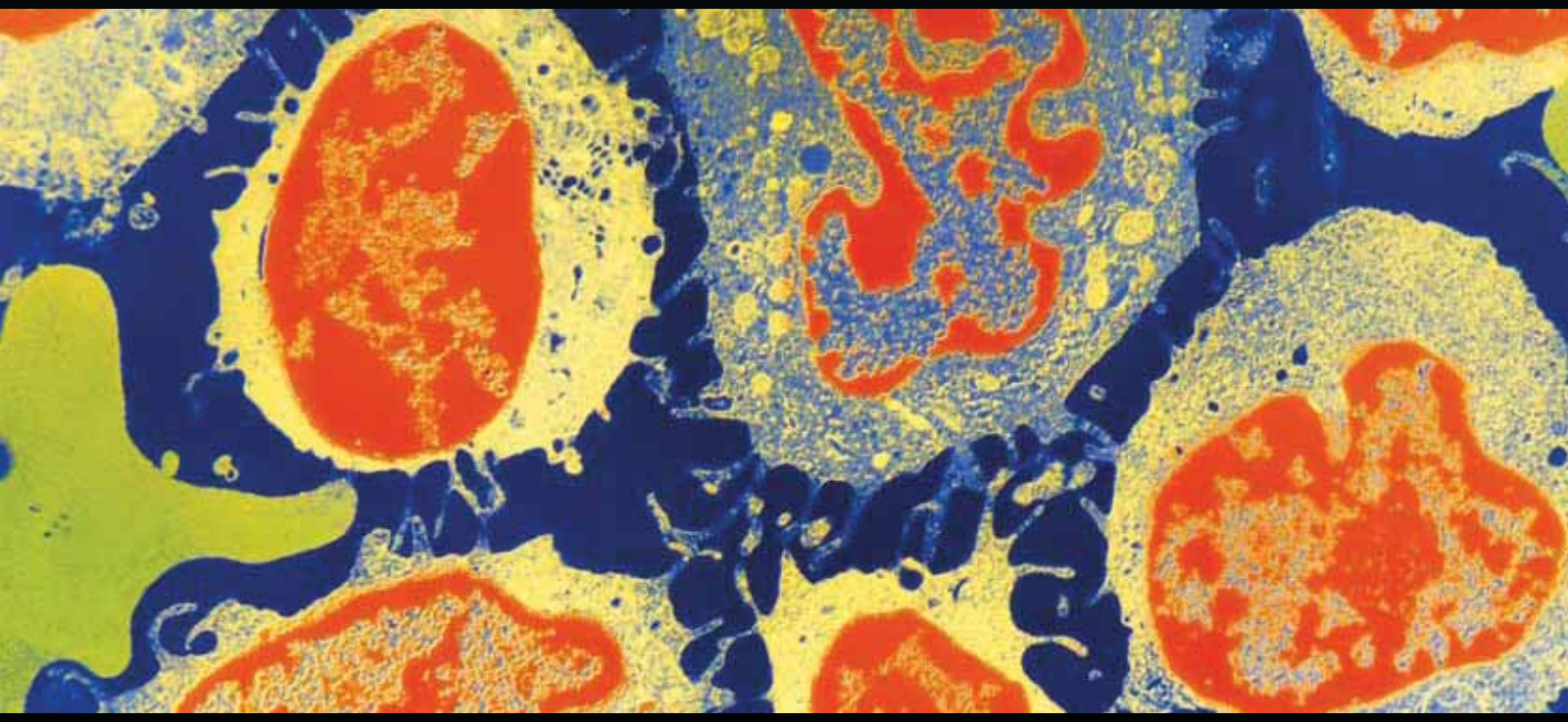
Journal of Oncology

Tumor Angiogenesis

Guest Editors: Arkadiusz Dudek, Kalpna Gupta,
Sundaram Ramakrishnan, and Debabrata Mukhopadhyay



Tumor Angiogenesis

Journal of Oncology

Tumor Angiogenesis

Guest Editors: Arkadiusz Dudek, Kalpna Gupta,
Sundaram Ramakrishnan, and Debabrata Mukhopadhyay

Editorial Board

Contents

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 761671, 2 pages
doi:10.1155/2010/761671

Editorial
Tumor Angiogenesis

Arkadiusz Dudek,[1] Kalpna Gupta,[1] Sundaram Ramakrishnan,[2] and Debabrata Mukhopadhyay[3]

[1] *Division of Hematology, Oncology and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN, USA*
[2] *Department of Pharmacology, University of Minnesota, Minneapolis, MN, USA*
[3] *Department of Biochemistry and Molecular Biology, Tumor Angiogenesis, Vascular Biology and Nanotechnology Laboratory, Mayo Clinic Foundation, Rochester, MN, USA*

Correspondence should be addressed to Arkadiusz Dudek, dudek002@umn.edu

Received 30 August 2010; Accepted 30 August 2010

In a seminal *New England Journal of Medicine* article, published 40 years ago, Folkman proposed the concept that angiogenesis could be a target for cancer therapy [1]. This proposal emerged from early observations that some tumors cells were capable of stimulating endothelial cells to form new capillary sprouts and that nonvascularized tumors were held in a dormant state, unable to grow beyond a size larger than 2-3 mm^3. These and related observations led Folkman to cautiously conclude that "...the mechanism by which tumor implants stimulate neovascularization must be well understood before therapy based upon interference with angiogenesis can be devised." Since then, a wide variety of antiangiogenic therapies have been tested in clinical trails, with relatively modest improvements in patient outcomes and unforeseen therapeutic challenges. These initial setbacks call for a reinvigorated research effort to better understand the complex molecular and biological relations between tumor cells and endothelial cells within a neoplasm and the development of new, more effective, therapeutic tools targeting tumor angiogenesis. Encouraging this research is the theme of this special issue of the *Journal of Oncology*.

Folkman's original proposal to target angiogenesis for cancer therapy relied on several assumptions. One key assumption was that solid tumors would only grow beyond a size of 2-3 mm^3 after vascularization was established, leading to more efficient diffusion of oxygen, nutrients, and wastes. A second assumption was that tumor cells produce angiogenesis in part by stimulating the growth of endothelial cells from surrounding vessels. A third assumption was that blocking angiogenesis would suppress tumor growth and result in resumption of tumor dormancy. The fourth assumption was that antiangiogenic cancer therapy could be delivered chronically because angiogenic activity is of minimal importance to healthy tissues.

Since Folkman's landmark paper, several factors have been recognized as critical for the induction of tumor angiogenesis, with one of these being vascular endothelial growth factor (VEGF) and its interaction with VEGF receptors. Many therapeutic strategies were developed to either block VEGF, block VEGF binding to its receptor, or interfere with intracellular signaling in the VEGF receptor pathway. Early work in preclinical models led to clinical studies and the development of a multitude of antibodies and small molecules that target tumor angiogenesis. Several clinical trials provided encouraging evidence supporting the use of these agents in the treatment of breast cancer, lung cancer, kidney cancer, and colon cancer. However, resistance to antiangiogenic agents was seen in clinical trials, challenging the notion that endothelial cells supplying the tumor vasculature are genetically stable and, therefore, unlikely to develop mutations that lead to such resistance. Furthermore, there are indications that the initial response to clinical antiangiogenic agents may lead to the development of more aggressive tumors. Since then we have identified several other pathways involved in the biology of angiogenesis and also identified several mechanisms leading to the resistance.

Lymphangiogenesis and lymph node metastases are another critical determinant of tumor progression and may even be responsible for the emergence of resistance to cancer therapy. Yu and colleagues and Kitadai review the literature

pertaining to lymphangiogenesis in breast and gastric cancers, respectively. Though endothelial in origin, lymphatic endothelial cells are distinct with specific cell surface receptors. Their origin, proliferation, and survival may pose a major challenge in treating cancer with angiogenesis-based therapy. Therefore, therapies targeting lymphangiogenesis, in addition to angiogenesis, offer promising prospects to achieve improved cancer management.

This special issue set out to address several issues related to tumor angiogenesis, such as, the mechanisms by which tumor cells acquire the capability to "turn on" the angiogenic switch, the influence of the tumor microenvironment on angiogenesis (Campbell et al., Schmidt and Varner, Tilan and Kitlinska, Rigogliuso et al., Oettgen), types of resistance to antiangiogenic therapy (Grepin and Pages, Rahman et al.), and the development of novel angiogenic strategies (Colema et al., Corti et al., Otten, Bokemeyer, and Fiedler, Seyfried and Mukherjee). Invited papers address several of the above topics and this issue is divided into the following chapter subgroups: biology of tumor angiogenesis, discovery of new angiogenesis targets for cancer therapy, disease-specific tumor angiogenesis, and disease-specific cancer therapies. There will be several review and research papers addressing all those issues.

Arkadiusz Dudek
Kalpna Gupta
Sundaram Ramakrishnan
Debabrata Mukhopadhyay

References

[1] J. Folkman, "Tumor angiogenesis: therapeutic implications," *New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 132641, 24 pages
doi:10.1155/2010/132641

Review Article

Tumor Angiogenesis: Insights and Innovations

Fernando Nussenbaum[1] and Ira M. Herman[2]

[1] *Tulane University School of Medicine, 257 Cherokee st., Unit 2, New Orleans, LA 70118, USA*
[2] *Center for Innovations in Wound Healing Research, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111, USA*

Correspondence should be addressed to Ira M. Herman, ira.herman@tufts.edu

Received 29 September 2009; Revised 12 February 2010; Accepted 12 February 2010

Academic Editor: Arkadiusz Dudek

Angiogenesis is a vital process resulting in the formation of new blood vessels. It is normally a highly regulated process that occurs during human development, reproduction, and wound repair. However, angiogenesis can also become a fundamental pathogenic process found in cancer and several other diseases. To date, the inhibition of angiogenesis has been researched at both the bench and the bedside. While several studies have found moderate improvements when treating with angiogenesis inhibitors, greater success is being seen when the inhibition of angiogenesis is combined with other traditional forms of available therapy. This review summarizes several important angiogenic factors, examines new research and ongoing clinical trials for such factors, and attempts to explain how this new knowledge may be applied in the fight against cancer and other angiogenic-related diseases.

1. Introduction

For over 35 years, scientists have been trying to fully understand the process of both normal and pathogenic angiogeneses, hoping to apply their findings to the world of clinical medicine and therapeutics. Angiogeneses is a critical process involving the formation of new blood vessels from preexisting vessels [1]. Normal angiogeneses is an essential process the body employs during fetal development, wound healing, ovulation, as well as growth and development [2]. Angiogeneses provides developing and healing tissues with vital nutrients and oxygen [3]. When angiogeneses goes awry, pathological problems often ensue. The understanding of normal and pathogenic angiogeneses has been a major focus of both cancer biology and clinical medicine for the past few decades.

In the past, research in angiogeneses was closely intertwined with cancer biology. The importance of angiogeneses in tumor growth was initially hypothesized in 1971, when Judah Folkman theorized that solid tumors possess limited resources that the many actively proliferating cancer cells fight for. Increased interstitial pressure within the tumor also inhibits the diffusion of metabolites and nutrients essential to the growth and survival of tumor cells [4]. This environment causes tumor cells to induce the sprouting of new blood vessels from the established vasculature, creating a vascular system within the tumor, thus enabling tumor cells to obtain the oxygen and nutrients they need to survive and multiply. Understanding these principles led to the idea that the inhibition of tumor angiogeneses could be a valuable therapy against cancer [1]. This sparked research into the proteins that regulate this process, both angiogeneses inhibitors and promoters. Since that time, many proteins and regulators of angiogeneses have been discovered and their role in the process defined.

Although cancer has traditionally been the most extensively studied angiogenic-dependent disease, several other conditions have also shown a reliance on angiogeneses. Some of these include psoriasis, endometriosis, arthritis, macular degeneration, regional ileitis, and atherosclerosis [5]. The emergence of other diseases connected to angiogeneses has led to increased research on angiogeneses as a whole. Recently, new drugs have been developed that are capable of targeting many of the regulators of angiogeneses [6]. Currently, several drugs have been approved by the FDA for the treatment of angiogeneses-dependent diseases including Avastin for colorectal cancer, Tarceva for lung cancer, and Lucentis for macular degeneration [5, 7]. Many other drugs

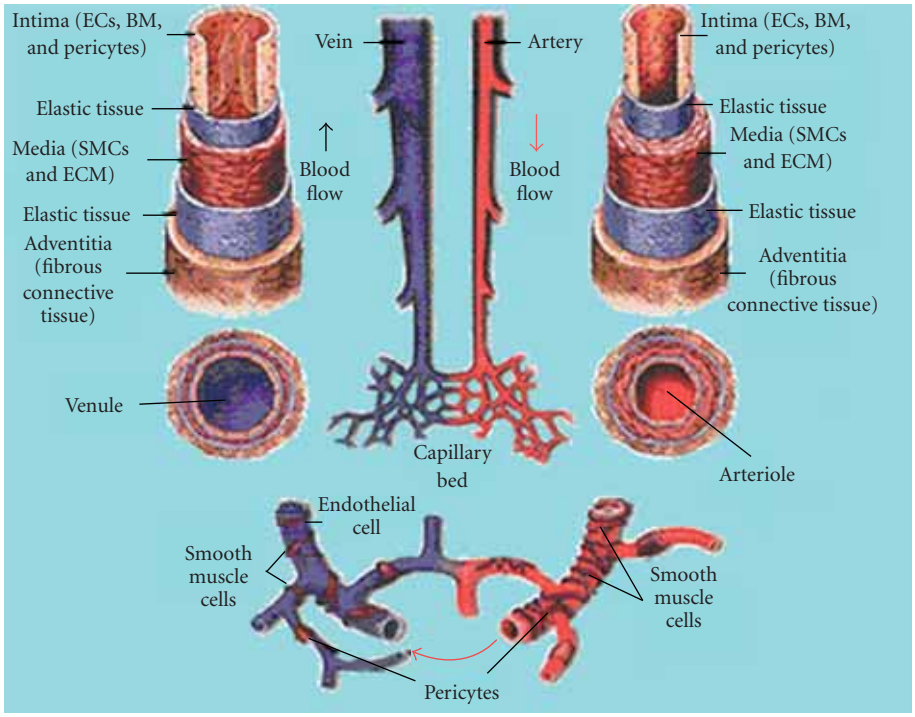


Figure 1: *Blood Vessels*. The cardiovascular system main components include arteries, arterioles, capillaries, venules, and veins. Each vessel has cellular differences from the other types of vessels and this is highlighted above.

are in late-stage clinical testing. This review will focus on the current knowledge of angiogeneses in health and disease, some important angiogenic promoters and inhibitors, and ongoing research and developments as they relate to oncology. Increasing the mechanistic understanding of these processes will improve the development of more efficient angiostatic treatments in cancers.

2. Normal Blood Vessel Formation

The cardiovascular system distributes blood, and thus oxygen and nutrients, throughout the body. The system consists of arteries, arterioles, capillaries, venules, and veins. The microvasculature is considered the portion of the circulatory system composed of the smallest vessels, such as the capillaries, arterioles, and venules. The microvasculature is a very dynamic and complex system, capable of constant change, while the larger blood vessels are more permanent structures with very little plasticity. As illustrated in Figure 1, capillaries are hollow tubes composed of endothelial cells (ECs) which are supported by pericytes. Unlike capillaries, arteries and veins have several distinct layers including the tunica intima, the tunica media, and tunica adventitia in the largest vessels (composition of each detailed in Figure 1). Due to the thickness of these structures, arteries, arterioles, venules, and veins are all considered conduit vessels. Capillaries are the most important vessels in cardiovascular system. The thin walls of these microscopic vessels allow for the exchange of oxygen and nutrients between the blood and tissues [7]. The formation of the initial vascular plexus within each tissue and the formation of the major blood vessels conducting blood to and from the heart are hard wired into the developmental system [8]; these networks are formed independent of oxygen concentration. In contrast, the pattern of capillary (microvasculature) development within each tissue is driven by local oxygen demand, and is therefore unique to each tissue [8].

Blood vessels comprising the microvasculature are formed in adults via two different mechanisms: vasculogenesis and angiogeneses. Both processes normally occur during embryonic development; however, special circumstances allow these processes to be initiated during adult life. Vasculogenesis is the de novo formation of ECs from angioblasts. This process helps form a primitive vascular labyrinth of small capillaries [9]. Angiogeneses is the process in which ECs sprout from preexisting blood vessels. The ECs then migrate and proliferate to form a cord-like structure.

2.1. Vasculogenesis: Current Concepts and Challenges. The establishment of fetal vasculature begins with hemangioblasts, primitive cells of mesodermal origin [10]. Hemangioblasts help form "blood islands", clusters of cells that have a designated spatial arrangement that facilitates their function. Hematopoietic stem cells (HSCs), which later become hematopoietic cells, are found at the center of these islands. Angioblasts, cells that differentiate into ECs, are found at the periphery of the blood islands [11]. The adult stem cells found within bone marrow (instead of blood islands) were discovered to contain much greater plasticity

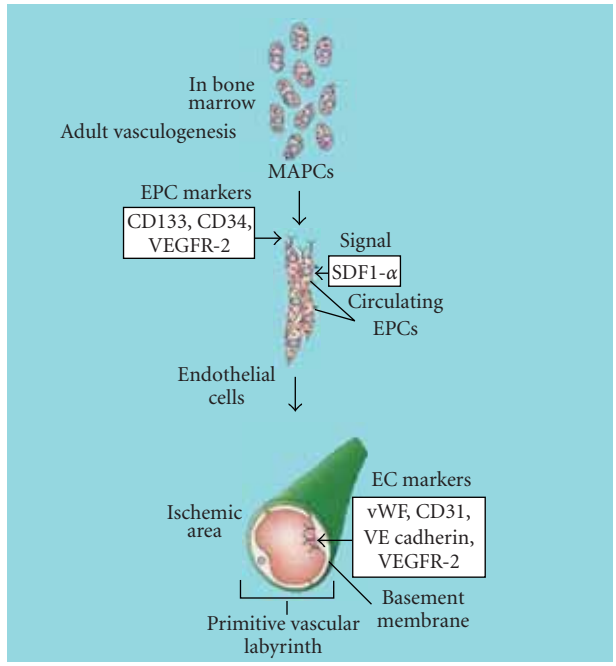


Figure 2: *Adult Vasculogenesis.* The figure illustrates the process where MAPCs become angioblasts, then circulating EPCs and ECs as part of the primitive vascular labyrinth.

than originally thought, and are now considered multipotent adult progenitor cells (MAPCs) [12]. MAPCs are capable of differentiating into ECs when removed from bone marrow and cultured on fibronectin with vascular endothelial growth factor-A (VEGF-A) [13]. In addition, MAPCs are capable of differentiating into skeletal muscle, cardiac muscle, and vascular endothelium after bone marrow transplantation [12].

As demonstrated in Figure 2, it is currently believed that vasculogenesis originates when MAPCs in bone marrow differentiate into early endothelial progenitor cells (EPCs) [13, 14]. As MAPCs evolve into EPCs, they gain hematopoietic and endothelial lineage-specific markers such as VEGF receptor-2 (VEGFR-2) and CD34 [15, 16]. EPCs in the bone marrow remain undifferentiated in one of two zones. The first zone is known as the vascular zone, and it consists of EPCs in either the S phase or G2M phase of the cell cycle. These cells are capable of differentiating and entering peripheral circulation upon receiving the correct signals [14, 17]. The second zone is known as the osteoblastic zone, where EPCs are maintained in the G0 phase of the cell cycle. These cells are not actively dividing, and therefore not readily available for release into circulation [14, 18]. The balance between these two functional compartments is maintained by cytokines present in the bone marrow's extracellular matrix (ECM) and on bone marrow stromal cells [19]. The bone marrow stromal cells and ECM preserve levels of cytokines bound to either ECM proteins or cell membranes. The cytokines can be cleaved by proteinases and activated. To illustrate, matrix metalloproteinases (MMPs) help mediate the digestion of ECM which leads to the release of membrane-bound cytokines. This allows the release of VEGF-A, an important regulator of both angiogeneses and vasculogenesis [20]. Evidence has suggested that MMP-9 is capable of mobilizing EPCs to enter the vascular zone and eventually be released into the peripheral circulation [14, 17]. Malignant transformation, tissue injury, or ischemia can induce systemic release of VEGF-A, activating bone marrow progenitor stem cells [15].

A recent study verified altered levels of MMP-9 and VEGF-A in patients with early-stage breast and colorectal cancer when compared to normal patients [57]. Healthy volunteers showed VEGF-A plasma levels averaging 37.6 μg/mL, while the breast cancer patients had average plasma concentrations of 52.9 μg/mL and colorectal cancer patients plasma concentrations averaged 109.6 μg/mL. MMP-9 plasma concentrations for healthy volunteers averaged 169 ng/mL, while breast cancer patients had plasma concentrations averaging 237.8 ng/mL and colorectal cancer patients averaged 370.1 ng/mL. The patients underwent surgical resection of their primary tumors and the levels of MMP-9 and VEGF-A were measured between 7 and 8 weeks postsurgery. These same patients saw a decrease in their MMP-9 and VEGF-A plasma levels [57]. The breast cancer patients saw a decrease in the average VEGF-A plasma concentration from 52.9 μg/mL to 43.8 μg/mL and their MMP-9 levels dropped from an average of 237.8 ng/mL to 109.6 ng/mL. The colorectal cancer patients saw similar changes after their tumors were removed with their average VEGF-A plasma levels dropping from 109.6 μg/mL to 57.6 μg/mL and their MMP-9 plasma levels going from 370.1 ng/mL to 190.3 ng/mL [57]. This evidence shows the direct effect that malignancy can have on factors important to both vasculogenesis and angiogeneses.

Table 1: Factors regulating angiogenesis.

Angiogenesis factors	Biological activities
Vascular endothelial growth factor (VEGF)	(i) Promoter of angiogeneses and vasculogenesis [21] (ii) Stimulates microvascular EC proliferation [22, 23] (iii) Enhances EC migration and sprouting [24, 25] (iv) Inhibits EC apoptosis [26] (v) Increases EC permeability [27–29]
Fibroblast growth factor (FGF)	(i) Stimulates EC proliferation [30] (ii) Promotes microvessel tube formation [30] (iii) Promotes EC migration [30] (iv) Important promoter of blood vessel remodeling after tissue injury [31]
Angiopoieten-1 (Ang-1)	(i) Recruits pericytes to recently created blood vessels [32] (ii) Helps promote EC survival and sprout formation [32, 33] (iii) Increases the diameter of blood vessels endothelium [34]
Angiopoieten-2 (Ang-2)	(i) Antagonist of Tie-2 receptor, reduces levels of pericytes [35] (ii) Increases plasticity of newly formed blood vessels [36]
Platelet-derived growth factor (PDGF)	(i) Increases capillary wall stability [3] (ii) Stimulates the proliferation of cultured pericytes and SMCs [37] (iii) Increases DNA synthesis on capillary ECs [38] (iv) Stimulate formation of angiogenic sprouts in vitro [38]
Transforming growth factor-β (TGF-β)	(i) At low doses upregulates angiogenic factors and proteinases [39] (ii) At high doses, inhibits EC growth, promotes reformation of BM and stimulates SMC reformation [39] (iii) Stimulates or inhibits EC tube growth [39] (iv) Signals inflammatory mediators such as fibroblasts and monocytes [3, 40, 41] (v) Enhances integrity of vessel walls [42]
Integrin $\alpha V\beta 3$	(i) Binds and activates MMP2 to help break down ECM [43] (ii) Helps regulate cell attachment, spreading, and migration [44] (iii) Shows Increased activity near wound sites [45] (iv) Localized to ECs at ends of growing vessels during EC sprouting [46]
Integrin $\alpha V\beta 5$	(i) Interacts with VEGF to promote angiogeneses [47]
VE cadherin	(i) Thought to mediate passage of molecules across endothelium [28, 29] (ii) Regulates CD growth through contact inhibition [48] (iii) Helps prevent EC apoptosis by promoting VEGFs signal [49] (iv) Helps stabilize the branches and sprouts produced during angiogeneses [48]
Tumor necrosis factor-α (TNF-α)	(i) Stimulates angiogeneses in vivo [50] (ii) Stimulates EC tube formation in vitro [51]
Transforming growth factor-α (TGF-α)	(i) Promotes EC proliferation [50] (ii) Stimulates angiogeneses in vivo [50, 52]
Angiogenin	(i) Promotes angiogeneses in vivo [53] (ii) Assists EC adhesion and spreading in vitro [54]
Angiotropin	(i) Helps activate microvascular ECs during wound healing [38] (ii) Stimulates angiogeneses in vivo [38] (iii) Randomly induces capillary EC migration [55]
Matrix metalloproteinase-9 (MMP-9)	(i) Thought to help mobilize EPCs by cleaving ECM [17]
Stromal-cell-derived factor-1 (SDF-1)	(i) Helps guide EPCs to ischemic areas during angiogeneses [56]

During the process of vasculogenesis, EPCs that enter the peripheral circulation migrate to the areas where the vasculature will be established. The chemokine stromal-cell-derived factor-1α (SDF-1α) helps mediate the migration of many stem cells, including EPCs. SDF-1α is upregulated during hypoxic conditions due to increased levels of VEGF-1 [56]. Once released, SDF-1α acts as a key homing signal, helping to guide EPCs to areas of ischemia [58, 59]. The guided cells are still considered early EPCs because they are positive for CD133, CD34, and VEGFR-2 (as demonstrated in Figure 2) [19]. While in circulation, the EPCs continue to differentiate. They begin this process by losing the CD133 marker, and gaining EC-specific markers such as von Willebrand Factor (vWF), CD31, and VE cadherin [60]. EPCs normally compose approximately 0.002% of the mononuclear cell fraction of blood [61]. However,

if neovascularization is required, vasculogenic stimuli are released, increasing the circulating concentration of EPCs [62–64]. Clinical trials have demonstrated this phenomenon using patients who either suffered burns or underwent coronary artery bypass graft surgery [65]. Both patient groups saw a 50-fold increase in EPC levels within the first 6 hours after the initiating event, with a return to basal levels within 72 hours. It is believed that this transient increase in EPC levels caused by the vascular and tissue trauma induces the release of several cytokines, including VEGF, promoting EPC mobilization and the initiation of vasculogenesis [65].

