cultured in DMEM/F-12 medium (Sigma-Aldrich) with 10% FBS (Euroclone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. H295R need DMEM/F-12 medium enriched with a mixture of insulin/transferrin/selenium (Sigma-Aldrich). Cells were incubated at 37°C in a humidified 5% CO$_2$ atmosphere.

Subconfluent cells starved 24 hours were treated with different stimuli added to serum-free medium. Rosiglitazone was added simultaneously with IGF-I, at the doses and for the time intervals (15 minutes for western blot analysis and from 24 hours up to 7 days for proliferation experiments) indicated in figure legends.

Cells were pretreated 1 hour with NVP-AEW541 before addition of other stimuli. For incubations longer than 24 hours, media and stimuli were replaced every day.

2.3. MTS assay

SW13 and H295R were seeded in 96-well plates at the density of $3·10^3$ and $8·10^3$ cells/well, respectively. After 24-hour starvation in serum-free (SF) medium, cells were treated with the different stimuli in SF medium for the indicated times (see figure legends) and cell number in each well was evaluated by MTS assay (Promega), according to the manufacturer's instructions. The samples were analyzed by an ELISA plate reader (Wallac 1420 - PerkinElmer) at 490 nm wavelength to measure optical density (OD). Each experimental point was performed in quintuplicate in at least three independent experiments.

2.4. Cell proliferation assays

2.4.1. Viable cell counting

Cells were seeded in 12-well plates ($1,8·10^4$ and $5·10^4$ cells/well for SW13 and H295R, resp.), and after 24-hour starvation were treated for 2 or 4 days in SF medium, then trypsinizedand counted in the haemocytometer. Mean cell number was obtained by counting triplicate in three

different experiments. Dead cells were excluded by trypan blue exclusion test.

2.4.2. DNA synthesis assay: [³H]thymidine uptake

DNA synthesis was evaluated according to the amount of [³H]TdR incorporated into trichloroacetic acid (TCA)-precipitated materials. Cells seeded at different density ($5 \cdot 10^4$ or $2,5 \cdot 10^4$ H295R cells/well in 12 or 24 well plates, and $1,8 \cdot 10^4$ SW13 cells/well in 12 well plates) were grown in 10% FBS complete medium till 70% confluence. After 24-hour starvation, cells were treated with IGF-I or RGZ in 1% FBS medium for the indicated times (1–7 days) pulsing them with 1.0μCi/ml [³H]TdR (6.7 Ci/mmol) 4 hours before stopping proliferation in ice-cold 10% TCA. After washing in TCA and then in methanol, cells were solubilized in 0.2 N NaOH, and radioactivity was measured in the scintillation counter. Experiments were performed in triplicate and repeated at least three times.

2.5. Western blot analysis

Treated cells were extracted in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF). Thirty μg of proteins measured by Coomassie reagent (BIO-RAD Labs, Hercules, Calif, USA) were loaded onto 8–10% reducing SDS-PAGE. After separation, proteins transferred to nitrocellulose membranes were 1 hour blocked at room temperature in 5% skimmed milk in TTBS (0.1% Tween-20, 20 mM Tris, 150 mM NaCl) and incubated overnight with primary antibodies at appropriate dilutions followed by peroxidase-secondary IgG (1:3000). Proteins were revealed by BM-enhanced chemiluminescence system (Roche Diagnostics, Milan, Italy). Image acquisition anddensitometric analysis were performed with Quantity One software on a ChemiDoc XRS instrument (BIO-RAD Labs, Hercules, Calif, USA). All western blots were repeated in at least 3 independent experiments. Membrane re-probing was performed after stripping procedure (Pierce, Rockford, Il, USA).

PI3 kinase assay

Treated cells were extracted in lysis buffer A (20 mM Tris, pH 7.4, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF). After protein measurement, aliquots containing equal amount of proteins (300 μg) were precleared with 50 μl of Protein G-Sepharose. Precleared lysates were then immunoprecipitated overnight at 4°C with 3 μg of rabbit anti-p85 PI3K antibody in the presence of 50 μl of Protein A-Sepharose. Sepharose beads washed in a 10 mM Tris-HCl (pH 7.4) containing 0.1 mM EGTA and 5 mM LiCl, were suspended in a kinase buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA) containing 20 μg of L-α-phosphatidyl inositol (Sigma-Aldrich, St. Louis, Mo, USA), 25 mM $MgCl_2$ and 10 μCi of [γ^{32}P]ATP and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of 60 μl of 6 M HCl + 160 μl of chloroform:methanol (1:1). Lipids were then resolved by thin layer chromatography

plates (TLC silica gel 60) (Merck Laborchimica, Florence, Italy) in chloroform, methanol, water and ammonium hydroxide (60:47:11, 3:2). Dried TLC sheets were developed by autoradiography. Band quantification was performed with ChemiDoc XRS instrument (BIO-RAD Labs, Hercules, Calif, USA).

2.6. Statistical analysis

Results are expressed as mean $\pm$ SE. The effect of different concentrations of IGF-I and RGZ on cell proliferation was tested by One-Way ANOVA. Multiple *Post Hoc* comparisons were performed by Bonferroni's correction. Student's τ-test for paired or unpaired data was applied when appropriate for comparison of two sets of data. $P < .05$ was taken as significant.

3. RESULTS

Adrenal cancer is characterized by an increased expression of the IGF-IR compared to both nontumoral and adenomal adrenal tissues as demonstrated by western blot analysis with a specific antibody against the β subunit of IGF-IR (Figure 1(a)). A marked expression of this receptor isalsopresent in SW13 and in particular in H295R adrenocortical cancer cells (Figure 1(a)), suggesting that both tissue and cell systems may be highly responsive to the effects of IGF-I and IGF-II.

In order to study the role of IGF-IR and of its downstream intracellular signaling pathways on cell proliferation, we stimulated both SW13 and H295R cells with increasing concentrations of the receptor's elective ligand, IGF-I. IGF-I is able to induce cell proliferation in a time- and dose-dependent manner as demonstrated by evaluating cell viability through MTS assay in both SW13 (Figure 1(b)) and H295R (Figure 1(c)) cells stimulated with increasing concentrations of IGF-I (1 to 50 nM) for 24 hours up to 7 days. Such a stimulatory effect is confirmed by [³H]thymidine uptake experiments in H295R cells (Figure 1(d)).

Following 24-hour treatment, RGZ reduces cell viability in a dose-dependent manner in SW13 cells treated (Figure 2(b)) or not (Figure 2(a)) with 10 nM IGF-I, showing an IC_{50} of $22.48 \pm 1.54 \mu$M (coefficient of variation 6.9%) as calculated with ALLFIT program [17], Figure 2(c). Interestingly, the IC_{50}s derived fromthe two RGZ dose response viability curves obtained for IGF-I-treated and untreated cells are not statistically different, suggesting that the effect of RGZ is similar independently of exogenous IGF-I stimulation. Thus, we chose to use 20 μM RGZ in all the experiments. The inhibitory effect of RGZ on cell proliferation in basal conditions is significantly increased with the time of incubation as evaluated by [³H]thymidine uptake experiments in both SW13 (Figure 2(d)) and H295R (Figure 2(e)) cells. Again, thenegativeeffect on cell viability exerted by RGZ is similar in IGF-I treated and untreated SW13 cells (Figures 3(a)–3(e)). Conversely, in H295R cells where the effect of RGZ requires longer times to become evident, RGZ is also able to revert IGF-I-stimulation (Figures 3(f)–3(h)). Both IGF-I-stimulated proliferation and the

(a)

(b)

(c)

(d)
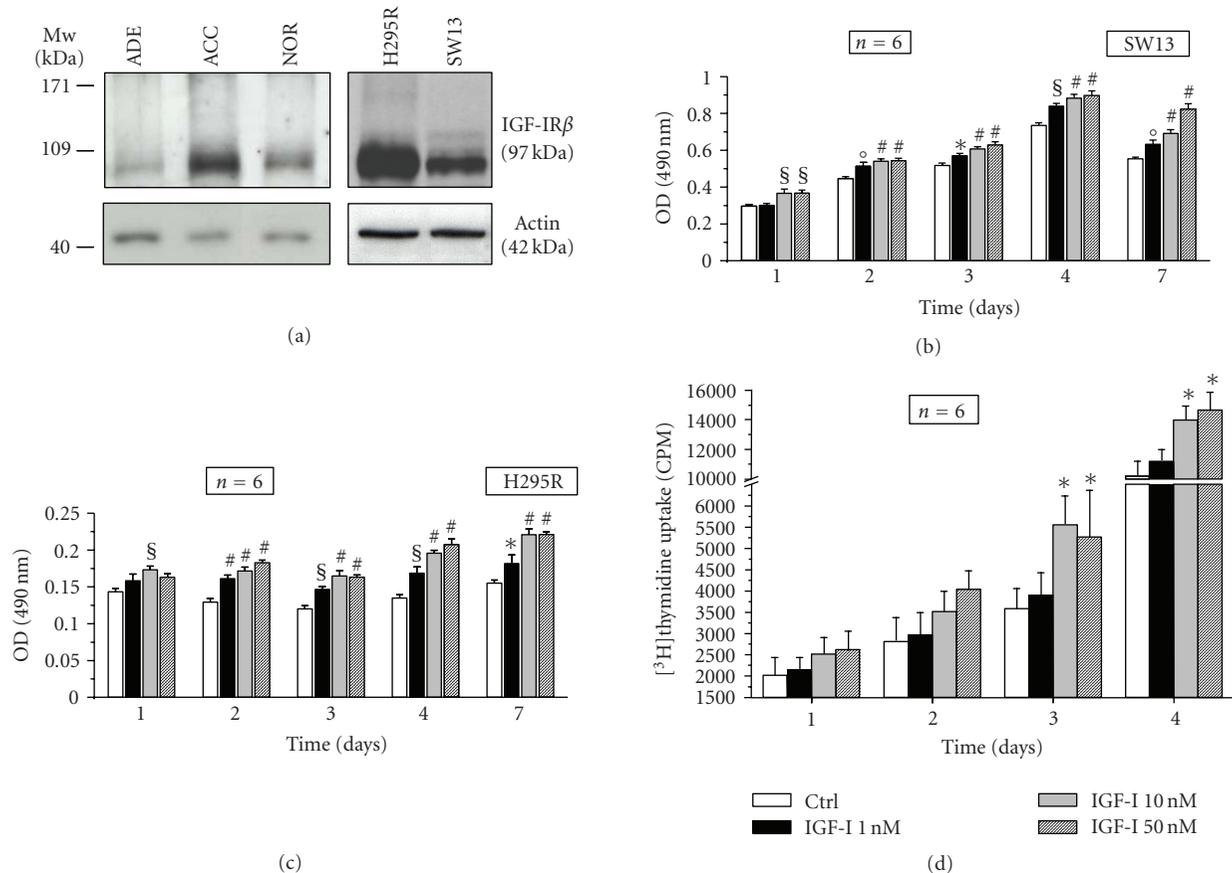
Figure 1: *Adrenocortical cancer is responsive to IGFs.* (a) Western blot analysis of adrenocortical tissues (adrenocortical carcinoma: ACC; normal adrenal tissue: NOR; adrenocortical adenoma: ADE) and adrenocortical cancer cell lines (SW13 and H295R) reveals a high expression of IGF-IR in cancer tissue and cells and lower levels in normal and adenoma tissues. Western blot for actin was performed for lane protein normalization. Molecular weight (Mw) markers are indicated. The effects of cell incubation with increasing doses of IGF-I (1, 10 and 50 nM) for the indicated times on cell proliferation were evaluated by MTS in SW13 (b) and H295R (c) and confirmed in H295R by evaluation of [^{3}H]TdR incorporation in cell DNA content (d). Data represent mean ± SE OD (b), (c) or mean ± SE of [^{3}H]TdR incorporation (counts per minute, cpm) (d). Statistical significance versus respective control: $^*P < .05$, $^\circ P < .01$, $^\S P < .005$, $^\# P < .001$.

inhibitory effect of RGZ are confirmed by SW13 (Figure 3(i)) and H295R (Figure 3(j)) cell counting at 2- and 4-day incubation, respectively.

To elucidate the intracellular mechanism by which RGZ affects adrenal cancer cell proliferation, we investigated the effects of the drug on the two main intracellular signaling pathways engaged by the activated IGF-IR, namely, the phosphatidyl inositol 3 kinase (PI3K)-Akt cascade and the extracellular signal-regulated (ERK) signaling [18].

Rapid stimulation (15 minutes) of SW13 (Figure 4(a)) and H295R (Figure 4(b)) cells with 10 nM IGF-I determines an increased phosphorylation of Akt in Ser473, resulting in the activation of the enzyme (Figure 4, upper and lower panels). Concomitant addition of 20 μM RGZ for 15 minutes in the presence or absence of IGF-I interferes with Akt phosphorylation/activation in SW13 (Figure 4(a)) and H295R (Figure 4(b)) cells. The inhibitory effect of RGZ is statistically significant versus IGF-I only, but this trend is also present on active Akt in basal conditions (Figure 4 lower panels). The in vitro immunokinase assay for PI3K performed on SW13 (Figure 5(a)) and on H295R

(Figure 5(b)) lysates demonstrates a rapid activation (15 minutes) of the enzyme by IGF-I, which is reverted by co-incubation with RGZ. The inhibitory effect of RGZ is evident in basal conditions in H295R cells only (Figures 5(a), 5(b)). Similarly, RGZ interferes with IGF-I-rapid stimulation of phosphorylation/activation of ERK1/2 in SW13 (Figure 6(a)) and H295R (Figure 6(b)), but, conversely, the effect of RGZ in basal conditions is evident in SW13 cells only (Figures 6(a), 6(b)).

In order to further demonstrate the involvement of Akt and ERK signaling downstream of the IGF-IR in mediating cell proliferation, we use the NVP-AEW541 (NVP) inhibitor of the IGF-IR tyrosine kinase activity. As shown in Figure 7(a), NVP is able to block phosphorylation of Akt (upper panel) and ERK1/2 (middle panel) both in the basal conditions and following 15-minute IGF-I stimulation. The block of the IGF-IR system by NVP results in inhibition of IGF-I-stimulated cell proliferation evaluated at 2 and 4 days of treatment in SW13 (Figure 7(b)) and H295R, respectively, (Figure 7(c)). Moreover, a further addition of RGZ to the inhibitor results in no significant reduction of

(a)

(b)

(c)

(d)

(e)
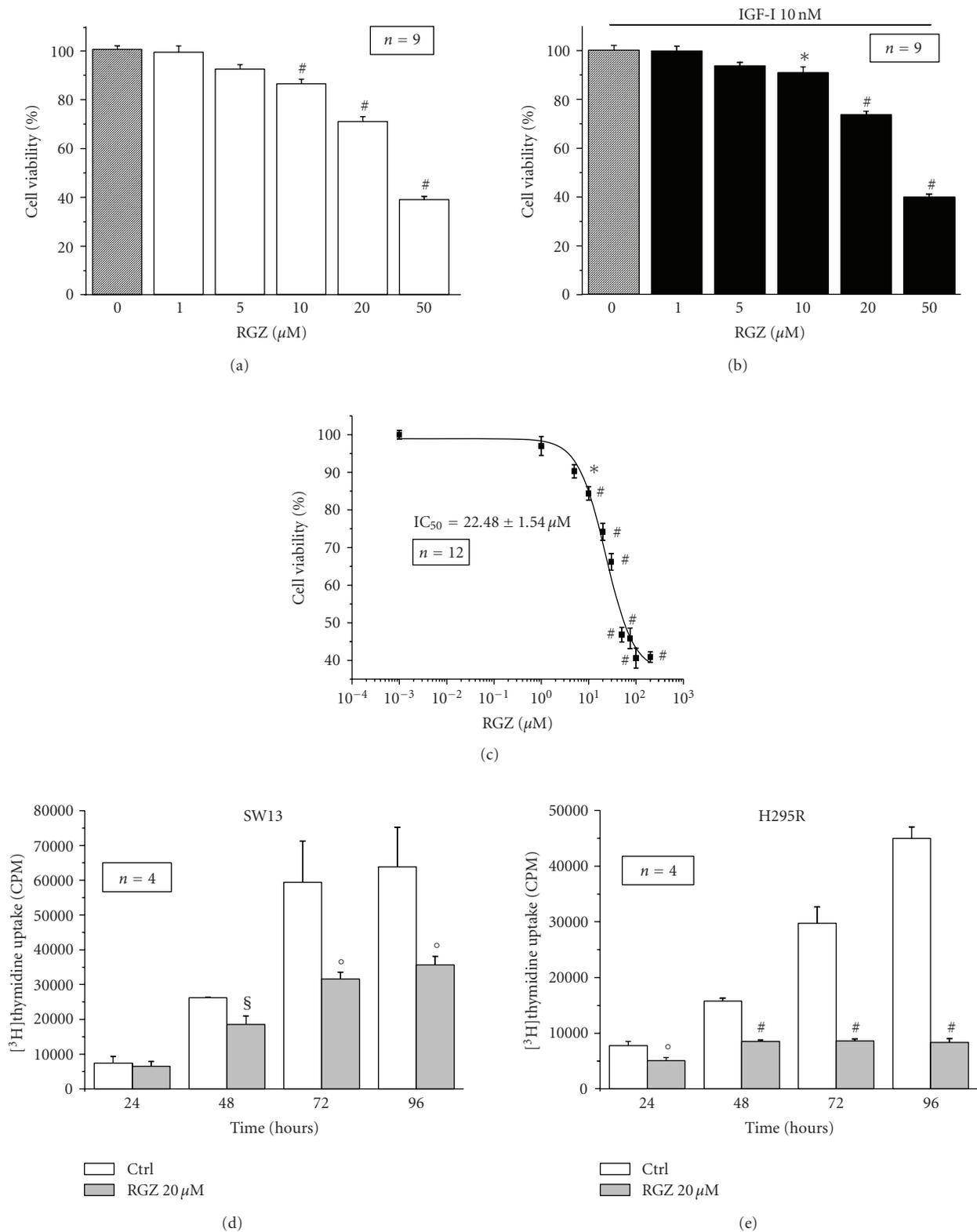
FIGURE 2: *Dose dependent effects of RGZ on adrenocortical carcinoma cell proliferation.* The dose dependent effect of RGZ on SW13 cell proliferation was evaluated by MTS assay at 48-hour stimulation in the absence (a), (c) or presence (b) of 10 nM IGF-I. A complete dose response curve for RGZ (10^{-3} to $2 \cdot 10^2$ μM) on cell proliferation in SW13 is reported (c), showing the IC$_{50}$ for RGZ calculated by ALLFIT program [17]. Data represent mean $\pm$ SE percentage of OD over controls (RGZ 0 μM). Statistical significance versus respective control (RGZ 0 μM): $^*P < 0.05$, $^\#P < 0.001$. The time dependent effect of RGZ administration (20 μM) on cell proliferation was evaluated at the indicated times in SW13 (d) and H295R (e) by [^{3}H]TdR incorporation. Data represent mean $\pm$ SE percentage of cpm over untreated control. Statistical significance versus respective Ctrl: $^\circ P < 0.01$, $^\S P < 0.005$, $^\#P < 0.001$.
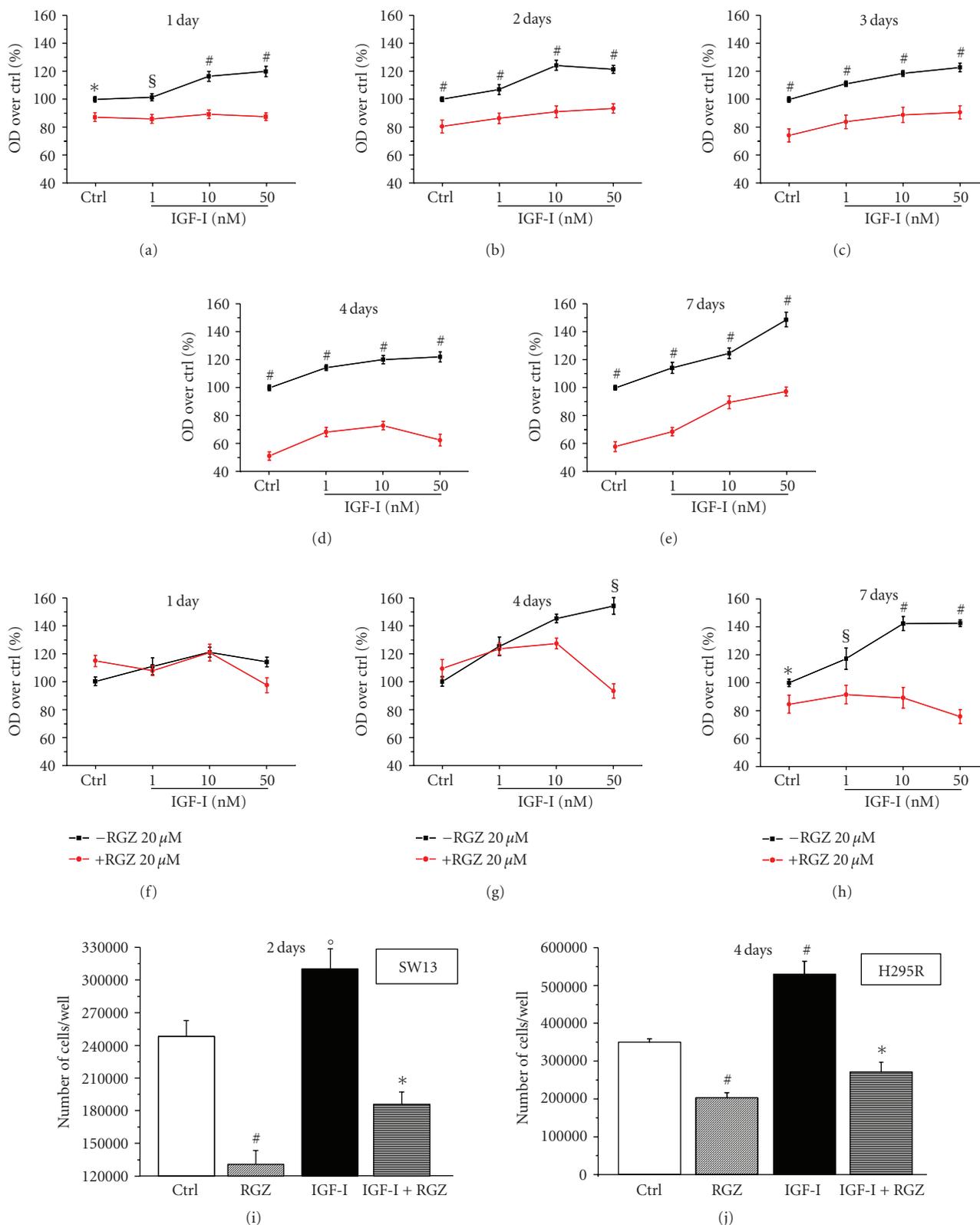
FIGURE 3: *IGF-induced cell proliferation is differently affected by RGZ in SW13 and H295R cell lines.* SW13 (a)–(e) and H295R (f)–(h) proliferation was evaluated by MTS assay following stimulation with increasing doses of IGF-I in the presence or absence of 20 μM RGZ for the indicated times. Mean $\pm$ SE percentage of OD over respective control. Statistical significance: $^*P < 0.05$, $\S P < 0.005$, $\# P < 0.001$ versus the corresponding points with RGZ, $n = 6$. Total number/well of SW13 (i) and H295R (j) cells treated with 10 nM IGF-I and 20 μM RGZ for 2 and 4 days, respectively, was evaluated by cell counting under optical microscope. Data are expressed as mean $\pm$ SE of cell number evaluated in triplicate in three independent experiments. $^\circ P < .01$, $\# P < .001$ versus Ctrl; $^*P < .001$ versus IGF-I.