After EPCs arrive and enter the target tissue, some continue their differentiation into mature ECs [19]. It is thought that the cells that do not differentiate into mature ECs act as a source of proangiogenic cytokines [19]. The maturation into ECs is marked by the loss of the CD34 marker on the cell surface.

Vasculogenesis that occurs during postfetal life in response to angiogenic cytokines has a few key differences from the vasculogenesis that occurs during embryonic life. One major difference is that the formation of the initial vascular plexus in embryonic life is not driven by insufficient oxygen like the vasculogenesis that occurs later in life [8]. An imbalance in oxygen supply and demand can cause hypoxia resulting in an induction of cytokine production or release from cells throughout the body. One such cytokine released during hypoxic conditions is VEGF. After VEGF is released, it binds to VEGF receptors on ECs. This leads to the activation of signal transduction pathways capable of stimulating both angiogeneses and vasculogenesis. The hypoxic conditions seen in tumor cells have been studied and factors associated with vasculogenesis and angiogeneses have been monitored to detail their relationships. Many of these factors have been listed in Table 1.

As discussed above, Zaman's work on colorectal and breast cancer patients specifically showed increased concentrations in VEGF-A and MMP-9 plasma levels due to malignancy. These levels are reduced after the tumor is removed, and thus levels of angiogeneses and vasculogenesis are decreased. Another difference in the two types of vasculogenesis is that most of the cells recruited to sites of vasculogenesis during postembryonic life are inflammatory cells and are not incorporated into the new capillaries or remodeling arteries. The postembryonic form of vasculogenesis is much more similar to angiogeneses then the embryonic form of vasculogenesis and this is illustrated by the similar factors that are vital to postembryonic vasculogenesis and angiogeneses.

2.2. Angiogeneses: Current Concepts, Known Factors, and Challenges. In contrast to vasculogenesis, angiogeneses is the expansion of preexisting vasculature, such as a vascular labyrinth of capillaries, by means of budding and branching into a functional capillary bed which is illustrated in Figure 3. This normally occurs in very organized manner forming what is known as primary vascular trees [66]. Like vasculogenesis, angiogeneses occurs most often during embryonic development; however, it can also occur in adult life in response to specific stimulations. Nonpathogenic angiogeneses can be seen in adults during the ovarian cycle, in skeletal and cardiac muscle during times of exercise and training, as well as during the process of wound healing [3].

The process of angiogeneses is very closely regulated. Stimulation of angiogeneses occurs by growth factors such as VEGF and FGF (see Figure 3, Table 1). New blood vessel formation actually begins with the removal of mural cells (pericytes) from preexisting blood vessels. The absence of these pericytes initiates the degradation of the EC basement membrane and extracellular matrix, a process which is aided by MMPs [67].

As the basement membrane and extracellular matrix are being degraded, ECs begin proliferating and migrating with the help of soluble growth factors. The ECs will continue to grow until they form an unstable microvessel. Following the formation of this small blood vessel, mesenchymal cells are recruited to the vessel, where they are subsequently differentiated into pericytes. After differentiation, cell-cell contact between pericytes and ECs occurs. Stable blood vessels are then formed and blood flow can be established. This process of angiogeneses is visualized in Figure 3. Vessels made from ECs not covered with pericytes are unstable, and undergo regression [3, 21]. There are many known factors that help regulate angiogeneses. Some of the known factors are touched upon in Table 1 and discussed in further detail below.

2.3. Angiogenic Promoters: Current Research and Clinical Implications

2.3.1. Vascular Endothelial Growth Factor. VEGF is an important regulator of both vasculogenesis and angiogeneses [21]. Several cell types including fibroblasts, ECs, and keratinocytes release a small amount of VEGF throughout life. The loss of a VEGF allele always results in embryonic lethality [35]. Increased levels of VEGF are seen when angiogeneses is necessary, such as during active wound healing [68].

There are currently six known monomers of VEGF that arise from alternative splicing of a single gene with eight exons. The documented isoforms contain 121, 145, 165, 183, 189 or 206 amino acids [69–72]. Some of these isoforms remain associated with cells or membranes, while others are released extracellularly. Despite these differences, all of them have identical biological activities [73].

VEGF interacts with two different receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), to alter angiogeneses. VEGFR-1 interacts very strongly with VEGF, but this interaction plays a minor role in the events of angiogeneses [74]. The interaction of VEGFR-2 with VEGF is a major contributor to the mitogenic, chemotactic, angiogenic, and increased permeability effects of VEGF. VEGFR-2 expression has been observed on both endothelial and hematopoietic precursors [22].

Experiments have shown that VEGF has the ability to stimulate microvascular EC proliferation [22, 23]. VEGF is also capable of enhancing EC migration [24], inhibiting EC apoptosis [26], and inducing the growth of new capillaries

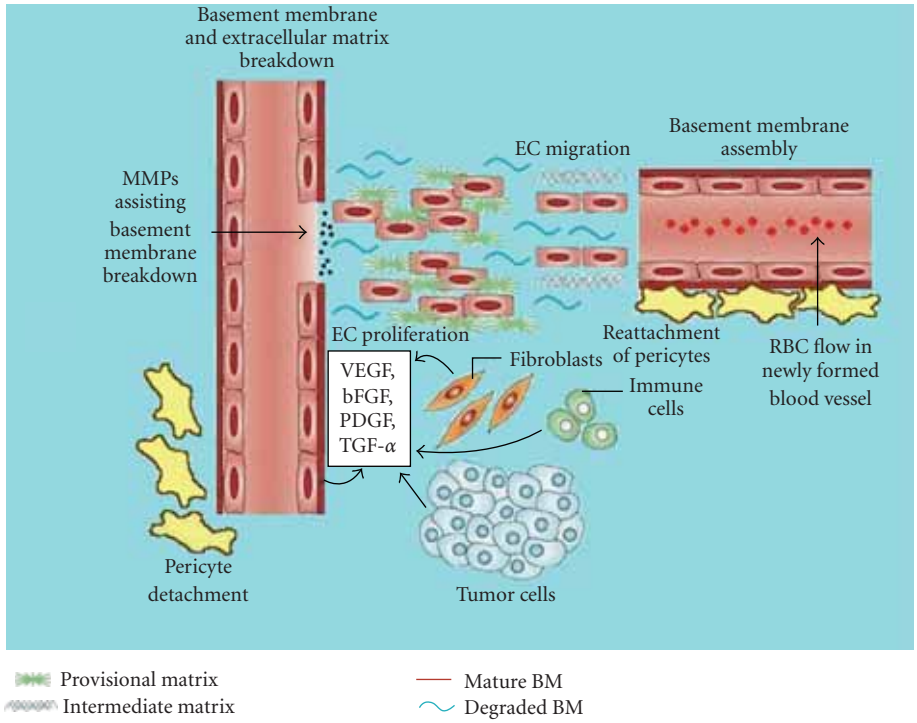


Figure 3: *Tumor Influenced Angiogeneses*. The stepwise process of angiogeneses begins with ECM and BM breakdown, followed by EC proliferation, EC migration and finally re-formation of stable blood vessel. Tumor cells will secrete a variety of factors to ensure that the new blood vessels formed are fed directly to the tumor tissue.

from preexisting vasculature [25]. VEGF helps induce EC migration and sprouting by upregulating integrin receptors $\alpha V\beta 3$, $\alpha 1\beta 1$, and $\alpha 1\beta 2$ (discussed below) [27, 75]. VEGF also helps activate MMPs, an important step in the initial stages of angiogeneses.

VEGF is upregulated during hypoxic conditions via the following mechanism, which involves hypoxia-inducible factor-1 (HIF-1), a protein released during oxygen stress [76]. Responses to ischemia are mostly regulated by cells within the ischemic area monitoring oxygen concentrations. After sensing reduced concentrations, these cells increase their expression of genes encoding vascular growth factors. At the heart of this pathway is HIF-1, a heterodimeric transcription factor composed of the constitutively expressed HIF-1β, and the oxygen-sensitive HIF-1α subunits [77, 78]. Although HIF-1α is also constitutively expressed, it is broken down within adequately oxygenated and perfused tissue. When tissues are deprived of oxygen, the breakdown of HIF-1α is inhibited. HIF-1α begins to build up and eventually dimerizes with HIF-1β. This complex then binds to DNA, helps recruit coactivators, and activates transcription of its target genes [79]. This system induces expression of several vasculogenic and angiogenic growth factors including VEGF and PLG in response to hypoxia [80]. HIF-1 is also required for expression of VEGFR-1 on EPCs in the bone marrow and the chemotactic migration of EPCs towards a VEGF gradient [79].

The use of anti-VEGF drugs has been applied to many different fields of medicine. The American Academy of Ophthalmology recently published a report stating that the use of anti-VEGF pharmacotherapy is a safe and effective treatment for neovascular age-related macular degeneration (AMD) [81]. Anti-VEGF signaling pathway drugs have also been tested in a large number of clinical and laboratory studies aimed at preventing angiogeneses associated with cancer. Some of these drugs target VEGF (Avastin), while others target the VEGFRs (Nexavar and Sutent) [82]. Although these drugs have seen dramatic results in animal models [83–85], the results in many of the clinical trials have been mixed [86]. There have been clinical trials which show as many as 94% of invasive carcinomas and 88% of in situ carcinomas having a complete response [87]. These same patients saw no recurrence during the five-year followup [87]. However, many other angiogeneses inhibitors targeting VEGF signaling pathways have failed to produce the same long-term responses in a majority of their patients [86, 88, 89]. A short-term response of either tumor stasis or increased survival was normally observed in these patients [90]; however after the initial benefit, most patients experienced tumor growth after several months [90]. These contradictory results have changed the philosophy on the resistance of tumors to antiangiogenic treatments, as well as the vascular makeup thought to be associated with the blood vessels that support tumors. These concepts will be further explored in the future directions section.

Inducing neovascularization in ischemic diseases such as chronic wounds and myocardial infarction is also a very active area of research and is leading to a greater

understanding of how VEGF works in the body. Several groups have attempted to induce angiogeneses in ischemic tissue through local delivery of the VEGF gene or protein. In clinical trials, local delivery of pro-VEGF growth factors has induced modest levels of neovascularization in ischemic tissue [91]. However, the amount of angiogeneses was inadequate as a monotherapeutic treatment [91]. This information is also valuable for the field of tumor angiogeneses. It is evident that although VEGF is an important part of angiogeneses, controlling its level alone is not enough to regulate angiogeneses in normal tissues or tumors.

2.3.2. Fibroblast Growth Factor. Another important set of proteins mediating angiogeneses is the FGF family. FGFs are soluble growth factors that come in an acidic (aFGF) and basic (bFGF) variety. Both types consist of widespread polypeptides that are powerful inducers of EC migration, proliferation, and microvessel tube formation (Figure 3) [3, 30]. While VEGF is a specific mitogen for ECs, the same cannot be said for FGF. FGF is pleiotropic; it stimulates proliferation in nearly all cells derived from embryonic mesoderm or neuroectoderm [92]. Recent evidence suggests that FGF does not play a major role in generalized angiogeneses in vivo, as mice deficient in both forms of FGF underwent normal development [31]. Instead, FGF seems to be more important in the remodeling of damaged blood vessels [31] which can occur during both wound healing and tumor angiogeneses. FGF is generally found in the cytoplasm of cells or bound to heparin in the ECM [93, 94]. After tissue damage occurs, it appears that FGF is released from the damaged cell(s). This local release of FGF is thought to help promote angiogeneses at the site of the damaged vessel.

One group recently studied the role of FGF in vascular integrity and human saphenous vein ECs in vitro by disrupting FGF signaling in bovine aortic endothelial cells [95]. They also disrupted the FGF signaling pathway in adult mouse and rat ECs in vivo using soluble FGF traps or a dominant inhibitor of all FGF receptors [95]. Inhibition of this signaling pathway led to a loss of function in the adherens and tight junctions, which caused the loss of EC's, severe impairment of the endothelial barrier function, and finally, disintegration of the vasculature [95]. This experiment showed another possible mechanism for the inhibition of angiogeneses. A targeted approach could possibly allow the breakdown of specific vasculature. In addition to this study, another possible inhibitor of both FGF and VEGF was examined. A plasma glycoprotein, Beta-2 glycoprotein-1, was found to have inhibitory effects on Human Umbilical Vein Endothelial Cells (HUVEC) proliferation, migration, and tubule formation in a dose-dependent manner [96]. This was accomplished by the downregulation of VEGFR-2, the main mediator of angiogenic signals from VEGF which is also activated by bFGF [96]. When low doses of Beta-2 glycoprotein-1 were applied, HUVEC proliferation was decreased by 11.5% [96]. As this dosage was increased, proliferation continued to decrease until the highest dose, which displayed a 68.9% reduction in proliferation [96].

2.3.3. Tie Receptors. The Tie receptors are a family of tyrosine kinases expressed by ECs that mimic the behavior of VEGF receptors [33, 97]. To date, Tie1 and Tie2 have been identified, and their mechanisms of action studied. Genetically altered mice bred without either receptor underwent normal vasculogenesis, but their ECs lacked normal integrity causing the mice to die from widespread edema and hemorrhage due to a lack of adequate angiogeneses [98]. While both Tie1 and Tie2 receptors are important for vascular integrity [32, 99, 100], only Tie2 appears to be vital to vascular sprouting and branching occurring during angiogeneses [3].

Tie Receptor inhibitors have recently been produced in the lab. A research group has developed several Tie inhibitors and tested them in vitro to find one with the best selectivity, potency, and pharmacokinetic parameters [101]. Although preliminary studies for Tie2 inhibitors have begun, further animal and possibly clinical trials still remain.

2.3.4. Angiopoietins. Angiopoietins are protein growth factors that act as ligands for the Tie Receptors on ECs [21]. There are two important angiopoietins that play a role in angiogeneses, Ang-1 and Ang-2. Ang-1 is a well-characterized regulator of angiogeneses. It is an important agonist of and ligand for Tie2 receptors [32]. Experiments have shown that mice that lack Ang-1 or Tie2 receptors will develop normal primary vasculature, but will eventually die because vascular remodeling was never completed [32, 102]. The interaction between Ang-1 and Tie2 is important in angiogeneses as it helps recruit pericytes to newly created blood vessels, increasing the stability of the new vasculature and making it less permeable [32]. Ang-1 also helps induce formation of capillary sprouts and promote survival of ECs [21]. Overexpression of Ang-1 in transgenic mice led to a greater number of blood vessels, with larger diameters, and more vascular branches [34], illustrating the important role Ang-1 plays in vascular sprout formation.

Preliminary studies have been completed to determine whether Ang-1 inhibitors would be an effective method for reducing levels of angiogeneses. A recombinant human Ang-1 antisense strand was made and used to inhibit Ang-1 expression levels in mice with implanted tumor tissue [103]. Microvascular density in the antisense Ang-1-treated group averaged 6.02 vessels/mm in implanted tumor tissue while the group not receiving the antisense strand averaged 8.44 vessels/mm in the implanted tumor tissue [103]. The group concluded that although Ang-1 inhibitors may help reduce the levels of angiogeneses, it was unlikely that Ang-1 inhibitors alone would be an effective method to inhibit pathogenic angiogeneses. The study suggested that several angiogeneses modulators would need to be inhibited simultaneously to have a noticeable effect on angiogeneses [103].

The role of Ang-2 is more complicated than that of Ang-1. It appears that Ang-2 is at least a partial antagonist of Tie2, resulting in pericyte loss [35]. The result of this pericyte loss is destabilization of the blood vessel. Under normal conditions this helps prevent excessive angiogeneses. However, the destabilization also allows newly formed blood

vessels to be more plastic. These destabilized vessels show increased endothelial sprouting and tube formation in the presence of VEGF [36]. This fact has made Ang-2 blockers an important area of study for tumor angiogeneses inhibition. A recent study created Ang-2-selective peptide-Fc fusion proteins and antibodies; this enabled a controlled pharmacological inactivation of endogenous Ang-2, allowing the group to study Ang-2 inactivation without using the lethal knockout approach [104]. The use of these inhibitors demonstrated tumor inhibition in mice as well as corneal angiogeneses inhibition in rats [104]. Although the study demonstrated the properties of Ang-2 in vivo and gave therapeutic possibilities to Ang-2 inhibitors, many questions still remain. How Ang-2 inhibition blocks EC proliferation is still not completely understood. In addition, it is unknown if inhibition of both Ang-1 and Ang-2 would further reduce angiogeneses or if blocking one will offset the inhibition of the other. Additionally, these studies have only been completed using animal models, and we can only hope that a similar effect will be seen in humans.

Despite the current problems understanding Ang-2, the selective inhibition of Ang-2 could have a very high clinical value. Studies have demonstrated that Ang-2 upregulation is seen in several diseases that involve pathogenic angiogeneses including cancer, macular degeneration, rheumatoid arthritis, osteoarthritis, and psoriasis [105–107]. One recent study found that mRNA levels of Ang-2 in metastatic liver cells and lymph nodes are between 1.5 and 2 times greater than normal levels [108].

2.3.5. Platelet-Derived Growth Factor. PDGF is another important signaling molecule with several different roles in angiogeneses. Although originally purified from platelets, it has also been identified in fibroblasts, astrocytes, ECs, and several other cell types [38]. To date, both hetero and homodimeric versions of PDGF (PDGF-AA, -BB, or -AB) have been studied. Capillary ECs express PDGF-BB receptors, and when the receptors are stimulated, increased DNA synthesis and angiogenic sprouting can be seen in vitro (Figure 3) [38]. Although pericytes are initially recruited to growing microvessels independently of PDGF, pericyte proliferation and migration at a growing blood vessel is enhanced by interaction with PDGF [109]. Mice bred without PDGF-BB or its receptor exhibited a large increase in the permeability of their blood vessels and died prenatally [110]. The interaction of PDGF with its receptor on pericytes increases the expression of Ang-1. This increase in Ang-1 leads to a signaling cascade that helps establish the interaction between pericytes and ECs [97]. This interaction is important for maintaining the stability of newly formed capillary walls [3], a vital part of new blood vessel formation.

It has been hypothesized that blocking PDGF from interacting with its receptor will reduce the stability of the growing capillaries rendering them incapable of delivering nutrients to the cancer cells [111]. Inhibition of PDGF has been attempted with compounds such as CP-673,451 [111]. Rat glioblastomas treated with CP-673,451 showed a 47% decrease in microvascular density and a 55% decrease in tumor growth. Although a decrease in tumor growth and angiogeneses has occurred through the treatment of cancers with CP-673,451 during in vitro and in vivo animal studies, clinical trials have not been performed to demonstrate its efficacy in humans. Another recent study indicated that lycopene, a carotenoid found in tomatoes, may inhibit PDGF-BB-induced signaling [112] reducing levels of unwanted angiogeneses.

2.3.6. Transforming Growth Factor-Beta. Transforming Growth Factor-Betas (TFG-β) are a family of homodimeric cytokines that help control many different processes in the body, including angiogeneses. TGF-β's are normally found in the ECM of many different cells types [113, 114]. Within the microvasculature, both ECs and pericytes produce and display receptors for TGF-β [99], illustrating the variety of cells capable TGF-β expression. To date, both pro- and antiangiogenic properties have been ascribed to TGF-β. At low doses TGF-β helps initiate the angiogenic switch by upregulating angiogenic factors and proteinases. However, at high doses TGF-β inhibits EC growth, promotes basement membrane reformation, and stimulates SMC's differentiation and recruitment [39]. Genetic studies in mice have shown that the loss of TGF-β leads to leaky vessels lacking structural integrity leading to premature death [42]. Stimulation of angiogeneses through TGF-β is mostly via indirect mechanisms. TGF-β signals inflammatory mediators to the site of angiogeneses, where inflammatory cells release proangiogenic factors such as VEGF, FGF, and PDGF [40, 41].

Recent phase I/II clinical trials attempting to use TGF-β inhibitors have been completed. These studies employed a TGF-β antisense oligonucleotide, termed AP12009, as a treatment for patients with malignant gliomas [115]. Despite the late stage of the glioma in the patient population, positive efficacy results were observed. Two of the 24 patients saw complete remission of their disease after treatment and remained cancer free 4.5 years after completion of the trial [115]. Seven of the 24 patients also found their disease stabilized after beginning treatment, a larger number compared to controls in other clinical trials [115]. In addition, the median survival time of the AP12009-treated patients was longer than the controls reported by recent literature [115]. After the initial positive results of this trial, a Phase IIB trial was initiated and is still ongoing [115]. These results indicate that the targeted inhibition of TGF-β may provide an excellent mechanism to reduce unwanted angiogeneses in a variety of diseases.

2.3.7. Integrins. Integrins are heterodimeric cell surface receptors for ECM proteins that also play a role in cell-cell attachment. They contain various α-and β-subunits, with over 20 different combinations of subunits known. Integrins are important regulators for many different cell processes including both vasculogenesis and angiogeneses [116].

Of the integrins, $\alpha V\beta 3$ is one of the most extensively studied, and has an important role in angiogeneses. It binds and activates MMP-2 at the tips of growing blood vessels

to help break down the ECM [43]. Integrin $\alpha V\beta 3$ shows increased expression in vitro when exposed to VEGF [27] and bFGF [117]. Cell attachment, spreading, and migration are all regulated by integrin $\alpha V\beta 3$ in vitro [44]. Angiogenic blood vessels near granulation tissue showed much greater levels of $\alpha V\beta 3$ than vessels in uninjured normal skin [45]. During wound repair, $\alpha V\beta 3$ is localized to the ECs at the ends of the growing vessels [46]. Anti-$\alpha V\beta 3$ monoclonal antibodies reduce bFGF-stimulated angiogeneses, demonstrating an important relationship between the two proteins [47].

Several other integrins have also been implicated in angiogeneses. Inhibition of integrin $\alpha V\beta 5$ hindered angiogeneses stimulated by VEGF [47]. Abs to collagen receptor integrins ($\alpha 1\beta 1$ and $\alpha 2\beta 1$) also reduced VEGF-mediated angiogeneses [75]. A variety of integrins play important roles in angiogeneses including EC adhesion to ECM, protease localization, and increased EC survival [3]. This diversity suggests a number of different integrins; each plays a distinct role that uniquely contributes to the process of angiogeneses.

There are currently three classes of integrin inhibitors in preclinical and clinical trials. Some of these include a synthetic peptide Cilengitide (a $\alpha V\beta 3/\alpha V\beta 5$ inhibitor), a monoclonal Ab Abergin (a $\alpha V\beta 3$ antagonist), and a peptidomimetic compound S247 (a $\alpha V\beta 3/\alpha V\beta 5$) antagonist [118]. Phase I trials using Vitaxin (similar to Abergin) were unsuccessful in reducing tumor growth [119]. A second generation Phase II trial which altered Vitaxin to give it greater affinity for $\alpha V\beta 3$ also failed to reduce tumor growth [120, 121]. Phase I and II trials with Cilengitide have also been completed. Although some antitumor effects in the treatment of gliomas were seen with Cilengitide, the study concluded that its action appeared to be antitumor cell specific as opposed to angiostatic [118]. Trials in other cancer patients failed to show any reduction in tumor load using Cilengitide [122, 123]. The low efficacy of the $\alpha V\beta 3/\alpha V\beta 5$ antagonists demonstrates that the mechanistic understanding of integrins in angiogeneses is not yet fully understood. At this time, antagonists for $\alpha V\beta 3$ or $\alpha V\beta 5$ alone do not appear to prevent angiogeneses, and other strategies need to be examined. Currently, integrin inhibitors for $\alpha 2\beta 1$ [118, 124, 125] and $\alpha 5\beta 1$ [118, 126] are being tested in phase I and phase II clinical trials.

2.3.8. Cadherins. Cadherins are a class of calcium-binding transmembrane proteins that play an important role in cell-cell interactions. Several studies have underlined the important role of one particular cadherin, the vascular endothelial (VE) cadherin, in neovascularization. VE-cadherins are localized exclusively to the adherens junctions in ECs [127]. It has been suggested that VE-cadherins are important in regulating the passage of various molecules across the endothelium [28, 29]. In addition, VE-cadherin plays an important role in mediating EC growth through contact inhibition [48]. Mice deficient in VE-Cadherin showed extreme vascular abnormalities including diminished branching and sprouting, as well as disconnected ECs [48]. The vascular problems continued to progress until the vessels finally regressed or disintegrated [49]. This is thought to occur because the VE-cadherins establish EC junctional stability in the vessel walls. The cadherins also enhance EC survival by increasing the transmission of the antiapoptotic signal of VEGF [49]. Therefore, despite VE-cadherins nonexistent role in vasculogenesis, it is vital to the maturation of blood vessels associated with angiogeneses [38].

Inhibition of VE-cadherins to prevent angiogeneses has been examined in several different animal models. Monoclonal Abs designed to recognize certain sections of extracellular repeats found in active VE-cadherins have been designed [128]. These Abs prevented EC junctional assembly and induced the disassembly of already existing EC junctions in vitro, abilities that could help prevent unwanted angiogeneses [128]. Although these original Abs were found to have some inhibitory effects on angiogeneses at low doses, significant vascular permeability was found in the heart and lungs of the mice at moderate to high doses, illustrating toxic side effects of the Abs [129, 130]. Since these original studies, other monoclonal Abs have been developed that do not exhibit the vascular permeability problems of the original version; these include BV14 and E4G10 [131, 132]. It is currently hypothesized that these second-generation monoclonal Abs will be effective because angiogenic junctions are weaker and contain different epitopes which are open to monoclonal Ab targeting [133]. Other groups have focused on the gene sequence of VE-cadherins, which may allow researchers to produce more specific monoclonal Abs for VE-cadherins and prevent unwanted angiogeneses [134].

VE-cadherins role in retinal neovascularization was also recently examined in mice. A group induced retinal neovascularization in newborn mice by exposure to oxygen [135]. Some of the mice were then treated with a VE-cadherin antagonist while others were treated with a control peptide. The mice treated with the VE-cadherin antagonist saw significantly reduced retinal angiogeneses compared to the control group [135]. In addition, the group treated with the antagonist had reduced levels of EC migration and proliferation as well as suppressed tubule formation from ECs [135]. As studies designed to better comprehend VE-cadherins role in angiogeneses have been completed, it has become clear that the role of cadherins in the angiogenic pathway is larger than just their adhesive activity. The ability of VE-cadherins to interact with various signaling molecules suggests that it has a role in EC growth, migration, survival, and morphogenesis [133]. Although VE-cadherin inhibitors alone are not capable of suppressing angiogeneses at this time, combining a VE-cadherin inhibitor with other inhibitors of angiogeneses may provide more complete suppression.

2.3.9. Endoglin. Endoglin (Eng, CD-105) is a homodimeric cell surface glycoprotein that serves as a coreceptor for TGF-β [136]. Eng is found on proliferating ECs and also serves as an EPC marker [137]. It has been observed that Eng expression is greatly increased during angiogeneses and inflammation [138]. Studies have shown that Eng can regulate TGF-β, but the mechanism remains unknown [139]. With this function in mind, researchers have investigated the use of anti-Eng-based therapies in several different forms of cancer with

the hopes of preventing tumor-based angiogeneses. Early in vitro studies using anti-Eng mAbs in the presence of human ECs showed that the mAbs greatly reduced growth of the ECs [140]. Following this work, in vivo studies showed that injections of anti-Eng mAbs into mice with colon or breast cancer xenografts demonstrated significant reductions in tumor size and had much greater survival rates than controls [141, 142].

The United States FDA approved a multicenter phase I clinical trial in 2008 using a naked anti-Eng mAB (TRC105) in patients with advanced and/or metastatic cancers [143]. Preliminary results from this trial have suggested clinical activity and tolerability of the mAb TRC105 in 17 patients [143]. As we await further results, no conclusive decision can be made about the clinical value of anti-Eng mAbs, though there does appear a reason for optimism. While this trial continues, several other groups have suggested conjugating the anti-Eng mABs with toxic molecules to ensure that the targeted ECs are killed. This model has had success in mice with breast cancer without any measurable toxicity [144]. In either case, Eng has been shown to be an important regulator of angiogeneses and a better understanding of its mechanistic course of action may help the drug design process in the future.

2.3.10. Additional Factors. In addition to the factors mentioned above, many others have been shown to play important roles in angiogeneses, but their effects on the vasculature are not as widespread or as understood as the previously mentioned factors. An example of this is Tumor Necrosis Factor-α (TNF-α), a cytokine usually secreted by activated macrophages. TNF-α has been shown to help stimulate angiogeneses in vivo [50] and stimulate EC tube formation in vitro [51]. Transforming Growth Factor-α (TGF-α) is another cytokine secreted by macrophages and is capable of stimulating angiogeneses and EC proliferation in vivo [52]; however its role is still not completely understood. Angiogenin is another small polypeptide whose role in angiogeneses is still being investigated. It promotes angiogeneses in chorioallantoic membrane and rabbit cornea [53], but it is not mitogenic or chemotactic for ECs in vitro [53]. Angiogenin helps support EC adhesion and spreading in vitro [54]; however, its levels of synthesis are inconsistent with the timing of neovascularization in vivo [145]. Angiotropin is a polyribonucleopeptide originally isolated from peripheral monocytes [55]. Angiotropin is able to randomly induce capillary EC migration and tube formation in rabbit skin [55] and may trigger proliferative reactions in wound healing by activating microvascular ECs [38].

2.4. Angiogeneses Inhibitors: Current Research and Clinical Implications

2.4.1. Angiostatin. Angiostatin is a 38 kDa internal fragment of plasminogen that displays inhibitory effects against tumor angiogeneses [146]. When plasminogen is in the vicinity of an implanted and/or primary tumor, it is cleaved by an unknown protease; a product of this cleavage is the antiangiogenic protein angiostatin [5, 147]. Research groups have shown that the endogenous protein is capable of inhibiting the growth of distant metastases [147, 148].

Experiments with angiostatin demonstrated that its activity leads to several different physiological results. As mentioned, angiostatin was shown to reduce the growth of remote metastasis [147]. This is accomplished by increasing the rate of apoptosis in metastatic tumors [149]. Apoptosis is increased because angiostatin attacks the energy system of the metastatic tissue by inhibiting ATP synthase F1F0, leading to caspase-mediated apoptosis [149, 150]. Another effect of activated angiostatin is the inhibition of capillary endothelial growth in vitro [151]. Mice with gliomas and melanomas experienced greatly reduced tumor growth and neovascularization when they were genetically engineered to express angiostatin [152, 153]. The activity of angiostatin was also examined on a global level using microarray techniques. A total of 189 genes had their expression levels altered with treatment of angiostatin. Most of these genes were involved in growth, apoptosis, and migration of ECs, as well as inflammation [6, 154], demonstrating the wide range of effects that angiostatin has within the body.

To date, several clinical trials have tested the efficacy of angiostatin as a treatment for several forms of cancer. Subcutaneous injections of recombinant human angiostatin showed little to no toxicity in phase I clinical trials [155]. Phase II trials using angiostatin in combination with both paclitaxel and carboplatin have been completed in non-small-cell lung cancer (NSCLC) patients [156]. The response rate of the combinational therapy was higher than previous studies using the chemotherapy alone [156]. The group reported that the overall response rate to the combined treatment was 39.1%, another 39.1% of the patients remained stable, while the last 21.7% of the patients saw their disease progress [156]. Although the group did see improved rates of treatment, they fell below the expected levels. Several groups are working on alternate methods of administering angiostatin to increase its success as a treatment. Experiments using intravenous administration of angiostatin genes complexed to cationic liposomes are ongoing [157].

2.4.2. Endostatin. Endostatin is an angiostatic 20-kD internal fragment of the carboxy terminus of collagen XVIII [151], an important proteoglycan in basement membranes. It was originally discovered in the blood and urine of tumor-bearing mice [151]. Two of the enzymes responsible for the release of endostatin include elastase [158] and cathepsin L [159]. Endostatin interacts with many different cell surface proteins including the integrins ($\alpha 5\beta 1$ and to an extent also $\alpha V\beta 3$ and $\alpha V\beta 5$) [151] and several glycpians [160]. These interactions result in altered EC adhesion and migration [6].

In vitro, endostatin inhibits EC migration, proliferation, and tube formation [151], three key aspects of angiogeneses. Inhibition of angiogeneses via endostatin leads to a reduction of tumor growth in vivo [161]. It appears that this inhibition is partially accomplished by reducing the expression of VEGF [162]. Endostatin also has the ability to block existing

VEGF from interacting with its receptor VEGFR-2 [163]. Endostatin reduces EC proliferation by arresting the EC cell cycle through the downregulation of cyclin-D1 promoter transcriptional activity [164]. As a result, the cell is unable to progress through the G1/S transition. Recent studies have also shown that endostatin disturbs the survival/death balance via activation of the proapoptotic pathway through the induction of caspase-9 activation [165]. These pathways induction is due to the endostatin-led decrease of the anti-apoptotic proteins Bcl-2, Bcl-Xl, and Bad [165]. Gene array and proteonomic analysis have given insight into the vast number of genes that can be affected by endostatin treatment in human dermal microvascular ECs. Approximately 12% of the 74,834 genes represented on the microarray chip had altered expression levels when treated with endostatin [166]. Both angiostatin and endostatin cause apoptotic pathway activation [166]. However, the identity and number of genes regulated by endostatin differ from angiostatin, suggesting alternative pathways of action.

Evidence for endostatin's importance can been seen by studying individuals with Down Syndrome. People with Down Syndrome have a third copy of collagen XVIII due to a trisomy of chromosome 21. These individuals tend to have a 1.6–2 fold elevation of endostatin levels [167] and have greatly reduced levels of malignant tumors (except testicular cancer and megakaryocytic leukemia) [168], atherosclerosis [169], and diabetic retinopathy due to neovascularization [170]. These three diseases are all angiogeneses-dependent [5], and showcase the important role that endostatin may play in inhibiting unwanted pathogenic angiogeneses in humans.

Endostatin is currently being analyzed for therapeutic potential in several forms of cancer. Using animal studies, a group recently demonstrated that it may be used as a possible treatment to boost the post-operative prognosis of osteosarcoma patients [171]. The study was designed to determine whether antiangiogenic treatment could help prevent the progression of pulmonary metastasis, a secondary problem often associated with postoperative osteosarcoma [171]. The group injected an adenovirus encoding endostatin vector (Ad5CMV-mEnd) two weeks after tumor inoculation [171]. The group found statistically significant differences in the size and prevalence of pulmonary metastasis between the control and treatment groups two weeks after the administration of the vector [171].

A recently completed animal study investigated the use of an endostatin-angiostatin fusion protein in Renal Cell Carcinomas (RCCs). The group tested the fusion protein's ability to inhibit tumor angiogeneses, tumor growth, and metastasis [172]. All animals underwent postmortem histopathological analysis of the liver, kidney, lung, spleen, and brain to determine levels of metastasis. The mice treated with the angiostatin-endostatin fusion protein had a 97% primary tumor growth reduction compared to the controls. In vivo tumor vascular imaging showed that the fusion-treated group had fewer blood vessels, and decreased lumen diameter [172]. These results indicate that sustained angiostatin-endostatin gene therapy may provide a novel treatment method for metastatic RCCs [172].

Clinical trials using endostatin for the treatment of several types of cancer are ongoing. Phase I clinical trials showed that endostatin is well tolerated by patients, but its antitumoral activity was minimal at best [173–175]. A multicenter phase II study of recombinant human endostatin use in carcinoid neuroendocrine tumors and pancreatic neuroendocrine tumors was recently completed. The endostatin vector was given subcutaneously to 42 patients with the advanced form of either disease [176]. None of these patients experienced significant toxicity; however they did not demonstrate a clinically relevant radiological response either [176]. Eighty percent of the patients receiving treatment experienced disease stabilization, while the other 20% had further disease progression [176]. Although the study found minimal benefit from the treatment, the group admitted that the optimal therapeutic dosage and form of administration are still unknown [176]. In addition, it has been suggested that adding endostatin to current chemotherapeutic strategies may enhance the efficacy of the treatment for carcinoid and pancreatic neuroendocrine tumors [176]. Another group examined the use of novel recombinant endothelial endostatin (YH-16) also known as "endostar," for advanced NSCLC patients in a phase III trial [177]. The phase III trial treated one group of patients with endostar in combination with vinorelbine and cisplatin, while the other group of patients only received only vinorelbine and cisplatin [177]. The group that received endostar saw a response rate of 35.4%, while the group that received the chemotherapeutics alone saw response rates of only 19.5%. The median time to progression was 6.3 months in the endostar-treated group compared to 3.6 months in the control group. The clinical benefit rates for the chemotherapeutic and endostar-treated group were 73.3% while the rates for group receiving vinorelbine and cisplatin alone were 64% [177]. Overall, the study found that the addition of endostar to the vinorelbine and cisplatin treatment resulted in significant and clinically meaningful improvement compared to the vinorelbine and cisplatin treatment alone.

2.4.3. Tumstatin. Degradation of type IV collagen releases a 28 kDa fragment known as tumstatin, a compound that also displays antiangiogenic properties [178]. Tumstatin binds the $\alpha V\beta 3$ integrin, which results in G1 arrest and the induction of EC apoptosis [179]. Mice models have shown that exogenous tumstatin is able to inhibit the growth of tumors [179]. In addition, tumstatin-deficient mice had a much greater microvessel density near implanted murine tumors, and the mice had a 300% increase in overall tumor growth [5].

Animal studies determining the viability of tumstatin as an antiangiogenic-drug have been and continue to be completed. Tumstatin treated mice with teratocarcinomas showed over a 90% reduction in tumor size compared to controls [180]. The same group also examined a combination of anti-VEGF (Avastin) and tumstatin treatment compared to the tumstatin treatment alone. The animals receiving the combination treatment saw a statistically significant reduction in tumor growth when compared to the tumstatin

alone or a placebo [180]. These findings demonstrated strong preclinical evidence for a future treatment of cancer with an anti-VEGF Ab alongside a tumstatin peptide [180]. Another recently completed animal study examined gene delivery of a tumstatin fragment into hepatocellular carcinomas (HCCs) [181]. In vivo intratumoral injection of the tumstatin fragment (pSecTag2B-tum-1) greatly diminished the growth of preestablished human HCCs [181]. In addition, there was a decrease in the amount of CD-34 positive vessels in the tumor [181].

2.4.4. Platelet Factor-4. Platelet Factor-4 (PF-4) is a chemokine naturally secreted by platelets that normally promotes blood coagulation. In addition to this role, PF-4 is also known to be an inhibitor of angiogeneses. PF-4 is secreted from the alpha-granules of activated platelets and binds with high affinity to heparin-like glycosaminoglycans on the surface on ECs blocking them from further activity [6]. Studies have also shown that PF-4 blocks the upregulation of MMP-1 and MMP-3, inhibiting EC migration [182]. Finally, PF-4 is also capable of inhibiting the EC cell cycle by impairing pRB phosphorylation [183].

Despite the antiangiogenic and antitumoral effects of PF-4 in murine human tumor implant models [184], PF-4 has not proven to be an effective treatment for human cancers [185]. The early failure of PF-4 as a monotherapeutic treatment led researchers to examine different approaches for the use of PF-4 as an angiostatic agent. Recently, researchers produced a novel peptide containing the active fragment of PF-4 along with vasostatin, an inhibitor of EC proliferation [186]. This peptide was examined as a potential angiostatic agent in chick embryos. In vivo neovascular growth was compared between a group receiving the novel peptide, a group only receiving vasostatin, another receiving PF-4 alone, as well as a control. The chick embryos receiving the novel peptide showed a statistically significant reduction in angiogeneses when compared to the other groups [186]. Although the study was only completed in animals, it demonstrates another possible drug that may be used to inhibit tumor angiogeneses.

2.4.5. Thrombospondin. Thrombospondin-1 (TSP-1), the first naturally occurring angiostatic protein discovered, is a multidomain matrix glycoprotein that has been shown to be a natural inhibitor of neovascularization [187]. Unlike previous angiostatic agents discussed, TSP-1 is a native, full-length protein. TSP-1 is stored in α-granules of platelets, where it is complexed with TGFβ1 [188]. When released from the platelets and free from TGFβ1, TSP-1 inhibits the migration of ECs [189] and induces EC apoptosis [190]. To slow migration, TSP-1 binds to EC surface receptors capable of promoting promigratory signals [189]. The induction of apoptosis in ECs is associated with TSP-1's ability to alter the concentrations of several important apoptotic factors. TSP-1 upregulates Bax, downregulates Bcl-2, and activates the caspase-3 intrinsic pathway, leading to programmed EC death [191]. Other experiments have shown that mice depleted of TSP-1 saw a 250%–300% increase in tumor growth rate when implanted with murine tumors [1]. Microvessel density was also increased near the tumor in these mice [5].

A phase I clinical trial using ABT-510, an angiogenic inhibitor derived from TSP-1, has been completed. This drug was delivered subcutaneously in patients with advanced solid malignancies. Although the phase I trial was not designed to test efficacy, 6 of the 39 patients saw disease stabilization after the treatment [192]. The toxicity effects associated with ABT-510 were minimal and deemed safe for future trials [192]. The use of ABT-510 alongside chemotherapeutic agents has also been examined. A phase I clinical trial investigated the use of ABT-510 with gemcitabine-cisplatin chemotherapy in patients with solid tumors [193]. This study found no clinically significant pharmacokinetic interactions between the combined drugs. Despite the low doses, 3 of the 12 patients tested saw a partial response with the drug treatment [193]. Another phase I trial examined the toxicity profile of ABT-510 along with the chemotherapeutics 5-fluorouracil and leucovorin [194]. Minimal toxicity was found by combining the drugs, and 4 of the 12 patients had tumor stabilization posttreatment [194]. Both of the combined phase I trials stated that the optimal dosage for ABT-510 has not been established yet, and determining these values would be important in future clinical trials.

2.4.6. Tissue Inhibitors of Metalloproteinases. Tissue Inhibitors of Metalloproteinases (TIMPs) are a family of proteases, derived from cartilage, which inhibit MMPs. As previously mentioned, MMPs play an integral role in the initiation of angiogeneses. They are responsible for EC basement membrane degradation and EC remodeling [3, 67]. The newly formed ECM developed by MMPs during the angiogenic response provides a scaffold for ECs to adhere, migrate, and form tubes for nutrient delivery. The inhibition of MMPs by TIMPs reduces the angiogenic capacity of ECs [195, 196]. High levels of TIMP-1 greatly inhibit migration of ECs through gelatin in vitro [197]. The invasive potential, growth, and neovascularization of metastatic murine melanoma cells were inhibited in vivo when transfected with TIMP-2 [195].

The role of TIMP-3 in tumor angiogeneses was examined using mice models. The study found that TIMP-3 treatment of mice with lung cancer led to reduced angiogeneses in vivo through the inhibition of the VEGF-VEGFR-2 signaling pathway and the induction of EC-apoptosis [198]. The inhibition of this pathway and the EC associated death are in part due to the inhibition of MMP-2 by TIMP-3 [198]. Another study investigated the use of TIMP-1 gene transfer through an adenovirus as a way to treat established gastric cancer in nude mice. Compared to controls, mice transfected with TIMP-1 gene saw a significant reduction in the mean number of tumor vessels [199]. Although the exact mechanism of TIMPs inhibition of cancer progression remains unclear, TIMPs should still be considered for therapeutic trials because of their success in animal models.

2.4.7. Interleukins. Interleukins (ILs) are a group of cytokines that are released by leukocytes and control a wide range of biological activities. A few of these ILs have been shown to affect the growth of blood vessels [200]. The ability to either enhance or suppress angiogeneses is based on a Glu-Leu-Arg (ELR) motif at the NH2 terminus. IL-8 possesses this sequence (discussed later), and therefore enhances angiogeneses, while IL-4 does not contain the motif, and is an inhibitor of angiogeneses [200].

IL-4 acts as an inhibitor of tumor growth [201], but its mechanism of action likely varies with different tumor cells. For example, IL-4 is thought to directly inhibit proliferation of cells from cancers such as colon tumors, head and neck tumors, and glioblastomas [202], while in other cases it is thought to induce a host immune response against the tumor cells such as in B-cell lymphomas and melanomas [203]. There is also evidence that IL-4 inhibits neovascularization, thus inhibiting tumor growth. In vitro, IL-4 inhibits migration of ECs towards bFGF. In vivo, IL-4 has been shown to inhibit neovascularization in rat corneas that should have been induced by the high concentration of bFGF present [204]. These experiments demonstrate that IL-4-mediated suppression of tumor growth may be due to IL-4s ability to inhibit angiogenic processes. Other noncancer-related animal studies have shown IL-4's antiangiogenic capabilities in vivo. One such study examined rats with adjuvant-induced arthritis. One set of animals received an adenovirus capable of producing IL-4, another group received a control virus without the IL-4 producing capabilities, and the last received a saline injection [205]. The group treated with the IL-4 producing adenovirus saw a statistically significant reduction in blood vessel growth [205]. The reduction in angiogeneses from the IL-4 treatment appears to be associated with a change in the pro- and antiangiogenic cytokine levels [205]. Although the study was designed to study inflammatory arthritis, knowledge gained about IL-4 could be used for the treatment of cancer in the future.

2.4.8. Interferons (IFNs). Interferons (IFNs) belong to a large family of secreted glycoproteins known as cytokines. They are produced and secreted by a wide variety of immune-related cells and IFN-α has been shown to inhibit angiogeneses in vivo [206]. It is thought that both IFN-α and IFN-β are able to inhibit angiogeneses by repressing bFGF mRNA and protein levels [207]. In addition to the downregulation of key angiogeneses signaling factors, IFN-α also inhibits the migration of EC cells in vivo [208].

Several clinical trials have been performed investigating the administration of IFN-α in combination with chemotherapeutics in different forms of cancer. Many studies have used PEG IFN-α, a form of IFN-α that has been modified to have a longer half-life and thus have a greater opportunity to reduce angiogeneses [209]. A phase I trial completed tested the effect of subcutaneous injections of PEG IFN-α and recombinant IL-2 in patients with metastatic RCC [210]. Minimum toxicity was found at most levels, and an unsafe level of the drug combination was determined. Of the 34 patients in the study, 15% saw a partial response, 68% had disease stabilization, and 18% had their disease continue to progress. Overall, the median survival was 31.9 months for patients treated and their median progression-free survival was 9.0 months [210]. Both the median survival time and the median progression-free survival time were slightly greater than the average control reported in the literature [210]. It was determined from this study, though, that IFN-α alone or in combination with IL-2 is not the optimal treatment of RCCs. The group did suspect that the use of IFN-α in combination with Avastin (a monoclonal Ab that targets VEGF) may provide a better treatment for RCC [210]. This theory was tested in a clinical trial using patients who also had metastatic RCCs. They reported that the median progression-free survival in patients receiving the combined Avastin and IFN-α treatment was double that of patients only receiving IFN-α and a placebo [211]. The combined therapy also showed improvement in overall response rate from 13% in the IFN-α alone group to 32% in the combined treatment arm of the study [211]. A clinical benefit was seen in 79% of the patients receiving combined treatment versus 65% in the IFN-α alone treatment group [211]. Lastly, the median duration of tumor response was 13.5 months in the combined drug group while it was only 11.1 months in the IFN-α only group [211]. After completion of the study, it was observed that the levels of IFN-α used were higher than the optimal dosage, and it is likely that treatment with slightly lower levels of IFN-α would have seen greater clinical benefit in both groups [211]. The study determined that the combination therapy was an effective first-line treatment option for metastatic RCC [211], and further trials should be conducted to determine optimal dosages.

In addition to metastatic RCCs, clinical trials have also tested the use of PEG IFN-α as a treatment for metastatic melanomas. A phase I trial using PEG IFN-α determined that it was safe and effective in humans [212]. A phase II trial then investigated the use of Dacarbazine (DTIC) alongside PEG IFN-α for the treatment of metastatic melanomas. Of the 25 patients who completed the study, 8% had complete remission from the treatment, while another 16% had partial responses [209]. Stable disease was found in 4% of patients while 72% had their disease progress. Although the treatment proved that the combinational treatment was not toxic, the efficacy of the treatment could not be determined because the study did not compare different regimens of treatment [209]. Despite this, the 24% overall response rate, including two long-term complete responses, is promising and warrants more clinical investigation [209].

2.4.9. Tumor Angiogeneses. Tumors begin as an avascular mass of host-derived cells that proliferate atypically because they have lost the ability to control their growth [3]. Tumors initially survive and thrive on vasculature that is already available in the surrounding host environment [36]. In order for tumors to grow beyond 2-3 mm^3, they need a continual supply of blood to remove waste and deliver nutrients [1]. Hypoxia of tumor cells will occur if the tumor grows beyond the maximum distance of effusion

from local vessels (around 200 μm) [36]. In order to counter this lack of oxygen, tumor cells will attempt to create new blood vessels to supply their needs in a mechanism that closely resembles normal angiogeneses [3]. This process and several important tumor-derived factors are illustrated in Figure 3.

The blood vessels formed during tumor-induced angiogeneses are abnormal. The walls of tumor vessels are usually made of a combination of both tumor cells and ECs [213]. Functional pericytes are often absent from the peripheral blood vessels [214], leaving an incomplete basement membrane. This causes those vessels to be especially leaky and dilated [215]. Recently, it was found that, although the majority of tumor vasculature does not possess pericytes, some tumors keep a core of blood vessels alive and functional because the vessels are protected by pericytes [216, 217]. This concept emerged from several studies that showed VEGF signaling inhibition led to a large reduction in tumor vascularity. However, functional vessels that remained after treatment were small in diameter and covered with pericytes [216, 218]. The morphology of these surviving tumor vessels was very different from normal dilated tumor vessels which are sparsely populated with pericytes [216, 218]. These observations support the prevailing thought that tumor vessels lacking pericytes are more vulnerable to anti-VEGF treatment [218, 219].

Like the normal angiogenic process, tumor angiogeneses is reliant on VEGF and other angiogenic proteins. Increased levels of VEGF and its receptor VEGFR-2 have been observed in many cancers, including metastatic human colon carcinomas, where increased levels were shown to directly increase tumor vascularization [220]. Breast cancer patients with higher levels of VEGF expression have increased intratumoral vascularization and a worse prognosis [221]. Experiments with monoclonal Abs against VEGF, or genetic inactivation of VEGF (or VEGFR-2), have dramatic decreases in angiogeneses and neovascularization in several different forms of cancer [83–85]. VEGF is stimulated by the hypoxic conditions near the central necrotic tissue of solid tumors [222]. The mechanism of tumor blood vessel growth activated by VEGF is similar to the normal angiogenic response to hypoxic conditions. Neovascularization occurs in both cases to help meet the metabolic needs of cells [2, 3]. In addition to the endovascular stimulation attributed to VEGF, it can also increase vascular permeability [28, 29, 223, 224], explaining the leaky blood vessels observed in tumors. VEGF has many important roles in tumor angiogeneses and therefore its inactivation has often been a target of tumor therapy.