(a) SW13
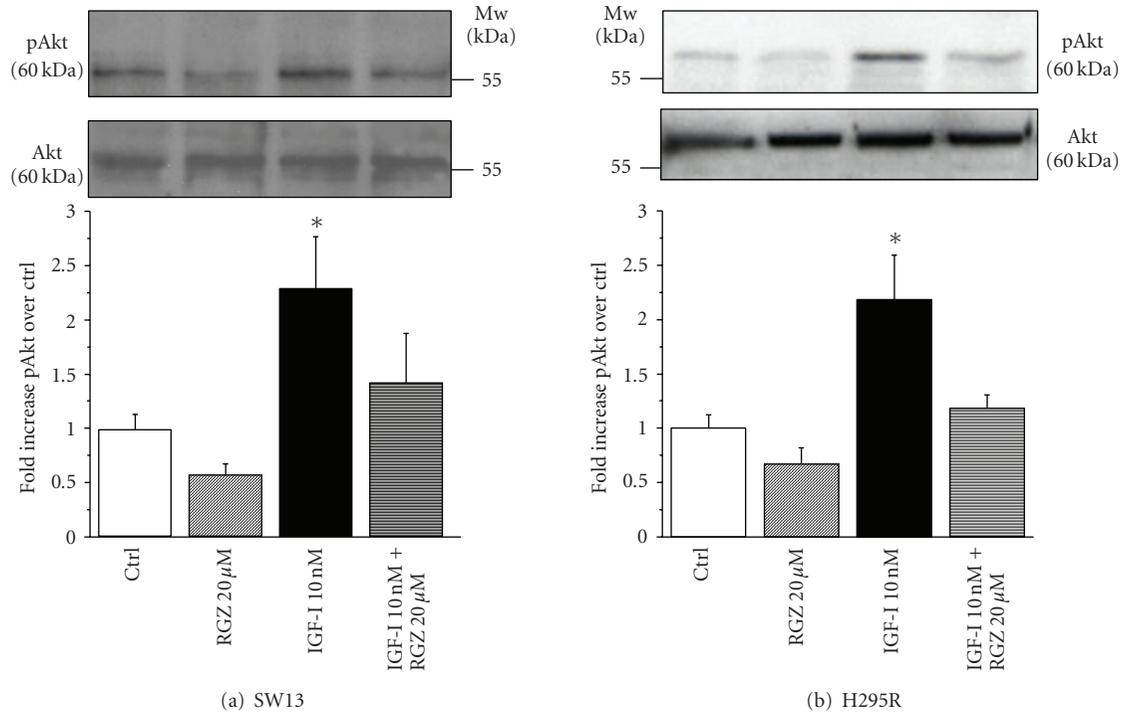
(b) H295R

Figure 4: *RGZ blunts IGF-I stimulation of Akt phosphorylation/activation.* Western blot analysis of SW13 (a) and H295R (b) cell lysates following 15-minutestimulation with 10 nM IGF-I and 20 μM RGZ, reveals an increased phosphorylation of Akt in Ser473 following IGF-I, which is reverted by RGZ. Lane protein normalization for Akt is shown in the middle panels. Molecular weight markers are indicated. Mean $\pm$ SE of phospho-Akt band intensity over Ctrl is shown in 4 independent experiments for SW13 (a) and H295R (b) cells (lower panels). Statistical significance: $^*P < .05$ versus Ctrl or IGF + RGZ.


(a) SW13
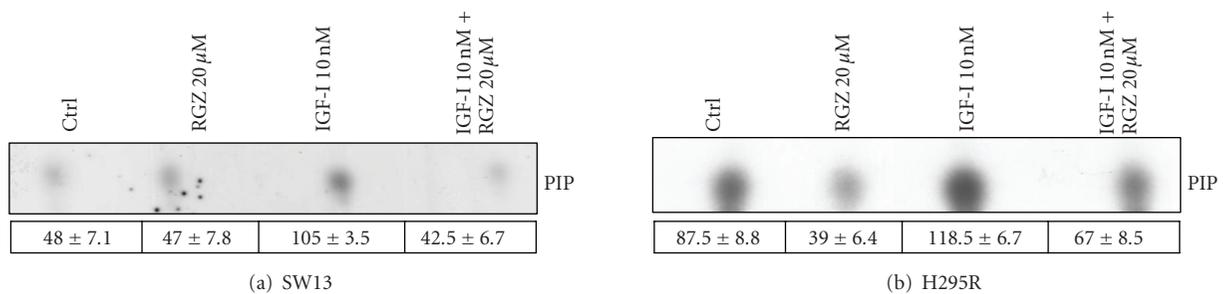
(b) H295R

Figure 5: *RGZ inhibits IGF-I stimulated PI3K.* PI3K activity in SW13 (a) and H295R (b) treated or not for 15 minutes with 10 nM IGF-I and 20 μM RGZ was evaluated by an in vitro assay after immunoprecipitation with an antibody against the PI3K regulatory subunit p85. The spots correspond to PI3K catalytic product [^{32}P]phosphatidyl inositol phosphate (PIP). Representative of two similar experiments. Mean $\pm$ SE ($n = 2$) of phospho-Akt band intensity (arbitrary units) is shown in the table under each blot.

cell proliferation in the presence of IGF-I compared to NVP + IGF-I (Figures 7(b), 7(c)) suggesting that RGZ growth inhibition is mediated via IGF-IR signaling.

Finally, we investigated whether RGZ could affect not only the downstream signaling of IGF-IR, but also the levels of the receptor itself. Figure 8 shows that the IGF-IR levels do not change following up to 4-day stimulation of SW13 (a) d H295R (b) cells with 20 μM RGZ.

4. DISCUSSION

Although adrenocortical carcinomas are very rare tumors, they are very aggressive and highly resistant to chemo- and radiotherapy. Moreover, the use of the adrenolytic agent, mitotane (o,p-DDD), is the only medical therapy available at present. Thus, a better knowledge of the molecular mechanisms underlying the tumor growth and progression is mandatory in order to develop more selective and specific treatments.

Recently, PPARγ ligands have been described as suppressing tumor cell proliferation as well as inducing apoptosis and a more differentiated phenotype in several types of cancers [15], including adrenocortical carcinoma [10, 16], thus suggesting the use of these drugs as a potential new anticancer therapy. However, all these studies have been performed either in vivo, on animal models, or in vitro, on

(a) SW13
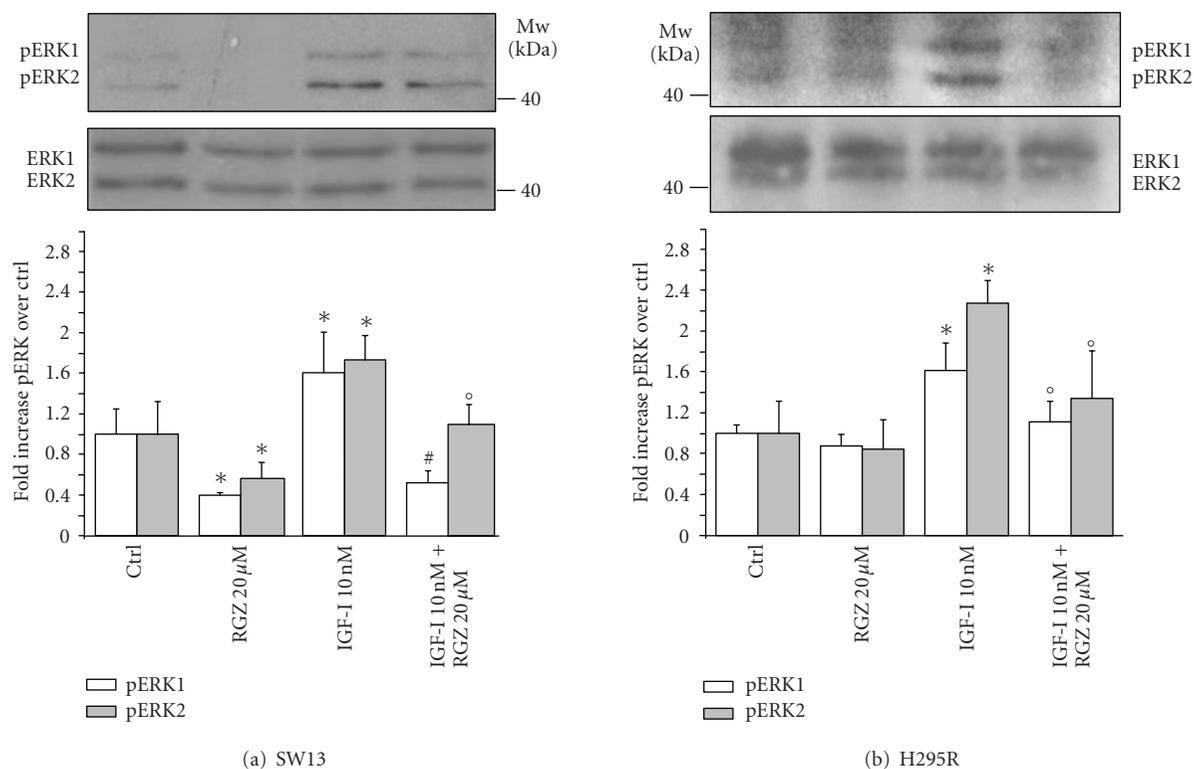
(b) H295R

Figure 6: *RGZ dampens IGF-I stimulated ERK1/2 phosphorylation*. Western blot analysis of SW13 (a) and H295R (b) cell lysates following 15-minutes stimulation with 10 nM IGF-I and 20 μM RGZ, reveals an increased phosphorylation of ERK1/2 following IGF-I, which is reverted by RGZ. Lane protein normalization for ERK1/2 is shown in the middle panels. Molecular weight markers are indicated. Mean ± SE of phospho-ERK1/2 band intensity over respective Ctrl is shown in 3 independent experiments for SW13 (a) and H295R (b) cells (lower panels). *$P < 0.05$ versus respective Ctrl; °$P < .05$ and #$P < .005$ versus IGF-I.

human cancer cells. Moreover, these anticancer effects have been observed with concentrations of PPARγ agonists which are not only higher than the clinical doses used for T2D treatment but also affect PPAR α and δ isoforms, being no longer selective for PPARγ. However, treatment with RGZ doses higher than the therapeutical 8 mg/die has been well tolerated and did not result in any increase in the percentage of adverse events compared to the placebo group in a double-blind clinical trial [19].

At present, only three papers address the effects of PPARγ ligands in ACC using H295R cells as an in vitro model [10, 16, 20]. Both RGZ and pioglitazone (PIO), the most used PPARγ TZD ligands, inhibit cell growth by affecting key cell cycle elements and inducing apoptosis [10, 16], also dampening cell invasiveness through reduction of metalloproteinase 2 (MMP2) expression and activity [16]. Moreover, in these cells, RGZ and PIO induce a more differentiated phenotype where steroidogenesis is increased due to a significant upregulation of MC2-R and Star expression [10].

In this paper, we use two different cell models of ACC, H295R, and SW13. H295R cells retain the ability to synthesize steroid hormones, while SW13, derived from a stage IV tumor, do not, thus suggesting them to be less differentiated than H295R. Both cell lines seem to be suitable models for studying the effects of RGZ on IGF-I/IGF-IR axis, since they express high levels of the IGF-IR, similarly to ACC tissue. In order to stimulate IGF-IR and its downstream signaling cascade, we added to the cells increasing concentrations of its elective ligand, IGF-I. Indeed, IGF-IR binds IGF-I with a 15-fold higher affinity than IGF-II [21]. However, H295R cells have been described as producing high levels of IGF-II, which acts in an autocrine-paracrine loop stimulating the IGF-IR axis even in basal conditions [8]. For this reason, in our experimental procedure, we changed cell incubation media every day to remove the endogenously produced IGF-II thus making the receptor more responsive to the exogenously added IGF-I.

In this condition, IGF-I was able to stimulate cell proliferation in a dose- and a time-dependent manner in both cell models through activation of the two main downstream intracellular signaling cascades involving phosphatidyl inositol 3-kinase (PI3K)-Akt and extracellular signal-regulated kinase (ERK1/2) [21], as described also for other tumors [18]. However, these two pathways are already active even in basal conditions, as demonstrated by the rather high levels of ERK and Akt phosphorylation as well as of PI3K activity found in the absence of IGF-I stimulation, probably due to the paracrine-autocrine effects of endogenously produced IGF-II [8].

RGZ has been demonstrated to impair IGF-I system both in vivo and in vitro by reducing IGF-I production in bone

(a)

(b)

(c)
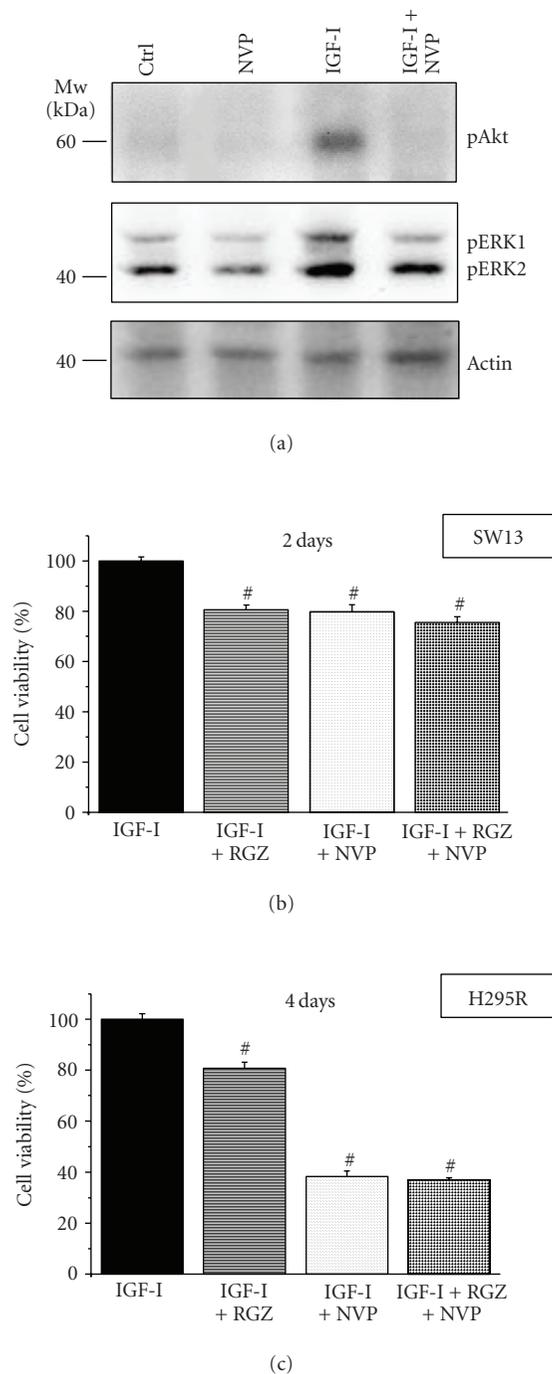
FIGURE 7: *ERK and Akt activation downstream of the IGF-IR mediates IGF-I stimulation of cell proliferation.* Western blot analysis of H295R cell lysates following 15-minute stimulation with 10 nM IGF-I in the presence or absence of 1μM NVP-AEW541 (NVP) inhibitor of the tyrosine kinase activity of IGF-IR (a). The inhibitor blocks phosphorylation of Akt (upper panel) and of ERK1/2 (middle panel) both in basal conditions and following IGF-I stimulation. Lane protein normalization for actin is shown in the lower panel. Molecular weight markers are indicated. Cell proliferation was evaluated by MTS assay following 2 or 4 day stimulation with the indicated treatments (10 nM IGF-I, 20μM RGZ, 1μM NVP) in SW13 (b) or H295R (c) cells, respectively. Mean ± SE percentage of OD over respective control IGF-I taken as 100%. #$P < .001$ versus IGF-I, $n = 6$.


(a) SW13
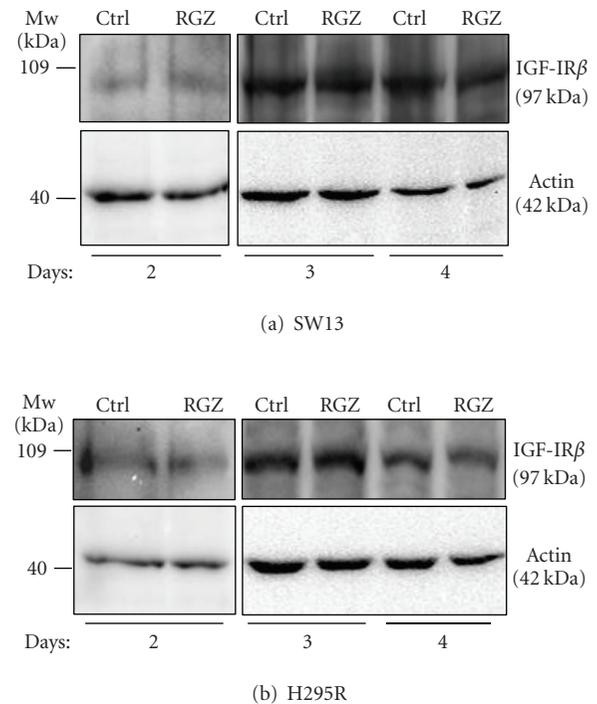
(b) H295R

FIGURE 8: *RGZ does not affect IGF-IR levels.* Western blot analysis of SW13 (a) and H295R (b) cell lysates following 2, 3, and 4 day cell incubation in the absence (Ctrl) or in the presence of 20μM RGZ reveals no significant change in IGF-IR (upper panels). Lane protein normalization for actin is shown in the lower panels. Molecular weight markers are indicated. Representative of two independent experiments.

marrow cells and in the liver [22]. Moreover, troglitazone inhibits IGF-I tumor-promoting activity in mouse skin by affecting the intracellular pathway involving AMP kinase [23].

In SW13 as well as in H295R cells, RGZ was able to significantly reduce cell proliferation in a dose- and time-dependent manner as evaluated by different techniques (MTS, thymidine uptake and cell counting), with a calculated IC$_{50}$ of $22.48 \pm 1.54 \mu$M. This concentration is higher than the RGZ plasma levels estimated from the total area under the plasma concentration-time curve (Cmax 1.33μM) obtained by pharmacokinetic studies on subjects undergoing oral RGZ administration with the therapeutical dosage of 8 mg/die [24]. However, it is in perfect agreement with the doses currently used for in vitro studies, in particular in cancer cells [10, 16]. Although the RGZ inhibitory effect on cell proliferation and viability has already been reported in H295R cells [10, 16], this is the first time that RGZ IC$_{50}$ and maximal effect have been calculated with an appropriate statistical analysis [17], thus validating the current dose of 20μM used in cancer studies and suggesting that the anti-proliferative effects of RGZ are obtained at higher concentrations than the ones effective on insulin sensitivity [13]. Toxic studies associated with a pharmacokinetic studies to define the RGZ oral dose capable of resulting in a range of 20μM circulating concentration of RGZ and to

investigate the possible toxic effect associated to such doses, are mandatory in order to hypothesize a possible therapeutic use of RGZ and other TZD for ACC treatment.