Ang-2 plays a more important role in tumor angiogeneses than it does in normal angiogeneses. As an antagonist for Ang-1, it is largely responsible for blood vessel destabilization seen in vasculature surrounding tumors. Normally, destabilization leads to blood vessel breakdown, but in the presence of VEGF, the vasculature is readily receptive to VEGF-mediated growth [225]. Ang-2 expression in ECs of tumor vessels greatly exceeds that of ECs in normal blood vessels and can be used as an early biomarker of tumor-induced vascularization [226]. Besides giving the growing vasculature plasticity in the presence of VEGF, it also plays an important role in the initial stages of tumor angiogeneses. During early tumor development, VEGF levels are greatly reduced, but Ang-2 mRNA levels are high [227]. At this stage, Ang-2 is responsible for the degradation and regression of blood vessels associated with early stages of tumor angiogeneses. As the tumor continues to grow in size, it eventually reaches a point where it requires more nutrients, thus levels of VEGF rise and new capillary growth can begin.

FGF was the first tumor-derived factor found to stimulate neovascularization and EC proliferation in vivo [228]. The importance of bFGF in tumor angiogeneses was confirmed by the use of bFGF receptor inhibition in tumor-injected mice. Inactivation of bFGF receptor led to decreased tumor growth [228] and blood vessel density [229]. Based on the time that bFGF is active during tumor angiogeneses, it has been suggested that bFGF is important in maintaining this process, as opposed to VEGF which likely initiates tumor angiogeneses [229]. However, bFGF has also been shown to help increase VEGF production [230], upregulate VEGF mRNA in vascular smooth muscle [231], and increase VEGF receptor density in microvascular ECs [232].

It was recently discovered that TGF-β signaling behaves as a strong activator of tumor growth and metastasis through stimulation of angiogenic processes [233, 234]. It is believed that TGF-β expression by neoplastic cells acts to induce the stromal reaction, which results in the formation of a reactive stroma microenvironment that is thought to promote angiogeneses and tumor growth [235]. It has also been shown that the use of neutralizing Abs against TGF-β leads to a reduction in the amount of blood vessels surrounding implanted tumors and greatly inhibits angiogeneses in these regions [235].

Heparanase is highly reactive 50 kDa protein known to induce tumor angiogeneses [236]. Heparanase is preferentially expressed in both melanoma and carcinoma [237]. Transfection of both nonmetastatic T lymphoma and melanoma cell lines with the heparanase gene caused both cell lines to become highly metastatic in vivo [236]. In addition, T lymphoma cells transfected with the heparanase gene saw a considerable increase in neovascularization near implanted tumors when compared to the nontransfected T lymphoma cells [236]. Heparanase stimulates angiogeneses directly by promoting EC invasion and vascular sprouting [3]. Heparanase also helps release bFGF that is bound to heparin sulfate at the ECM [236], increasing local bFGF levels. The increase in the local bFGF concentration is thought to contribute to the increased neovascularization measured near implanted tumors.

Interleukin-8, which is produced by macrophages, is not an important factor in normal angiogeneses. However, IL-8 appears to be a central mediator of tumor-derived angiogeneses. Elevated levels of IL-8 have been documented in several types of neoplastic tissues [238]. The increased expression of IL-8 correlates with amplified neovascularization density [238, 239] as well as an increase in tumor growth [238]. Melanoma cells forced to

continually express IL-8 were highly tumorigenic and had greater metastatic potential compared with parental and control transfected cells [238]. An important characteristic of IL-8 is its ability to increase levels of MMP-2 which degrades the EC basement membrane and remodels the ECM, initiating the early phase of tumor angiogeneses [21]. IL-8-transfected melanoma cells displayed greatly increased levels of MMP-2, while transfection of identical melanoma cells with VEGF and bFGF did not affect MMP-2 levels [240, 241] demonstrating that this is an important but separate mechanism involved in tumor-induced angiogeneses.

It is obvious that many different factors play an important role in tumor angiogeneses. To date, VEGF has been shown to play the most dominant role, but many other factors such as IL-8, MMP-2, heparanase, TGF-β, and bFGF also play an essential part in the process. Because so many factors are involved with tumor angiogeneses, it is likely that several of these factors must be inhibited simultaneously in order to significantly reduce the unwanted angiogeneses and eventual tumor metastasis.

3. Future Directions

Currently, investigation into mechanisms of angiogeneses inhibition in cancer is an important and promising area of research. Prohibiting angiogeneses is an important therapeutic approach for fighting cancer, reducing atherosclerosis, and preventing blindness due to retinal neovascularization in diabetic patients. In recent years, several new angiostatic therapies have been tested and approved by the FDA; examples include Avastin, Tarceva, and Lucentis [242]; several others are currently being tested in phase III trials throughout the country. Among them are possible angiostatic treatments for many different types of cancer including esophageal cancer, pancreatic cancer, lymphoma, renal cell cancer, gastric cancer, and many others [82]. Important advances have also taken place in defining the molecular understanding of angiogeneses. This includes a greater understanding of both angiogeneses as a whole as well as the mechanism of antiangiogenic drugs currently being used. However, the many studies using anti-VEGF treatments illustrate that our knowledge of the angiogenic pathway remains incomplete. In the past, it was thought that angiostatic treatment would create a form of cancer treatment that would evade the problem of resistance [243, 244]. As clinical trials continue, it now appears that many tumors can overcome the use of angiogenic inhibitors, thereby acquiring a way to bypass the therapeutic angiogeneses blockade [9, 82, 90]. Although there are several different adaptive mechanisms that tumors may employ to overcome antiangiogenic therapy, two concepts have emerged as the most likely candidates [82]. The first is that tumors are able to activate or upregulate alternative proangiogenic pathways after the first pathway is inhibited. An example was observed in animal models when a monoclonal Ab that specifically blocked VEGFR signaling was used in mice with tumors. The mice saw an initial response to the treatment, and the tumors possessed reduced vascularity [245]. After a short period of time, however, the tumors saw a reinitiation of tumor angiogeneses. When the tumors were resected and studied, they were found to express greater levels of mRNA for the proangiogenic factors FGF and Ang-1. This change in expression helps explain one possible method the tumors are employing to overcome VEGF inhibition. To further test the effect of upregulation of alternative proangiogenic factors, a similar study was conducted in which some mice were treated with VEGF inhibitor alone while others were treated with a VEGF inhibitor as well as an FGF trap. Mice treated with combinational therapy saw a great reduction in vascularization and slowed tumor growth [245]. Yet another study in mice showed that the induction of IL-8 was able to maintain angiogenic capability in tumors that did not express HIF-1α, an inducer of VEGF expression [246]. This alternative pathway illustrates another proangiogenic pathway that tumors may use to increase vascularization. Together, these studies have begun to shed light on why anti-VEGF treatments alone may have seen limited results in clinical trials.

The other theory to why tumors have been able to withstand the anti-VEGF treatment is that resistant tumors have increased pericyte support on their tumor vasculature. The pericytes are believed to protect the remaining vessels and defend against the anti-VEGF treatment [219, 247]. The hypothesis states that tumor pericytes are most likely expressing appreciable levels of VEGF and possibly other proangiogenic factors [247]. In addition, pericytes are capable of reducing the rate of EC proliferation which allows EC maturation and stabilization in newly formed blood vessels [248]. Currently, several ongoing clinical trials are attempting to prevent tumor angiogeneses by inhibiting pericyte association with tumor vasculature along with angiogenic factors [82, 219].

These two ongoing theories showcase the current problems in the field of antiangiogenic research in cancer. After several years of clinical trials, it appears that targeting one angiogenic factor is not enough to permanently halt neovascularization in most tumors. Although these results were initially disheartening, they also opened up the possibility of other angiostatic therapies. Many clinical trials now use existing chemotherapeutic drugs or radiation along with antiangiogenic drugs. This two-front attack has had more success than antiangiogenic drugs or chemotherapy alone in a majority of patients [5, 118, 249].

As research continues, more information is also being uncovered about the angiogenic pathway. Increased understanding of the angiogenic pathway will allow for development and use of drugs that can target several angiogenic factors concurrently, allowing greater inhibition of angiogeneses, and increasing the likelihood of therapeutic success. Although the benefit of antiangiogenic treatments has not been as great as initially anticipated, many advances have come from their development and clinical use. However, with time, it is likely that the success of angiogenic treatment in cancer will continue to improve and we will come ever closer to the original goal of curing cancer and other angiogenic-related diseases.

Abbreviations

Ang: Angiopoieten
Ab: Antibody
AMD: Age related macular degeneration
CD: Cluster of differentiation
Eng: Endoglin
EC: Endothelial cell
EPC: Endothelial progenitor cell
ECM: Extra cellular matrix
FDA: Food and drug administration
FGF: Fibroblast growth factor
HSC: Hematopoietic stem cell
HCC: Hepatocellular carcinoma
HUVEC: Human umbilical vein endothelial cell
HIF: Hypoxia inducible factor
IFN: Interferon, IL-interleukin
MMP: Matrix metalloproteinase
mAB: monoclonal Antibody
MAPC: Multipotent adult progenitor cell
NSCLC: Non-small-cell lung cancer
PDGF: Platelet derived growth factor
PF: Platelet factor, rcc-renal cell carcinoma
RCC: Renal cell carcinoma
TSP: Thrombospondin
TIMP: Tissue inhibitor of metalloproteinase
TGF: Transforming growth factor
TNF: Tumor necrosis factor
VE: Vascular endothelial
VEGF: Vascular endothelial growth factor
VWF: von Willenbrand factor.

References

[1] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[2] J. Folkman and Y. Shing, "Angiogenesis," *Journal of Biological Chemistry*, vol. 267, no. 16, pp. 10931–10934, 1992.

[3] M. Papetti and I. M. Herman, "Mechanisms of normal and tumor-derived angiogenesis," *American Journal of Physiology*, vol. 282, no. 5, pp. C947–C970, 2002.

[4] R. K. Jain, "Transport of molecules in the tumor interstitium: a review," *Cancer Research*, vol. 47, no. 12, pp. 3039–3051, 1987.

[5] J. Folkman, "Angiogenesis," *Annual Review of Medicine*, vol. 57, pp. 1–18, 2006.

[6] S. P. Tabruyn and A. W. Griffioen, "Molecular pathways of angiogenesis inhibition," *Biochemical and Biophysical Research Communications*, vol. 355, no. 1, pp. 1–5, 2007.

[7] A. Maton, J. Hopkins, C. W. J. S. McLaughlin, M. Warner, and D. W. J. LaHart, *Human Biology and Health*, Prentice Hall, Englewood Cliffs, NJ, USA, 1993.

[8] G. L. Semenza, "Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling," *Journal of Cellular Biochemistry*, vol. 102, no. 4, pp. 840–847, 2007.

[9] P. Carmeliet, "Angiogenesis in life, disease and medicine," *Nature*, vol. 438, no. 7070, pp. 932–936, 2005.

[10] D. G. Tang and C. J. Conti, "Endothelial cell development, vasculogenesis, angiogenesis, and tumor neovascularization: an update," *Seminars in Thrombosis and Hemostasis*, vol. 30, no. 1, pp. 109–117, 2004.

[11] D. Baksh, J. E. Davies, and P. W. Zandstra, "Adult human bone marrow-derived mesenchymal progenitor cells are capable of adhesion-independent survival and expansion," *Experimental Hematology*, vol. 31, no. 8, pp. 723–732, 2003.

[12] K. A. Jackson, S. M. Majka, H. Wang, et al., "Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells," *Journal of Clinical Investigation*, vol. 107, no. 11, pp. 1395–1402, 2001.

[13] Y. Jiang, B. N. Jahagirdar, R. L. Reinhardt, et al., "Pluripotency of mesenchymal stem cells derived from adult marrow," *Nature*, vol. 418, no. 6893, pp. 41–49, 2002.

[14] S. Rafii, S. Avecilla, S. Shmelkov, et al., "Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment," in *Annals of the New York Academy of Sciences*, vol. 996, pp. 49–60, 2003.

[15] A. Aicher, A. M. Zeiher, and S. Dimmeler, "Mobilizing endothelial progenitor cells," *Hypertension*, vol. 45, no. 3, pp. 321–325, 2005.

[16] A. Janowska-Wieczorek, L. A. Marquez, J.-M. Nabholtz, et al., "Growth factors and cytokines upregulate gelatinase expression in bone marrow $CD34^+$ cells and their transmigration through reconstituted basement membrane," *Blood*, vol. 93, no. 10, pp. 3379–3390, 1999.

[17] B. Heissig, Z. Werb, S. Rafii, and K. Hattori, "Role of c-kit/Kit ligand signaling in regulating vasculogenesis," *Thrombosis and Haemostasis*, vol. 90, no. 4, pp. 570–576, 2003.

[18] T. Cheng, N. Rodrigues, H. Shen, et al., "Hematopoietic stem cell quiescence maintained by p21(cip1/waf1)," *Science*, vol. 287, no. 5459, pp. 1804–1809, 2000.

[19] S. M. Bauer, R. J. Bauer, and O. C. Velazquez, "Angiogenesis, vasculogenesis, and induction of healing in chronic wounds," *Vascular and Endovascular Surgery*, vol. 39, no. 4, pp. 293–306, 2005.

[20] G. Bergers, R. Brekken, G. McMahon, et al., "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis," *Nature Cell Biology*, vol. 2, no. 10, pp. 737–744, 2000.

[21] M. Klagsbrun and M. A. Moses, "Molecular angiogenesis," *Chemistry and Biology*, vol. 6, no. 8, pp. R217–R224, 1999.

[22] A. Eichmann, C. Corbel, V. Nataf, P. Vaigot, C. Breant, and N. M. Le Douarin, "Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 10, pp. 5141–5146, 1997.

[23] G. Conn, D. D. Soderman, M.-T. Schaeffer, M. Wile, V. B. Hatcher, and K. A. Thomas, "Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 4, pp. 1323–1327, 1990.

[24] S. Dimmeler, E. Dernbach, and A. M. Zeiher, "Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration," *FEBS Letters*, vol. 477, no. 3, pp. 258–262, 2000.

[25] D. T. Connolly, D. M. Heuvelman, R. Nelson, et al., "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis," *Journal of Clinical Investigation*, vol. 84, no. 5, pp. 1470–1478, 1989.

[26] H.-P. Gerber, A. McMurtrey, J. Kowalski, et al., "Vascular endothelial growth factor regulates endothelial cell survival

through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway: requirement for Flk-1/KDR activation," *Journal of Biological Chemistry*, vol. 273, no. 46, pp. 30336–30343, 1998.

[27] D. R. Senger, S. R. Ledbetter, K. P. Claffey, A. Papadopoulos-Sergiou, C. A. Perruzzi, and M. Detmar, "Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha v\beta 3$ integrin, osteopontin, and thrombin," *American Journal of Pathology*, vol. 149, no. 1, pp. 293–305, 1996.

[28] S. Esser, M. G. Lampugnani, M. Corada, E. Dejana, and W. Risau, "Vascular endothelial growth factor induces VE-cadherin tyrosine," *Journal of Cell Science*, vol. 111, no. 13, pp. 1853–1865, 1998.

[29] C. G. Kevil, D. Keith Payne, E. Mire, and J. S. Alexander, "Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins," *Journal of Biological Chemistry*, vol. 273, no. 24, pp. 15099–15103, 1998.

[30] J. Denekamp, "Endothelial cell proliferation as a novel approach to targeting tumor therapy," *British Journal of Cancer*, vol. 45, no. 1, pp. 136–139, 1982.

[31] D. L. Miller, S. Ortega, O. Bashayan, R. Basch, and C. Basilico, "Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice," *Molecular and Cellular Biology*, vol. 20, no. 6, pp. 2260–2268, 2000.

[32] C. Suri, P. F. Jones, S. Patan, et al., "Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis," *Cell*, vol. 87, no. 7, pp. 1171–1180, 1996.

[33] G. D. Yancopoulos, M. Klagsbrun, and J. Folkman, "Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border," *Cell*, vol. 93, no. 5, pp. 661–664, 1998.

[34] C. Suri, J. McClain, G. Thurston, et al., "Increased vascularization in mice overexpressing angiopoietin-1," *Science*, vol. 282, no. 5388, pp. 468–471, 1998.

[35] N. Ferrara and R. S. Kerbel, "Angiogenesis as a therapeutic target," *Nature*, vol. 438, no. 7070, pp. 967–974, 2005.

[36] L. C. L. van Kempen and W. P. J. Leenders, "Tumours can adapt to anti-angiogenic therapy depending on the stromal context: lessons from endothelial cell biology," *European Journal of Cell Biology*, vol. 85, no. 2, pp. 61–68, 2006.

[37] P. A. D'Amore and S. R. Smith, "Growth factor effects on cells of the vascular wall: a survey," *Growth Factors*, vol. 8, no. 1, pp. 61–75, 1993.

[38] Z. K. Otrock, R. A. R. Mahfouz, J. A. Makarem, and A. I. Shamseddine, "Understanding the biology of angiogenesis: review of the most important molecular mechanisms," *Blood Cells, Molecules, and Diseases*, vol. 39, no. 2, pp. 212–220, 2007.

[39] P. Carmeliet, "Angiogenesis in health and disease," *Nature Medicine*, vol. 9, no. 6, pp. 653–660, 2003.

[40] D. J. Falcone, T. A. McCaffrey, A. Haimovitz-Friedman, and M. Garcia, "Transforming growth factor-$\beta 1$ stimulates macrophage urokinase expression and release of matrix-bound basic fibroblast growth factor," *Journal of Cellular Physiology*, vol. 155, no. 3, pp. 595–605, 1993.

[41] K.-Y. Kim, S.-Y. Jeong, J. Won, P.-D. Ryu, and M.-J. Nam, "Induction of angiogenesis by expression of soluble type II transforming growth factor-β receptor in mouse hepatoma," *Journal of Biological Chemistry*, vol. 276, no. 42, pp. 38781–38786, 2001.

[42] S. van den Driesche, C. L. Mummery, and C. J. J. Westermann, "Hereditary hemorrhagic telangiectasia: an update on transforming growth factor β signaling in vasculogenesis and angiogenesis," *Cardiovascular Research*, vol. 58, no. 1, pp. 20–31, 2003.

[43] P. C. Brooks, S. Stromblad, L. C. Sanders, et al., "Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$," *Cell*, vol. 85, no. 5, pp. 683–693, 1996.

[44] D. I. Leavesley, M. A. Schwartz, M. Rosenfeld, and D. A. Cheresh, "Integrin $\beta 1$- and $\beta 3$-mediated endothelial cell migration is triggered through distinct signaling mechanisms," *Journal of Cell Biology*, vol. 121, no. 1, pp. 163–170, 1993.

[45] P. C. Brooks, R. A. F. Clark, and D. A. Cheresh, "Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis," *Science*, vol. 264, no. 5158, pp. 569–571, 1994.

[46] R. A. F. Clark, M. G. Tonnesen, J. Gailit, and D. A. Cheresh, "Transient functional expression of $\alpha_v\beta_3$ on vascular cells during wound repair," *American Journal of Pathology*, vol. 148, no. 5, pp. 1407–1421, 1996.

[47] M. Friedlander, P. C. Brooks, R. W. Shaffer, C. M. Kincaid, J. A. Varner, and D. A. Cheresh, "Definition of two angiogenic pathways by distinct α_v integrins," *Science*, vol. 270, no. 5241, pp. 1500–1502, 1995.

[48] L. Caveda, I. Martin-Padura, P. Navarro, et al., "Inhibition of cultured cell growth by vascular endothelial cadherin (cadherin-5/VE-cadherin)," *Journal of Clinical Investigation*, vol. 98, no. 4, pp. 886–893, 1996.

[49] P. Carmeliet, M.-G. Lampugnani, L. Moons, et al., "Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis," *Cell*, vol. 98, no. 2, pp. 147–157, 1999.

[50] M. Frater-Schroder, W. Risau, and R. Hallmann, "Tumor necrosis factor type α, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 15, pp. 5277–5281, 1987.

[51] S. J. Leibovich, P. J. Polverini, H. M. Shepard, D. M. Wiseman, V. Shively, and N. Nuseir, "Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α," *Nature*, vol. 329, no. 6140, pp. 630–632, 1987.

[52] A. B. Schreiber, M. E. Winkler, and R. Derynck, "Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor," *Science*, vol. 232, no. 4755, pp. 1250–1253, 1986.

[53] J. W. Fett, D. J. Strydom, R. R. Lobb, et al., "Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells," *Biochemistry*, vol. 24, no. 20, pp. 5480–5486, 1985.

[54] F. Soncin, "Angiogenin supports endothelial and fibroblast cell adhesion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 6, pp. 2232–2236, 1992.

[55] M. Hockel, W. Jung, P. Vaupel, H. Rabes, C. Khaledpour, and J. H. Wissler, "Purified monocyte-derived angiogenic substance (angiotropin) induces controlled angiogenesis associated with regulated tissue proliferation in rabbit skin," *Journal of Clinical Investigation*, vol. 82, no. 3, pp. 1075–1090, 1988.

[56] D. J. Ceradini, A. R. Kulkarni, M. J. Callaghan, et al., "Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1," *Nature Medicine*, vol. 10, no. 8, pp. 858–864, 2004.

[57] K. Zaman, R. Driscoll, D. Hahn, et al., "Monitoring multiple angiogenesis-related molecules in the blood of cancer patients shows a correlation between VEGF-A and MMP-9 levels before treatment and divergent changes after surgical vs. conservative therapy," *International Journal of Cancer*, vol. 118, no. 3, pp. 755–764, 2006.

[58] K. Dumstrei, R. Mennecke, and E. Raz, "Signaling pathways controlling primordial germ cell migration in zebrafish," *Journal of Cell Science*, vol. 117, no. 20, pp. 4787–4795, 2004.

[59] M. Reichman-Fried, S. Minina, and E. Raz, "Autonomous modes of behavior in primordial germ cell migration," *Developmental Cell*, vol. 6, no. 4, pp. 589–596, 2004.

[60] H. Bompais, J. Chagraoui, X. Canron, et al., "Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells," *Blood*, vol. 103, no. 7, pp. 2577–2584, 2004.

[61] T. Asahara, T. Murohara, A. Sullivan, et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[62] T. Asahara, H. Masuda, T. Takahashi, et al., "Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization," *Circulation Research*, vol. 85, no. 3, pp. 221–228, 1999.

[63] M. Hristov, W. Erl, and P. C. Weber, "Endothelial progenitor cells: isolation and characterization," *Trends in Cardiovascular Medicine*, vol. 13, no. 5, pp. 201–206, 2003.

[64] M. Pesce, A. Orlandi, M. G. Iachininoto, et al., "Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues," *Circulation Research*, vol. 93, no. 5, pp. e51–e62, 2003.

[65] M. Gill, S. Dias, K. Hattori, et al., "Vascular trauma induces rapid but transient mobilization of VEGFR2$^+$ AC133$^+$ endothelial precursor cells," *Circulation Research*, vol. 88, no. 2, pp. 167–174, 2001.

[66] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.

[67] M. A. Moses, "The regulation of neovascularization by matrix metalloproteinases and their inhibitors," *Stem Cells*, vol. 15, no. 3, pp. 180–189, 1997.

[68] J. Li, Y.-P. Zhang, and R. S. Kirsner, "Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix," *Microscopy Research and Technique*, vol. 60, no. 1, pp. 107–114, 2003.

[69] E. Tischer, R. Mitchell, T. Hartman, et al., "The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing," *Journal of Biological Chemistry*, vol. 266, no. 18, pp. 11947–11954, 1991.

[70] K. A. Houck, N. Ferrara, J. Winer, G. Cachianes, B. Li, and D. W. Leung, "The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA," *Molecular Endocrinology*, vol. 5, no. 12, pp. 1806–1814, 1991.

[71] Z. Poltorak, T. Cohen, R. Sivan, et al., "VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix," *Journal of Biological Chemistry*, vol. 272, no. 11, pp. 7151–7158, 1997.

[72] L. Jingjing, Y. Xue, N. Agarwal, and R. S. Roque, "Human Muller cells express VEGF183, a novel spliced variant of vascular endothelial growth factor," *Investigative Ophthalmology and Visual Science*, vol. 40, no. 3, pp. 752–759, 1999.

[73] S. Hughes, H. Yang, and T. Chan-Ling, "Vascularization of the human fetal retina: roles of vasculogenesis and angiogenesis," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 5, pp. 1217–1228, 2000.

[74] N. Ferrara, "Vascular endothelial growth factor: basic science and clinical progress," *Endocrine Reviews*, vol. 25, no. 4, pp. 581–611, 2004.

[75] D. R. Senger, K. P. Claffey, J. E. Benes, C. A. Perruzzi, A. P. Sergiou, and M. Detmar, "Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13612–13617, 1997.

[76] D. Shweiki, A. Itin, D. Soffer, and E. Keshet, "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis," *Nature*, vol. 359, no. 6398, pp. 843–845, 1992.

[77] G. L. Wang, B.-H. Jiang, E. A. Rue, and G. L. Semenza, "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 12, pp. 5510–5514, 1995.

[78] G. L. Wang and G. L. Semenza, "Purification and characterization of hypoxia-inducible factor 1," *Journal of Biological Chemistry*, vol. 270, no. 3, pp. 1230–1237, 1995.

[79] H. Kimura, A. Weisz, T. Ogura, et al., "Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide," *Journal of Biological Chemistry*, vol. 276, no. 3, pp. 2292–2298, 2001.

[80] B. D. Kelly, S. F. Hackett, K. Hirota, et al., "Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1," *Circulation Research*, vol. 93, no. 11, pp. 1074–1081, 2003.

[81] M. S. Ip, I. U. Scott, G. C. Brown, et al., "Anti-vascular endothelial growth factor pharmacotherapy for age-related macular degeneration. A report by the American Academy of Ophthalmology," *Ophthalmology*, vol. 115, no. 10, pp. 1837–1846, 2008.

[82] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[83] B. Millauer, L. K. Shawver, K. H. Plate, W. Risau, and A. Ullrich, "Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant," *Nature*, vol. 367, no. 6463, pp. 576–579, 1994.

[84] B. Millauer, M. P. Longhi, K. H. Plate, et al., "Dominant-negative inhibition of Flk-1 suppresses the growth of many tumor types in vivo," *Cancer Research*, vol. 56, no. 7, pp. 1615–1620, 1996.

[85] N. Ferrara, K. Carver-Moore, H. Chen, et al., "Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene," *Nature*, vol. 380, no. 6573, pp. 439–442, 1996.

[86] F. Shojaei and N. Ferrara, "Antiangiogenic therapy for cancer: an update," *Cancer Journal*, vol. 13, no. 6, pp. 345–348, 2007.

[87] V. W. Li, R. A. Ball, N. Vassan, et al., "Antiangiogenic therapy of squamous cell carcinoma using combinaorial agents," *Journal of Clinical Oncology*, vol. 23, p. 3032, 2005.

[88] L. B. Saltz, H.-J. Lenz, H. L. Kindler, et al., "Randomized phase II trial of cetuximab, bevacizumab, and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer: the BOND-2 study,"

Journal of Clinical Oncology, vol. 25, no. 29, pp. 4557–4561, 2007.

[89] R. K. Jain, "Antiangiogenic therapy for cancer: current and emerging concepts," *Oncology*, vol. 19, no. 4, supplement 3, pp. 7–16, 2005.

[90] J. C. Miller, H. H. Pien, D. Sahani, A. G. Sorensen, and J. H. Thrall, "Imaging angiogenesis: application and potential for drug development," *Journal of the National Cancer Institute*, vol. 97, no. 3, pp. 172–187, 2005.

[91] N. Maulik and M. Thirunavukkarasu, "Growth factor/s and cell therapy in myocardial regeneration," *Journal of Molecular and Cellular Cardiology*, vol. 44, no. 2, pp. 219–227, 2008.

[92] K. A. Thomas, "Fibroblast growth factors," *FASEB Journal*, vol. 1, no. 6, pp. 434–440, 1987.

[93] J. Folkman, M. Klagsbrun, J. Sasse, M. Wadzinski, D. Ingber, and I. Vlodavsky, "A heparin-binding angiogenic protein-basic fibroblast growth factor-is stored within basement membrane," *American Journal of Pathology*, vol. 130, no. 2, pp. 393–400, 1988.

[94] A. M. Healy and I. M. Herman, "Density-dependent accumulation of basic fibroblast growth factor in the subendothelial matrix," *European Journal of Cell Biology*, vol. 59, no. 1, pp. 56–67, 1992.

[95] M. Murakami, L. T. Nguyen, Z. W. Zhang, et al., "The FGF system has a key role in regulating vascular integrity," *Journal of Clinical Investigation*, vol. 118, no. 10, pp. 3355–3366, 2008.

[96] P. Yu, F. H. Passam, D. M. Yu, G. Denyer, and S. A. Krilis, "β2-glycoprotein I inhibits vascular endothelial growth factor and basic fibroblast growth factor induced angiogenesis through its amino terminal domain," *Journal of Thrombosis and Haemostasis*, vol. 6, no. 7, pp. 1215–1223, 2008.

[97] P. Lindblom, H. Gerhardt, S. Liebner, et al., "Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall," *Genes and Development*, vol. 17, no. 15, pp. 1835–1840, 2003.

[98] M. C. Puri, J. Rossant, K. Alitalo, A. Bernstein, and J. Partanen, "The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells," *EMBO Journal*, vol. 14, no. 23, pp. 5884–5891, 1995.

[99] Y. Sato and D. B. Rifkin, "Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture," *Journal of Cell Biology*, vol. 109, no. 1, pp. 309–315, 1989.

[100] D. J. Dumont, T. P. Yamaguchi, R. A. Conlon, J. Rossant, and M. L. Breitman, "Tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors," *Oncogene*, vol. 7, no. 8, pp. 1471–1480, 1992.

[101] B. L. Hodous, S. D. Geuns-Meyer, P. E. Hughes, et al., "Synthesis, structural analysis, and SAR studies of triazine derivatives as potent, selective Tie-2 inhibitors," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 10, pp. 2886–2889, 2007.

[102] G. D. Yancopoulos, S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash, "Vascular-specific growth factors and blood vessel formation," *Nature*, vol. 407, no. 6801, pp. 242–248, 2000.

[103] J. Wang, K.-C. Wu, D.-X. Zhang, and D.-M. Fan, "Antisense angiopoietin-1 inhibits tumorigenesis and angiogenesis of gastric cancer," *World Journal of Gastroenterology*, vol. 12, no. 15, pp. 2450–2454, 2006.

[104] J. Oliner, H. Min, J. Leal, et al., "Suppression of angiogenesis and tumor growth by selective inhibition of angiopoietin-2," *Cancer Cell*, vol. 6, no. 5, pp. 507–516, 2004.

[105] M. Hangai, T. Murata, N. Miyawaki, et al., "Angiopoietin-1 upregulation by vascular endothelial growth factor in human retinal pigment epithelial cells," *Investigative Ophthalmology and Visual Science*, vol. 42, no. 7, pp. 1617–1625, 2001.

[106] U. Fearon, K. Griosios, A. Fraser, et al., "Angiopoietins, growth factors, and vascular morphology in early arthritis," *Journal of Rheumatology*, vol. 30, no. 2, pp. 260–268, 2003.

[107] K. Kuroda, A. Sapadin, T. Shoji, R. Fleischmajer, and M. Lebwohl, "Altered expression of angiopoietins and Tie2 endothelium receptor in psoriasis," *Journal of Investigative Dermatology*, vol. 116, no. 5, pp. 713–720, 2001.

[108] C. Morrissey, L. D. True, M. P. Roudier, et al., "Differential expression of angiogenesis associated genes in prostate cancer bone, liver and lymph node metastases," *Clinical and Experimental Metastasis*, vol. 25, no. 4, pp. 377–388, 2008.

[109] M. Hellstrom, M. Kalen, P. Lindahl, A. Abramsson, and C. Betsholtz, "Role of PDGF-B and PDGFR-β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse," *Development*, vol. 126, no. 14, pp. 3047–3055, 1999.

[110] P. Lindahl, B. R. Johansson, P. Leveen, and C. Betsholtz, "Pericyte loss and microaneurysm formation in PDGF-B-deficient mice," *Science*, vol. 277, no. 5323, pp. 242–245, 1997.

[111] W. G. Roberts, P. M. Whalen, E. Soderstrom, et al., "Antiangiogenic and antitumor activity of a selective PDGFR tyrosine kinase inhibitor, CP-673,451," *Cancer Research*, vol. 65, no. 3, pp. 957–966, 2005.

[112] H.-S. Chiang, W.-B. Wu, J.-Y. Fang, et al., "Lycopene inhibits PDGF-BB-induced signaling and migration in human dermal fibroblasts through interaction with PDGF-BB," *Life Sciences*, vol. 81, no. 21-22, pp. 1509–1517, 2007.

[113] J. Massague, "The transforming growth factor-beta family," *Annual Review of Cell Biology*, vol. 6, pp. 597–641, 1990.

[114] N. L. Thompson, K. C. Flanders, J. M. Smith, L. R. Ellingsworth, A. B. Roberts, and M. B. Sporn, "Expression of transforming growth factor-β1 in specific cells and tissues of adult and neonatal mice," *Journal of Cell Biology*, vol. 108, no. 2, pp. 661–669, 1989.

[115] P. Hau, P. Jachimczak, R. Schlingensiepen, et al., "Inhibition of TGF-β2 with ap 12009 in recurrent malignant gliomas: from preclinical to phase I/II studies," *Oligonucleotides*, vol. 17, no. 2, pp. 201–212, 2007.

[116] C. J. Drake, D. A. Cheresh, and C. D. Little, "An antagonist of integrin $\alpha_v\beta_3$ prevents maturation of blood vessels during embryonic neovascularization," *Journal of Cell Science*, vol. 108, no. 7, pp. 2655–2661, 1995.

[117] R. A. Swerlick, E. J. Brown, Y. Xu, K. H. Lee, S. Manos, and T. J. Lawley, "Expression and modulation of the vitronectin receptor on human dermal microvascular endothelial cells," *Journal of Investigative Dermatology*, vol. 99, no. 6, pp. 715–722, 1992.

[118] R. Silva, G. D'Amico, K. M. Hodivala-Dilke, and L. E. Reynolds, "Integrins: the keys to unlocking angiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 10, pp. 1703–1713, 2008.

[119] J. C. Gutheil, T. N. Campbell, P. R. Pierce, et al., "Targeted antiangiogenic therapy for cancer using vitaxin: a humanized monoclonal antibody to the integrin $\alpha_v\beta_3$," *Clinical Cancer Research*, vol. 6, no. 8, pp. 3056–3061, 2000.

[120] J. A. Posey, M. B. Khazaeli, A. DelGrosso, et al., "A pilot trial of Vitaxin, a humanized anti-vitronectin receptor (anti $\alpha_v\beta_3$) antibody in patients with metastatic cancer," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 16, no. 2, pp. 125–132, 2001.

[121] K. Mulgrew, K. Kinneer, X.-T. Yao, et al., "Direct targeting of $\alpha_v\beta_3$ integrin on tumor cells with a monoclonal antibody, Abegrin," *Molecular Cancer Therapeutics*, vol. 5, no. 12, pp. 3122–3129, 2006.

[122] L. B. Nabors, T. Mikkelsen, S. S. Rosenfeld, et al., "Phase I and correlative biology study of cilengitide in patients with recurrent malignant glioma," *Journal of Clinical Oncology*, vol. 25, no. 13, pp. 1651–1657, 2007.

[123] F. A. L. M. Eskens, H. Dumez, R. Hoekstra, et al., "Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of Cilengitide (EMD 121974), a novel inhibitor of the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in patients with advanced solid tumours," *European Journal of Cancer*, vol. 39, no. 7, pp. 917–926, 2003.

[124] Y. Funahashi, N. H. Sugi, T. Semba, et al., "Sulfonamide derivative, E7820, is a unique angiogenesis inhibitor suppressing an expression of integrin α2 subunit on endothelium," *Cancer Research*, vol. 62, no. 21, pp. 6116–6123, 2002.

[125] T. Semba, Y. Funahashi, N. Ono, et al., "An angiogenesis inhibitor E7820 shows broad-spectrum tumor growth inhibition in a xenograft model: possible value of integrin α2 on platelets as a biological marker," *Clinical Cancer Research*, vol. 10, no. 4, pp. 1430–1438, 2004.

[126] H. Jin and J. Varner, "Integrins: roles in cancer development and as treatment targets," *British Journal of Cancer*, vol. 90, no. 3, pp. 561–565, 2004.

[127] M. Klagsbrun and P. A. D'Amore, "Regulators of angiogenesis," *Annual Review of Physiology*, vol. 53, pp. 217–239, 1991.

[128] M. Corada, F. Liao, M. Lindgren, et al., "Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability," *Blood*, vol. 97, no. 6, pp. 1679–1684, 2001.

[129] M. Corada, M. Mariotti, G. Thurston, et al., "Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 17, pp. 9815–9820, 1999.

[130] F. Liao, Y. Li, W. O'Connor, et al., "Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis," *Cancer Research*, vol. 60, no. 24, pp. 6805–6810, 2000.

[131] M. Corada, L. Zanetta, F. Orsenigo, et al., "A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability," *Blood*, vol. 100, no. 3, pp. 905–911, 2002.

[132] F. Liao, J. F. Doody, J. Overholser, et al., "Selective targeting of angiogenic tumor vasculature by vascular endothelial-cadherin antibody inhibits tumor growth without affecting vascular permeability," *Cancer Research*, vol. 62, no. 9, pp. 2567–2575, 2002.

[133] U. Cavallaro, S. Liebner, and E. Dejana, "Endothelial cadherins and tumor angiogenesis," *Experimental Cell Research*, vol. 312, no. 5, pp. 659–667, 2006.

[134] C. May, J. R. Doody, R. Abdullah, et al., "Identification of a transiently exposed VE-cadherin epitope that allows for specific targeting of an antibody to the tumor neovasculature," *Blood*, vol. 105, no. 11, pp. 4337–4344, 2005.

[135] D. Navaratna, J. Maestas, P. G. McGuire, and A. Das, "Suppression of retinal neovascularization with an antagonist to vascular endothelial cadherin," *Archives of Ophthalmology*, vol. 126, no. 8, pp. 1082–1088, 2008.

[136] P. Lastres, J. Martín-Perez, C. Langa, and C. Bernabéu, "Phosphorylation of the human-transforming-growth-factor-β-binding protein endoglin," *Biochemical Journal*, vol. 301, no. 3, pp. 765–768, 1994.

[137] C. B. Pizarro, M. C. Oliveira, J. F. S. Pereira-Lima, et al., "Evaluation of angiogenesis in 77 pituitary adenomas using endoglin as a marker," *Neuropathology*, vol. 29, no. 1, pp. 40–44, 2009.

[138] C. Li, B. Guo, C. Bernabeu, and S. Kumar, "Angiogenesis in breast cancer: the role of transforming growth factor β and CD105," *Microscopy Research and Technique*, vol. 52, no. 4, pp. 437–449, 2001.

[139] K. J. Gordon and G. C. Blobe, "Role of transforming growth factor-β superfamily signaling pathways in human disease," *Biochimica et Biophysica Acta*, vol. 1782, no. 4, pp. 197–228, 2008.

[140] X. She, F. Matsuno, N. Harada, H. Tsai, and B. K. Seon, "Synergy between anti-endoglin (CD105) monoclonal antibodies and TGF-β in suppression of growth of human endothelial cells," *International Journal of Cancer*, vol. 108, no. 2, pp. 251–257, 2004.

[141] F. Matsuno, Y. Haruta, M. Kondo, H. Tsai, M. Barcos, and B. K. Seon, "Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies," *Clinical Cancer Research*, vol. 5, no. 2, pp. 371–382, 1999.

[142] M. Tsujie, S. Uneda, H. Tsai, and B. K. Seon, "Effective anti-angiogenic therapy of established tumors in mice by naked anti-human endoglin (CD105) antibody: differences in growth rate and therapeutic response between tumors growing at different sites," *International Journal of Oncology*, vol. 29, no. 5, pp. 1087–1094, 2006.

[143] L. Rosen, M. Gordon, H. Hurwitz, et al., "Early evidence of tolerability and clinical activity from a phase 1 study of TRC105 (anti-CD105 antibody) in patients with advanced refractory cancer," *European Journal of Cancer*, vol. 6, p. 126, 2008.

[144] D. M. Nettelbeck, D. W. Miller, V. Jérôme, et al., "Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105)," *Molecular Therapy*, vol. 3, no. 6, pp. 882–891, 2001.

[145] H. L. Weiner, L. H. Weiner, and J. L. Swain, "Tissue distribution and developmental expression of the messenger RNA encoding angiogenin," *Science*, vol. 237, no. 4812, pp. 280–282, 1987.

[146] M. L. Wahl, D. J. Kenan, M. Gonzalez-Gronow, and S. V. Pizzo, "Angiostatin's molecular mechanism: aspects of specificity and regulation elucidated," *Journal of Cellular Biochemistry*, vol. 96, no. 2, pp. 242–261, 2005.

[147] M. S. O'Reilly, L. Holmgren, Y. Shing, et al., "Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma," *Cell*, vol. 79, no. 2, pp. 315–328, 1994.

[148] Y. Cao, R. W. Ji, D. Davidson, et al., "Kringle domains of human angiostatin: characterization of the anti-proliferative activity on endothelial cells," *Journal of Biological Chemistry*, vol. 271, no. 46, pp. 29461–29467, 1996.

[149] N. Veitonmaki, R. Cao, L.-H. Wu, et al., "Endothelial cell surface ATP synthase-triggered caspase-apoptotic pathway is essential for K1-5-induced antiangiogenesis," *Cancer Research*, vol. 64, no. 10, pp. 3679–3686, 2004.

[150] B. Troyanovsky, T. Levchenko, G. Mansson, O. Matvijenko, and L. Holmgren, "Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation," *Journal of Cell Biology*, vol. 152, no. 6, pp. 1247–1254, 2001.

[151] M. S. O'Reilly, T. Boehm, Y. Shing, et al., "Endostatin: an endogenous inhibitor of angiogenesis and tumor growth," *Cell*, vol. 88, no. 2, pp. 277–285, 1997.

[152] T. Tanaka, Y. Cao, J. Folkman, and H. A. Fine, "Viral vector-targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA," *Cancer Research*, vol. 58, no. 15, pp. 3362–3369, 1998.

[153] S. Ambs, S. Dennis, J. Fairman, M. Wright, and J. Papkoff, "Inhibition of tumor growth correlates with the expression level of a human angiostatin transgene in transfected B16f10 melanoma cells," *Cancer Research*, vol. 59, no. 22, pp. 5773–5777, 1999.

[154] Y.-H. Chen, H.-L. Wu, C. Li, et al., "Anti-angiogenesis mediated by angiostatin K1-3, K1-4 and K1-4.5. Involvement of p53, FasL, AKT and mRNA deregulation," *Thrombosis and Haemostasis*, vol. 95, no. 4, pp. 668–677, 2006.

[155] L. V. Beerepoot, E. O. Witteveen, G. Groenewegen, et al., "Recombinant human angiostatin by twice-daily subcutaneous injection in advanced cancer: a pharmacokinetic and long-term safety study," *Clinical Cancer Research*, vol. 9, no. 11, pp. 4025–4033, 2003.

[156] A. Kurup, C.-W. Lin, D. J. Murry, et al., "Recombinant human angiostatin (rhAngiostatin) in combination with paclitaxel and carboplatin in patients with advanced non-small-cell lung cancer: a phase II study from Indiana University," *Annals of Oncology*, vol. 17, no. 1, pp. 97–103, 2006.

[157] K. S. Kim, S. Kim, J. S. Park, Y. G. Kwon, and Y. S. Park, "Inhibition of B16BL6 tumor progression by coadministration of recombinant angiostatin K1-3 and endostatin genes with cationic liposomes," *Cancer Gene Therapy*, vol. 11, no. 6, pp. 441–449, 2004.

[158] W. Wen, M. A. Moses, D. Wiederschain, J. L. Arbiser, and J. Folkman, "The generation of endostatin is mediated by elastase," *Cancer Research*, vol. 59, no. 24, pp. 6052–6056, 1999.

[159] U. Felbor, L. Dreier, R. A. R. Bryant, H. L. Ploegh, B. R. Olsen, and W. Mothes, "Secreted cathepsin L generates endostatin from collagen XVIII," *EMBO Journal*, vol. 19, no. 6, pp. 1187–1194, 2000.

[160] S. A. Karumanchi, V. Jha, R. Ramchandran, et al., "Cell surface glypicans are low-affinity endostatin receptors," *Molecular Cell*, vol. 7, no. 4, pp. 811–822, 2001.

[161] M. Dhanabal, R. Ramchandran, R. Volk, et al., "Endostatin: yeast production, mutants, and antitumor effect in renal cell carcinoma," *Cancer Research*, vol. 59, no. 1, pp. 189–197, 1999.

[162] A. Hajitou, C. Grignet, L. Devy, et al., "The antitumoral effect of endostatin and angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cells," *The FASEB Journal*, vol. 16, no. 13, pp. 1802–1804, 2002.

[163] Y.-M. Kim, S. Hwang, Y.-M. Kim, et al., "Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1," *Journal of Biological Chemistry*, vol. 277, no. 31, pp. 27872–27879, 2002.

[164] J.-I. Hanai, M. Dhanabal, S. Ananth Karumanchi, et al., "Endostatin causes G1 arrest of endothelial cells through inhibition of cyclin D1," *Journal of Biological Chemistry*, vol. 277, no. 19, pp. 16464–16469, 2002.

[165] H. Y. Kang, D. Shim, S. S. Kang, S.-I. Chang, and H. Y. Kim, "Protein kinase B inhibits endostatin-induced apoptosis in HUVECs," *Journal of Biochemistry and Molecular Biology*, vol. 39, no. 1, pp. 97–104, 2006.

[166] A. Abdollahi, P. Hahnfeldt, C. Maercker, et al., "Endostatin's antiangiogenic signaling network," *Molecular Cell*, vol. 13, no. 5, pp. 649–663, 2004.

[167] T. S. Zorick, Z. Mustacchi, S. Y. Bando, et al., "High serum endostatin levels in Down syndrome: implications for improved treatment and prevention of solid tumours," *European Journal of Human Genetics*, vol. 9, no. 11, pp. 811–814, 2001.

[168] Q. Yang, S. A. Rasmussen, and J. M. Friedman, "Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study," *The Lancet*, vol. 359, no. 9311, pp. 1019–1025, 2002.

[169] B. Chadefaux, I. Ceballos, M. Hamet, et al., "Is absence of atheroma in down syndrome due to decreased homocysteine levels?" *The Lancet*, vol. 2, no. 8613, p. 741, 1988.

[170] T. Fulcher, M. Griffin, S. Crowley, R. Firth, R. Acheson, and N. O'Meara, "Diabetic retinopathy in Down's syndrome," *British Journal of Ophthalmology*, vol. 82, no. 4, pp. 407–409, 1998.

[171] M. Kaya, T. Wada, S. Nagoya, and T. Yamashita, "Prevention of postoperative progression of pulmonary metastases in osteosarcoma by antiangiogenic therapy using endostatin," *Journal of Orthopaedic Science*, vol. 12, no. 6, pp. 562–567, 2007.

[172] M. J. Mellon, K.-H. Bae, C. E. Steding, J. A. Jimenez, C. Kao, and T. A. Gardner, "Suppression of renal cell carcinoma growth and metastasis with sustained antiangiogenic gene therapy," *Human Gene Therapy*, vol. 19, no. 5, pp. 487–495, 2008.

[173] R. Twombly, "First clinical trials of endostatin yield lukewarm results," *Journal of the National Cancer Institute*, vol. 94, no. 20, pp. 1520–1521, 2002.

[174] J. P. Thomas, R. Z. Arzoomanian, D. Alberti, et al., "Phase I pharmacokinetic and pharmacodynamic study of recombinant human endostatin in patients with advanced solid tumors," *Journal of Clinical Oncology*, vol. 21, no. 2, pp. 223–231, 2003.

[175] R. S. Herbst, K. R. Hess, H. T. Tran, et al., "Phase I study of recombinant human endostatin in patients with advanced solid tumors," *Journal of Clinical Oncology*, vol. 20, no. 18, pp. 3792–3803, 2002.

[176] M. H. Kulke, E. K. Bergsland, D. P. Ryan, et al., "Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors," *Journal of Clinical Oncology*, vol. 24, no. 22, pp. 3555–3561, 2006.

[177] Y. Sun, J. Wang, Y. Liu, et al., "Results of phase III trial of rh-endostatin (YH-16) in advanced non-small cell lung cancer (NSCLC) patients," *Journal of Clinical Oncology*, vol. 23, p. 7138, 2005.

[178] Y. Hamano and R. Kalluri, "Tumstatin, the NC1 domain of $\alpha 3$ chain of type IV collagen, is an endogenous inhibitor of pathological angiogenesis and suppresses tumor growth," *Biochemical and Biophysical Research Communications*, vol. 333, no. 2, pp. 292–298, 2005.

[179] Y. Maeshima, P. C. Colorado, and R. Kalluri, "Two RGD-independent $\alpha_v\beta_3$ integrin binding sites on tumstatin regulate distinct anti-tumor properties," *Journal of Biological Chemistry*, vol. 275, no. 31, pp. 23745–23750, 2000.

[180] H. P. Eikesdal, H. Sugimoto, G. Birrane, et al., "Identification of amino acids essential for the antiangiogenic activity of tumstatin and its use in combination antitumor activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 39, pp. 15040–15045, 2008.

[181] T. Goto, H. Ishikawa, K. Matsumoto, et al., "Tum-1, a tumstatin fragment, gene delivery into hepatocellular carcinoma suppresses tumor growth through inhibiting angiogenesis," *International Journal of Oncology*, vol. 33, no. 1, pp. 33–40, 2008.

[182] A. Bikfalvi, "Platelet factor 4: an inhibitor of angiogenesis," *Seminars in Thrombosis and Hemostasis*, vol. 30, no. 3, pp. 379–385, 2004.

[183] G. Gentilini, N. E. Kirschbaum, J. A. Augustine, R. H. Aster, and G. P. Visentin, "Inhibition of human umbilical vein endothelial cell proliferation by the CXC chemokine, platelet factor 4 (PF4), is associated with impaired downregulation of p21(Cip1/WAF1)," *Blood*, vol. 93, no. 1, pp. 25–33, 1999.

[184] M. B. Zucker and I. R. Katz, "Platelet factor 4: production, structure, and physiologic and immunologic action," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 198, no. 2, pp. 693–702, 1991.

[185] G. R. Bernard, J.-L. Vincent, P.-F. Laterre, et al., "Efficacy and safety of recombinant human activated protein C for severe sepsis," *The New England Journal of Medicine*, vol. 344, no. 10, pp. 699–709, 2001.

[186] X. Li, L. Jiang, Y. Wang, et al., "Inhibition of angiogenesis by a novel small peptide consisting of the active fragments of platelet factor-4 and vasostatin," *Cancer Letters*, vol. 256, no. 1, pp. 29–32, 2007.

[187] D. J. Good, P. J. Polverini, F. Rastinejad, et al., "A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 17, pp. 6624–6628, 1990.

[188] L. C. Armstrong and P. Bornstein, "Thrombospondins 1 and 2 function as inhibitors of angiogenesis," *Matrix Biology*, vol. 22, no. 1, pp. 63–71, 2003.