In addition to its effect on cell proliferation in basal conditions, RGZ was also effective on cell growth induced by addition of increasing concentrations of IGF-I. Interestingly, such an inhibitory effect was similar in the presence or in the absence of IGF-I only in SW13 cells, while in H295R, RGZ also prevents the stimulatory effect of IGF-I. However, the inhibitory effect of RGZ was reached more slowly in H295R (4–7 days) than in SW13 (1-2 days), probably due to the differences in the kinetic of duplication between the two cell lines. Moreover, in H295R RGZ inhibition increases with the dose of IGF-I, while in SW13, the effect was similar independently of the dose of the growth factor. Consequently, the IC_{50} calculated for RGZ curve on SW13 cell proliferation was similar in the presence or absence of IGF-I.

In order to elucidate at which level RGZ interferes with the activated IGF-I axis both in basal conditions (IGF-IR activated by endogenously produced IGF-II) and following a further activation of the receptor by exogenous addition of IGF-I, we investigated the signaling pathways acting downstream of the receptor. RGZ was able to block the rapid activation of the PI3K-Akt axis induced by IGF-I. In agreement with the experiments on cell proliferation, we found that in H295R cells only, RGZ was more effective on IGF-I-treated than on untreated cells. RGZ also affected the ERK pathway through inhibition of the rapid phosphorylation-activation of ERK1 and 2 isoforms. A similar effect of RGZ on rapid phosphorylation of ERKs has been recently described in the inflammatory response of endothelial cells [14]. Such an effect is too rapid (15 minutes) to be ascribed to the classical transactivation mechanism of PPARγ on specific target genes, and suggests a PPARγ independent mechanism or a nongenomic activity of the receptor. Interestingly, among the anticancer action exerted by RGZ in H295R, both PPARγ dependent and independent effects seem to coexist in these cells, since the PPARγ antagonist GW9662 has been demonstrated to block RGZ induction of MC2R expression and cortisol secretion but not RGZ inhibition of cell proliferation (Betz et al., 2005). Further studies are required to elucidate the precise mechanism by which PPARγ ligands affect rapid signaling in these cells. Interestingly, no effect of RGZ was detected up to 4 day stimulationon the level of IGF-IR in both cell lines. In accordance, Betz and colleagues described that IGF-II production increased with time despite the clear growth-suppressive effects of RGZ and PIO in H295R [10]. Our findings suggest that RGZ inhibition acts on the signaling pathways downstream but not at the level of the IGF-IR.

In conclusion, our result shed new light on the molecular mechanisms underlying cell proliferation and progression in adrenal carcinoma, contributing to demonstrate that the inhibitory effect exerted by RGZ on cell growth is due to the TDZ interferences with the two main signaling pathways downstream of the activated IGF-IR. RGZ ability to block the IGF-IR axis suggests the potential application of this molecule for the treatment of ACC.

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Review Article

PPARγ and Proline Oxidase in Cancer

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Proline is metabolized by its own specialized enzymes with their own tissue and subcellular localizations and mechanisms of regulation. The central enzyme in this metabolic system is proline oxidase, a flavin adenine dinucleotide-containing enzyme which is tightly bound to mitochondrial inner membranes. The electrons from proline can be used to generate ATP or can directly reduce oxygen to form superoxide. Although proline may be derived from the diet and biosynthesized endogenously, an important source in the microenvironment is from degradation of extracellular matrix by matrix metalloproteinases. Previous studies showed that proline oxidase is a p53-induced gene and its overexpression can initiate proline-dependent apoptosis by both intrinsic and extrinsic pathways. Another important factor regulating proline oxidase is peroxisome proliferator activated receptor gamma (PPARγ). Importantly, in several cancer cells, proline oxidase may be an important mediator of the PPARγ-stimulated generation of ROS and induction of apoptosis. Knockdown of proline oxidase expression by antisense RNA markedly decreased these PPARγ-stimulated effects. These findings suggest an important role in the proposed antitumor effects of PPARγ. Moreover, it is possible that proline oxidase may contribute to the other metabolic effects of PPARγ.

1. INTRODUCTION

PPARγ can regulate inflammatory responses to prevent chronic inflammation [1], but more importantly, it plays an important role in the sensing and regulation of metabolism [2]. These functions, especially the regulation of metabolism, may be involved in the documented ability of PPARγ to modulate the malignant phenotype [3]. This aspect of PPARγ articulates with the resurgence of interest in metabolism and cancer [4, 5] which has underscored the 50-year old findings of Warburg that the metabolism of tumor cells is deranged; aerobic glycolysis rather than oxidative phosphorylation is the mode of tumor metabolism [6]. Recent findings suggest that many oncogenes and suppressor proteins target metabolic pathways, and in the context of Warburg's early discovery, they form a new, revealing paradigm [7]. The survival and malignant potential of a tumor are critically dependent on its adaptation to a variety of stress situations and nutrient limitations. To generate adequate energy from the relatively inefficient glycolytic pathway, the flux from glucose to lactate must be maintained at a high rate [8].

Thus, vascularity and neoangiogenesis as a response not only to hypoxia but also to the depletion of nutrients play a critical role in tumor progression [9]. In this context, the mobilization of proline from the degradation of extracellular matrix in the tumor microenvironment has come to our attention. The use of proline as alternative stress substrate and the regulation of this response by stress signals has been a focus of our research effort.

2. PROLINE METABOLISM

Proline is the only secondary amino acid incorporated into protein. Because the alpha nitrogen is contained within a pyrrolidine ring, proline cannot be metabolized by generic amino acid enzymes, that is, aminotransferases, decarboxylases, and racemases [10, 11]. Instead, a special family of enzymes evolved with their own subcellular localizations and mechanisms of regulation. There is a little overlap between the activity of these enzymes and that for generic amino acids. Thus, the metabolic system is distinct and can be responsive to special metabolic requirements. The enzymes
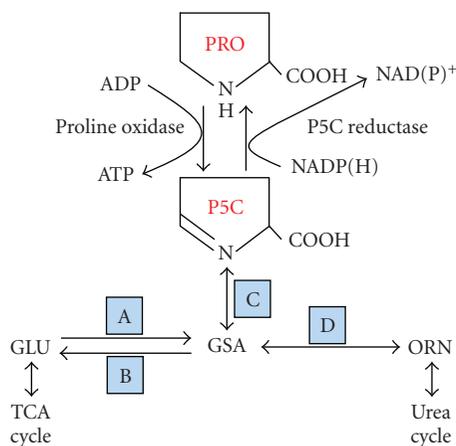
FIGURE 1: Proline metabolic pathway. Abbreviations: PRO, proline; P5C, Δ^1-pyrroline-5-carboxylate; GLU, glutamate; GSA, glutamic-gamma-semialdehyde; ORN, ornithine. Enzyme names not shown: A, P5C Synthase; B, P5C dehydrogenase; C, spontaneous; D, ornithine aminotransferase.

for the proline metabolic scheme had been characterized by the 1960s and the general system is shown schematically in Figure 1. Pyrroline-5-carboxylate, in tautomeric equilibrium with glutamic-γ-semialdehyde, is a central intermediate. It is not only the committed precursor of proline but also the immediate product of proline degradation. Importantly, it is an obligate intermediate bridging the urea cycle and the tricarboxylic acid cycle and can play an anaplerotic role for both metabolic cycles [10, 11]. The complete metabolic system is not present in all tissues.

The role of proline in proteins has been characterized and reviewed by others [12], and the topic is outside the scope of this review. However, functions beyond its contribution to proteins have also been recognized in a variety of animal and plant species. In prokaryotes, proline is thought to have antioxidant and osmoprotective functions [11]. Regulatory roles have been proposed for parasitic trematodes although the mechanisms are not understood [13]. In a variety of higher plants, proline is thought to be an osmoprotectant and the metabolism of proline has been linked to the synthesis of polyphenolic compounds [14]. Proline has been identified as a critical metabolic substrate in the initiation of flight in insects. In addition, insects can detect and are attracted to proline. The finding that proline is at high concentrations in plant floral nectar has led to the proposal that proline is the basis of a coevolution to optimize insect-mediated pollination [15]. During the molecular biological explosion of the 1990s, the genes for proline metabolism were cloned from a variety of sources, making possible studies defining functions for this special metabolic system.

An interesting feature of proline metabolism is that the interconversions of proline and pyrroline-5-carboxylate form a *proline cycle*. Proline oxidase (POX), a.k.a. proline dehydrogenase (PRODH), is tightly bound to mitochondrial inner membranes (the enzyme will be designated POX, but the gene will be referred to as *PRODH*). The enzyme is a flavoprotein and electrons from proline are passed into

the electron transport chain at site II with cytochrome c as the electron acceptor [10, 11]. Pyrroline-5-carboxylate, the product of proline degradation, can be converted to glutamate and α-ketoglutarate to contribute anaplerotically to the TCA cycle [11]. However, it is also converted back to proline by pyrroline-5-carboxylate reductase in the cytosol to form a metabolic cycle. Coupled by pyridine nucleotides (NADP/NADPH preferentially over NAD/NADH), the proline cycle forms a metabolic interlock with glucose-6-phosphate dehydrogenase and the pentose phosphate pathway and serves as a redox shuttle to convert reducing potential from the pentose phosphate pathway into an ATP-generating system in mitochondria [16–18]. The magnitude of ATP generation, however, is small compared to the TCA cycle and oxidative phosphorylation. The glycolytic pathway, with optimized flux, also can generate ATP more efficiently. Thus, the contribution of the proline cycle to redox and energetics was considered trivial previously. However, as the mechanisms for upregulating POX were elucidated, it became clear that the system serves as an important accessory source for energy under stress conditions.

Proline is available from dietary proteins and can be biosynthesized from either glutamate or ornithine [10, 11]. However, an abundant source is from degradation of collagen in the extracellular matrix, connective tissue, and bone [19]. Since 25% of the residues in collagen is either proline or hydroxyproline and collagen is the most abundant (by mass) protein in the body, it serves as an ample reservoir of proline. Additionally, matrix metalloproteinases (MMPs), the family of enzymes which degrade collagen and other proteins in the extracellular matrix, are markedly upregulated under a variety of conditions. Importantly, upregulation of MMPs occurs during tumor progression and invasion [20, 21] as well as during inflammation and wound healing [22, 23]. MMP upregulation has been considered an important physical component of invasion, that is allowing for tumor cells to escape from their basement membrane site and migrate through tissue. Recently, it has been shown that a variety of biologically active factors are released from binding sites on ECM with activation of the MMPs [24]. However, the utilization of proline or hydroxyproline as a source of metabolic substrate has not been considered. That degradation of collagen occurs during carcinogenesis in the skin tumor model has been convincingly demonstrated [25]. Recently, using breast and prostate cancer xenografts and novel imaging methodology, investigators have shown that hypoxia mediates collagen fiber breakdown and restructuring [26].

3. POX AND APOPTOSIS

P53 is considered the most important cancer suppressor protein [27]. It is mutated in 85% of all human tumors and germ-line mutations in p53 result in the Li-Fraumeni syndrome, a familial syndrome with predisposition to early cancers in a variety of tissues [28]. To screen for p53 target genes, Polyak et al. [29] used an adenoviral-p53 expression construct and serial analysis of gene expression. Only 14 out of 7202 genes monitored were induced more than 7-fold,

and POX was one of these and designated as p53-induced gene-6 (PIG6). Using a construct where POX expression was under the control of tetracycline, the overexpression of POX produced proline-dependent ROS [30] and induced proline-dependent apoptosis [31–35]. Subsequently, it was shown that POX overexpression produced its effects through generation of proline-dependent mitochondrial superoxide (**Figure 2**) [34]. It is this superoxide which plays a critical role in signaling to produce not only the release of cytochrome c from mitochondria and the activation of the caspases in the intrinsic (mitochondrial) limb of programmed cell death, but also it activated the extrinsic (death receptor) limb by increasing the production of TRAIL [35]. A number of other signaling systems respond to POX-mediated signaling including downregulation of MEK/ERK phosphorylation [35], downregulation of COX-2 with decreased PGE2 production, and blockade of the progression through the cell cycle [36].

The findings from the tissue culture system have been translated into an animal model. In studies using DLD-POX cells to form xenografts in athymic mice, the expression of POX markedly inhibited tumor formation [37]. In mice given doxycycline to suppress POX expression in DLD-POX cells, or in animals injected with DLD-vector cells, tumors formed rapidly. By week 2 all these animals developed palpable tumors and by week 3, the animals had to be sacrificed due to the size of the tumors. By contrast, in mice without doxycycline in which POX was overexpressed, few tumors were detected. By week 2, only 1 out of 16 animals had palpable tumors. Thus, the expression of POX markedly inhibited the formation of xenografts.

The relevance of these changes in POX was pursued by immunohistochemical studies in human tissues. Ninety-two paired normal and cancer tissues from a variety of tumors were examined using immunohistochemistry. The findings were striking from gastrointestinal tumors (stomach, colon, pancreas) in which the level of POX expression was markedly decreased or undetectable in 79% of the tumors [36]. We are currently investigating the genetic or epigenetic mechanism for the decrease in POX expression, but based on these findings, we propose that POX is a potential cancer suppressor protein.

The mechanism for the POX-mediated, proline-dependent generation of superoxide may be due to leakage of electrons from the electron transport chain, a mechanism proposed for other sources of mitochondrial superoxide. However, recent studies from structural biology suggest that the generation of superoxide is an intrinsic property of the enzyme. White et al. [38] described interesting findings using recombinant *Thermus thermophilus* POX/PRODH. Unlike the POX/PRODH from certain prokaryotic species, for example *Escherichia coli*, which have a bifunctional enzyme, embodying the activities of POX and pyrroline-5-carboxylate dehydrogenase in a single protein, the enzyme from *T. thermophilus* is monofunctional and produces pyrroline-5-carboxylate in a manner similar to the enzyme in animal tissues, and thus may serve as a good model for human POX [38]. These workers found that the flavin adenine dinucleotide is located in a domain exposed to solvent
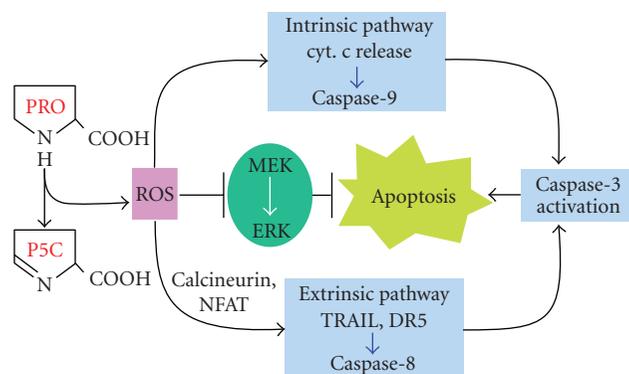
Figure 2: Proline oxidase-induced apoptosis. Abbreviations: ROS, reactive oxygen species; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; DR5, death receptor 5 NFAT, nuclear factor of activated T cells; MEK, MAP kinase; ERK, extracellular-signal regulated kinase.
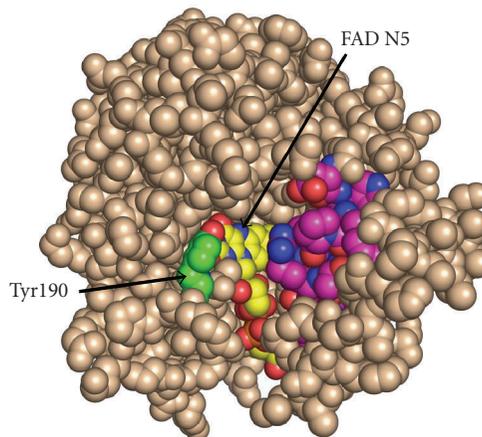
Figure 3: Structure of proline dehydrogenase (proline oxidase) from *Thermus thermophilus*. The flavine adenine dinucleotide at the active site is shown in yellow. The flexible alpha helix adjacent to the FAD is shown in violet and blue. Access of the FAD to solvent O_2 allows direct reduction of O_2 to form superoxide radicals. The figure is used with permission from Dr. Jack Tanner, University of Missouri-Columbia, and the Journal of Biological Chemistry.

oxygen. Thus, the electrons from proline can be used to reduce oxygen to superoxide (**Figure 3**). In addition, they found an adjacent α-helix which can shield the FAD and block its access to solvent oxygen. The interpretation of these findings includes the intriguing possibility that POX can be switched from an ATP-generating function to a superoxide-producing function. Although a number of enzymes have been proposed as generators of superoxide, these enzymes are cytosolic (xanthine oxidase) or are associated with cell membranes (NADPH oxidase) with their own specified functions.

These aforementioned functions of POX have been emphasized for their relevance to cancer, but another function deserves mention. Proline functions as a neurotransmitter, inhibiting glutamatergic neurons [39]. Additionally,

a high-affinity transporter has been discovered and cloned from the brain [39]. The relevance to neurological systems extends to lower species. Mutations in POX/PRODH result in "sluggishness" in *Drosophila melanogaster* [40] and the PRO/Re mice, defective in POX/PRODH, exhibit "gating" defects, a functional neurologic defect [41]. In humans, mutations in *PRODH* have been associated with risk for early schizophrenia [42]. Although there has been a number of studies supporting or contradicting this conclusion, evidence supports the relevance of POX mutations. It has been shown that the mutations in PRODH associated with the neuropsychiatric syndrome have a biochemical phenotype with markedly decreased activity in the enzyme [43].

4. REGULATION OF POX

The induction of POX by p53 suggested that it served special functions and was not simply a "housekeeping enzyme." To screen for potential regulators, Pandhare et al. [44] made a POX-promoter, luciferase-reporter construct, and cotransfected a variety of transcriptional factors corresponding to binding sites identified in the *PRODH* promoter. Although Jun, Fos, and p65 of NF-κB produced modest stimulatory effects (<2-fold), a marked activation of the *PRODH* promoter was observed with cotransfection of PPARγ. This finding was interesting, indeed, since this pleiotropic factor not only plays an important role in metabolism [2], especially of adipocytes, but also it is an important modulator of inflammatory responses [1]. The wide use of the thiazolidinediones (TZDs) in the management of hyperglycemia in type 2 diabetes mellitus is an example of the former [45]. For the latter, some investigators have suggested that PPARγ provides a mechanism to downregulate inflammatory stress responses and avoid the pathologic consequences of chronic inflammation [46]. Attracting considerable attention recently is the finding in a variety of cultured cancer cells that TZDs will block cell proliferation and induce apoptosis [47–49]. Epidemiologic data from patients with type 2 DM treated with TZDs suggest that these ligands of PPARγ are protective against lung cancer but not against colon or prostate cancer [50]. With the impressive in vitro data and suggestive findings from epidemiology, oncologists have proposed that PPARγ is an attractive target for cancer treatment.

5. MECHANISM OF TZDs IN INDUCING *PRODH*

Pandhare et al. [44] showed that cotransfection of PPARγ activated the *PRODH* promoter 8-fold, and troglitazone, a widely used TZD before it was taken off the market because of side effects, further increased the magnitude of this activation. The combination of PPARγ expression and troglitazone treatment activated the *PRODH* promoter more than 10-fold (Figure 4). The effect could be generalized to a variety of colorectal cancer cells and could be elicited by four different TZDs. That troglitazone induced POX through a PPARγ mediated binding to the peroxisomal proliferator response element was shown using several methods. First, an electrophoretic shift mobility assay showed a troglitazone-

stimulated formation of a nuclear complex with the labeled PPRE sequence from the *PRODH* promoter. That PPARγ was present in this complex was shown with chromatin immunoprecipitation assays. In this assay, formaldehyde was used to cross-link DNA-protein complexes and then the DNA was sheared by sonication. After immunoprecipitation with specific anti-PPARγ antibody, the PPRE sequences of the *PRODH* promoter were amplified using polymerase chain reaction.

Although these studies showed that PPARγ and its pharmacologic ligands are directly involved in the activation of the *PRODH* promoter, the integration of signaling by the PPARγ assembly to physiologically regulate *PRODH* expression may be more complex. The interaction with retinoid-X receptors (RXR) is a requisite for PPARγ function [51]. Moreover, a number of coactivators interact with liganded PPARγ and RXR to form an active transcriptional complex. These include steroid receptor PPARγ-coactivator-1 (PGC-1) and steroid receptor coactivator-1 (SRC-1) [52]. The specific coactivator may depend on the cell type and stimuli. In the context of metabolism, PGC-1 may be especially relevant since it responds to signaling from other metabolism-regulating hormones and cytokines [53]. The specific effect of these coactivators on *PRODH* expression, however, has not been elucidated, but it is an area of emphasis of our current work.

6. CONTRIBUTION OF POX TO THE PPARγ EFFECTS ON ROS AND APOPTOSIS

The discovery that PPARγ has a marked inhibitory effect on cultured cancer cells stimulated a large number of studies using a variety of cancer cells. The TZDs augmented differentiation, slowed proliferation, and induced apoptosis. Although this effect was generally observed, there were a few reports of TZDs actually stimulating the growth of certain cultured cancer cells [54]. Nevertheless, the preponderance of studies showed that TZDs inhibited growth [47–49]. Although the mechanism of this effect was not well understood, several investigators found that TZDs induced the generation of ROS, and they concluded that ROS was the mechanism for inducing apoptosis as has been reported for many experimental models. The actual mechanism by which ROS production was induced by TZDs, however, remained unknown.