[189] D. W. Dawson, S. F. A. Pearce, R. Zhong, R. L. Silverstein, W. A. Frazier, and N. P. Bouck, "CD36 mediates the in vitro inhibitory effects of thrombospondin-1 on endothelial cells," *Journal of Cell Biology*, vol. 138, no. 3, pp. 707–717, 1997.

[190] B. Jimenez, O. V. Volpert, S. E. Crawford, M. Febbraio, R. L. Silverstein, and N. Bouck, "Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1," *Nature Medicine*, vol. 6, no. 1, pp. 41–48, 2000.

[191] J. E. Nor, R. S. Mitra, M. M. Sutorik, D. J. Mooney, V. P. Castle, and P. J. Polverini, "Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway," *Journal of Vascular Research*, vol. 37, no. 3, pp. 209–218, 2000.

[192] R. Hoekstra, F. Y. de Vos, F. A. Eskens, et al., "Phase I safety, pharmacokinetic, and pharmacodynamic study of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 in patients with advanced cancer," *Journal of Clinical Oncology*, vol. 23, no. 22, pp. 5188–5197, 2005.

[193] J. A. Gietema, R. Hoekstra, F. Y. F. L. de Vos, et al., "A phase I study assessing the safety and pharmacokinetics of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 with gemcitabine and cisplatin in patients with solid tumors," *Annals of Oncology*, vol. 17, no. 8, pp. 1320–1327, 2006.

[194] R. Hoekstra, F. Y. F. L. de Vos, F. A. L. M. Eskens, et al., "Phase I study of the thrombospondin-1-mimetic angiogenesis inhibitor ABT-510 with 5-fluorouracil and leucovorin: a safe combination," *European Journal of Cancer*, vol. 42, no. 4, pp. 467–472, 2006.

[195] P. Valente, G. Fassina, A. Melchiori, et al., "TIMP-2 overexpression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis," *International Journal of Cancer*, vol. 75, no. 2, pp. 246–253, 1998.

[196] K. Brew, D. Dinakarpandian, and H. Nagase, "Tissue inhibitors of metalloproteinases: evolution, structure and function," *Biochimica et Biophysica Acta*, vol. 1477, no. 1-2, pp. 267–283, 2000.

[197] H. A. Fernandez, K. Kallenbach, G. Seghezzi, et al., "Inhibition of endothelial cell migration by gene transfer of tissue inhibitor of metalloproteinases-1," *Journal of Surgical Research*, vol. 82, no. 2, pp. 156–162, 1999.

[198] C. Chetty, S. S. Lakka, P. Bhoopathi, S. Kunigal, R. Geiss, and J. S. Rao, "Tissue inhibitor of metalloproteinase 3 suppresses tumor angiogenesis in matrix metalloproteinase 2-down-regulated lung cancer," *Cancer Research*, vol. 68, no. 12, pp. 4736–4745, 2008.

[199] M. Miyagi, K. Aoyagi, S. Kato, and K. Shirouzu, "The TIMP-1 gene transferred through adenovirus mediation shows a suppressive effect on peritoneal metastases from gastric cancer," *International Journal of Clinical Oncology*, vol. 12, no. 1, pp. 17–24, 2007.

[200] R. M. Strieter, P. J. Polverini, S. L. Kunkel, et al., "The functional role of the ELR motif in CXC chemokine-mediated angiogenesis," *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 27348–27357, 1995.

[201] R. K. Puri and J. P. Siegel, "Interleukin-4 and cancer therapy," *Cancer Investigation*, vol. 11, no. 4, pp. 473–486, 1993.

[202] M. S. Topp, C. A. Papadimitriou, F. Eitelbach, et al., "Recombinant human interleukin 4 has antiproliferative activity on human tumor cell lines derived from epithelial and nonepithelial histologies," *Cancer Research*, vol. 55, no. 10, pp. 2173–2176, 1995.

[203] R. I. Tepper, R. L. Coffman, and P. Leder, "An eosinophil-dependent mechanism for the antitumor effect of interleukin-4," *Science*, vol. 257, no. 5069, pp. 548–551, 1992.

[204] O. V. Volpert, T. Fong, A. E. Koch, et al., "Inhibition of angiogenesis by interleukin 4," *Journal of Experimental Medicine*, vol. 188, no. 6, pp. 1039–1046, 1998.

[205] C. S. Haas, M. A. Amin, B. B. Allen, et al., "Inhibition of angiogenesis by interleukin-4 gene therapy in rat adjuvant-induced arthritis," *Arthritis and Rheumatism*, vol. 54, no. 8, pp. 2402–2414, 2006.

[206] D. Ribatti, A. Vacca, M. Iurlaro, R. Ria, L. Roncali, and F. Dammacco, "Human recombinant interferon alpha-2a inhibits angiogenesis of chick area vasculosa in shell-less culture," *International Journal of Microcirculation-Clinical and Experimental*, vol. 16, no. 4, pp. 165–169, 1996.

[207] R. K. Singh, M. Gutman, C. D. Bucana, R. Sanchez, N. Llansa, and I. J. Fidler, "Interferons α and β down-regulate the expression of basic fibroblast growth factor in human carcinomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 10, pp. 4562–4566, 1995.

[208] T. N. Sato, Y. Tozawa, U. Deutsch, et al., "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation," *Nature*, vol. 376, no. 6535, pp. 70–74, 1995.

[209] A. Hauschild, R. Dummer, S. Ugurel, et al., "Combined treatment with pegylated interferon-a-2a and dacarbazine in patients with advanced metastatic melanoma a phase 2 study," *Cancer*, vol. 113, no. 6, pp. 1404–1411, 2008.

[210] S. George, T. E. Hutson, T. Mekhail, et al., "Phase I trial of PEG-interferon and recombinant IL-2 in patients with metastatic renal cell carcinoma," *Cancer Chemotherapy and Pharmacology*, vol. 62, no. 2, pp. 347–354, 2008.

[211] B. Melichar, P. Koralewski, A. Ravaud, et al., "First-line bevacizumab combined with reduced dose interferon-α2a is active in patients with metastatic renal cell carcinoma," *Annals of Oncology*, vol. 19, no. 8, pp. 1470–1476, 2008.

[212] R. M. Bukowski, C. Tendler, D. Cutler, E. Rose, M. M. Laughlin, and P. Statkevich, "Treating cancer with PEG intron: pharmacokinetic profile and dosing guidelines for an improved interferon-alpha-2b formulation," *Cancer*, vol. 95, no. 2, pp. 389–396, 2002.

[213] Y. S. Chang, E. di Tomaso, D. M. McDonald, R. Jones, R. K. Jain, and L. L. Munn, "Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 26, pp. 14608–14613, 2000.

[214] L. E. Benjamin, D. Golijanin, A. Itin, D. Pode, and E. Keshet, "Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal," *Journal of Clinical Investigation*, vol. 103, no. 2, pp. 159–165, 1999.

[215] P. Carmeliet, N. Mackman, L. Moons, et al., "Role of tissue factor in embryonic blood vessel development," *Nature*, vol. 383, no. 6595, pp. 73–75, 1996.

[216] M. R. Mancuso, R. Davis, S. M. Norberg, et al., "Rapid vascular regrowth in tumors after reversal of VEGF inhibition," *Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2610–2621, 2006.

[217] R. K. Jain and M. F. Booth, "What brings pericytes to tumor vessels?" *Journal of Clinical Investigation*, vol. 112, no. 8, pp. 1134–1136, 2003.

[218] T. Kamba and D. M. McDonald, "Mechanisms of adverse effects of anti-VEGF therapy for cancer," *British Journal of Cancer*, vol. 96, no. 12, pp. 1788–1795, 2007.

[219] G. Bergers, S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan, "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1287–1295, 2003.

[220] Y. Takahashi, Y. Kitadai, C. D. Bucana, K. R. Cleary, and L. M. Ellis, "Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer," *Cancer Research*, vol. 55, no. 18, pp. 3964–3968, 1995.

[221] M. Toi, S. Kondo, H. Suzuki, et al., "Quantitative analysis of vascular endothelial growth factor in primary breast cancer," *Cancer*, vol. 77, no. 6, pp. 1101–1106, 1996.

[222] T. G. Graeber, C. Osmanian, T. Jacks, et al., "Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours," *Nature*, vol. 379, no. 6560, pp. 88–91, 1996.

[223] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. Susan Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, 1983.

[224] S. Kohn, J. A. Nagy, H. F. Dvorak, and A. M. Dvorak, "Pathways of macromolecular tracer transport across venules and small veins: structural basis for the hyperpermeability of tumor blood vessels," *Laboratory Investigation*, vol. 67, no. 5, pp. 596–607, 1992.

[225] P. C. Maisonpierre, C. Suri, P. F. Jones, et al., "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55–60, 1997.

[226] D. Zagzag, A. Hooper, D. R. Friedlander, et al., "In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis," *Experimental Neurology*, vol. 159, no. 2, pp. 391–400, 1999.

[227] J. Holash, P. C. Maisonpierre, D. Compton, et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF," *Science*, vol. 284, no. 5422, pp. 1994–1998, 1999.

[228] J. Folkman, E. Merler, C. Abernathy, and G. Williams, "Isolation of a tumor factor responsible or angiogenesis," *Journal of Experimental Medicine*, vol. 133, no. 2, pp. 275–288, 1971.

[229] A. Compagni, P. Wilgenbus, M.-A. Impagnatiello, M. Cotten, and G. Christofori, "Fibroblast growth factors are required for efficient tumor angiogenesis," *Cancer Research*, vol. 60, no. 24, pp. 7163–7169, 2000.

[230] C. F. Deroanne, A. Hajitou, C.-M. Calberg-Bacq, B. V. Nusgens, and C. M. Lapiere, "Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression," *Cancer Research*, vol. 57, no. 24, pp. 5590–5597, 1997.

[231] G. T. Stavri, I. C. Zachary, P. A. Baskerville, J. F. Martin, and J. D. Erusalimsky, "Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells: synergistic interaction with hypoxia," *Circulation*, vol. 92, no. 1, pp. 11–14, 1995.

[232] S. J. Mandriota and M. S. Pepper, "Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor," *Journal of Cell Science*, vol. 110, no. 18, pp. 2293–2302, 1997.

[233] K. A. Waite and C. Eng, "From developmental disorder to heritable cancer: it's all in the BMP/TGF-β family," *Nature Reviews Genetics*, vol. 4, no. 10, pp. 763–773, 2003.

[234] T. Ota, M. Fujii, T. Sugizaki, et al., "Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells," *Journal of Cellular Physiology*, vol. 193, no. 3, pp. 299–318, 2002.

[235] J. A. Tuxhorn, S. J. McAlhany, F. Yang, T. D. Dang, and D. R. Rowley, "Inhibition of transforming growth factor-β activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model," *Cancer Research*, vol. 62, no. 21, pp. 6021–6025, 2002.

[236] I. Vlodavsky, M. Elkin, O. Pappo, et al., "Mammalian heparanase as mediator of tumor metastasis and angiogenesis," *Israel Medical Association Journal*, vol. 2, pp. 37–45, 2000.

[237] I. Vlodavsky, O. Goldshmidt, E. Zcharia, et al., "Mammalian heparanase: involvement in cancer metastasis, angiogenesis and normal development," *Seminars in Cancer Biology*, vol. 12, no. 2, pp. 121–129, 2002.

[238] M. Luca, S. Huang, J. E. Gershenwald, R. K. Singh, R. Reich, and M. Bar-Eli, "Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis," *American Journal of Pathology*, vol. 151, no. 4, pp. 1105–1113, 1997.

[239] A. Yuan, P.-C. Yang, C.-J. Yu, et al., "Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer," *American Journal of Respiratory and Critical Care Medicine*, vol. 162, no. 5, pp. 1957–1963, 2000.

[240] M. Bar-Eli, "Role of interleukin-8 in tumor growth and metastasis of human melanoma," *Pathobiology*, vol. 67, no. 1, pp. 12–18, 1999.

[241] Y. Kitadai, Y. Takahashi, K. Haruma, et al., "Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice," *British Journal of Cancer*, vol. 81, no. 4, pp. 647–653, 1999.

[242] J. Folkman, "Is angiogenesis an organizing principle in biology and medicine?" *Journal of Pediatric Surgery*, vol. 42, no. 1, pp. 1–11, 2007.

[243] R. S. Kerbel, "Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents," *BioEssays*, vol. 13, no. 1, pp. 31–36, 1991.

[244] R. S. Kerbel, "A cancer therapy resistant to resistance," *Nature*, vol. 390, no. 6658, pp. 335–336, 1997.

[245] O. Casanovas, D. J. Hicklin, G. Bergers, and D. Hanahan, "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors," *Cancer Cell*, vol. 8, no. 4, pp. 299–309, 2005.

[246] Y. Mizukami, W.-S. Jo, E.-M. Duerr, et al., "Induction of interleukin-8 preserves the angiogenic response in HIF-1α-deficient colon cancer cells," *Nature Medicine*, vol. 11, no. 9, pp. 992–997, 2005.

[247] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ^+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[248] K. K. Hirschi and P. A. D'Amore, "Control of angiogenesis by the pericyte: molecular mechanisms and significance," *EXS*, vol. 79, pp. 419–428, 1997.

[249] J.-D. Raguse, H. J. Gath, J. Bier, H. Riess, and H. Oettle, "Cilengitide (EMD 121974) arrests the growth of a heavily pretreated highly vascularised head and neck tumour," *Oral Oncology*, vol. 40, no. 2, pp. 228–230, 2004.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 586905, 13 pages
doi:10.1155/2010/586905

Review Article

Extracellular Matrix Proteins and Tumor Angiogenesis

N. E. Campbell,[1] L. Kellenberger,[1] J. Greenaway,[2] R. A. Moorehead,[1] N. M. Linnerth-Petrik,[3] and J. Petrik[1]

[1] *Department of Biomedical Sciences, University of Guelph, Guelph, ON, Canada N1G 2W1*
[2] *CIHR Group in Matrix Biology, University of Toronto, ON, Canada M5G 1G6*
[3] *Department of Pathobiology, University of Guelph, Guelph, ON, Canada N1G 2W1*

Correspondence should be addressed to J. Petrik, jpetrik@uoguelph.ca

Received 2 November 2009; Accepted 26 May 2010

Academic Editor: Kalpna Gupta

Tumor development is a complex process that relies on interaction and communication between a number of cellular compartments. Much of the mass of a solid tumor is comprised of the stroma which is richly invested with extracellular matrix. Within this matrix are a host of matricellular proteins that regulate the expression and function of a myriad of proteins that regulate tumorigenic processes. One of the processes that is vital to tumor growth and progression is angiogenesis, or the formation of new blood vessels from preexisting vasculature. Within the extracellular matrix are structural proteins, a host of proteases, and resident pro- and antiangiogenic factors that control tumor angiogenesis in a tightly regulated fashion. This paper discusses the role that the extracellular matrix and ECM proteins play in the regulation of tumor angiogenesis.

1. Introduction

Conventional cancer treatments typically target the epithelial component of carcinomas, which represent a varying proportion of tumors. More recently, a paradigm shift has occurred wherein epithelial cells are being evaluated as a functional and complex system along with stromal components [1]. These stromal cells are typically recruited by tumors and include fibroblasts, endothelial cells, smooth muscle cells and immune cells. Recruitment of stromal factors not only provide a structural extracellular matrix (ECM) scaffold that provides structural support, but also generates pleiotrophic effects which contribute to tumorigenecity, thus a tumor phenotype is not only characteristic of the transformed cells, but also the ECM and stroma surrounding the cells. Stromal recruitment and alterations in the ECM result in complex communication networks between cancerous cells which may provide ideal targets for future therapies [2, 3].

The extracellular matrix provides structural support for cells within a tumor providing anchorage for cells and separating tissues, however it also acts homoestatically to mediate communication between cells and contributes survival and differentiation signals. The ECM contains a basement membrane that separates cells from the interstitial matrix. At this junction, molecular components of the ECM can be found including proteoglycan, nonproteoglycan polysaccharides, and various fibrous proteins. The carbohydrate polymers and proteins are organized in such a way that an interlocking meshwork exists and is the basic framework for the ECM.

It is known that the ECM has structural components that neoplastic cells can exploit to create a protumor environment. Studies have found that injecting tumorigenic cells into the site of origin (orthotopically) results in a more replicable onset and progression in a variety of tumors in different mouse models [2, 3]. Others have reported the necessity of the native microenvironment in order to accurately mimic the metastatic disease [4]. Our laboratory has investigated this relationship between tumor cells and the host stroma using an orthotopic model of epithelial ovarian cancer. In this model, transformed epithelial cells are injected directly under the ovarian bursa where they can then colonize and grow. In this model, the ovarian epithelial cells interact with the ovarian stroma and result in the formation of primary serous adenocarcinoma, numerous secondary

peritoneal lesions, and the formation of abdominal ascites which closely replicate the features of human epithelial ovarian cancer. The importance of the interaction between the epithelial cells and the tumor stroma is apparent as this interaction causes a reprogramming of the epithelial cells, increasing their metastatic potential; when the tumorigenic epithelial cells were injected intraperitoneally instead of orthotopically, the lack of stromal interaction resulted in the formation of small spheroids within the abdomen, as opposed to well-differentiated peritoneal lesions generated with the orthotopic model [5].

2. Tumor Angiogenesis

Investigation into the roles of the tumor stroma have established that the ECM plays an important role in tumor vascularization [6]. Cancer cells which have acquired several mutations have the ability to be: self-sufficient in growth signalling via activation of oncogenes or loss of tumor suppressor genes, insensitive to antigrowth signals, unresponsive to apoptotic events, capable of limitless replication, and tumorigenic. Although all of these neoplastic properties are necessary for tumor development, they are not sufficient to become clinically relevant cancers unless the tumor is able to recruit its own blood supply [8]. In most tumors, new blood vessels are formed through a process called angiogenesis, in which new blood vessels form from preexisting vasculature [7, 8]. Tumors require the ability to establish an angiogenic phenotype, which occurs via the angiogenic switch [9]. The angiogenic switch is regulated by a balance between pro and antiangiogenic factors and when the balance is disrupted, pathological conditions such as cancer can result [10].

Proangiogenic factors such as growth factors and cytokines stimulate the formation of tumor blood vessels. Two of the most critical and widely studied proangiogenic factors include vascular endothelial growth factor (VEGF) [11] and basic fibroblast growth factor (bFGF) [12, 13] (reviewed in [14]). These factors stimulate endothelial cells to produce and export various proteolytic enzymes that enable cell invasion and metastasis by degradation of the extracellular and intracellular proteins of the ECM, allowing endothelial cells to proliferate, and migrate into surrounding tissues, [15]. Under normal physiologic conditions these proteolytic enzymes are involved in wound healing and matrix remodelling. Overexpression of VEGF [16, 17] and bFGF [18–21] have been shown to promote tumorigenic properties by triggering angiogenesis. Therefore, inhibition of proangiogenic factors or upregulation of antiangiogenic factors could lead to an effective therapeutic approach. Ultimately, the angiogenic shift must favour expression of antiangiogenic factors in order for vessel regression to occur.

3. Regulation of Angiogenesis by the ECM

The angiogenic process is complex and involves endothelial cell proliferation and migration, degradation of the blood vessel basement membrane and associated extracellular matrix. Following endothelial cell proliferation and early tube formation, newly formed vessels differentiate into arterioles and venules, necessary to provide blood supply to tumors [8, 22]. Remodelling of the ECM is an integral component of the angiogenic process. A variety of mechanisms have been documented about how the ECM plays a pivotal role in regulating angiogenesis (reviewed by [23]). The ECM is composed of a network of fibrous proteins and glycosaminoglycans (GAGs). GAGs are carbohydrate polymers that form proteoglycans that are involved in both keeping the EMC and surrounding cells hydrated and trapping and storing growth factors. Therefore, GAG molecules may employ a variety of regulatory effects on the accessibility of angiogenic factors [24, 25]. Release of proteolytic enzymes leading to the degradation of the ECM results in the release of ECM-bound growth factors such as VEGF ([26]; reviewed by [25]). Heparan sulfate glycosaminoglycans (HSGAGs) are a diverse family of GAGs that include the syndecans, glypicans, perlecans and agrins. Members of this group of proteins play a key role in the modulation of angiogenesis. HSGAGs that are present on the surface of endothelial cells have the ability to either inhibit or promote neovascularization by mediating signalling through VEGF receptors [27] or bFGF [28, 29]. As well, HSGAGs can also act as a binding site for antiangiogenic factor endostatin [30]. Fibrous proteins include collagen and elastin both of which are well characterized structural proteins components of the skin, connective tissue and blood vessel walls. Collagen involvement in angiogenesis has recently received a great deal of attention. Metabolic inhibition of the synthesis of type I and IV inhibits capillary formation on the CAM [31]. Data has shown that components of the ECM can have both pro and antiangiogenic effects. Proteases involved in degrading the ECM and often activated during remodelling can promote angiogenesis by stimulating migration of endothelial cells or by releasing proangiogenic growth factors [32, 33]. Angiogenesis can also be inhibited when antiangiogenic compounds are secreted from the fragments formed during proteolytic cleavage of matrix molecules [34, 35]. This paper will focus on proteases and matrix-related molecules that have been found to influence tumor angiogenesis.

4. ECM Proteins Involved in Remodeling and Tumor Angiogenesis

Major ECM proteins that promote angiogenesis include collagen, laminin and fibronectin. Collagen IV and laminin are predominate proteins of the basal lamina, a 50 nm wide ECM that provides structural support for endothelial cells and creates a separation from the adjacent perivascular cells. The majority of ECM proteins mediate angiogenesis through arginine-glycine-aspartic acid (RGD) motifs which bind to integrins that mediate outside in signalling. Endothelial cells in a resting quiescent state exhibit the lowest mitotic index of cells within the body [36]. Induction of angiogenesis and remodelling of the ECM is characterized by increased permeability and cytoskeletal and cell-to-cell contact changes which results in newly formed focal contacts mediated

primarily by integrins. Fibronectin is produced by both activated endothelial and smooth muscle cells, levels are augmented during angiogenesis by delivery of fibronectin from circulation by increased vascular permeability. Fibronectin contains the arginine-glycine-aspartic acid (RGD) protein motifs that bind to the integrin $\alpha5\beta1$. This integrin receptor is markedly up regulated during angiogenesis and is overexpressed in endothelial cells in tumors. Mice genetically engineered to lack the $\alpha5$ integrin subunit die during embryogenesis due to fail of the yolk sac vasculature to form properly [37–39]. The collagen integrin receptors ($\alpha1\beta1$ and $\alpha2\beta1$) also play a positive role for angiogenesis. Use of a potent and specific $\alpha1\beta1$ inhibitor Obtustatin, is able to inhibit angiogenesis in the chick chorioallantoic membrane (CAM) assay and in the Lewis lung syngeneic model [40, 41]. Finally laminin peptides derived from the $\alpha1$ chain mediate angiogenesis *in vitro* [41–43]. Receptors involved in laminins proangiogenic properties have not been fully elucidated, $\alpha6\beta1$ receptor may play important role in tube formation [44].

Proteolytic activity of the ECM facilitates degradation of the basement membrane, matrix remodelling, and cell migration and invasion, all of which are essential for angiogenesis. In order for angiogenesis to occur, activation of proteases is essential. However, aberrantly excessive degradation of the ECM does not permit developing vessels [32]. Therefore, in a similar fashion to the regulation of angiogenic processes by angiogenesis by pro and antiangiogenic factors, activation of proteolytic enzymes of the ECM is also tightly regulated. There are two main classes of enzymes that have been studied for their abilities to degrade and remodel the ECM: the plasminogen activator (PA)/plasmin system and matrix metalloproteinases (MMPs), which have been reviewed for their roles in angiogenesis [45]. To date, a number of MMPs have been shown to degrade the vascular basement membrane and matrix in order to permit vascular sprouting. The activity of these proteases is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs) which generally have antiangiogenic properties.

MMPs are a group of zinc-dependent proteases that are involved in the degradation and remodelling of the ECM in order for processes such as angiogenesis to occur. The MMP family consists of over 20 proteases and many of them have been implicated in tumorigenesis [32]. Those that have been reported to have proangiogenic actions are described below. By degrading the matrix, MMPs not only provide physical space within the matrix for migration, but also provide proliferation and differentiation signals to endothelial cells by releasing cryptic sites on ECM proteins and soluble growth factors. The involvement of MMPs in angiogenesis has been supported through the use of knockout mice. Studies involving knockout mice of the gelatinase type MMPs (MMP-2 and MMP-9) have shown tumor angiogenesis [46]. Researchers have subsequently shown that MMP-2 promotes an angiogenic phenotype, while suppression of the protease inhibited angiogenesis [47]. MMPs cleave ECM bound growth factors including proangiogenic factors [48]. Various MMPs have been found to cleave heparin bound growth factors such as VEGF and bFGF, releasing soluble forms which then exert proangiogenic actions and stimulate the formation of new blood vessels [49]. In particular, it was reported that MMP-9 stimulates the production of the proangiogenic growth factor VEGF [50, 51]. Other members of the MMP family have also been shown to enhance the effectiveness of proangiogenic growth factors. Membrane bound MMPs also mediate proangiogenic effects. Corneal pocket implantation assays revealed that MT1-MMP can potentiate the neovascularization effects of basic fibroblast growth factor (bFGF) [52]. When cells that do not normally express MT1-MMP were transfected with the matrix protease, angiogenesis was stimulated and *in vivo*, neovascularization was associated with an increase in expression of VEGF [53]. In a xenograft model of Glioma, cells that overexpressed MT1-MMP were capable of remodelling a matrix *in vitro* and had increased levels of angiogenesis in vivo. Consistent with other studies, these changes in angiogenesis were correlated with an increase in VEGF [54]. This is maintained during situations in which MMPs are decreased, ultimately resulting in a reduction in the levels of proangiogenic growth factors [55]. Stromal recruitment of fibroblasts and immune cells such as macrophages can also modulate MMP remodelling of the stroma altering the signalling that ultimately results in increases and decreases of angiogenesis. Recently loss of PTEN signalling in stromal fibroblasts results in the induction of ECM remodelling by the increase of the transcriptional factor Ets2 which is an upstream target of MMP9 [56]. Protease mediated cleavage of the ECM also results in the release of cryptic antiangiogenic factors. Cleavage of basement membrane proteins, collagen XVIII and the $\alpha1$, $\alpha2$ and $\alpha3$ chains of collagen IV release the angiogenic inhibitors endostatin, arrestin, canstatin and tumstatin, respectively [57]. Endostatin once release from mature collagen XVIII binds to cell surface proteoglycans, VEGFR-2 and the $\alpha5\beta1$ integrin to inhibit angiogenesis *in vitro* and *in vivo* [57]. Due to the longer half-life on these endogenous inhibitors of angiogenesis, it has been hypothesized that they accumulate in the serum of patients with larger primary neoplasia and inhibit angiogenesis at distal sites and limit the growth of metastatic foci until resection of the primary lesion [58].