Since POX is a p53-induced gene and has been established as a mechanism for generating superoxide that initiates apoptosis, the PPARγ induction of POX raised the attractive hypothesis that POX may be involved in the apoptotic mechanism observed with the TZDs. To answer this question, Pandhare et al. [44] showed that in colorectal cancer cells, troglitazone not only induced POX, but also markedly increased the production of ROS as has been shown by others in other cultured cells. More importantly, the knockdown of POX with antisense RNA markedly decreased the generation of troglitazone-stimulated ROS. These studies strongly suggested that the ROS presumed to be the mechanism for TZD-stimualted apoptosis was due, at least in part, to its induction of POX. Thus, POX plays an
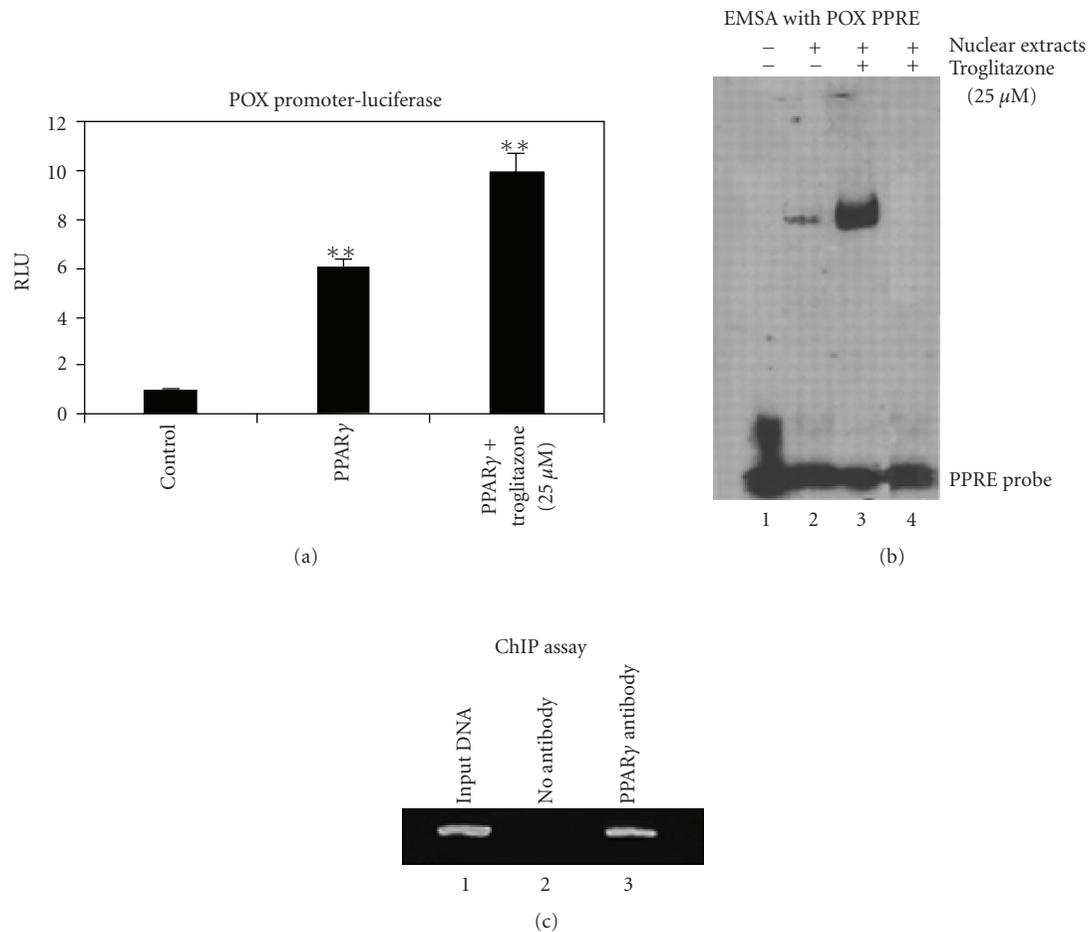
FIGURE 4: Induction of proline oxidase by PPARγ and its pharmacologic ligand, troglitazone. (a) Activation of the POX promoter using a luciferase reporter assay. HEK 293 colorectal cancer cells were transfected with equivalent amounts of cDNA of PPARγ or vector plasmid as control. The cells were also transfected with *POX*-Luc and pRL-null. Troglitazone (25 μM) or Me$_2$SO in control was added after 10 hours as indicated. At 24–36 hours after transfection, the cell lysates were harvested, and the *POX* promoter luciferase activity was determined using the Dual Luciferase Assay kit. (b) Troglitazone increases the binding of PPARγ to the PPRE in the *POX* promoter. HCT 116 colorectal cancer cells were treated with or without 25 μM troglitazone for 36 hours and nuclear extracts were prepared. The binding of PPARγ to the PPRE was evaluated by an electrophoretic mobility shift analysis assay using the double-stranded POX-PPRE oligonucleotide probe. Unlabeled POX-PPRE probe (100x) was used as a competitor (lane 4). (c) Chromatin immunoprecipitation assay of the *POX* promoter in troglitazone-treated HCT 116 cells. HCT 116 cells were incubated with 1% formaldehyde to fix protein-DNA complexes. DNA was sheared by sonication. Soluble chromatin-DNA complexes were immunoprecipitated using PPARγ antibody and immunoprecipitates were analyzed by PCR with specific primers for the *POX* promoter region containing the POX-PPRE.

important role in the apoptotic effect of TZDs, at least in tissue culture. This finding was soon confirmed by others. Working with nonsmall cell lung cancer cells, Kim et al. [55] showed that rosiglitazone induced apoptosis through an ROS-dependent mechanism, and that the induction of POX by rosiglitazone played a critical role in the production of apoptosis. These are exciting findings but require further corroboration and extension to other cultured cancer cells.

The effects of TZDs in cultured cells have been extended to several tumor models in animals and the results are encouraging. In athymic mice, the growth rates of xenografts of ovarian, thyroid, and bladder cancer are markedly affected by a variety of PPARγ-stimulating agents [47–49]. Not only is tumor growth inhibited but survival of the host animal is prolonged. Although the mechanism underlying these effects

remains unclear, it appears that the cells in the tumors are apoptotic perhaps due to decreased expression of COX-2 [56]. Recent work in our laboratory links POX expression to downregulation of COX-2 [36]. There are direct effects on the tumor as well as effects on angiogenesis. There are no studies of the effects of PPARγ on POX expression in animals or on the role of POX in mediating the PPARγ-mediated antitumor effects.

7. PARADOXES AND POSSIBLE SOLUTIONS

The enthusiasm generated by these antitumor effects of PPARγ and the TZDs was somewhat blunted by the finding that in C57Bl/6J-APC$^{Min/+}$ mice, activation of PPARγ-mediated signaling promotes rather than inhibits

the development of colon tumors [57]. APC is the tumor suppressor protein in adenomatous polyposis coli and is an integral part of the Wnt/β-catenin signaling system. The Min mutation blocks the formation of the tetrameric complex (APC, axin, GSK-3β, β-catenin) which allows for phosphorylation of β-catenin leading to its proteasomal degradation. Accumulated β-catenin translocates into the nucleus to form transcriptional complexes with TCF/LEF to induce target genes involved in proliferation [58]. However, in keeping with the earlier reports that activation of PPARγ or its ligands had antitumor effects, recent studies have shown marked reduction in tumor growth or survival of animals with peritoneal carcinomatosis with various PPARγ ligands. These recent studies include ovarian cancers [47], anaplastic thyroid carcinomas [48], and bladder tumors [49]. Thus, the debate continues: ". . . the action of PPARγ on cell cycle, proliferation, differentiation, and apoptosis seems to depend on the cell type and/or the mutational events that predispose tissue to cancer development" [58]. The importance of coactivators or corepressors cannot be overemphasized. Interactions with and contributions of the microenvironment must also be considered in understanding these different effects.

A common target of these signaling pathways is the matrix metalloproteinases (MMP) [59, 60]. Differential effects on these enzymes may explain, in part, the variability in the aforementioned effects of PPARγ activation. Increased PPARγ signaling will downregulate MMP whereas certain MMP are target genes of β-catenin/TCF-LEF. The transcriptional system constitutively upregulated by the APC^{Min} mutation increases the expression of MMP-7. Just how these mechanisms articulate for regulating MMP remains unclear. However, in the context of the aforementioned induction of POX by PPARγ, the differential effects on MMP may be relevant. In a given experimental model, the availability of ECM and the effects on MMP may determine the relative availability of proline as a stress substrate for POX. Furthermore, the consequences of POX induction may also be two-edged. Under stimulation of p53, POX can use proline to generate mitochondrial superoxide to initiate apoptosis by both intrinsic and extrinsic pathways [34]. Recent work has shown that POX overexpression will also blockade the cell cycle [61]. Thus, upregulation of POX in the presence of MMP to generate free proline will activate antitumor mechanisms. On the other hand, POX also can generate ATP and it is upregulated by downregulation of mTOR signaling under nutrient stress. With the availability of proline, upregulation of POX can support cell survival [62]. Like several mediators of metabolic regulators, for example, p53 and PPARγ, POX also can play a two-edged regulatory role.

8. THE ROLE OF POX IN ANTITUMOR EFFECTS OF PPARγ

Additional work is needed to translate these findings in cultured cancer cells to animal models and eventually to clinical trials. As a first step, studies are being undertaken

to monitor the expression of POX in mice administered TZDs. Assuming that certain tissues in intact animals will respond as in cultured cells, the effect of POX upregulation on spontaneous tumors in that tissue can be investigated. The inhibition of POX by proline analogues or the blockade of MMPs, specifically prolidase, may limit the availability of proline in that tissue. Also, control of dietary proline could be important. With the insights gained by these animal studies, it may be possible to design clinical trials in which perturbations of the POX-mediated effects can be pharmacologically attacked as an adjunct to the use of TZDs or other PPARγ activators. Furthermore, PPARγ activation with or without POX can be used in combination with other chemotherapeutic modalities.

9. CONTRIBUTION OF POX TO OTHER PPARγ-MEDIATED EFFECTS

The consequences of POX induction and its role in PPARγ-mediated metabolic effects other than that on cancer have not been explored. However, it is intriguing that the well-established metabolic effects of PPARγ could be mediated in part by induction of POX. Nevertheless, the known effects of POX and PPARγ invite speculation, but these specific questions have not been experimentally addressed. Thus, these questions remain in the realm of future plans. Of special consideration are the following effects of PPARγ: (1) increased insulin sensitivity, (2) decreased inflammation, and (3) increased osteopenia.

There are potential links between degradation of proline and insulin-related metabolic effects. Certainly, POX uses proline to generate intermediates for anaplerosis of the TCA cycle which could make oxidative metabolism more efficient. Investigators have cited the importance of these intermediates as building blocks rather than as energy substrates. Furthermore, the metabolic interlock of the proline cycle and glucose metabolism through the pentose phosphate pathway could affect insulin sensitivity since it opens an alternative pathway for glucose metabolism. Thus, glucose would not only be metabolized by oxidative phosphorylation in the TCA cycle and converted to lactate by glycolysis, but also would be converted to CO_2 by interconversions and cycling through the pentose phosphate shunt.

The PPARγ signaling pathway is frequently considered as a response to inflammatory stress, that is, to prevent chronic inflammation. Inflammatory cells such as macrophages will respond to inflammatory signals such as prostaglandins and this will induce POX in macrophages and induce apoptosis. Furthermore, COX-2 may be regulated by the expression of POX and the generation of proline-mediated ROS [36].

The final metabolic consideration is the demonstrated effects in animals and in humans that TZDs will result in osteopenia [63]. From histologic and metabolic studies, PPARγ appears to decrease osteogenesis and increase osteoloysis. There are decreased numbers of osteoblasts and increased numbers of osteoclasts [64]. Since bone is primarily made up of calcified collagen, it is not surprising that collagen synthesis is decreased and collagen degradation is

increased. Since collagen synthesis requires the incorporation of proline, the degradation of proline by increased POX would be a biochemical process consistent with osteoclastic function.

Another interesting area involves a physiologic/pathophysiologic source of natural ligands for PPARγ, that is, oxidized low-density lipoproteins (oxLDL). Their precursor, low-density lipoproteins (LDL) are synthesized in the liver and are the carriers for 60% of total serum cholesterol, and they are widely known as the "bad cholesterol." Recent studies suggest that LDL is oxidized in human blood and tissues under various pathological conditions. OxLDL may be an important player in the development of atherosclerosis, promoting apoptosis in endothelial cells, increasing proliferation of smooth muscle cells, and upregulating inflammatory signaling in macrophages. The result is the formation of atheromatous plaques. Mechanisms of oxLDL-induced effects are being intensively investigated, but there is a considerable evidence supporting a role for PPARγ activation [65]. Additionally, oxidized LDL activates p53 [66, 67] and stimulates the formation of mitochondrial ROS [68] to induce cell death. Since all these mechanisms are linked to POX activity, it is tempting to speculate that POX may be involved.

Although oxLDL is mainly associated with atherosclerosis, several studies point to the correlation between serum oxLDL levels and cancer risk in humans [69, 70]. This prompted us to study the possible role of POX in the oxLDL-mediated effects on carcinogenic pathways. First, we transfected breast, prostate, colon, cervical, ovarian, and lung cancer cell lines with the POX promoter-luciferase reporter and found that oxLDL treatment activated the POX promoter in a dose- and time-dependent manner. This effect was further augmented by the addition of 2.5 mM proline. We also found that oxLDL treatment increased POX gene expression as compared to nonoxidized LDL, or a solvent control [Zabirnyk O and Phang JM, unpublished results]. These preliminary studies suggest a role of proline oxidase in the oxLDL-mediated effects on PPARγ activation and initiation of apoptotic cell death.

In summary, POX, a p53-induced gene, is markedly upregulated by overexpression of PPARγ or by the addition of TZDs. The effect is generalizable to a variety of cells and to all the TZDs. The mechanism of this effect appears to be by transcriptional activation by activating the POX promoter at the PPRE site. The PPARγ effect on apoptosis is mediated by the generation of ROS, and knockdown of POX by siRNA markedly decreases or blocks the effects of PPARγ on ROS formation and apoptosis in colorectal cancer cell or nonsmall cell lung cancer cell, respectively. These findings suggest that POX may play a critical role in PPARγ-mediated antitumor effects. Furthermore, it may offer an explanation for the inconsistent findings observed in different animal systems. It also may offer an adjunctive therapeutic approach to optimize the PPARγ-mediated antitumor effects. Finally, a speculative proposal for the articulation of POX-dependent metabolic effects on the metabolic syndrome with PPARγ activation is presented.

ABBREVIATIONS

NFAT: nuclear factor of activated T-cells
P5C: Δ^1-pyrroline-5-carboxylic acid
POX: proline oxidase
PPARγ: peroxisome proliferator-activated receptor gamma
PRODH: proline dehydrogenase
ROS: reactive oxygen species
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand
TZDs: thiazolidinediones

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Review Article

The Role of PPARγ in the Cyclooxygenase Pathway in Lung Cancer

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Decreased expression of peroxisome proliferator activated receptor-γ (PPARγ) and high levels of the proinflammatory enzyme cyclooxygenase-2 (COX-2) have been observed in many tumor types. Both reduced (PPARγ) expression and elevated COX-2 within the tumor are associated with poor prognosis in lung cancer patients, and recent work has indicated that these signaling pathways may be interrelated. Synthetic (PPARγ) agonists such as the thiazolidinedione (TZD) class of anti-diabetic drugs can decrease COX-2 levels, inhibit growth of non-small-cell lung cancer (NSCLC) cells in vitro, and block tumor progression in xenograft models. TZDs alter the expression of COX-2 and consequent production of the protumorigenic inflammatory molecule prostaglandin E2 (PGE2) through both (PPARγ) dependent and independent mechanisms. Certain TZDs also reduce expression of PGE2 receptors or upregulate the PGE2 catabolic enzyme 15-prostaglandin dehydrogenase. As several COX-2 enzymatic products have antitumor properties and specific COX-2 inhibition has been associated with increased risk of adverse cardiac events, directly reducing the effects or concentration of PGE2 may provide a more safe and effective strategy for lung cancer treatment. Understanding the mechanisms underlying these effects may be helpful for designing anticancer therapies. This article summarizes recent research on the relationship between (PPARγ), TZDs, and the COX-2/PGE2 pathways in lung cancer.

Despite the many advances made in diagnostic and treatment strategies, lung cancer remains the leading cause of cancer-related mortality in the United States and is responsible for more deaths than prostate, colon, and breast cancers combined [1]. Investigating the molecular mechanisms underlying the pathogenesis of lung cancer provides opportunities to develop innovative therapies that may reduce suffering due to this devastating disease. Decreased expression of peroxisome proliferator activated receptor (PPARγ) originally identified as a regulator of glucose metabolism and adipocyte differentiation [2] has been associated with poor prognosis in lung cancer patients [3]. PPARγ affects inflammatory gene expression, cell division, apoptosis, invasion, release of proangiogenic cytokines, and differentiation in many cancer types including lung cancer [4–8]. These properties have prompted extensive research on PPARγ in cancer treatment and prevention. Members of the thiazolidinedione (TZD) class of PPARγ agonists are currently approved for treatment of diabetes, and elicit many of the antitumor properties of

PPARγ activation through both PPARγ dependent and independent pathways [9–14]. Several studies have demonstrated elevated constitutive expression of the inducible proinflammatory enzyme, cyclooxygenase-2 (COX-2) in human lung cancer [15–19]. Mounting evidence from investigations into the molecular effects of COX-2 over-expression in lung tumor cells indicates that this enzyme has a multifaceted role in conferring the malignant and metastatic phenotypes. The COX-2 enzymatic product prostaglandin E2 (PGE2) has been implicated in apoptosis resistance [20–22], angiogenesis [23, 24], decreased host immunity [25, 26], and enhanced invasion and metastasis [27–29]. This review summarizes investigations in the relationship between PPARγ, its ligands, and COX-2 and PGE$_2$ in lung cancer.

The PPAR family consists of three isoforms: PPARα, PPARγ, and PPARδ, each encoded by different genes. PPARs are members of the nuclear hormone class of receptors and are involved in energy metabolism through transcriptional regulation of specific gene sets. Observations

regarding high PPARγ expression in adipose tissue in combination with its role in lipid and glucose homeostasis led to the development of the TZD class of PPARγ agonists, including troglitazone, ciglitazone, rosiglitazone, and pioglitazone as antidiabetic and insulin-sensitizing drugs. Rosiglitazone and pioglitazone are currently approved for treatment of type 2 diabetes mellitus [30], and this class of drugs has been clinically available for approximately a decade. Some of the TZDs have been shown to exert anti-inflammatory [31], antiproliferative [32], and antiangiogenic effects [4]. The COX metabolite 15d-PGJ$_2$ is a natural PPARγ ligand and is considered a negative regulator of inflammatory and immune responses [33]. More recent results indicating that PPARγ activation may attenuate inflammatory responses and cancer progression have led to extensive investigation into the role of this protein in inflammation and carcinogenesis.

PPARγ is expressed in human non-small-cell lung cancer (NSCLC) and small cell lung carcinoma [34], and the expression of PPARγ has been correlated with tumor histological type and grade [35]. In NSCLC, decreased PPARγ expression was correlated with poor prognosis [3]. TZDs inhibit tumor formation in a variety of animal models, including colon [36] and lung cancers [37], and PPARγ over-expression protects against tumor development in a mouse model of lung tumorigenesis [38]. Further, increased PPARγ activity promotes epithelial differentiation of NSCLC cells in 3D culture [5]. It has also been shown that PPARγ inhibits the growth of NSCLC in vitro and in vivo [5, 39, 40].

Cyclooxygenase is the rate-limiting enzyme for production of prostaglandins and thromboxanes from free arachidonic acid [41, 42]. Two COX isoforms, COX-1 and COX-2, have been extensively studied. COX-1 is constitutively expressed in most cells and tissues. COX-2 is an inducible enzyme that acts to produce prostaglandins and/or thromboxanes during an acute inflammatory response. The direct enzymatic product of COX-2 and PGH$_2$ is converted to prostaglandins or thromboxanes by individual isomerases or prostaglandin synthases, and relative production of the various COX-2 products depends upon cellular concentrations of down-stream metabolic and catabolic enzymes within the COX-2 pathway. In NSCLC, the major eicosanoid produced is prostaglandin E2 (PGE$_2$) through microsomal PGE$_2$ synthase (mPGES) activity. The nicotinamide adenine dinucleotide positive-dependent catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) metabolizes PGE$_2$ to biologically inactive 15-keto derivatives. The final PGE2 concentration experienced by NSCLC cells depends upon expression of PGES and 15-PGDH. A large body of evidence indicates that increased PGE2 production contributes to tumorigenesis. COX-2 over-expression is frequently observed in NSCLC, and the accompanying increased proliferation, invasion, angiogenesis, and resistance to apoptosis have been attributed in part to elevated PGE$_2$ production in the vicinity of the tumor. Thus, COX-2 and its downstream signaling pathways represent potential targets for lung cancer chemoprevention and therapy.

Studies indicate that COX-2 and PPARγ signaling pathways are intertwined. PPARγ ligands suppress COX-2 expression induced by LPS and PMA in macrophages, astrocytes, and epithelial cells [43–45]. The COX-2 metabolite 15d-PGJ2 is an endogenous ligand for PPARγ [46], and during resolution of inflammation elevated 15d-PGJ2 production downregulates COX-2 through a negative feedback loop involving PPARγ and NF-κB [44, 47]. Synthetic and endogenous PPARγ ligands decrease the high COX-2 expression associated with several malignancies including cervical [48] and liver cancers [49] and forced PPAR over-expression decreases COX-2 levels in lung cancer cells [38]. While PPARγ agonists decrease COX-2 expression or prevent COX-2 induction in most settings, COX-2 expression is increased in some studies [50, 51]. For example, Ikawa et al. reported that rosiglitazone (also known as BRL49653) increases COX-2 expression in human colorectal carcinoma cells [52]. PPARγ ligands also have been shown to induce COX-2 expression in mammary epithelial cells [53], monocytes [54], and human synovial fibroblasts [55]. The effect of PPARγ agonists on COX-2 expression may vary based upon the cell type, the specific agonist molecule, and the presence of additional inflammatory mediators. Off-target effects of TZDs may also partially account for differences in the effects of these molecules on COX-2 expression.

Although TZDs are widely known as ligands for PPARγ, they may mediate receptor-independent effects, as previously reported [56–58]. For example, by using embryonic stem cells from PPARγ-null mice, Chawla et al. found that neither macrophage differentiation nor anti-inflammatory effects of synthetic PPARγ ligands are PPARγ receptor-dependent. To distinguish the effects of PPARγ from off-target effects of PPARγ ligands in lung cancer cells, Bren-Mattison et al. utilized a molecular approach to over-express PPARγ in two NSCLC cell lines and assessed the direct effect of PPARγ. Their goal was to determine whether the antitumorigenic effects of PPARγ were mediated via COX-2 pathways in NSCLC. Their results clearly demonstrated that exogenously expressed PPARγ suppresses COX-2 promoter activity and protein expression resulting in suppression of PGE$_2$ production [38]. The COX-2 promoter has binding sites for cAMP response element, NF-IL-6, and NF-κB [59]. Although the COX-2 promoter contains multiple regulatory elements, their data indicate that the inhibitory effects of PPARγ are mediated through NF-κB. Several studies have demonstrated that constitutive activation of NF-κB in tumor cells is mediated through activation of Akt [60, 61]. Bren-Mattison et al. demonstrated that the inhibitory effects of PPARγ on COX-2 were mediated via increased activity of PTEN leading to decreased phospho-Akt and inhibition of NF-κβ [38]. These authors further demonstrated that transgenic mice over-expressing PPARγ exhibited reduced COX-2 in type II alveolar epithelial cells of lung, and those mice were protected against lung cancer development in a chemical carcinogenesis mouse model [38]. In summary, these data indicate that COX-2 downregulation may mediate some of the antitumorigenic effects of PPARγ over-expression.

The PPARγ agonists may also affect COX-2 in a PPARγ independent manner (see Table 1). For example, in A549 NSCLC cells troglitazone enhanced both COX-2 and mPGES expression in a concentration dependent manner, resulting

Table 1: Off-target effects of TZDs in NSCLC.

| Thiazolidinediones	Molecular effects	Mechanisms	Reference
Troglitazone	↑ PGE2	↑ COX-2, ERK and PI3K phosphorylation	[62]
Pioglitazone, Rosiglitazone	↓ PGE2	↑ 15-PGDH	[14]
Ciglitazone	↓PGE2	↓ COX-2	[13]
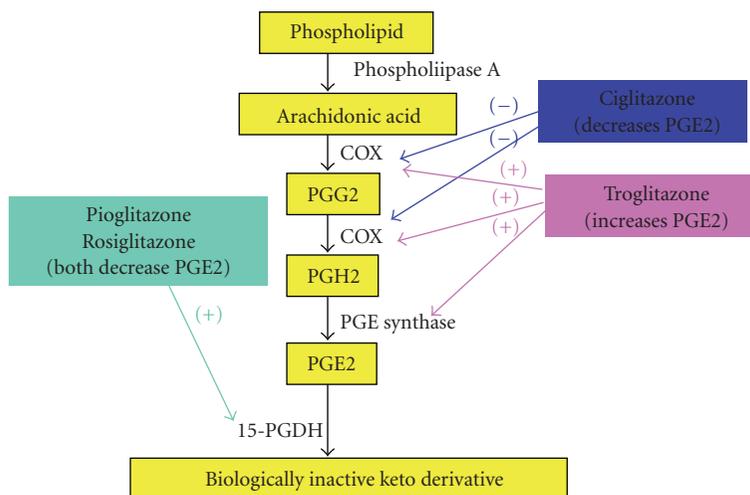
Figure 1: Effects of various TZDs on the PGE2 pathway.

in a marked increase in PGE$_2$ production [62]. Cotreatment with the PPARγ antagonists GW9662 and bisphenol A diglycidyl ether (BADGE) had no effect on COX-2 induction by troglitazone indicating that this event is PPARγ independent. Troglitazone increased COX-2 expression at least in part by activating ERK and PI3K pathways, which have been previously demonstrated to influence COX-2 levels [63–65]. Combined troglitazone and TNFα stimulation resulted in additive enhancement of COX-2 expression. The COX-2 metabolite 15d-PGJ$_2$ had no detectable effects on COX-2 or mPGES expression or PGE$_2$ production in A549 cells. This is consistent with the hypothesis that PPARγ-independent mechanisms can partially account for discrepancies in the effects of different TZD drugs on COX-2 expression. Thus, in lung cancer, PPARγ agonists appear to regulate COX-2 expression and affiliated protumorigenic cellular phenotypes through both PPARγ dependent and independent means.

We recently examined the effect of the pioglitazone and rosiglitazone on COX-2 and PGE$_2$ levels in A427 and A549 NSCLC cells. Both TZDs inhibited PGE$_2$ production in NSCLC cells via a COX-2 independent pathway. To define the mechanism underlying COX-2 independent suppression of PGE$_2$ production, we focused on the prostaglandin synthases that are responsible for the PGE$_2$ production and on 15PGDH the catabolic enzyme responsible for its degradation to biologically inactive15-keto derivatives [66]. None of the three prostaglandin synthases (microsomal PGES1, PGES2, and cytosolic PGES) was downregulated by pioglitazone or rosiglitazone, however, 15-PGDH was induced by TZDs. TZD-mediated suppression of PGE$_2$

concentration was significantly inhibited by small interfering RNA to 15-PGDH. Studies using dominant-negative PPARγ over-expression or GW9662 revealed that the induction of 15-PGDH by both pioglitazone and rosiglitazone is PPARγ-independent.These findings indicate that it is possible to use a clinically available pharmacological intervention to suppress tumor-derived PGE$_2$ by enhancing catabolism rather than blocking synthesis. The potential benefits of inhibiting PGE$_2$ levels in a COX-2-independent manner include the following. First, promoting 15-PGDH activity could decrease PGE$_2$ without modifying other prostaglandins such as PGI$_2$. This is potentially important because the latter has been noted to have antitumor properties [67]. It has been suggested that a ratio of PGs may be important in regulating the malignant phenotype. Thus, inhibiting COX-2 activity would diminish both PGE$_2$ and PGI$_2$, whereas selective induction of 15-PGDH could lead to a more favorable PGI$_2$/PGE$_2$ ratio. Second, suppression of PGE$_2$ levels without alteration in COX-2 may limit some of the cardiovascular toxicities associated with COX-2 inhibition [68]. Finally, unlike COX-2 inhibition, which may lead to upregulation of certain leukotrienes that favor malignant progression [69], 15-PGDH induction may lead only to a decrement of PGE$_2$. This speculation will require further investigation.

Different TZDs have the capacity to modulate arachidonic acid metabolism by a variety of pathways (see Figure 1). Recent evidence indicates that ciglitazone induces differentiation and apoptosis in NSCLC [7]. The mechanisms of ciglitazone's capacity to modulate PGE2 levels in lung adenocarcinoma cells were recently reported [13].

In contrast to pioglitazone and rosiglitazone, ciglitazone mediates COX-2 dependent suppression of PGE_2 in NSCLC. Ciglitazone treatment suppressed COX-2 mRNA expression and COX-2 promoter activity but did not modify the expression of enzymes downstream of COX-2 including PGES and 15-PGDH. Utilization of dominant-negative PPARγ showed that suppression of COX-2 and PGE2 by ciglitazone is mediated via non-PPAR pathways.

PPARγ ligands may also interfere with protumorigenic signals derived from COX-2 by interrupting the function of PGE_2 G-protein coupled receptors (GPCRs) designated E-prostanoid (EP) receptors 1–4 [70]. Han and Roman found that in NSCLC cell lines, the PPARγ ligands GW1929, 15 $dPGJ_2$, ciglitazone, troglitazone, and rosiglitazone significantly decreased EP2 mRNA and protein levels causing growth inhibition in NSCLC cells [71]. The inhibitory effects of rosiglitazone and ciglitazone but not 15d-PGJ2 were suppressed by the PPARγ antagonist GW9662 suggesting the involvement of PPARγ-dependent and independent mechanisms.

Recently, a retrospective study by Govindarajan et al. demonstrated a significant reduction in lung cancer risk in diabetic patients using the TZD rosiglitazone [72]. Importantly, several clinical studies in diabetes patients have demonstrated an increased risk of cardiovascular events associated with rosiglitazone or pioglitazone treatment [73–75]. This is of particular significance in light of cardiovascular toxicity associated with COX-2 inhibition. Recently, several chemoprevention trials are being initiated using TZDs [76]. However, adverse cardiac events are associated with chronic TZD treatment [74]. Based on these findings, future clinical studies attempting to utilize TZDs in prevention of cancer will require selection of patient populations without cardiovascular risk. Prospective clinical studies specifically designed to address the effects of TZDs on cancer, and cardiac outcomes are required. If the anti-inflammatory and antitumor effects of TZDs are derived through pathways distinct from those leading to cardiovascular toxicity, more selective candidate drug molecules may be therapeutically effective, without leading to adverse cardiac events. Thus, more research is required to define opportunities to specifically interfere with PGE_2 production, metabolism, or downstream effects. This could ultimately lead to reduction in lung cancer growth or prevention while leaving the steady-state concentrations of desirable eicosanoids in tact [77].

Both elevated COX-2 and reduced PPARγ expression are associated with poor prognosis in lung cancer patients [3, 78–80] and recent work has revealed multiple interactions between PPARγ signaling and the COX-2 pathway. The COX-2 product 15d-PGJ_2 is an endogenous ligand for PPARγ, and PPARγ activation as a result of elevated 15d-PGJ_2 results in COX-2 downregulation in an autoregulatory feedback loop that may contribute to natural resolution of the inflammatory response [46]. Forced expression of PPARγ decreases COX-2 expression in cultured human NSCLC cells and mouse lungs and protects against lung tumor development in a murine model [5, 38]. Synthetic PPARγ ligands, several of which are currently approved for treatment of diabetes, can interrupt several stages of the COX-2/PGE_2 protumorigenic pathway, although in certain cases PPARγ ligands may increase COX-2 expression. These effects are primarily mediated through PPARγ-independent pathways (see Table 1). PPARγ ligands may directly decrease COX-2 transcription in an NF-κB-dependent manner [38], or they can interfere with downstream targets such as the PGE_2 receptor EP2 [71] or the enzyme responsible for PGE2 catabolism, 15-PGDH [66]. The targets downstream of COX-2 may be useful in light of recent evidence that interfering with COX-2 enzymatic activity may increase risk of cardiovascular events [68]. The discovery that certain PPARγ agonists can specifically reduce PGE_2 concentration or expression of EP receptors may aid in the design of strategies to reduce the effects of harmful prostaglandins without impacting production of critical cardioprotective eicosanoids.

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Review Article

PPARγ and Apoptosis in Cancer

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Peroxisome proliferator-activated receptors (PPARs) are ligand binding transcription factors which function in many physiological roles including lipid metabolism, cell growth, differentiation, and apoptosis. PPARs and their ligands have been shown to play a role in cancer. In particular, PPARγ ligands including endogenous prostaglandins and the synthetic thiazolidinediones (TZDs) can induce apoptosis of cancer cells with antitumor activity. Thus, PPARγ ligands have a potential in both chemoprevention and therapy of several types of cancer either as single agents or in combination with other antitumor agents. Accordingly, the involvement of PPARγ and its ligands in regulation of apoptosis of cancer cells have been extensively studied. Depending on cell types or ligands, induction of apoptosis in cancer cells by PPARγ ligands can be either PPARγ-dependent or -independent. Through increasing our understanding of the mechanisms of PPARγ ligand-induced apoptosis, we can develop better strategies which may include combining other antitumor agents for PPARγ-targeted cancer chemoprevention and therapy. This review will highlight recent research advances on PPARγ and apoptosis in cancer.

1. INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand binding transcription factors belonging to the nuclear receptor superfamily which includes receptors for steroids, thyroid hormone, and retinoids [1, 2]. PPARs function in a variety of roles including regulation of lipid metabolism, immune function, cell growth, differentiation, and apoptosis [2]. PPARs are involved in several diseases including obesity, diabetes, cardiovascular disease, and cancer [3]. Three different subtypes of PPARs have been identified, PPARα, PPARβ/δ, and PPARγ, each encoded by separate genes. The three isoforms share functions as well as have distinct activities [2].

PPARs function by regulating gene transcription via binding to DNA sequences known as peroxisome proliferator response elements (PPREs) located in the promoter regions of target genes. PPREs are direct repeats of the consensus sequence with a spacing of one nucleotide (AGGTCA N AGGTCA) [4]. PPARs bind to PPREs as heterodimers with retinoid X receptors (RXR). The heterodimer PPAR/RXR can bind other transcriptional coactivators or corepressors to influence gene transcription [1]. Ligand binding to PPARs induces conformational changes that release corepressors from the heterodimer and recruit coactivators to allow for target gene transcription [5].

Synthetic and endogenous PPAR ligands have been used to elucidate the role of PPARs. Specifically, thiazolidinediones (TZDs) including pioglitazone, ciglitazone, troglitazone, and rosiglitazone are synthetic PPARγ ligands which are insulin-sensitizing agents developed to treat diabetes mellitus [2]. The naturally occurring prostaglandin, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$(15d-PGJ$_2$), is generally considered to be an endogenous PPARγ ligand [6, 7]. The promiscuous nature of PPARs may lead to the binding of multiple ligands resulting in the activation of many cellular pathways. These ligands have been extensively studied and shown to exert antineoplastic properties including induction of apoptosis.

Apoptosis or programed cell death is a highly regulated process critical for normal development and tissue homeostasis. Aberrant regulation of apoptosis can lead to cancer. Apoptosis is induced from signals inside or outside the cell including radiation, viral infection, growth factors, and

TABLE 1: PPARγ agonists induce apoptosis in cancer.

PPARγ agonist	PPARγ	Tumor type	Molecular mediator(s) of apoptosis	Reference
15d-PGJ$_2$	Independent	Breast	Unknown	[8]
Troglitazone and 15d-PGJ$_2$	Dependent	Thyroid	c-myc	[9]
Ciglitazone	Dependent	Thyroid	PPARγ	[10]
Rosiglitazone	Dependent	Thyroid	NF-κB, cyclinD1, caspase-3	[11]
Troglitazone and 15d-PGJ$_2$	Unknown	Colon	c-myc, c-jun, GADD153	[12]
Troglitazone	Dependent	Lung	GADD153	[13]
Troglitazone	Independent	Colon	EGR-1, NAG-1	[14, 15]
15d-PGJ$_2$	Dependent	Colon	EGR-1, NAG-1	[15]
Troglitazone	Dependent	Lung	ERK1/2	[16]
Troglitazone	Dependent and independent	Colon	p53, POX	[17]
Troglitazone	Independent	Prostate	Bcl-2, Bcl-X$_L$	[18]
15d-PGJ$_2$	Independent	Oral	Stat3	[19]
15d-PGJ$_2$	Independent	Prostate, bladder	Caspase-3, -7	[20]
15d-PGJ$_2$	Independent	Multiple myeloma, burkitt lymphoma	NF-kappa-B, cIAP-1, XIAP, c-FLIP	[21]
Rosiglitazone	Dependent	Breast	PPARγ, p53	[22]

hormones [23]. Apoptosis involves signature morphological changes induced by caspases, which are activated upon induction of apoptotic signaling and cleave downstream molecules to facilitate the apoptotic cascade [24]. The induction of apoptosis can occur through two pathways: the intrinsic apoptotic pathway which involves signaling through the mitochondria and the extrinsic apoptotic pathway which is initiated through activation of cell surface death receptors [25]. Apoptotic signaling through the intrinsic pathway primarily involves activation of the proapoptotic Bcl-2 family members Bax and Bak, which facilitate release of cytochome C from the mitochondria and subsequent caspase-9 cleavage or activation. The activated caspase-9 will finally cleave or activate the downstream effector caspases such as caspase-3 and -7, leading to apoptosis. This pathway is negatively regulated by several antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-X$_L$ [26]. Apoptotic signaling through the extrinsic pathway is initiated by ligand binding to death receptors or by induction of trimerization of the receptors [27]. The death receptors belong to the tumor necrosis factor (TNF) receptor superfamily, which includes Fas, TNFR1, DR3, DR4 (TRAIL-R1), DR5 (TRAIL-R2), and DR6. Upon ligand binding and trimerization of death receptors, the intracellular death domain of the death receptors recruits adapter proteins such as Fas-associated death domain (FADD), forming a death-inducing signaling complex (DISC) which helps recruit procaspase-8 to the DISC. Caspase-8 is then activated, leading to activation of the downstream effector caspases such as caspase-3 and -7. The effector caspases can also be activated by death receptors indirectly through caspase-8-mediated cleavage of Bid, which facilitates Bax activation and subsequent release of cytochome C from the mitochondria. Thus, the Bid

cleavage links the two apoptotic pathways [28]. Cellular FLICE inhibitory protein (c-FLIP), an inactive homolog of caspase-8, primarily functions as an inhibitor of the extrinsic apoptotic pathway by preventing caspase-8 activation, whereas inhibitors of apoptosis protein (IAPs) such as survivin mainly suppress the intrinsic apoptotic pathway by inhibiting caspase-9 as well as caspase-3 activation (Figure 1).

PPARs, particularly PPARγ, and their ligands play a role in regulation of both apoptotic pathways. Thus, this review will specifically focus on the role of PPARγ and its ligands in regulation of tumor cell apoptosis. Some of the underlying mechanisms resulting in apoptosis of tumor cells in PPARγ-dependent and -independent manners will be highlighted.

2. PPARγ AGONISTS INDUCE APOPTOSIS OF CANCER CELLS

PPARγ agonists (e.g., TZDs) have been shown to induce apoptosis in a variety of cancer cells including lymphoma, multiple myeloma, bladder, gastric, esophageal, pancreatic, hepatoma, colon, breast, brain, and lung cancer cells [8, 12, 29–39]. However, many of the underlying mechanisms of the apoptotic properties of TZDs remain unknown. In general, this induction of apoptosis is PPARγ-dependent and/or -independent depending on cell types or ligands (Table 1).