As mentioned, remodelling of the ECM is a tightly regulated process. The inhibitory influence that the TIMPs have on MMP expression and function therefore is an important regulator of matrix degradation. Overexpression of TIMP-1 has shown to suppress tumorigenesis, in part due to its effects on the tumor vasculature. Immunostaining revealed that mice that overexpressed the endogenous TIMP-1 had significantly reduced tumor vessel density compared to controls (REF). *In vitro* treatment with TIMP-1 showed that tube formation was altered despite no significant changes in endothelial cell proliferation. Decreased expression of MMP-2 and MMP-9 in the tumors of TIMP-1 transgenic mice but not in the *in vitro* experiments, suggest that inhibition of the matrix degradation is not a direct effect on MMPs, but may require the presence of a reactive stroma [59]. *In vitro*, TIMP-2 decreases proliferation of endothelial cells and inhibits angiogenesis *in vivo* [60, 61]. Other *in vivo* experiments involving immunohistochemical analysis

of tumors overexpressing TIMP-2 reported a decrease in microvessel density compared to controls [62]. Although the mechanisms by which protease inhibitors such as TIMP-2 inhibit angiogenesis are not well understood, it is thought to be the result of a decrease in proangiogenic factors such as VEGF [63] and bFGF [64]. TIMP-3 has also shown to decrease angiogenesis, particularly through inhibition of endothelial tube formation and disaggregation of endothelial cells [65–67]. Support for TIMP-3 as a therapeutic comes from studies involving animals that were deficient in the protease inhibitor exhibited an increased angiogenic phenotype [68]. TIMPs have been reported to not only influence the vasculature, but in some cases, exert their antiangiogenic properties through an MMP-independent mechanism [69–71]. In 2003, Fernández et al. characterized the antiangiogenic domains of TIMP-2, a protease inhibitor which decreases endothelial cell proliferation. In this study, they found that both terminal domains of the protein were capable of inhibiting angiogenesis. It was also noted that only the domain which does not function with MMPs was able to inhibit mitogen-driven angiogenesis. This can be interpreted that therapeutics that solely target MMPs to inhibit angiogenesis might not be as effective as TIMPs [72].

Another group of ECM proteins which have recently been studied for their role in tumor angiogenesis are a disintegrin and metalloproteinases (ADAMs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs). Besides the addition of thrombospondin (TSP) motifs on these proteases, the ADAMs are associated with the membrane while the ADAMTs are secreted [73]. These proteins belong to a similar family as MMPs and many of them have been found to regulate angiogenesis directly or through expression of MMPs. ADAM-17 has been reported to play a role in angiogenesis as was evident from *in vitro* experiments on endothelial cells. Inhibiting ADAM-17 not only altered morphology of the endothelial cells but it also decreased proliferation, leaving apoptosis unchanged. In terms of elucidating how ADAM-17 influences angiogenesis, this was determined to be the result of MMP-2 activation via VEGF. The involvement of ADAM-17 was confirmed when endothelial cells lacking the protein did not have an increase in MMP-2 following VEGF treatment [74]. Evaluation of the first thrombospondin repeat (TSR1) in ADAMTS5 revealed inhibition of endothelial tube formation and proliferation [75]. Unlike ADAM-17, ADAMTS5 induced endothelial cell death even in the presence of VEGF, a potent proangiogenic and growth promoting factor. ADAMTS1 and ADAMTS8 have also been identified for containing the antiangiogenic domain (TSR1) of thrombospondin. Both these proteins inhibited endothelial cell proliferation and suppressed growth factor induced vascularization of various assays [76]. Luque et al., elaborated on the function of ADAMTS1 to inhibit angiogenesis by decreasing VEGF. They found that ADAMTS1 binds VEGF which ultimately interferes with its ability to interact with its receptor VEGFR2, as was evident from lack of phosphorylation [77].

The ECM protein SPARC is a multifunctional matricellular glycoprotein that has been evaluated in various cancers but its role as either a tumor promoter or inhibitor has been controversial (reviewed by [78]). However, with respect to its effect on blood vessels, SPARC has been reported to be an antiangiogenic factor [79]. In this study, over-expression of SPARC in a glioma cell line resulted in a decrease in vascularity of xenograft tumors. It was also determined that the antiangiogenic effects of SPARC were associated with reduced tumor levels of VEGF [80]. SPARC also directly inhibits endothelial cell binding to the extracellular matrix by modulating extracellular calcium levels, effectively inhibiting blood vessel migration through the tumor stroma [81]. Other studies have shown that SPARC interferes with the growth promoting effects of VEGF on endothelial cells [82], and promotes the assembly of tightly organized stroma that does not permit blood vessel formation or tumor progression [81].

Lastly is a group of ECM proteins classified as endogenous inhibitors of angiogenesis which include various antiangiogenic peptides, hormone metabolites and modulators of apoptosis [83]. Therapeutic drugs have been developed based on matrix derived and nonmatrix-derived endogenous angiogenic inhibitors [83, 84]. These inhibitors can be classified based on whether they have solely angiogenic actions or whether they have functions in addition to angiogenic actions [84]. Of these, thrombospondin-1 was the first protein recognized as an endogenous inhibitor of angiogenesis [85] and has since become a popular target for the treatment of various cancers. The role of TSP-1 as an antiangiogenic protein will be extensively reviewed.

Another class of proteins which have recently gained recognition for their potential role in tumor angiogenesis are the bone morphogenic proteins (BMPs). BMPs belong to the transforming growth factor beta (TGFb) superfamily of proteins and to date, more than 20 members have been identified [86]. An angiogenic role of the BMPs has been suggested due to a number of BMP mutations found in various vascular diseases and abnormal angiogenesis that occurs when BMP signalling is disrupted (reviewed by [87]). One of the mechanisms by which the BMPs have been reported to influence angiogenesis is by stimulating the secretion of proangiogenic growth factors such as VEGF [88]. BMP expression is associated with promoting tube formation and endothelial cell migration, whereas these activities are inhibited when BMP function is impaired [89]. Although BMPs are thought to stimulate angiogenesis, there have also been reports that demonstrate an antiangiogenic role [90, 91].

Aside from a direct role on blood vessel formation, recent studies have implicated interactions between BMPs and proteins of the ECM which can ultimately affect tumor growth and development. ECM proteins have been reported to interfere with BMP signalling by altering the bioavailability of TGFb (reviewed by [92]). BMPs are also thought to play a critical role in metastatic processes. In a model of prostate cancer, BMP-7 was shown to be highly expressed in bone and soft tissue metastases compared to the primary tumor and subcutaneous tumors formed from prostate adenocarcinoma cells overexpressing BMP-7 had a significantly reduced tumor volume compared to those with

normal expression [93]. Similar results were reported in a model of lung carcinoma [94] and these results illustrate the relevance of the tissue microenvironment when studying how BMPs affect tumor growth. It is thought that tumor cells secrete BMPs which creates an environment that promotes tumor cell growth and metastasis [95]. Studies have shown that BMPs can contribute to decreased expression of various MMPs [96–98]. A reduction in MMP expression would permit metastatic cells to colonize and propagate in the tissue. The role of BMPs in primary and secondary tumor formation is not completely clear, however. Some evidence points to the need for BMP inhibition before metastatic spread can occur and it may be the case that the effects of members of the BMP family may be context-specific. In an ovarian cancer model, it was shown that overexpression of the BMP receptor, ALK3 decreases adhesion of epithelial ovarian cancer cells *in vitro* reduces formation of intraperitoneal tumors and ascites fluid *in vivo* [99]. Further understanding of the role which BMPs play in the ECM and with tumor angiogenesis will benefit therapeutic studies which target angiogenesis, tumour growth, and metastatic spread of disease.

5. TSP Family of Proteins

Thrombospondin was originally identified as thrombin-sensitive protein by Baenziger et al., in the early 1970s. It was later realized that the protein was a subunit of a larger protein released from α granules of platelets in response to activation by thrombin. The native protein was officially named thrombospondin (TSP) [100, 101]. TSPs belong to a family of multifunctional glycoproteins that have a high affinity for matrix molecules, plasma proteins, ions, and various cell surfaces. They are capable of binding to heparin [102–104], fibronectin [105, 106], fibrinogen [106–108], plasminogen [109], histidine-rich glycoprotein [110], type IV collagen [111], and calcium [112, 113]. (For an extensive list of macromolecules that interacts with TSP see [114]. TSP is also capable of associating with various cell types and their corresponding extracellular matrices [115–119]. Combined, these diverse interactions allow TSP to be involved in cell-to-cell and cell-to-matrix communications (reviewed by [120]).

TSP is a 450 kDA protein which is composed of three 150 kDA disulfide-linked polypeptide chains [101, 121–125]. Each subunit of the trimer consists of multiple domains: an N-terminal globular domain, a region homologous to procollagen, three types of repeated sequence motifs (type 1, type 2, and type 3 repeats) and a C-terminal globular domain [126]. There are five family members, TSP-1, -2, -3, -4 and -5 [127–129]. The TSP family can be divided into two subgroups on the basis of their oligomerization and molecular architecture. TSP-1 and -2 are trimers that have the same set of structural domains and belong to subgroup A. They are members of the thrombospondin type-1 repeat (TSR) supergene family whereas the remaining members of the family lack the TSR and the procollagen domain, are pentamers and are part of subgroup B [130].

6. Thrombospondin-1 (TSP-1) and Tumor Angiogenesis

Thrombospondin-1 (TSP-1) was the first of the five members to be identified as a major component of blood platelets. Since its discovery, TSP-1 has been implicated in the regulation of cell growth and proliferation [131, 132], cell motility [85, 133, 134], cytoskeletal organization [135, 136], inflammatory responses [137, 138], development and differentiation of various cell types [139], regulation of angiogenesis during wound healing [140], and tumorigenesis [141] (reviewed by [114]).

Due to the complex structure of TSP-1, there are multiple receptor binding domains located throughout the peptide that are capable of various functions [126]. These receptors include, low density lipoprotein receptor-related protein (LRP), proteoglycans and sulfatides, CD36, integrins, integrin-associated protein (IAP), and an unidentified receptor located in the C-terminus [142]. Many membrane proteins can also act as receptors for TSP-1 and activate downstream signalling pathways [143].

Many human tumor cell lines express relatively low levels of TSP-1 compared to normal or benign lines. It has also been observed that cell lines with low metastatic potential express higher levels of TSP-1 compared to metastatic lines [144, 145]. This has further been validated in experiments where TSP-1 transfection into human cancer cell lines inhibited primary tumor formation [145, 146] and decreased metastasis *in vivo* [145]. These results propose an inverse correlation between TSP-1 expression and tumor aggressiveness, whereby malignant progression is associated with reduced levels of TSP-1 in certain cancers.

Overexpression of TSP-1 in cancer cell lines has been shown to suppress tumor formation by targeting the vasculature [145–150]. Studies involving introduction of TSP-1 into cell lines derived from gliobastoma multiforme induced the angiogenic switch to an antiangiogenic phenotype. Angiogenesis was measured by *in vitro* endothelial cell migration and *in vivo* corneal neovascularization assays [148]. Other *in vivo* studies have manipulated TSP-1 via transfection into human cancer cells lines and subsequent injection into nude mice. Angiogenesis is typically assessed based on microvessel density (MVD) which utilizes endothelial cell-specific markers, such as cluster of differentiation 31 (CD31) in order to perform vessel staining and counts. A decrease in MVD was observed in primary tumors that formed from the TSP-1 transfectant cell lines [145]. TSP-1 overexpression experiments in a model of human squamous cell carcinoma revealed consistent results in that tumor growth, vessel number, and size were drastically decreased. Histological examination demonstrated that tumors derived from TSP-1 stable transfected cells exhibited extensive areas of tumor cell necrosis which might have been due to the antiangiogenic effects of TSP-1 on tumor vasculature [149].

Clinical studies of patients with bladder, papillary thyroid and epithelial ovarian cancer have investigated levels of TSP-1 and correlated it with angiogenesis. This relationship was established based on a significant association between

TSP-1 expression and MVD count. Tumors from patients that expressed high levels of TSP-1 had low MVD counts and were therefore more likely to exhibit a decrease in angiogenesis compared to control tissue. The inverse has also been documented; a decrease in TSP-1 expression was accompanied with high MVD counts which may contribute to an angiogenic phenotype [151–155].

Most studies have attempted to relate vascularity with expression of TSP-1 by probing tumor tissue with endothelial cell specific markers. Few studies have evaluated the expression of TSP-1 in hypo- compared to hypervascular carcinomas. There are however various human carcinomas with a varying degree of vascularization and have been used to determine how diminished vascularity relates to expression of TSP-1. It was found that a hypovascularized human carcinoma had increased levels of TSP-1 [156]. These results defend the inverse relationship that exists between the degree of vascularization and TSP-1 expression. They also support TSP-1 as an antiangiogenic protein that regulates tumorigenesis.

TSP-1 expression has also been a predictor of tumor recurrence and overall survival. In clinical studies of patients with invasive bladder cancer and papillary thyroid carcinoma, low TSP-1 expression, as determined by immunohistochemistry, was associated with an increased probability of disease recurrence and decreased overall survival [151, 154]. Clinical studies of invasive epithelial and cervical cancer have revealed that TSP-1 expression is a valuable prognostic factor [155, 157]. In another study of invasive epithelial ovarian cancer, the majority of cases expressed high levels of TSP-1 which was associated with a higher survival rate compared to cases where tumors expressed lower levels of TSP-1 [152]. The 5-year survival rate of oral squamous cell carcinoma patients has also been shown to be significantly higher in tumors that express high levels of TSP-1 [153]. Based on this evidence, it is likely that TSP-1 possesses a tumor inhibitory function in some cancers and it may be a useful tool to assess prognosis.

7. Antiangiogenic Compounds and Vessel Normalization

The concept of antiangiogenic therapy for the treatment of various cancers was postulated in 1971 by Judah Folkman. It was hypothesized that solid tumor growth depends on angiogenesis in order to grow beyond 1-2 mm^3. Therefore, it was thought that angiogenic inhibitors might be a potential therapeutic target; by blocking angiogenesis, tumor dormancy could be initiated [7, 158]. In 1996, Teicher proposed that antiangiogenic therapy would be most effective if used in combination with chemotherapy. The rationale was that the combinatorial effects would diminish the tumor cells as well as the endothelial cells associated with the tumor [159]. A hallmark of tumor angiogenesis is that blood vessels are formed so rapidly that they often become disorganized, torturous, and as a result have reduced functional capacity [160]. These immature blood vessels typically lack pericyte coverage, which may render them more vulnerable to apoptotic signals [161]. In 2001, Jain proposed the idea of tumor vasculature normalization as the product of antiangiogenic treatment. Because of the abnormal anatomy of tumor vessels perfusion is restricted, resulting in areas of tumor hypoxia and necrosis. This reduced blood flow to the tumor impairs the delivery of cytotoxic chemotherapeutic agents to the tumor interior, inhibiting their effectiveness, facilitating drug resistance and tumor regrowth [162, 163]. Anti-angiogenic therapy designed to target this abnormal, immature vasculature could effectively prune back vessels and increase blood flow, nutrient delivery, and waste removal. Combining vessel normalization with chemotherapy would provide better tissue perfusion of cytotoxic agents which induce apoptosis of the tumor [164]. It has since been reported that agents that inhibit proangiogenic factors alter the tumor vasculature and increase the delivery of chemotherapeutics when used in combination [165]. Recent studies have utilized the antiangiogenic peptide, TSP-1 for the treatment of various cancers and have found that the compounds are capable of normalizing tumor vasculature [166]. We have shown that TSP-1 directly inhibits VEGF and reduces its availability to ovarian cells [167]. In addition, we have shown that treatment with the TSP-1 mimetic peptide ABT-510 significantly reduces ovarian tumor volume and vascularity [168]. Importantly, treatment with ABT-510 decreased overall blood vessel density, but increased the proportion of mature, pericyte-covered blood vessels and decreased tumor tissue hypoxia.

If normalized vessels increase the uptake of chemotherapeutic agents they may allow the drugs to be administered at lower doses, which would minimize their many deleterious side effects. The benefits are also supported by the fact that if the tumor vasculature is normalized instead of completely diminished, the tumor will not undergo hypoxia which is the major activator of VEGF, a potent proangiogenic factor.

8. The Thrombospondins and Other EMC Proteins

The TSPs are also known to directly interact with other ECM proteins in their regulation of tumor progression and tumor angiogenesis. The Type 1 repeats of the TSP-1 and -2 genes inhibit MMP activity by preventing activation of the MMP-2 and -9 zymogens [169]. Conversely, others have reported that TSP-1 increases MMP-9 activity and tumor cell invasion [170], suggesting that the interaction between matricellular proteins may be context specific. TSP-1 null mice have reduced expression of TGFβ, lower collagen content and delayed wound closure [171]. We also discovered that the ovaries of TSP-1 null mice were hypervascularized, with increased expression of VEGF [5]. *In vivo*, TSP-1 binds to a number of matrix glycosaminoglycans including heparan sulfate [172] chondroitin sulfate [173] and binds to members of the syndecan family, versican, and cerbroglycan [172–174] and these proteoglycans are thought to be important mediators of TSP-1. Aside from MMPs, TSP-1 is also known to inhibit the activity of plasmin,

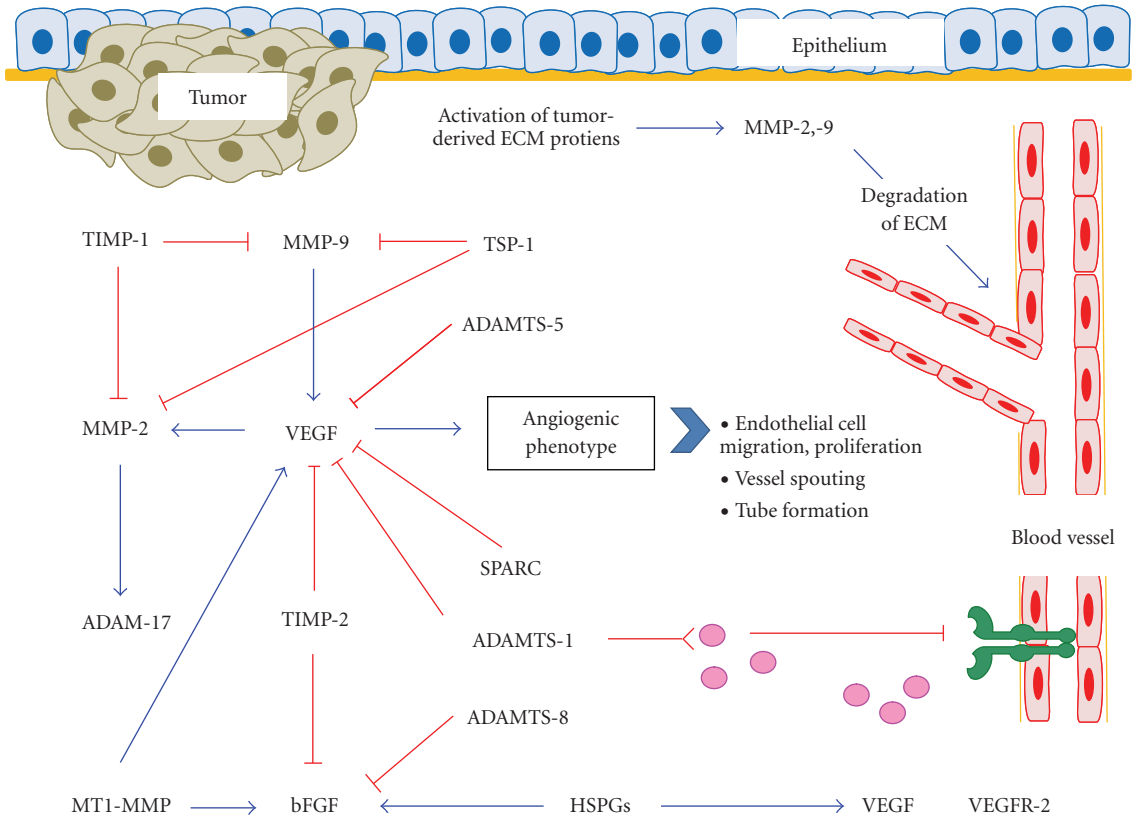


Figure 1: Summary of various extracellular matrix (ECM) proteins that are involved in tumour angiogenesis. Activation of tumor-derived ECM proteins permits communication between the tumor and its surrounding microenvironment. Regulating of angiogenesis can be directly through MMP activation and degradation of the ECM or through and indirect mechanism. This involves interactions between various ECM proteins and pro or antiangiogenic growth factors in order to alter angiogenesis. Ultimately, expression of the proangiogenic growth factor can influence the angiogenic phenotype and determine whether vascular sprouting occurs in order to provide the tumor with the necessary nutrients to survive.

urokinase plasminogen activator, and elastin [175, 176], which are all key components of the extracellular matrix and are important in facilitating vessel invasion into the stroma. Aside from direct effects on VEGF expression and endothelial cells, TSP-1 appears to have potent antiangiogenic effects through its interaction with the extracellular matrix and on a host of matricellular proteins.

9. Conclusion

Solid tumors exhibit significant structural complexity and progression of the disease is regulated by a host of different factors. This paper focused on the extracellular matrix as a major contributor to tumorigenesis. Once cells have undergone transformation and initiated the formation of a tumor, they must interact with the surrounding environment in order for tumor progression to occur [1]. This interaction activates tumor-derived ECM proteins which can have multiple effects on the tumor stroma in order to promote angiogenesis, a process which is essential for tumor growth [6]. The involvement of the ECM in tumor angiogenesis includes degradation of the basement membrane, matrix remodelling, and cell migration and invasion [32]. In this paper, we focused on ECM proteins that have both direct and indirect roles on the regulation of angiogenesis. Many of the ECM proteins appear to affect angiogenesis by altering expression of proangiogenic growth factors such as VEGF and bFGF. Other ECM proteins such as SPARC, ADAMs, and ADAMTs have also been investigated for their role in angiogenesis. These proteins have been shown to have direct antiangiogenic properties through their ability to inhibit proangiogenic growth factors. Lastly, we reported on the role of TSP-1 as it has been extensively studied with respect to tumor angiogenesis. It has been well documented that *in vitro*, TSP-1 decreases endothelial cell migration and invasion and decreases tumor vasculature *in vivo*. The involvement of ECM proteins in tumour angiogenesis is summarized in Figure 1. The potent antiangiogenic effects of TSP-1 have led to the development of mimetic peptides that have shown significant antiangiogenic and antitumorigenic effects *in vivo*. This review demonstrates the necessity for investigation of the microenvironment of the tumor and also supports the development of various therapeutics which can target ECM proteins.

References

[1] D. Radisky, C. Hagios, and M. J. Bissell, "Tumors are unique organs defined by abnormal signaling and context," *Seminars in Cancer Biology*, vol. 11, no. 2, pp. 87–95, 2001.

[2] C. Brekken, O. S. Bruland, and C. de Lange Davies, "Interstitial fluid pressure in human osteosarcoma xenografts: significance of implantation site and the response to intratumoral injection of hyaluronidase," *Anticancer Research*, vol. 20, no. 5, pp. 3503–3512, 2000.

[3] D. Fukumura, F. Yuan, W. L. Monsky, Y. Chen, and R. K. Jain, "Effect of host microenvironment on the microcirculation of human colon adenocarcinoma," *American Journal of Pathology*, vol. 151, no. 3, pp. 679–688, 1997.

[4] J. J. Killion, R. Radinsky, and I. J. Fidler, "Orthotopic models are necessary to predict therapy of transplantable tumors in mice," *Cancer and Metastasis Reviews*, vol. 17, no. 3, pp. 279–284, 1998.

[5] J. Greenaway, R. Moorehead, P. Shaw, and J. Petrik, "Epithelial-stromal interaction increases cell proliferation, survival and tumorigenicity in a mouse model of human epithelial ovarian cancer," *Gynecologic Oncology*, vol. 108, no. 2, pp. 385–394, 2008.

[6] N. Wernert, "The multiple roles of tumour stroma," *Virchows Archiv*, vol. 430, no. 6, pp. 433–443, 1997.

[7] J. Folkman, "Tumor angiogenesis: therapeutic implications," *New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[8] J. Folkman and M. Klagsbrun, "Angiogenic factors," *Science*, vol. 235, no. 4787, pp. 442–447, 1987.

[9] J. Folkman, K. Watson, D. Ingber, and D. Hanahan, "Induction of angiogenesis during the transition from hyperplasia to neoplasia," *Nature*, vol. 339, no. 6219, pp. 58–61, 1989.

[10] P. Carmeliet, "Angiogenesis in health and disease," *Nature Medicine*, vol. 9, no. 6, pp. 653–660, 2003.