2.1. PPARγ-dependent apoptosis

In thyroid cancer cell lines, it has been shown that the expression of PPARγ correlates with the sensitivity of troglitazone and 15d-PGJ$_2$ to cell death. Thyroid cancer cells that did not express PPARγ showed no growth inhibition
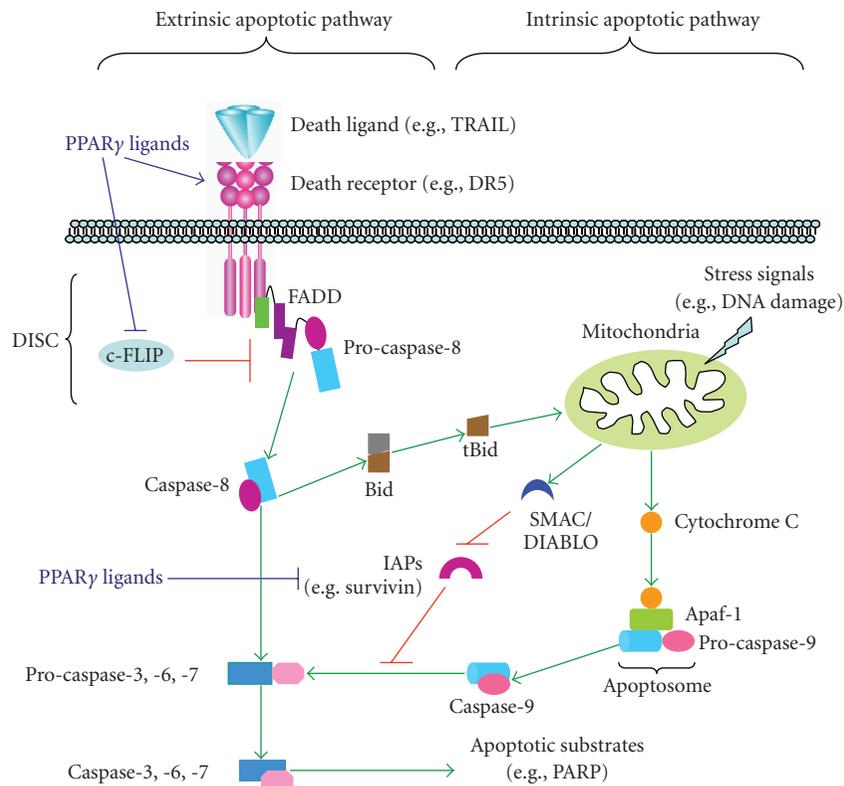
FIGURE 1: *Schema for basic apoptotic signaling pathways and possible mechanisms underlying PPARγ ligand-induced apoptosis.* Ligation of death ligands (e.g., TRAIL) with their receptors (e.g., DR5) results in formation of the death-inducing signaling complex (DISC), in which procaspase-8 will be recruited through the death adaptor protein FADD and cleaved to generate activated caspase-8. This process is inhibited by c-FLIP. Certain stress signals (e.g., DNA damage) can target mitochondria and induce cytochrome C release from the mitochondria into the cytosol leading to caspase-9 activation by forming an apoptosome via binding to Apaf-1. Both caspase-8 and caspase-9 activate downstream procaspase-3, -6, and -7, leading to cleavages of their target death proteins such as PARP. In addition, truncated Bid (tBid), activated by caspase-8 via cleavage, facilitates insertion of Bax into the mitochondrial membrane leading to cytochrome C release. Therefore, tBid may serve as a link between the extrinsic and intrinsic apoptotic pathways. Inhibitors of apoptosis proteins (IAPs) such as survivin can bind to activated caspase-9 and prevent its action on effector caspases, whereas SMAC/DIABLO binds to IAPs, leaving caspase-9 free to activate the effector caspases. PPARγ ligands may induce apoptosis through induction of DR5 and/or downregulation of c-FLIP and/or survivin.

after treatment with troglitazone and 15d-PGJ$_2$ compared with thyroid cancer cells that did express PPARγ and were sensitive to growth inhibition by troglitazone and 15d-PGJ$_2$, suggesting PPARγ-dependent growth inhibition. Growth inhibition by troglitazone was due to apoptosis as was seen by DNA laddering [9]. Another study in thyroid cancer cell lines also implicates PPARγ as an important target. In this study, ciglitazone was effective in reducing the growth of thyroid cancer cells that expressed PPARγ, but had no effect in reducing growth in a thyroid cancer cell line that do not express PPARγ [10]. After introduction of wild-type PPARγ into the PPARγ-deficient cells, these cells became responsive to ciglitazone. Moreover, overexpression of PPARγ in thyroid cancer cells significantly increased apoptosis compared to cells transfected with empty vector or with a vector carrying a mutated nonfunctional PPARγ cDNA [10]. Collectively, it appears that the presence of PPARγ at least partly contributes to the induction of apoptosis by PPARγ ligands in thyroid cancer. The recent findings from a transgenic

mouse study [11] may provide an explanation for why thyroid cancer is susceptible to treatment with PPARγ agonists. Mice harboring a knockin dominant negative mutant thyroid hormone receptor β (TRβPV/PV mouse) spontaneously develop follicular thyroid carcinoma similar to human thyroid cancer. Using the offspring from the cross of TRβPV/+ and PPARγ+/− mice, Kato et al. [11] found that thyroid carcinogenesis progressed significantly faster in TRβPV/PV mice with PPARγ insufficiency from increased cell proliferation and reduced apoptosis. Reduced PPARγ protein activated the NF-κB signaling pathway, resulting in the activation of cyclin D1 and repression of critical genes involved in apoptosis. Treatment of TRβPV/PV mice with a PPARγ agonist, rosiglitazone, delayed the progression of thyroid carcinogenesis by decreasing cell proliferation and activating apoptosis. These results suggest that PPARγ is a critical modifier in thyroid carcinogenesis.

Other molecular mediators of apoptosis have been examined in PPARγ-dependent models. In thyroid cancer cells,

troglitazone increased c-Myc expression without changing the expression of Bcl-2 or Bax [9]. In contrast, in colon cancer cells, both troglitazone and 15d-PGJ$_2$ have been shown to downregulate c-Myc expression [12]. Thus, whether c-Myc is involved in mediating PPARγ agonist-induced apoptosis needs further investigation.

In colon and lung cancer cells, troglitazone was reported to increase the expression of growth arrest and DNA-damage inducible 153 (GADD153) [12, 13], a key apoptosis-regulated gene particularly involved in endoplasmic reticulum (ER) stress-induced apoptosis [40]. Further analysis revealed that troglitazone did not stimulate GADD153 mRNA levels in undifferentiated 3T3-L1 cells lacking PPARγ expression, whereas its induction was clearly observed in differentiated adipocytes expressing PPARγ, suggesting the importance of PPARγ in troglitazone-induced GADD153 expression. In lung cancer cells, inhibition of GADD153 gene expression by an antisense phosphorothionate oligonucleotide attenuated the troglitazone-induced growth inhibition [13]. These findings collectively suggest that GADD153 might be a candidate factor implicated in TZD-induced growth inhibition and apoptosis.

Several studies have demonstrated the importance of ERK and its regulated genes in PPARγ agonist-induced apoptosis [14–16, 41]. In human lung cancer cells, troglitazone induced apoptosis as well as PPARγ and ERK1/2 accumulation in the nucleus. Both PPARγ siRNA and U0126, a specific inhibitor of ERK1/2, blocked these effects of troglitazone, suggesting that troglitazone-induced apoptosis is PPARγ- and ERK1/2-dependent. Moreover, inhibition of ERK1/2 by U0126 also significantly decreased the levels of PPARγ, suggesting a positive crosstalk between PPARγ and ERK1/2 or an autoregulatory feedback mechanism to amplify the effect of ERK1/2 on cell growth arrest and apoptosis [16].

Proline oxidase (POX) is a redox enzyme localized in the mitochondrial inner membrane and functions as a p53-induced gene that can mediate apoptosis through generation of reactive oxygen species (ROS) [17]. A recent study in colon cancer cells showed that troglitazone enhanced the binding of PPARγ to PPRE in the POX promoter, activated the POX promoter, and increased endogenous POX expression. Blocking of PPARγ activation either by the antagonist GW9662 or deletion of the PPAR-responsive element in the POX promoter only partially decreased the POX promoter activation in response to troglitazone, suggesting also the involvement of PPARγ-independent mechanisms. Further, troglitazone induced p53 protein expression in HCT116 cells, which may be the possible mechanism for PPARγ-independent POX activation, since POX has been shown to be a downstream mediator in p53-induced apoptosis. In HCT15 cells, with both mutant p53 and mutant PPARγ, troglitazone did not activate POX, whereas it did in HT29 cells, with a mutant p53 and wild type PPARγ, indicating that both PPARγ-dependent and -independent mechanisms are involved in the troglitazone-induced POX expression [17]. Thus, this study suggests that troglitazone-induced apoptosis involves targeting POX gene expression for generation of ROS.

2.2. PPARγ-independent apoptosis

To help discern the PPARγ-dependent and -independent properties of TZDs, TZD derivatives lacking PPARγ activity were developed. These derivatives have a double bond adjoining the terminal thiazolidine-2,4-dione ring which abolishes ligand binding to PPARγ [42]. Shiau et al. [18] showed that the pioglitazone, troglitazone, and ciglitazone derivatives (Δ2-PG, Δ2-TG, Δ2-CG) were unable to activate PPARγ compared to pioglitazone, troglitazone, and ciglitazone which showed significant activation of PPARγ. When troglitazone and Δ2-TG were tested for growth inhibition in two prostate cancer cell lines: one cell line expressing high levels of PPARγ (PC-3) and one deficient of PPARγ expression (LNCaP), the LNCaP cells were more sensitive to troglitazone compared to PC-3 cells despite being deficient in PPARγ. As well, Δ2-TG which cannot activate PPARγ was more effective than troglitazone in suppressing growth in both PC-3 and LNCaP cells. Both troglitazone and Δ2-TG induced cytochome C release and DNA fragmentation in these cells, attributing the growth inhibition to apoptosis. These results suggest that TZDs can induce apoptosis independent of PPARγ activation. The induction of apoptosis in this study appears to be partly due to the inhibition of the antiapoptotic function of Bcl-2 and Bcl-X$_L$. It is thought that Bcl-2 and Bcl-xL sequester proapoptotic molecules such as Bax and Bak through heterodimerization through BH3 domain binding which inhibits the proapoptotic function of Bax and Bak [43, 44]. Both troglitazone and Δ2-TG reduced the association of Bak with Bcl-2 and Bcl-X$_L$ causing the cells to undergo apoptosis as shown by cytochrome C release and caspase-9 activation. Moreover, Bcl-X$_L$ overexpression protected LNCaP cells from troglitazone- and Δ2-TG-induced apoptosis [18]. Collectively, these results show that PPARγ ligands trigger apoptosis independent of PPARγ and primarily target activation of the intrinsic apoptotic pathway, at least in the tested prostate cancer cells.

It was shown that 15d-PGJ$_2$, but not rosiglitazone and ciglitazone, induced apoptosis in oral squamous cell carcinoma cells, suggesting that 15d-PGJ$_2$ is acting through pathways other than activation of PPARγ. In this study, the apoptotic effect of 15d-PGJ$_2$ was associated with downregulation of the oncogene Stat3 which was not seen with rosiglitazone or ciglitazone [19]. Similarly, in bladder and prostate cancer cells, 15d-PGJ$_2$ and troglitazone inhibited cell growth but rosiglitazone and pioglitazone had no effect on growth inhibition. 15d-PGJ$_2$ inhibited cell growth by induction of apoptosis, while troglitazone induced cell cycle arrest [20]. Thus, the induction of apoptosis can also be selective for certain PPARγ ligands.

Other mediators of apoptosis in PPARγ ligand-induced cell death include the early growth response-1 (EGR-1) transcription factor. EGR-1 has been linked to apoptosis and shown to be activated by ERK. In colon cancer cells, EGR-1 was induced dramatically by troglitazone but not by other PPARγ ligands [14]. Inhibition of ERK phosphorylation abolished EGR-1 induction by troglitazone, suggesting an ERK-dependent induction of EGR-1. Given that troglitazone-induced apoptosis is accompanied by the

biosynthesis of EGR-1, these results suggest that PPARγ-independent EGR-1 induction is a unique property of troglitazone compared with other PPARγ ligands and may play an important role in troglitazone-induced apoptosis [14]. One of the EGR-1-regulated genes is proapoptotic nonsteroidal anti-inflammatory drug (NSAID)-activated gene (NAG-1) [15]. A recent study has demonstrated that the novel TZD derivative MCC-555 exerts a PPARγ-independent upregulation of NAG-1. Moreover, NAG-1 induction contributes to MCC-555-induced apoptosis as downregulation of NAG-1 by siRNA suppressed MCC-555-induced apoptosis [41]. As well, NAG-1 induction was also observed in colon cancer cells treated with troglitazone or 15d-PGJ$_2$ [15]. Importantly, both agents induce NAG-1 expression through an EGR-1-dependent mechanism. However, troglitazone, but not 15d-PGJ$_2$, increases EGR-1 binding to the EGR-1 binding site located within region -73 to -51 of the NAG-1 promoter; this effect has an important role in the transactivation of TGZ-induced NAG-1 expression. The effect of 15d-PGJ$_2$ is probably PPARγ-dependent because a PPARγ antagonist inhibited the 15d-PGJ$_2$-induced expression of NAG-1, whereas TGZ-induced NAG-1 expression was not inhibited by the PPARγ antagonist [15].

Multiple myeloma and Burkitt lymphoma cells express constitutively active NF-κB. 15d-PGJ$_2$ was reported to suppress constitutive NF-κB activity and potently induce apoptosis in both types of B-cell malignancies. NF-κB inhibition is accompanied by rapid downregulation of NF-κB-dependent antiapoptotic gene products, including cellular inhibitor-of-apoptosis protein 1 (cIAP-1), cIAP-2, X-chromosome-linked inhibitor-of-apoptosis protein (XIAP), and c-FLIP. These effects were mimicked by the proteasome inhibitor MG-132, but not by troglitazone, suggesting that 15d-PGJ$_2$-induced apoptosis is independent of PPARγ [21]. Thus, the inhibition of NF-κB may play a major role in the proapoptotic activity of 15d-PGJ$_2$ in aggressive B-cell malignancies characterized by aberrant regulation of NF-κB. Another study in MCF7 breast cancer cells has shown that both PPARγ and p53 are involved in rosiglitazone-induced apoptosis. However, the NF-κB sequence in the p53 promoter region is required for rosiglitazone to increase p53 transcription in this study [22].

3. PPAR AGONISTS AUGMENT DEATH RECEPTOR-INDUCED APOPTOSIS

Apoptosis induced by death receptors can be initiated through binding of death receptor ligands such as TRAIL or Fas ligand. PPARγ ligands can increase death receptor expression and augment death receptor-induced apoptosis. The linkage between PPARγ and TRAIL/death receptor-induced apoptosis came from the early work showing that the PPARγ ligand pioglitazone enhances TRAIL-induced apoptosis through induction of p21 (WAF1) [45, 46]. Subsequently, there are multiple studies demonstrating that different PPARγ ligands have the ability to enhance TRAIL-induced apoptosis in various types of cancer cells both in vitro and in vivo [34, 47–51]. The majority of the studies using various approaches including PPARγ antagonists, PPARγ siRNA or dominant-negative PPARγ

mutants conclude that PPARγ ligands enhance TRAIL/death receptor-induced apoptosis through PPARγ-independent mechanisms [34, 47–49] (Table 2).

Among these studies, Kim et al. [50] first reported their important findings that a variety of natural and synthetic ligands of PPARγ including 15d-PGJ$_2$, ciglitazone, troglitazone, and the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) selectively reduce the levels of c-FLIP, and hence sensitize tumor but not normal cells to apoptosis induction by TRAIL. Both PPARγ agonists and antagonists displayed these effects, regardless of the levels of PPARγ expression and even in the presence of a PPARγ dominant-negative mutant, indicating a PPARγ-independent mechanism. Importantly, PPARγ agonists induced ubiquitination and proteasome-dependent degradation of c-FLIP, without concomitant reductions in c-FLIP mRNA, thus demonstrating a mechanism by which PPARγ agonists decrease c-FLIP through facilitating ubiquitin/proteasome-dependent c-FLIP degradation.

Our group has shown that PPARγ agonists including ciglitazone, troglitazone, and GW1929 induce the expression of death receptor 5 (DR5) including increasing the cell surface distribution of DR5, reducing the levels of c-FLIP, and enhancing TRAIL-induced apoptosis in human lung cancer cells [34]. When c-FLIP was overexpressed or DR5 was silenced, PPARγ ligands showed diminished ability to enhance TRAIL-induced apoptosis, indicating that both DR5 induction and c-FLIP downregulation are two critical events accounting for enhancement of TRAIL-induced apoptosis by PPARγ ligands. Moreover, we have shown that the modulation of DR5 and c-FLIP expression is independent of PPARγ because the use of a PPARγ antagonist and silencing of PPARγ did not effect the ability of the PPARγ ligands to induce DR5 or downregulate c-FLIP [34].

Consistently, 15d-PGJ$_2$ has also been shown to induce DR5 expression and augment TRAIL-induced apoptosis in Jurkat leukemia cells and PC-3 prostate cancer cells. This induction of DR5 by 15d-PGJ$_2$ occurs posttranscriptionally through increasing DR5 mRNA stability, which is independent of PPARγ activation because two other PPARγ agonists pioglitazone and rosiglitazone did not upregulate DR5 expression and pretreatment with GW9662, a PPARγ inhibitor did not block the induction of DR5 by 15d-PGJ$_2$ [47]. As well, DR5 upregulation contributes to the sensitization of TRAIL-induced apoptosis by 15d-PGJ$_2$ since the knockdown of DR5 using DR5 siRNA decreased apoptosis induced by the combination of TRAIL and 15d-PGJ$_2$ compared to control cells [47].

In renal cancer cells, rosiglitazone, in addition to downregulating c-FLIP expression, also increases DR5 expression at the mRNA level [49]. The use of a DR5/Fc chimeric protein or DR5 siRNA attenuated rosiglitazone and TRAIL-induced apoptosis, indicating a critical role of DR5 induction in this death process. Interestingly, rosiglitazone induced the generation of ROS, whereas cotreatment with glutathione, which can scavenge ROS, prevented ROS generation, DR5 upregulation, and enhancement of TRAIL-induced apoptosis by rosiglitazone, suggesting that ROS-mediated

Table 2: Combination of anticancer drugs with PPARγ ligands enhances tumor cell death.

PPARγ agonist + antitumor agent	PPARγ	Tumor type	Molecular mediator(s) of apoptosis	Reference
Troglitazone or ciglitazone or GW1929 + TRAIL	Independent	Lung	DR5, c-FLIP	[34]
15d-PGJ$_2$ + TRAIL	Independent	Leukemia, prostate	DR5	[47]
Troglitazone + TRAIL	Independent	Glioblastoma, neuroblastoma	c-FLIP, survivin, DR5	[52]
Rosiglitazone + TRAIL	Independent	Renal, glioma, breast, prostate	ROS, DR5, c-FLIP	[49]
15d-PGJ$_2$ or ciglitazone or troglitazone or CDDO or CDDO-Me + TRAIL	Independent	Prostate, ovarian, colon	c-FLIP	[50]
Troglitazone + TRAIL or troglitazone + etoposide or paclitaxel	Independent	Glioma	PTP1B, STAT3, c-FLIP, Bcl-2	[53] [54]
15d-PGJ$_2$ + MK886				
15d-PGJ$_2$ + Indomethacin	Dependent	Lung	PPARγ, RXRα	
Ciglitazone + MK886 + 13-cis-retinoic acid				[55]
Rosiglitazone + LG100268 or all transretinoic acid	Dependent and independent	Leukemia, lymphoma, myeloma	Bcl-2, caspase-9	
15d-PGJ$_2$ + LG100268 or all trans-retinoic acid				
CDDO + LG100268 or all transretinoic acid				
Rosiglitazone + carboplatin	Dependent	Lung, ovarian, colon	MT1H, MT1X, MTIIA	[56]
TZD18 + imatinib	Independent	Leukemia	Bax, NF-κB	[57, 58]
RS5444 + paclitaxel	Dependent	Thyroid	p21WAF1/CIP1	[59]
15dPGJ$_2$ + docetaxel	Independent	Lung	Bcl-2, BAD, cyclin D1, p53	[60]

transcriptional activation of DR5 is important for sensitization of renal cancer cells to TRAIL-induced apoptosis [49].

As well, glioblastoma and neuroblastoma cells can be sensitized to TRAIL-induced apoptosis by troglitazone [51]. In addition to upregulation of DR5 and reduction of c-FLIP, troglitazone downregulated survivin levels in these cells, all of which may explain the synergy observed with troglitazone and TRAIL treatment. Importantly, normal astrocytes did not become sensitive to TRAIL-induced apoptosis when treated with troglitazone, suggesting limited toxicity to normal tissues with this treatment [51]. Downregulation of survivin by PPARγ ligands was also observed in breast cancer cells; this event contributes to enhancement of TRAIL-induced apoptosis because enforced expression of ectopic survivin partially protected cells from troglitazone/TRAIL-induced apoptosis. Different from other studies, this study did not find that troglitazone altered the levels of the key proteins in the death receptor-mediate apoptotic pathway including DR4, DR5, and c-FLIP. Instead, they found that troglitazone decreased cyclin D3 levels through inducing ubiquitin/proteasome-mediated protein degradation. Importantly, cyclin D3 downregulation is associated with troglitazone-induced survivin reduction and enhancement of TRAIL-induced apoptosis in human breast cancer cells as silencing of cyclin D3 reduced the levels

of survivin and promoted TRAIL-induced apoptosis [48]. Currently, it is unclear how cyclin D3 regulates survivin expression.

In human glioma cells, it has been shown that troglitazone activates protein-tyrosine phosphatase-1B (PTP-1B), which subsequently reduces phosphotyrosine 705 STAT3 (pY705-STAT3) via a PPARγ-independent pathway [53]. Reduction of pY705-STAT3 in glioma cells caused downregulation of c-FLIP and Bcl-2. When given in combination with TRAIL or caspase-dependent chemotherapeutic agents, such as etoposide and paclitaxel, troglitazone exhibited a synergistic effect by facilitating caspase-8 and -9 activities. Thus, it appears that PTP-1B plays a critical role in the downregulation of activated STAT3, as well as c-FLIP and Bcl-2 [53]. However, it is also not clear how PTP-1B regulates the expression of c-FLIP and Bcl-2.

Although PPARγ agonists downregulate c-FLIP through promoting its degradation, the detailed mechanisms by which PPARγ agonists trigger ubiquitin/proteasome-dependent degradation of c-FLIP are unknown. Moreover, the mechanisms underlying PPARγ agonist-induced upregulation of DR5 expression have not been addressed as well. Nonetheless, the sensitization of TRAIL-induced death by PPARγ agonists may have relevant clinical implications as TRAIL is currently being tested in clinical trials for cancer.

Identification of tumors that can overcome TRAIL resistance by treatment with PPARγ agonists will enhance tumor specific targeting by TRAIL and reduce toxicity of normal tissues as TRAIL has been shown to induce death in tumor cells while sparing normal cells.

PPARγ agonists have also been shown to effect Fas-mediated apoptosis. In HT-29 colon cancer cells, there was a synergistic effect on induction of apoptosis when the anti-Fas agonistic antibody, CH11, was combined with 15d-PGJ$_2$ or ciglitazone [61]. As well, rosiglitazone sensitized the breast cancer cell line, MDA-MB-231, to the antitumor effects of CH11 as well as TNF-α [62]. In uterine leiomyoma cells, ciglitazone downregulated the antiapoptotic protein Bcl-2 and upregulated Bax and Fas while enhancing PARP cleavage and caspase-8 activation, suggesting that ciglitazone induces apoptosis in a Fas- and caspase-dependent mechanism [63].

4. PPARγ AGONISTS IN COMBINATION WITH OTHER ANTICANCER AGENTS ENHANCE APOPTOSIS

Combination therapy regimens are effective in the clinical treatment of various cancers. Many studies have shown that combining PPARγ agonists with anticancer agents can further sensitize tumor cells to apoptosis (Table 2). As we continue to elucidate the molecular mechanisms of PPARγ in the regulation of cancer formation and development, combining PPARγ agonists with other targeted anticancer agents may be an effective strategy for chemoprevention and treatment of various cancers.

One such combination involves the 5-lipoxygenase inhibitor MK866. MK866 blocks the 5-lipoxygenase pathway of arachidonic acid metabolism, increases the expression of PPARα and PPARγ in breast cancer cells, and induces apoptosis [64]. As well, in lung cancer cells, MK886 increased PPARγ reporter activity. The combination of MK886 with 15d-PGJ$_2$ generated greater growth-inhibitory effects including apoptosis than each single agent alone in A549 lung cancer cells [54]. Moreover, MK866 increased the expression of RXRα whose heterodimerization with PPARγ is thought to be necessary for the proapoptotic effect of PPARγ. When MK866 was combined with ciglitazone and the RXR agonist, 13-cis-retinoic acid, there was a superadditive growth inhibitory effect compared to each drug alone [54]. These results suggest that the induction of PPARγ and RXRα by MK866 sensitizes tumor cells to apoptosis by PPARγ ligands or retinoids. In leukemia, lymphoma, and myeloma cells, exposure to rosiglitazone, 15d-PGJ$_2$, or CDDO in combination with the RXR agonist, LG100268, or the retinoic acid receptor (RAR) agonist, all transretinoic acid, augmented the growth-inhibitory effects in these cells [55]. In agreement, treatment of breast cancer cells with another RXR selective ligand, AGN194204, and the PPARγ ligand γ-linolenic acid showed an additive growth inhibitory response [65]. Thus, combining retinoids with PPAR ligands may prove to be a successful treatment in some cancers. This approach may be useful in the clinic as lower doses of each drug can be used to inhibit growth and a more optimal therapeutic index can be achieved.

Other drug combinations that show synergy with PPARγ ligands include the platinum-based drugs. The combination of rosiglitazone with carboplatin in lung, ovarian, and colon cancer models showed a synergistic inhibition of growth. These results are PPARγ-dependent as a non-TZD PPARγ ligand was also able to enhance growth inhibition when combined with carboplatin, and the PPARγ antagonist GW9662 significantly reduced the synergistic effect of rosiglitazone and carboplatin [56]. This synergy is related to the metallothioneins which are heavy metal binding proteins that play a role in platinum drug resistance. Rosiglitazone reduces metallothionein gene expression through a PPARγ-dependent mechanism as treatment with the PPARγ antagonist GW9662 abrogated metallothionein reduction by rosiglitazone and the non-TZD PPARγ agonist GW1929 was also able to reduce metallothionein expression. Moreover, overexpression of the metallothionein MT1H reduced the synergistic effect of rosiglitazone and carboplatin. Therefore, it appears that the downregulation of metallothioneins contributes to the synergism of PPARγ ligands and carboplatin. This synergistic effect of the combination of rosiglitazone and carboplatin was also observed in vivo in xenograft models of lung and ovarian cancer as well as a carcinogen-induced model of colon cancer [56]. Platinum-based drugs are currently being used in the clinic to treat lung and ovarian cancer, therefore, the use of PPARγ ligands to enhance the efficacy of platinum drugs in the treatment of these cancers would be a great advancement in treating these two deadly diseases.

A dual PPARα/γ ligand, TZD18, can induce apoptosis in adult lymphocytic leukemia and chronic myeloid leukemia cell lines [57, 58]. When TZD18 was combined with the bcr-abl tyrosine kinase inhibitor, imatinib, in these cell lines, there was enhanced growth inhibition. Treatment of leukemia patients with imatinib has been a successful therapy, however resistance to imatinib is a problem. These data suggest that combining TZD18 with imatinib is a potential therapy for treating imatinib resistant disease. In these studies, the growth inhibitory effects of TZD18 appeared to be independent of PPARα or PPARγ [57, 58].

A common mutation found in anaplastic thyroid cancer is a PAX8/PPARγ rearrangement which results in down regulation of PPARγ, suggesting that PPARγ may be a tumor suppressor gene in this type of cancer [59]. In addition, the fusion protein, PAX8-PPARγ, resulting from this rearrangement can act as a dominant negative inhibitor of wild-type PPARγ [66]. Treatment with the novel PPARγ agonist RS5444 in anaplastic thyroid cancer cells lines resulted in growth inhibition and PPARγ activation. When RS5444 was combined with paclitaxel, a standard treatment for anaplastic thyroid cancer, enhanced apoptosis-inducing effects were observed [59]. Similarly, the combination of a PPARγ agonist and docetaxel also exerted enhanced apoptosis-inducing and antitumor effects in human lung cancer cells. In addition, 15d-PGJ$_2$ combined with docetaxel significantly reduced tumor volume compared with control, 15d-PGJ$_2$, or docetaxel alone in both A549 and H460 xenografts. This combination showed a significant increase in apoptosis associated with inhibition of Bcl-2 and cyclin D1 expression

and overexpression of caspase-3 and p53 pathway genes. However, enhanced expression of caspase 3 and inhibition of cyclin D1 by the combination was not reversed by GW9662, thus suggesting a possible PPARγ-independent mechanism underlying enhanced apoptosis-inducing and antitumor effects by the combination of 15d-PGJ$_2$ and docetaxel [60].

5. PPARγ ANTAGONISTS EXERT APOPTOSIS-INDUCING EFFECTS

Although most of the antitumor effects of PPARγ ligands are attributed to PPARγ agonists, there is evidence that PPARγ antagonists can have antiproliferative and apoptotic effects on tumor cells. In one study, two PPARγ antagonists, T0070907 and GW9662, were tested in a panel of cancer cell lines and were able to inhibit cell growth and induce apoptosis. Combining the PPARγ agonist, pioglitazone, with the PPARγ antagonists T0070907 or GW9662 actually increased growth inhibition in a colon cancer cell line compared to each agent alone [67]. In breast cancer cells, the PPARγ antagonist GW9662 inhibited growth and also surprisingly enhanced rosiglitazone-induced growth inhibition [68]. The effects of this enhanced growth inhibition appeared to be independent of PPARγ activity as the combination of GW9662 and rosiglitazone did not result in activation of PPARγ as compared to rosiglitazone alone which did activate PPARγ [68]. How the combination of PPARγ agonists and antagonists can enhance tumor growth inhibition needs further investigation.

Human primary squamous cellcarcinoma, lymph node metastasis, and squamous cell carcinoma cell lines express high levels of PPARγ [69]. The specific PPARγ antagonists T0070907, GW9662, and BADGE, but not agonists (i.e., pioglitazone and rosiglitazone) induced apoptosis in squamous cell carcinoma cell lines by interfering with adhesion to the extracellular matrix and disrupting survival signals, and thus inducing anoikis. Furthermore, the PPARγ antagonists strongly inhibited the invasion of squamous cell carcinomas. These results imply a potentially important and novel role for the inhibition of PPARγ function via the use of specific antagonists in the treatment of oral squamous cell carcinoma and the prevention of tumor invasion and metastasis [69]. Similarly, these antagonists also induced apoptosis in colorectal cancer cells as well as altered cell morphology which was linked to alterations in microtubules. The PPARγ antagonists reduced the levels of α and β tubulin which prevented microtubule formation. This mechanism is unique from the known antimicrotubule drugs for the treatment of cancer such as the taxanes which alter microtubule polymerization. These data suggest that PPARγ antagonists may be used as cancer therapy particularly in cancers that are not responsive to antimicrotubule therapy [70].

In contradiction to data suggesting that activation of PPARγ can reduce tumor growth, treatment with PPARγ ligands increased the number of colon tumors in the *Min* mouse model of familial adenomatous polyposis [71, 72]. In this model, PPARγ may be playing a role in tumor promotion. Thus, it appears that activation or inhibition of PPARγ

can have dual roles in tumorigenesis depending on the type of cancer models examined. Determining the mechanisms of PPARγ ligands in cancer either dependent or independent of PPARγ action will be critical to understanding how to best target tumor cells for effective therapy.

6. ACTIVATION OF PPARγ AS A MECHANISM FOR CERTAIN ANTICANCER AGENTS TO INDUCE APOPTOSIS

In addition to PPARγ ligands, certain antitumor agents induce apoptosis through activation of endogenous PPARγ. It was reported that the NSAID, sulindac, induced apoptosis, and upregulated PPARγ expression in oral squamous carcinoma cells [73]. When PPARγ was silenced with PPARγ antisense oligonucleotides, sulindac lost its growth-inhibitory effects compared to control cells transfected with PPARγ sense oligonucleotides in which significant growth inhibition was observed. Therefore, PPARγ is an important mediator of cell growth induced by sulindac [73]. Similarly, β-carotene was shown to induce apoptosis and increase PPARγ expression at both mRNA and protein levels in MCF-7 breast cancer cells. The presence of the PPARγ antagonist GW9662 partially attenuated β-carotene-induced cell death, thus suggesting that PPARγ is involved in β-carotene-induced apoptosis in this cell line [74].

Butyrate is a histone deacetylase inhibitor with the capacity to induce apoptosis of cancer cells. Its growth-inhibitory effects were suggested previously to be dependent on PPARγ activation [75]. A recent study has shown that stimulation of cells with butyrate increased PPARγ expression and activity as well as phospho-p38 MAPK protein levels and caspase-3 activity. Butyrate-induced upregulation of PPARγ was abrogated by coincubation with the p38 MAPK inhibitor SB203580. Treatment of cells with butyrate resulted in both increased caspase-8 and -9 activity and reduced expression of XIAP and survivin. Moreover, these effects were almost completely abolished in cells expressing a dominant-negative PPARγ mutant [76]. These results collectively suggest PPARγ as a key target in the butyrate-induced signaling cascade leading to apoptosis.

Capsaicin (N-vanillyl-8-methyl-alpha-nonenamide), a spicy component of hot pepper, is a homovanillic acid derivative that preferentially induces certain cancer cells to undergo apoptosis and has a putative role in cancer chemoprevention. In colon cancer cells, capsaicin induced apoptotic cell death; this effect was completely blocked by bisphenol A diglycidyl ether, a specific PPARγ antagonist, but not by capsazepine, a specific antagonist for vanilloid receptor [77]. Thus, it seems that capsaicin-induced apoptotic cell death in colon cancer cells is associated with the PPARγ pathway without the involvement of the vanilloid receptor.

Abnormally elevated expression or activation of cyclooxygenase-2 (COX-2) is often associated with cell proliferation and transformation. However, increased numbers of studies have suggested that induction of COX-2 can be proapoptotic [78–81]. In COX-2-mediated apoptosis, production of prostaglandin D$_2$(PGD$_2$) and 15d-PGJ$_2$ and activation of PPARγ have been considered important

mechanisms. For example, the alkylphospholipid type antitumor agent ET-18-O-CH3, at the same concentration ranges that induce apoptosis, induced COX-2 expression in H-ras transformed human breast epithelial cells (MCF10A-ras). The addition of a selective COX-2 inhibitor SC-58635 and COX-2 gene knockdown blocked ET-18-O-CH3-induced apoptosis, suggesting that COX-2 induction by this drug is causally linked to its apoptosis-inducing activity. ET-18-O-CH3 treatment resulted in elevated release of 15d-PGJ$_2$ and DNA binding and transcriptional activity of PPARγ. These data suggest that ET-18-O-CH3 likely induces COX-2 expression and production of 15d-PGJ2, leading to induction of apoptosis in MCF10A-ras cells [79].

In agreement, several chemotherapeutics including paclitaxel, cisplatin, and 5-fluorouracil induced COX-2 expression and prostaglandin (PG) synthesis, accompanied by a substantial decrease of viability and enhanced apoptosis [80]. Cells were significantly less sensitive to apoptotic death when either COX-2 expression or its activity was suppressed by siRNA or by the selective COX-2 inhibitor NS-398. Experiments performed to clarify how COX-2 leads to apoptosis revealed a profound proapoptotic action of PGD$_2$ and its dehydration product, 15d-PGJ$_2$, because chemotherapeutic-induced apoptosis was prevented by siRNA targeting lipocalin-type PGD synthase (L-PGDS), which catalyzes the isomerization of PGH$_2$ to PGD$_2$. Moreover, apoptosis by chemotherapeutics, PGD$_2$ and 15d-PGJ$_2$, was suppressed by the PPARγ antagonist, GW-9662 or PPARγ siRNA. Collectively, this study suggests that COX-2 induction and synthesis of L-PGDS-derived PPARγ-activating PGs are a decisive target by which several chemotherapeutics induce apoptosis [80]. As well, the novel natural compound, a cycloanthranilylproline derivative (Fuligocandin B) was recently reported to sensitize leukemia cells to TRAIL-induced apoptosis through COX-2-dependent 15d-PGJ$_2$ production. However, the synergy mediated by 15d-PGJ$_2$ works in a PPARγ-independent manner as PPARγ siRNA failed to block the synergy [82].

These findings have important clinical impact on the treatment of cancer patients if this mechanism, particularly to chemotherapeutic agents, is common in different types of cancer cells. Because NSAIDs possess COX-2-inhibitory activity and are commonly used by many people including cancer patients, caution should be taken during cancer chemotherapy to avoid potentially diminished therapeutic efficacy due to COX-2 inhibition.

7. CONCLUSIONS

The role of PPARs, particularly PPARγ, in cancer is an evolving field. Understanding of the molecular mechanisms underlying PPAR-mediated regulation of apoptosis of tumor cells will continue to expand. Accordingly, targeting PPARs, especially PPARγ for cancer chemoprevention and therapy may prove to be very effective and will remain an interesting research topic. As we continue to address specific signaling pathways that lead to cancer, we can further elucidate how PPARs and their ligands contribute to these pathways and design effective combinations of therapy that target multiple

steps in the oncogenic process. PPARγ ligands have the potential to sensitize cancer cells to or overcome resistance to chemotherapy or other anticancer drug-based therapies. Thus, exploring mechanism-driven PPARγ ligand-based combination regimens for both cancer chemoprevention and therapy should be the focus of this future study. Specific types of tumors and unique tumor microenvironments also behave differently to PPAR activation or inhibition. Therefore, a close examination of individual tumor types and their response to PPAR stimulation will be critical for successful cancer therapy targeting PPARs, particularly PPARγ. Many studies have revealed that TZDs exert PPARγ-independent effects on induction of apoptosis in various cancer cells. Although some of the TZDs are clinically used drugs for treatment of diabetes with acceptable or manageable side effects or toxicity, they were not originally developed as anticancer drugs and hence are not optimal for cancer treatment. Therefore, it is necessary to use them as lead compounds for synthesizing analogs as anticancer drugs that possess better or optimized cancer chemopreventive or therapeutic efficacy.

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Review Article

The Role of NF-κB in PPARα-Mediated Hepatocarcinogenesis

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In this review, the role of NF-κB in the induction of hepatocarcinogenesis by peroxisome proliferators is examined. The administration of peroxisome proliferators for more than a three-day period leads to the activation of NF-κB in the livers of rats and mice. On the other hand, peroxisome proliferator activated receptor-α (PPARα) activation in non-hepatic tissues can lead to the inhibition of NF-κB activation. Several lines of evidence support the hypothesis that the activation of NF-κB by peroxisome proliferators in the liver is mediated by oxidative stress. The role of NF-κB in peroxisome proliferator-induced hepatocarcinogenesis has been examined using NF-κB knockout models. Specifically, the induction of cell proliferation and the promotion of liver carcinogenesis are inhibited in mice lacking the p50 subunit of NF-κB. Overall, the activation of NF-κB appears to be important in the carcinogenic activity of peroxisome proliferators.

1. INTRODUCTION

Peroxisome proliferators (also known as PPARα agonists) are a group of chemically distinct compounds capable of eliciting persistent peroxisome proliferation in hepatocytes and inducing liver tumors in rats and mice [1, 2]. These chemicals activate the peroxisome proliferator-activated receptor α (PPARα) which is essential for the carcinogenic properties of these agents [3]. The administration of peroxisome proliferators increases the size and number of peroxisomes and activates genes encoding several enzymes of the peroxisomal β-oxidation pathway [4, 5]. The rate-limiting enzyme of this pathway, fatty acyl CoA oxidase (FAO), produces hydrogen peroxide (H_2O_2) as a by-product. In addition, the induction of cytochrome P450 4A enzymes by peroxisome proliferators, which is mediated through PPARα, produces superoxide anions as by-products [6]. Oxidative stress may be important in the toxicity and carcinogenicity of peroxisome proliferators, via the induction of lipid peroxidation, oxidative DNA damage, and/or changes in gene expression

[1, 7]. The changes in gene expression may be brought about in part by the activation of the transcription factor NF-κB, which is known to be induced by oxidative stress. In this review, we will discuss the evidence that peroxisome proliferators activate NF-κB, whether NF-κB activation is necessary for the induction of carcinogenesis by peroxisome proliferators, and the mechanisms by which peroxisome proliferators may influence NF-κB activation.

2. NF-κB

Nuclear factor-κB (NF-κB) is a eukaryotic transcription factor family consisting of the following proteins: p50 (NF-κB1), p65 (RelA), p52 (NF-κB2), c-Rel, and RelB. It is normally found in the cytoplasm as an inactive dimer, with the most common being the p50–p65 heterodimer, bound to an inhibitory subunit, IκB, which also has several family members, including IκBα, IκBβ, IκBγ, and IκBε [8]. Upon activation, NF-κB is released from IκB and translocates to

the nucleus, where it binds target sequences of responsive genes. This process requires the phosphorylation of IκB, followed by its subsequent degradation via the ubiquitin-mediated 26S proteosome pathway [8]. A 900 kDa complex, termed the IκB kinase (IKK) complex, has been identified and it consists of two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ [9, 10]. These two kinase subunits form homo- or heterodimers that phosphorylate IκB molecules, leading to their degradation. This activation pathway for the p50–p65 heterodimer has been referred to as the classical or canonical NF-κB signaling pathway, and is dependent on the IKKβ and IKKγ subunits of IKK [11]. An alternative NF-κB signaling pathway has also been identified, in which IKKα is required and it results in the activation of the p52-RelB heterodimer [11].

One of the many mechanisms by which NF-κB can be activated is by increased oxidative stress. NF-κB can be activated in vitro by H_2O_2, and its activation can be inhibited by antioxidants, such as vitamin E or N-acetyl cysteine (NAC), or by increased expression of antioxidant enzymes [12–18]. In addition, agents that activate NF-κB frequently also increase oxidative stress [19]. However, Hayakawa et al. [20] found that NAC inhibits NF-κB activation independently of its antioxidant function.

NF-κB has been shown to be important in the regulation of numerous genes, including many that regulate the immune response, inflammation, cell proliferation, and apoptosis [21–23]. Several inflammatory factors that are related to NF-κB activation have been identified, including TNF-α, interleukin (IL)-6, and IL-1β [23, 24]. Several studies have used genetically modified mice to examine the role of NF-κB subunits in these functions. Knockout mice have been developed for all NF-κB subunits [25–29]; in addition, knockouts for specific tissues, such as the liver, have been developed [30, 31]. Studies in which NF-κB activity has been inhibited by the deletion of one of its subunits, the inhibition of its translocation, or the expression of a dominant negative form of IκB have demonstrated a clear role for NF-κB in inhibiting apoptosis by tumor necrosis factor-α (TNF-α) or other apoptosis inducers in several cell types [25, 26, 32–36]. The deletion of the p65/relA subunit leads to embryonic lethality at 15-16 days of gestation, due to hepatocyte apoptosis [26]. The deletion of the p50 subunit leads to defects in the immune response involving B cells [25]. Hepatocyte apoptosis is higher in p50 −/− mice [37, 38], but it is not lethal as in the p65 knockout. However, DNA synthesis and liver regeneration were not affected by the absence of the p50 subunit following partial hepatectomy or carbon tetrachloride treatment; increased levels of p65 may have compensated for the lack of p50 [39]. Similarly, the hepatic-specific expression of a truncated IκBα superrepressor did not affect DNA synthesis, apoptosis, or liver regeneration following partial hepatectomy, but led to increased apoptosis after treatment with TNF-α [40]. Also, the hepatic inflammatory response after ischemia/reperfusion was not altered in p50 −/− mice [41]. The deletion of p52 led to defects in humoral immunity and splenic architecture [28]. In RelB −/− mice, multiorgan inflammation, impaired cellular immunity, and hematopoietic abnormalities were observed [29]. The deletion of c-rel led to defects in humoral immunity and in the proliferation of T cells in response to mitogens [27]. In addition, B cells lacking p50, RelB, or c-Rel (but not p52 or p65) exhibited decreased proliferation in response to lipopolysaccharide (LPS) [25, 27, 42–44].

3. HEPATIC ACTIVATION OF NF-κB BY PEROXISOME PROLIFERATORS

Our initial study examined whether peroxisome proliferators could activate NF-κB in the liver (Table 1) [45]. Rats were fed a diet containing 0.01% ciprofibrate; control rats received the same diet without ciprofibrate. Animals were sacrificed 3, 6, or 10 days after starting treatment. NF-κB DNA binding activity was monitored using electrophoretic mobility shift assays (EMSAs) with a radiolabeled NF-κB probe. Low levels of NF-κB were found in the liver nuclear extracts from control rats and remained unchanged over the 10-day period. Three days after the initiation of treatment, an increase in nuclear NF-κB DNA binding activity was observed in treated versus control rats. NF-κB levels continued to increase at six and ten days after treatment. Quantitative radioanalytic image analysis indicated that the level of induction was nearly two-fold by day 3 and increased to 4-fold by day 10. Hepatocyte nuclear factor 3 (HNF-3; foxa) is composed of a family of liver-enriched transcription factors that regulate the expression of many liver genes [46]. EMSAs with a radiolabeled HNF-3 binding motif derived from the rat transthyretin promoter showed that HNF-3 binding activity remained unchanged over the 10-day period in both treated and control rats. This indicates that ciprofibrate does not lead to a global, but rather a more restricted increase in hepatic transcription factor activity.

Following this initial observation, several additional studies have demonstrated that peroxisome proliferators activate NF-κB in the livers of rats and mice, species that are sensitive to the carcinogenic effects of peroxisome proliferators (Table 1). Ciprofibrate has been found to increase the DNA binding activity of NF-κB in both rats and mice [16, 17, 38, 57, 58]. Wy-14,643 and dicamba increased the DNA binding activity of NF-κB in rats and/or mice, while gemfibrozil and dibutyl phthalate activated NF-κB to a lesser extent in rats and not at all timepoints [49, 53, 54, 56]. Delerive et al. [52] observed that hepatic IκBα expression was increased by ciprofibrate in mice; this finding is somewhat difficult to interpret since IκB is an NF-κB-regulated gene but it is also associated with inhibiting NF-κB signaling. Nanji et al. [55] observed that clofibrate treatment did not affect the DNA binding activity of NF-κB (this study also observed that clofibrate decreased ethanol-induced NF-κB activation); however, this finding is also difficult to interpret since all rats were fed fish oil, which itself is a peroxisome proliferator [59]. All of the above studies examined NF-κB activation 3 or more days after beginning peroxisome proliferator administration. In short-term studies, Rusyn et al. observed that hepatic NF-κB DNA binding activity was increased shortly after a single dose of Wy-14,643, and that the increase was primarily due to increased DNA binding activity in Kupffer cells and to the presence of NADPH

TABLE 1: Effect of peroxisome proliferators on the activation of NF-κB in the liver in vivo.

Authors	Species	Agent	Dose	Time Points	Endpoint	Effect
Li et al., 1996 [45]	Rats	Ciprofibrate	0.01% in diet	3, 6, 10 days	EMSA	Increased
Ohmura et al., 1996 [47]	Rats	BR-931	250 mg/kg single p.o. dose	0.5–5 hr after single dose	EMSA	No effect
Menegazzi et al., 1997 [48]	Rats	Nafenopin	200 mg/kg single p.o. dose	0.5–24 hr after single dose	EMSA	No effect
Nilakantan et al., 1998 [16]	Mice	Ciprofibrate	0.01% in diet	21 days	EMSA	Increased
Espandiari et al., 1998 [49]	Rats	Dicamba	1 or 3% in diet	7 days	EMSA	Increased
Rusyn et al., 1998 [50]	Rats	Wy-14,643	100 mg/kg single p.o. dose	1–36 hr after single dose	EMSA	Increased at 2 and 8 hr; no change at 1, 24, and 36 hr
Rusyn et al., 2000 [51]	Rats	Wy-14,643	100 mg/kg single p.o. dose	2 hr after single dose	EMSA	Increased
	Mice	Wy-14,643	100 mg/kg single p.o. dose	2–24 hr after single dose	EMSA	Increased
Delerive et al., 2000 [52]	Mice	Ciprofibrate	0.05% in diet	2 weeks	IκBα expression	Increased in wild-type but not PPARα −/− mice
Tharappel et al., 2001 [53]	Rats	Wy-14,643	0.05 or 0.005% in diet	6, 34, 90 days	EMSA	Increased
		Gemfibrozil	0.1 or 1.6% in diet	6, 34 days	EMSA	No effect
		Gemfibrozil	0.1 or 1.6% in diet	90 days	EMSA	Increased only at lower dose
		Dibutyl phthalate	0.5 or 2.0% in diet	6 days	EMSA	No effect
		Dibutyl phthalate	0.5 or 2.0% in diet	34, 90 days	EMSA	Increased
	Hamsters	Wy-14,643	0.05 or 0.005% in diet	6, 34, 90 days	EMSA	No effect
		Gemfibrozil	0.6 or 2.4% in diet	6, 34, 90 days	EMSA	No effect
		Dibutyl phthalate	0.5 or 2.0% in diet	6, 34, 90 days	EMSA	No effect
Fischer et al., 2002 [54]	Rats	Wy-14,643	0.1% in diet	10 days	EMSA	Increased
Tharappel et al., 2003 [38]	Mice	Ciprofibrate	0.01% in diet	10 days	EMSA	Increased
Calfee-Mason et al., 2004 [17]	Rats	Ciprofibrate	0.01% in diet	10 days	EMSA	Increased
Nanji et al., 2004 [55]	Rats	Clofibrate	100 mg/kg p.o. daily + fish oil	4 weeks	EMSA	No effect compared to fish oil alone; decreased ethanol-induced activation
Woods et al., 2007 [56]	Mice	Wy-14,643	0.1% in diet	1 week, 5 weeks, 5 months	EMSA	Increased in wild-type and NADPH oxidase-deficient mice; no effect in PPARα-deficient mice
Calfee-Mason et al., 2008 [57]	Mice	Ciprofibrate	0.01% in diet	10 days	EMSA	Increased

oxidase in Kupffer cells [50, 51]. However, after a single dose of BR-931 [47] or nafenopin [48], the DNA binding activity of NF-κB in liver was not increased after 0.5–24 hours following exposure. Taken together, these data suggest that the early activation of hepatic NF-κB occurs in Kupffer cells, while the activation in hepatocytes does not appear until 3 days or later after the beginning of peroxisome proliferator administration. Finally, the presence of PPARα is necessary for these changes in NF-κB activation to occur, since neither Wy-14,643 nor ciprofibrate affected NF-κB activation in PPARα-deficient mice [52, 56].

The activation of hepatic NF-κB had also been examined in vitro. EMSAs demonstrated NF-κB induction by ciprofibrate in peroxisome proliferator-responsive H4IIEC3 rat hepatoma cells but not in peroxisome proliferator-insensitive HepG2 human hepatoma cell lines [18]. In addition, stably transfected NF-κB-regulated reporter genes were activated by ciprofibrate in H4IIEC3 cells. These changes were observed after 72 hours of exposure, with the increase in fatty acyl CoA oxidase activity being observed at 24 hours, the first time point tested. This reporter gene activation was blocked by the antioxidants N-acetylcysteine and vitamin E. West et al. [60] examined the activation of NF-κB in cultured mouse hepatocytes in response to nafenopin. After a 4-hour exposure, the DNA binding activity of NF-κB was increased. Using human HuH7 hepatoma cells, Kleemann et al. [61] found that Wy-14,643 increased IκBα protein levels and decreased the nuclear translocation of NF-κB. In addition, the peroxisome proliferators Wy-14,643 and fenofibrate decreased interleukin- (IL-) 1β-induced C-reactive protein expression. Delerive et al. [52] also found that IκBα expression and protein levels were increased by Wy-14,643 in primary human hepatocytes and that IL-1β-induced cyclooxygenase- (COX-) 2 protein levels were decreased by Wy-14,643. These studies suggest that rodent hepatic NF-κB activation is due to, at least in part, activation in hepatocytes. In human liver cells and cell lines, however, NF-κB activation is not affected or is decreased by peroxisome proliferator administration.

4. IMPORTANCE OF NF-κB IN HEPATOCARCINOGENESIS BY PEROXISOME PROLIFERATORS

An important question is whether NF-κB activation by peroxisome proliferators is necessary for carcinogenesis by peroxisome proliferators, as well as the induction of changes in cell proliferation, apoptosis, and gene expression. If NF-κB activation does contribute to the promoting activity of peroxisome proliferators, one would predict that if the activity of NF-κB were diminished, the enhancement of cell proliferation and carcinogenesis as well as the inhibition of apoptosis by peroxisome proliferators would be decreased. Several studies have examined this question, using mice in which the p50 subunit of NF-κB has been deleted. In the first study, the effect of p50 deletion on cell proliferation, apoptosis, and related gene expression was examined [38]. Wild-type and p50 $-/-$ mice were fed a diet with or without 0.01% ciprofibrate for 10 days.

NF-κB DNA binding activity was present and increased after ciprofibrate treatment in wild-type mice, but was not detected in p50 $-/-$ mice. The untreated p50 $-/-$ mice had a higher level of hepatic cell proliferation, as measured by BrdU labeling, than did untreated wild-type mice. However, the increase in proliferation was greater in ciprofibrate-fed wild-type mice than in ciprofibrate-fed p50 $-/-$ mice. The apoptotic index was low in wild-type mice in the presence or absence of ciprofibrate. Apoptosis was increased in untreated p50 $-/-$ mice compared to wild-type mice; apoptosis was reduced in p50 $-/-$ mice after ciprofibrate feeding. Because increased cell proliferation in the liver is associated with increased activator protein-1 (AP-1) activity, the expression of genes in the Fos and Jun families of transcription factors was examined. The c-Jun and JunB mRNA levels were higher in untreated p50 $-/-$ mice than in untreated wild-type mice; c-Jun mRNA levels increased whereas JunB mRNA levels decreased in both groups after ciprofibrate treatment. However, c-Jun and JunB protein levels were the same in untreated wild-type and p50 $-/-$ mice, and increased in both groups after ciprofibrate treatment. Apoptosis-related gene expression was also examined, and several apoptosis-related mRNAs were higher in untreated p50 $-/-$ mice compared to untreated wild-type mice; expression of these genes increased in both groups after ciprofibrate treatment. These data indicate that NF-κB contributes to the proliferative and apoptotic changes that occur in the liver in response to ciprofibrate.

The role of NF-κB in the inhibition of apoptosis by peroxisome proliferators has also been examined in vitro. Using primary rat hepatocytes, Cosulich et al. [62] infected cells with an adenovirus containing a dominant negative form of IKK2. The dominant negative IKK2 induced apoptosis in the hepatocytes, which could not be inhibited by the addition of nafenopin. These data indicate that NF-κB activation is essential for the inhibition of apoptosis by peroxisome proliferators.

The role of NF-κB in the promotion of hepatocarcinogenesis by Wy-14,643 has been examined, using p50 $-/-$ mice [63]. The p50 $-/-$ and wild-type mice were first administered diethylnitrosamine (DEN) as an initiating agent. Mice were then fed a control diet or a diet containing 0.05% Wy-14,643 for 38 weeks. As expected, wild-type mice receiving DEN only developed a low incidence of tumors, and the majority of wild-type mice receiving both DEN and Wy-14,643 developed tumors. However, no tumors were seen in any of the p50 $-/-$ mice. Treatment with DEN/Wy-14,643 increased both cell proliferation and apoptosis in wild-type and p50 $-/-$ mice; DEN treatment alone had no effect. In the DEN/Wy-14,643-treated mice, cell proliferation and apoptosis were slightly lower in the p50 $-/-$ mice than in the wild-type mice. These data demonstrate that NF-κB is involved in the promotion of hepatic tumors by the peroxisome proliferator Wy-14,643; however, in this study, the difference in tumor incidence could not be attributed to alterations in either cell proliferation or apoptosis.

5. MECHANISMS BY WHICH PEROXISOME PROLIFERATORS INFLUENCE NF-κB ACTIVATION

The studies discussed above showed that peroxisome proliferators activate hepatic NF-κB, except possibly for very short exposure periods, and that NF-κB activation is necessary for the promoting activity and associated biochemical activities of peroxisome proliferators. The mechanisms by which peroxisome proliferators activate NF-κB have been examined in several studies. These studies can be divided into two main groups: (1) those taking place in nonhepatic cells or nonrodent hepatocytes, or in which the exposure time was short; and (2) those in liver, in which the exposure time was longer, usually greater than one week. The former studies involved alterations in NF-κB in the absence of changes in gene expression brought about by PPARα activation in rodent liver. For the longer studies, however, changes in gene expression and cell metabolism in response to PPARα activation have occurred. These include the induction of the peroxisomal β-oxidation pathway including fatty acyl CoA oxidase (FAO) and the cytochrome P-450 4A (CYP4A) family. FAO produces hydrogen peroxide as a by-product, and CYP4A may also produce reactive oxygen species. PPARα activation also results in a decrease in the activities of cellular antioxidant enzymes such as glutathione peroxidase, glutathione S-transferase, and DT-diaphorase, and in the concentrations of cellular antioxidants such as vitamin E [7]. Therefore, oxidative stress may be an important mechanism in the activation of NF-κB by peroxisome proliferators.

In tissues that are not responsive to the peroxisome proliferative and carcinogenic effects to peroxisome proliferators, such as human hepatocytes and nonhepatocyte tissues and cells, the administration of PPARα activators clearly leads to a decrease in NF-κB activation and NF-κB-regulated gene expression. These include kidney cells in vitro [64], human aortic smooth muscle cells [52, 65], human HuH7 hepatoma cells [61], primary human hepatocytes [52], human endothelial cells [66], Cos-1 cells [67], and mouse splenocytes in vivo [68]. In these cases, PPARα decreased NF-κB activation by the direct interaction with p65 [65] and/or by increasing IκBα expression [52]. The administration of peroxisome proliferators also decreased the expression and/or protein levels of NF-κB-regulated inflammatory genes, including IL-6 [65, 68], IL-12 [68], C-reactive protein [61], vascular cell adhesion molecule-1 (VCAM-1) [66], and COX-2 [67]. On the other hand, inhibition of the NF-κB signaling pathway by inactivating the NF-κB essential modulator (NEMO) gene in rodent liver leads to a decrease in the expression of PPARα [69].

Several lines of evidence support the hypothesis that NF-κB activation after one week or more of exposure to peroxisome proliferators is mediated by oxidative stress produced by peroxisome proliferators. First, overexpression of the hydrogen peroxide-producing enzyme that is induced by peroxisome proliferators, FAO, is sufficient to activate NF-κB in Cos-1 cells [70]. In addition, FAO overexpression in Cos-1 cells, in the presence of an H_2O_2-generating substrate, can activate an NF-κB-regulated reporter gene. Electrophoretic mobility shift assays further demonstrated that FAO expression increases nuclear NF-κB DNA binding activity in a dose-dependent manner. The antioxidants vitamin E and catalase can inhibit this activation [70].

Second, overexpression of the hydrogen peroxide-detoxifying enzyme catalase in the livers of transgenic mice inhibits the activation of NF-κB by ciprofibrate [16]. In this study, mice overexpressing catalase in the liver or non-transgenic littermates were fed either 0.01% ciprofibrate or a control diet for 21 days. FAO activity was not significantly affected by catalase overexpression although the ratio of FAO to catalase was significantly decreased in transgenic animals. Ciprofibrate increased NF-κB DNA binding activity in the livers of non-transgenic mice, but this increase was inhibited by catalase overexpression. In addition, the ciprofibrate-induced increase in hepatocyte proliferation was decreased by catalase overexpression, indicating a possible role for NF-κB in cell proliferation by peroxisome proliferators.

Third, studies in species with different responses to peroxisome proliferators support a role for oxidative stress in NF-κB activation. Rats and mice are sensitive to the hepatocarcinogenic and cell proliferation-inducing effects of peroxisome proliferators whereas other species, such as Syrian hamsters, are not [71, 72]. Therefore, we examined the effects of three different peroxisome proliferators on antioxidant enzymes, antioxidant vitamins, and NF-κB activation in rats and Syrian hamsters [53, 73, 74]. The peroxisome proliferators Wy-14,643, gemfibrozil, and dibutyl phthalate were administered to animals for 6, 34, or 90 days. In rats, decreases in glutathione reductase (GR), glutathione S-transferase (GST), and selenium-dependent glutathione peroxidase (GPx) were observed following peroxisome proliferator treatment at various time points. In hamsters, a higher basal level of activities for GR, GST, and selenium GPx was observed as compared to rats. In addition, hamsters showed decreases in GR and GST activities following peroxisome proliferator treatment. Interestingly, selenium-GPx activity was increased in hamsters following peroxisome proliferator treatment. Treatment for 90 days with Wy-14,643 resulted in no change in GPx1 mRNA in rats and increased GPx1 mRNA in hamsters. In both rats and hamsters treated with Wy-14,643, we observed decreases in α-tocopherol content and total superoxide dismutase (SOD) activity. Conversely, DT-diaphorase activity was decreased following Wy-14,643 treatment in rats at all time points and doses, but only sporadically affected in hamsters. Rats and hamsters treated with DBP demonstrated increased SOD activity at 6 days; however, in the rat, DBP decreased SOD activity at 90 days and α-tocopherol content was decreased throughout. In gemfibrozil-treated rats and hamsters, a decrease in α-tocopherol content and an increase in DT-diaphorase activity were observed. In either species, no consistent trend was observed in total ascorbic acid content after treatment with any of the peroxisome proliferators. NF-κB activation was evaluated by EMSA. Wy-14,643 increased the DNA binding activity of NF-κB at all three timepoints in rats and produced the highest activation of the three chemicals tested (Table 1). Gemfibrozil and DBP increased NF-κB activation to a less extent in rats and not at all times. There were no differences

in hepatic NF-κB levels between control hamsters and hamsters treated with any of the peroxisome proliferators. These studies show that NF-κB is not activated by peroxisome proliferators in hamsters, which have much higher levels of the antioxidant enzymes glutathione peroxidase, glutathione S-transferase, glutathione reductase, and DT-diaphorase, and which are not responsive to the carcinogenic effects of the peroxisome proliferators.

Finally, the antioxidant, vitamin E, inhibits ciprofibrate-induced NF-κB activation, both in vivo and in vitro. In an in vitro study [18], NF-κB-regulated reporter genes were stably transfected into rat hepatoma H4IIEC3 cells. The ciprofibrate-induced increase in luciferase activity after 72 hours of exposure was blocked by the addition of α-tocopheryl acetate. N-acetyl cysteine also inhibited the ciprofibrate-induced increase. In the in vivo study [17], thirty-six male Sprague-Dawley rats were fed a purified diet containing varying levels of vitamin E (10, 50, 250 ppm α-tocopheryl acetate). After 28 days, seven animals per vitamin E group received 0.01% ciprofibrate in the diet for 10 days. Increased dietary α-tocopherol acetate inhibited CIP-induced NF-κB DNA binding. Since NF-κB translocates to the nucleus upon the phosphorylation and degradation of IκB, we also used western blots to measure cytosolic protein levels of IκBα, IκBβ, and IκB kinases: IKKα and IKKβ. However, IκBα protein levels were decreased in all three CIP-treated groups, with the 10 ppm vitamin E diet also decreasing IκBα levels in control rats. No difference in IκBβ protein levels was observed among any of the groups. The CIP-treated rats generally had lower protein levels of IKKα and IKKβ.

An important question is whether vitamin E is exerting some of its effects by blocking the activation of NF-κB. The use of NF-κB knockout models may provide answers to this question. A study has addressed this question by examining if the inhibition of NF-κB by vitamin E is necessary for vitamin E's effects on the induction of cell proliferation by the peroxisome proliferator ciprofibrate and on the inhibition of apoptosis by ciprofibrate [57]. Wild-type and p50 $-/-$ mice were administered ciprofibrate and one of two levels of vitamin E (10 or 250 mg/kg diet). Vitamin E inhibited ciprofibrate-induced cell proliferation only in the p50 $-/-$ mice. Dietary vitamin E also increased apoptosis and increased the GSH/GSSG ratio in both wild-type and p50 $-/-$ mice. This study suggests that vitamin E does not act by blocking NF-κB activation, indicating that vitamin E is acting by other molecular mechanisms.

6. CONCLUSIONS

In summary, the administration of most peroxisome proliferators leads to the activation of NF-κB in the liver of rats and mice. This activation appears to be necessary for the tumor-promoting activity and for the induction of cell proliferation by peroxisome proliferators. The activation of NF-κB appears to be mediated at least in part by the induction of oxidative stress by peroxisome proliferators. Future studies examining the mechanisms by which NF-κB is altered by PPARα activation will need to clearly distinguish

between those changes brought about directly by PPARα and those brought about as a result of changes in gene expression through PPARα (such as the peroxisomal β-oxidation pathway). The identity of specific NF-κB-regulated genes after the administration of peroxisome proliferators, particularly genes related to cell proliferation and apoptosis, will also need to be determined in future studies.

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