[11] N. Ferrara, K. Houck, L. Jakeman, and D. W. Leung, "Molecular and biological properties of the vascular endothelial growth factor family of proteins," *Endocrine Reviews*, vol. 13, no. 1, pp. 18–32, 1992.

[12] Z. Galzie, A. R. Kinsella, and J. A. Smith, "Fibroblast growth factors and their receptors," *Biochemistry and Cell Biology*, vol. 75, no. 6, pp. 669–685, 1997.

[13] A. Compagni, P. Wilgenbus, M.-A. Impagnatiello, M. Cotten, and G. Christofori, "Fibroblast growth factors are required for efficient tumor angiogenesis," *Cancer Research*, vol. 60, no. 24, pp. 7163–7169, 2000.

[14] P. Gerwins, E. Sköldenberg, and L. Claesson-Welsh, "Function of fibroblast growth factors and vascular endothelial growth factors and their receptors in angiogenesis," *Critical Reviews in Oncology/Hematology*, vol. 34, no. 3, pp. 185–194, 2000.

[15] S. A. Rabbani, "Metalloproteases and urokinase in angiogenesis and tumor progression," *In Vivo*, vol. 12, no. 1, pp. 135–142, 1998.

[16] J. P. Geisler, G. A. Miller, J. R. Broshears, and K. J. Manahan, "Vascular endothelial growth factor staining and elevated INR in advanced epithelial ovarian carcinoma," *Journal of Surgical Oncology*, vol. 96, no. 6, pp. 514–517, 2007.

[17] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. Susan Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, 1983.

[18] L. De Cecco, L. Marchionni, M. Gariboldi et al., "Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2," *Oncogene*, vol. 23, no. 49, pp. 8171–8183, 2004.

[19] R. Salgado, I. Benoy, P. Vermeulen, P. van Dam, E. van Marck, and L. Dirix, "Circulating basic fibroblast growth factor is partly derived from the tumour in patients with colon, cervical and ovarian cancer," *Angiogenesis*, vol. 7, no. 1, pp. 29–32, 2004.

[20] A. M. Di Blasio, C. Carniti, P. Vigano, and M. Vignali, "Basic fibroblast growth factor and ovarian cancer," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 53, no. 1–6, pp. 375–379, 1995.

[21] M. K. Whitworth, A. C. Backen, A. R. Clamp et al., "Regulation of fibroblast growth factor-2 activity by human ovarian cancer tumor endothelium," *Clinical Cancer Research*, vol. 11, no. 12, pp. 4282–4288, 2005.

[22] M. Klagsbrun and P. A. D'Amore, "Regulators of angiogenesis," *Annual Review of Physiology*, vol. 53, pp. 217–239, 1991.

[23] J. Sottile, "Regulation of angiogenesis by extracellular matrix," *Biochimica et Biophysica Acta*, vol. 1654, no. 1, pp. 13–22, 2004.

[24] V. Rouet, Y. Hamma-Kourbali, E. Petit et al., "A synthetic glycosaminoglycan mimetic binds vascular endothelial growth factor and modulates angiogenesis," *Journal of Biological Chemistry*, vol. 280, no. 38, pp. 32792–32800, 2005.

[25] G. W. Yip, M. Smollich, and M. Götte, "Therapeutic value of glycosaminoglycans in cancer," *Molecular Cancer Therapeutics*, vol. 5, no. 9, pp. 2139–2148, 2006.

[26] M. I. Góth, E. Hubina, S. Raptis, G. M. Nagy, and B. E. Tóth, "Physiological and pathological angiogenesis in the endocrine system," *Microscopy Research and Technique*, vol. 60, no. 1, pp. 98–106, 2003.

[27] R. V. Iozzo and J. D. San Antonio, "Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena," *Journal of Clinical Investigation*, vol. 108, no. 3, pp. 349–355, 2001.

[28] R. Sasisekharan, M. A. Moses, M. A. Nugent, C. L. Cooney, and R. Langer, "Heparinase inhibits neovascularization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 4, pp. 1524–1528, 1994.

[29] A. Walker, J. E. Turnbull, and J. T. Gallagher, "Specific heparan sulfate saccharides mediate the activity of basic fibroblast growth factor," *Journal of Biological Chemistry*, vol. 269, no. 2, pp. 931–935, 1994.

[30] S. A. Karumanchi, V. Jha, R. Ramchandran et al., "Cell surface glypicans are low-affinity endostatin receptors," *Molecular Cell*, vol. 7, no. 4, pp. 811–822, 2001.

[31] T. M. Mundel and R. Kalluri, "Type IV collagen-derived angiogenesis inhibitors," *Microvascular Research*, vol. 74, no. 2-3, pp. 85–89, 2007.

[32] M. S. Pepper, "Extracellular proteolysis and angiogenesis," *Thrombosis and Haemostasis*, vol. 86, no. 1, pp. 346–355, 2001.

[33] O. Saksela and D. B. Rifkin, "Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity," *Journal of Cell Biology*, vol. 110, no. 3, pp. 767–775, 1990.

[34] B. Kaur, D. J. Brat, N. S. Devi, and E. G. Van Meir, "Vasculostatin, a proteolytic fragment of brain angiogenesis inhibitor 1, is an antiangiogenic and antitumorigenic factor," *Oncogene*, vol. 24, no. 22, pp. 3632–3642, 2005.

[35] H. A. Hanford, C. A. Wong, H. Kassan et al., "Angiostatin4.5-mediated apoptosis of vascular endothelial cells," *Cancer Research*, vol. 63, no. 14, pp. 4275–4280, 2003.

[36] J. Folkman, "Tumor suppression by p53 is mediated in part by the antiangiogenic activity of endostatin and tumstatin," *Science's STKE*, vol. 2006, no. 354, article pe35, 2006.

[37] J. T. Yang, H. Rayburn, and R. O. Hynes, "Embryonic mesodermal defects in α5 integrin-deficient mice," *Development*, vol. 119, no. 4, pp. 1093–1105, 1993.

[38] K. L. Goh, J. T. Yang, and R. O. Hynes, "Mesodermal defects and cranial neural crest apoptosis in α5 integrin-null embryos," *Development*, vol. 124, no. 21, pp. 4309–4319, 1997.

[39] S. E. Francis, K. L. Goh, K. Hodivala-Dilke et al., "Central roles of $\alpha 5\beta 1$ integrin and fibronectin in vascular development in mouse embryos and embryoid bodies," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 22, no. 6, pp. 927–933, 2002.

[40] C. Marcinkiewicz, P. H. Weinreb, J. J. Calvete et al., "Obtustatin: a potent selective inhibitor of $\alpha 1\beta 1$ integrin in vitro and angiogenesis in vivo," *Cancer Research*, vol. 63, no. 9, pp. 2020–2023, 2003.

[41] K. Tashiro, G. C. Sephel, B. Weeks et al., "A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration, and neurite outgrowth," *Journal of Biological Chemistry*, vol. 264, no. 27, pp. 16174–16182, 1989.

[42] M. C. Kibbey, D. S. Grant, and H. K. Kleinman, "Role of the SIKVAV site of laminin in promotion of angiogenesis and tumor growth: an in vivo Matrigel model," *Journal of the National Cancer Institute*, vol. 84, no. 21, pp. 1633–1638, 1992.

[43] K. M. Malinda, M. Nomizu, M. Chung et al., "Identification of laminin $\alpha 1$ and $\beta 1$ chain peptides active for endothelial cell adhesion, tube formation, and aortic sprouting," *FASEB Journal*, vol. 13, no. 1, pp. 53–62, 1999.

[44] A. Mettouchi, S. Klein, W. Guo et al., "Integrin-specific activation of Rac controls progression through the G1 phase of the cell cycle," *Molecular Cell*, vol. 8, no. 1, pp. 115–127, 2001.

[45] M. S. Pepper, "Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 21, no. 7, pp. 1104–1117, 2001.

[46] T. Itoh, M. Tanioka, H. Yoshida, T. Yoshioka, H. Nishimoto, and S. Itohara, "Reduced angiogenesis and tumor progression in gelatinase A-deficient mice," *Cancer Research*, vol. 58, no. 5, pp. 1048–1051, 1998.

[47] J. Fang, Y. Shing, D. Wiederschain et al., "Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 8, pp. 3884–3889, 2000.

[48] A. Zhang, L. Meng, Q. Wang et al., "Enhanced in vitro invasiveness of ovarian cancer cells through up-regulation of VEGF and induction of MMP-2," *Oncology Reports*, vol. 15, no. 4, pp. 831–836, 2006.

[49] S. Lee, S. M. Jilan, G. V. Nikolova, D. Carpizo, and M. Luisa Iruela-Arispe, "Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors," *Journal of Cell Biology*, vol. 169, no. 4, pp. 681–691, 2005.

[50] M. Hollborn, C. Stathopoulos, A. Steffen, P. Wiedemann, L. Kohen, and A. Bringmann, "Positive feedback regulation between MMP-9 and VEGF in human RPE cells," *Investigative Ophthalmology and Visual Science*, vol. 48, no. 9, pp. 4360–4367, 2007.

[51] L. J. A. C. Hawinkels, K. Zuidwijk, H. W. Verspaget et al., "VEGF release by MMP-9 mediated heparan sulphate cleavage induces colorectal cancer angiogenesis," *European Journal of Cancer*, vol. 44, no. 13, pp. 1904–1913, 2008.

[52] T. Onguchi, K. Y. Han, J.-H. Chang, and D. T. Azar, "Membrane type-1 matrix metalloproteinase potentiates basic fibroblast growth factor-induced corneal neovascularization," *American Journal of Pathology*, vol. 174, no. 4, pp. 1564–1571, 2009.

[53] N. E. Sounni, L. Devy, A. Hajitou et al., "MTI-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression," *FASEB Journal*, vol. 16, no. 6, pp. 555–564, 2002.

[54] E. I. Deryugina, L. Soroceanu, and A. Y. Strongin, "Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis," *Cancer Research*, vol. 62, no. 2, pp. 580–588, 2002.

[55] J. C. Rodríguez-Manzaneque, T. F. Lane, M. A. Ortega, R. O. Hynes, J. Lawler, and M. L. Iruela-Arispe, "Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 22, pp. 12485–12490, 2001.

[56] A. J. Trimboli, C. Z. Cantemir-Stone, F. Li et al., "Pten in stromal fibroblasts suppresses mammary epithelial tumours," *Nature*, vol. 461, no. 7267, pp. 1084–1091, 2009.

[57] R. Kalluri, "Basement membranes: structure, assembly and role in tumour angiogenesis," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 422–433, 2003.

[58] M. S. O'Reilly, L. Holmgren, Y. Shing et al., "Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma," *Cell*, vol. 79, no. 2, pp. 315–328, 1994.

[59] Y. Ikenaka, H. Yoshiji, S. Kuriyama et al., "Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model," *International Journal of Cancer*, vol. 105, no. 3, pp. 340–346, 2003.

[60] D.-W. Seo, H. Li, L. Guedez et al., "TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism," *Cell*, vol. 114, no. 2, pp. 171–180, 2003.

[61] P. Valente, G. Fassina, A. Melchiori et al., "TIMP-2 overexpression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis," *International Journal of Cancer*, vol. 75, no. 2, pp. 246–253, 1998.

[62] A. L. Feldman, W. G. Stetler-Stevenson, N. G. Costouros et al., "Modulation of tumor-host interactions, angiogenesis, and tumor growth by tissue inhibitor of metalloproteinase 2 via a novel mechanism," *Cancer Research*, vol. 64, no. 13, pp. 4481–4486, 2004.

[63] A. Hajitou, N.-E. Sounni, L. Devy et al., "Down-regulation of vascular endothelial growth factor by tissue inhibitor of metalloproteinase-2: effect on in vivo mammary tumor growth and angiogenesis," *Cancer Research*, vol. 61, no. 8, pp. 3450–3457, 2001.

[64] D.-W. Seo, S. H. Kim, S.-H. Eom et al., "TIMP-2 disrupts FGF-2-induced downstream signaling pathways," *Microvascular Research*, vol. 76, no. 3, pp. 145–151, 2008.

[65] W. W. Spurbeck, C. Y. C. Ng, T. S. Strom, E. F. Vanin, and A. M. Davidoff, "Enforced expression of tissue inhibitor of matrix metalloproteinase-3 affects functional capillary

morphogenesis and inhibits tumor growth in a murine tumor model," *Blood*, vol. 100, no. 9, pp. 3361–3368, 2002.

[66] K.-H. Kang, S.-Y. Park, S. B. Rho, and J.-H. Lee, "Tissue inhibitor of metalloproteinases-3 interacts with angiotensin II type 2 receptor and additively inhibits angiogenesis," *Cardiovascular Research*, vol. 79, no. 1, pp. 150–160, 2008.

[67] C. Chetty, S. S. Lakka, P. Bhoopathi, S. Kunigal, R. Geiss, and J. S. Rao, "Tissue inhibitor of metalloproteinase 3 suppresses tumor angiogenesis in matrix metalloproteinase 2-down-regulated lung cancer," *Cancer Research*, vol. 68, no. 12, pp. 4736–4745, 2008.

[68] W. Cruz-Muñoz, I. Kim, and R. Khokha, "TIMP-3 deficiency in the host, but not in the tumor, enhances tumor growth and angiogenesis," *Oncogene*, vol. 25, no. 4, pp. 650–655, 2006.

[69] M. L. Corcoran and W. G. Stetler-Stevenson, "Tissue inhibitor of metalloproteinase-2 stimulates fibroblast proliferation via a cAMP-dependent mechanism," *Journal of Biological Chemistry*, vol. 270, no. 22, pp. 13453–13459, 1995.

[70] S. E. Hoegy, H.-R. Oh, M. L. Corcoran, and W. G. Stetler-Stevenson, "Tissue inhibitor of metalloproteinases-2 (TIMP-2) suppresses TKR-growth factor signaling independent of metalloproteinase inhibition," *Journal of Biological Chemistry*, vol. 276, no. 5, pp. 3203–3214, 2001.

[71] J. Oh, B.-W. Seo, T. Diaz et al., "Tissue inhibitors of metalloproteinase 2 inhibits endothelial cell migration through increased expression of RECK," *Cancer Research*, vol. 64, no. 24, pp. 9062–9069, 2004.

[72] C. A. Fernández, C. Butterfield, G. Jackson, and M. A. Moses, "Structural and functional uncoupling of the enzymatic and angiogenic inhibitory activities of tissue inhibitor of metalloproteinase-2 (TIMP-2): loop 6 is a novel angiogenesis inhibitor," *Journal of Biological Chemistry*, vol. 278, no. 42, pp. 40989–40995, 2003.

[73] J. R. Dunn, J. E. Reed, D. G. du Plessis et al., "Expression of ADAMTS-8, a secreted protease with antiangiogenic properties, is downregulated in brain tumours," *British Journal of Cancer*, vol. 94, no. 8, pp. 1186–1193, 2006.

[74] P. Göoz, M. Göoz, A. Baldys, and S. Hoffman, "ADAM-17 regulates endothelial cell morphology, proliferation, and in vitro angiogenesis," *Biochemical and Biophysical Research Communications*, vol. 380, no. 1, pp. 33–38, 2009.

[75] S. Sharghi-Namini, H. Fan, K. N. Sulochana et al., "The first but not the second thrombospondin type 1 repeat of ADAMTS5 functions as an angiogenesis inhibitor," *Biochemical and Biophysical Research Communications*, vol. 371, no. 2, pp. 215–219, 2008.

[76] F. Vázquez, G. Hastings, M.-A. Ortega et al., "METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-inhibitory activity," *Journal of Biological Chemistry*, vol. 274, no. 33, pp. 23349–23357, 1999.

[77] A. Luque, D. R. Carpizo, and M. L. Iruela-Arispe, "ADAMTS1/METH1 inhibits endothelial cell proliferation by direct binding and sequestration of VEGF165," *Journal of Biological Chemistry*, vol. 278, no. 26, pp. 23656–23665, 2003.

[78] O. L. Podhajcer, L. G. Benedetti, M. R. Girotti, F. Prada, E. Salvatierra, and A. S. Llera, "The role of the matricellular protein SPARC in the dynamic interaction between the tumor and the host," *Cancer and Metastasis Reviews*, vol. 27, no. 4, pp. 691–705, 2008.

[79] A. Chlenski, S. Liu, L. J. Guerrero et al., "SPARC expression is associated with impaired tumor growth, inhibited angiogenesis and changes in the extracellular matrix," *International Journal of Cancer*, vol. 118, no. 2, pp. 310–316, 2006.

[80] C. K. Yunker, W. Golembieski, N. Lemke et al., "SPARC-induced increase in glioma matrix and decrease in vascularity are associated with reduced VEGF expression and secretion," *International Journal of Cancer*, vol. 122, no. 12, pp. 2735–2743, 2008.

[81] A. Chlenski, L. J. Guerrero, Q. Yang et al., "SPARC enhances tumor stroma formation and prevents fibroblast activation," *Oncogene*, vol. 26, no. 31, pp. 4513–4522, 2007.

[82] C. Kupprion, K. Motamed, and E. H. Sage, "SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells," *Journal of Biological Chemistry*, vol. 273, no. 45, pp. 29635–29640, 1998.

[83] P. Nyberg, L. Xie, and R. Kalluri, "Endogenous inhibitors of angiogenesis," *Cancer Research*, vol. 65, no. 10, pp. 3967–3979, 2005.

[84] J. Folkman, "Endogenous angiogenesis inhibitors," *APMIS*, vol. 112, no. 7-8, pp. 496–507, 2004.

[85] D. J. Good, P. J. Polverini, F. Rastinejad et al., "A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 17, pp. 6624–6628, 1990.

[86] B. Schmierer and C. S. Hill.

[87] L. David, J.-J. Feige, and S. Bailly, "Emerging role of bone morphogenetic proteins in angiogenesis," *Cytokine and Growth Factor Reviews*, vol. 20, no. 3, pp. 203–212, 2009.

[88] M. M. L. Deckers, R. L. van Bezooijen, D. E. R. van Geertje Horst et al., "Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A," *Endocrinology*, vol. 143, no. 4, pp. 1545–1553, 2002.

[89] T. Rothhammer, F. Bataille, T. Spruss, G. Eissner, and A.-K. Bosserhoff, "Functional implication of BMP4 expression on angiogenesis in malignant melanoma," *Oncogene*, vol. 26, no. 28, pp. 4158–4170, 2007.

[90] J. R. Mathura Jr., N. Jafari, J. T. Chang et al., "Bone morphogenetic proteins-2 and-4: negative growth regulators in adult retinal pigmented epithelium," *Investigative Ophthalmology and Visual Science*, vol. 41, no. 2, pp. 592–600, 2000.

[91] R. Kane, C. Godson, and C. O'Brien, "Chordin-like 1, a bone morphogenetic protein-4 antagonist, is upregulated by hypoxia in human retinal pericytes and plays a role in regulating angiogenesis," *Molecular Vision*, vol. 14, pp. 1138–1148, 2008.

[92] P. ten Dijke and H. M. Arthur.

[93] C. Morrissey, L. G. Brown, T. E. M. Pitts, R. L. Vessella, and E. Corey, "Bone morphogenetic protein 7 is expressed in prostate cancer metastases and its effects on prostate tumor cells depend on cell phenotype and the tumor microenvironment," *Neoplasia*, vol. 12, no. 2, pp. 192–205, 2010.

[94] E. M. Langenfeld, Y. Kong, and J. Langenfeld, "Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5," *Oncogene*, vol. 25, no. 5, pp. 685–692, 2006.

[95] J. M. Bailey, P. K. Singh, and M. A. Hollingsworth, "Cancer metastasis facilitated by developmental pathways: sonic hedgehog, notch, and bone morphogenic proteins," *Journal of Cellular Biochemistry*, vol. 102, no. 4, pp. 829–839, 2007.

[96] T. Takiguchi, M. Kobayashi, R. Suzuki et al., "Recombinant human bone morphogenetic protein-2 stimulates osteoblast differentiation and suppresses matrix metalloproteinase-1

production in human bone cells isolated from mandibulae," *Journal of Periodontal Research*, vol. 33, no. 8, pp. 476–485, 1998.

[97] T. Kumagai, T. Shimizu, and K. Takeda, "Bone morphogenetic protein-2 suppresses invasiveness of TSU-Pr1 cells with the inhibition of MMP-9 secretion," *Anticancer Research*, vol. 26, no. 1, pp. 293–298, 2006.

[98] T. C. Otto, R. R. Bowers, and M. D. Lane.

[99] T. G. Shepherd, M. L. Mujoomdar, and M. W. Nachtigal, "Constitutive activation of BMP signalling abrogates experimental metastasis of OVCA429 cells via reduced cell adhesion," *Journal of Ovarian Research*, vol. 3, no. 1, article 5, 2010.

[100] N. L. Baenziger, G. N. Brodie, and P. W. Majerus, "A thrombin-sensitive protein of human platelet membranes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 68, no. 1, pp. 240–243, 1971.

[101] N. L. Baenziger, G. N. Brodie, and P. W. Majerus, "Isolation and properties of a thrombin-sensitive protein of human platelets," *Journal of Biological Chemistry*, vol. 247, no. 9, pp. 2723–2731, 1972.

[102] J. W. Lawler and H. S. Slayter, "The release of heparin binding peptides from platelet thrombospondin by proteolytic action of thrombin, plasmin and trypsin," *Thrombosis Research*, vol. 22, no. 3, pp. 267–279, 1981.

[103] V. M. Dixit, G. A. Grant, S. A. Santoro, and W. A. Frazier, "Isolation and characterization of a heparin-binding domain from the amino terminus of platelet thrombospondin," *Journal of Biological Chemistry*, vol. 259, no. 16, pp. 10100–10105, 1984.

[104] G. O. Gogstad, N. O. Solum, and M. B. Krutnes, "Heparin-binding platelet proteins demonstrated by crossed affinity immunoelectrophoresis," *British Journal of Haematology*, vol. 53, no. 4, pp. 563–573, 1983.

[105] J. Lahav, M. A. Schwartz, and R. O. Hynes, "Analysis of platelet adhesion with a radioactive chemical crosslinking reagent: interaction of thrombospondin with fibronectin and collagen," *Cell*, vol. 31, no. 1, pp. 253–262, 1982.

[106] J. Lahav, J. Lawler, and M. A. Gimbrone, "Thrombospondin interactions with fibronectin and fibrinogen. Mutual inhibition in binding," *European Journal of Biochemistry*, vol. 145, no. 1, pp. 151–156, 1984.

[107] L. L. K. Leung and R. L. Nachman, "Complex formation of platelet thrombospondin with fibrinogen," *Journal of Clinical Investigation*, vol. 70, no. 3, pp. 542–549, 1982.

[108] G. P. Tuszynski, S. Srivastava, H. I. Switalska, J. C. Holt, C. S. Cierniewski, and S. Niewiarowski, "The interaction of human platelet thrombospondin with fibrinogen: thrombospondin purification and specificity of interaction," *Journal of Biological Chemistry*, vol. 260, no. 22, pp. 12240–12245, 1985.

[109] R. L. Silverstein, L. L. K. Leung, P. C. Harpel, and R. L. Nachman, "Platelet thrombospondin forms a trimolecular complex with plasminogen and histidine-rich glycoprotein," *Journal of Clinical Investigation*, vol. 75, no. 6, pp. 2065–2073, 1985.

[110] L. L. K. Leung, R. L. Nachman, and P. C. Harpel, "Complex formation of platelet thrombospondin with histidine-rich glycoprotein," *Journal of Clinical Investigation*, vol. 73, no. 1, pp. 5–12, 1984.

[111] S. M. Mumby, G. J. Raugi, and P. Bornstein, "Interactions of thrombospondin with extracellular matrix proteins: selective binding to type V collagen," *Journal of Cell Biology*, vol. 98, no. 2, pp. 646–652, 1984.

[112] J. Lawler and E. R. Simons, "Cooperative binding of calcium to thrombospondin. The effect of calcium on the circular dichroism and limited tryptic digestion of thrombospondin," *Journal of Biological Chemistry*, vol. 258, no. 20, pp. 12098–12101, 1983.

[113] D. R. Phillips, L. K. Jennings, and H. R. Prasanna, "Ca2+-mediated association of glycoprotein G (thrombin sensitive protein, thrombospondin) with human platelets," *Journal of Biological Chemistry*, vol. 255, no. 24, pp. 11629–11632, 1980.

[114] J. Lahav, "The functions of thrombospondin and its involvement in physiology and pathophysiology," *Biochimica et Biophysica Acta*, vol. 1182, no. 1, pp. 1–14, 1993.

[115] G. J. Raugi, S. M. Mumby, D. Abbott-Brown, and P. Bornstein, "Thrombospondin: synthesis and secretion by cells in culture," *Journal of Cell Biology*, vol. 95, no. 1, pp. 351–354, 1982.

[116] E. A. Jaffe, J. T. Ruggiero, L. L. K. Leung, M. J. Doyle, P. J. McKeown-Longo, and D. F. Mosher, "Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 80, no. 4, pp. 998–1002, 1983.

[117] P. J. McKeown-Longo, R. Hanning, and D. F. Mosher, "Binding and degradation of platelet thrombospondin by cultured fibroblasts," *Journal of Cell Biology*, vol. 98, no. 1, pp. 22–28, 1984.

[118] J. McPherson, H. Sage, and P. Bornstein, "Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture. Apparent identity with platelet thrombospondin," *Journal of Biological Chemistry*, vol. 256, no. 21, pp. 11330–11336, 1981.

[119] D. F. Mosher, M. J. Doyle, and E. A. Jaffe, "Synthesis and secretion of thrombospondin by cultured human endothelial cells," *Journal of Cell Biology*, vol. 93, no. 2, pp. 343–348, 1982.

[120] J. Lawler, "The structural and functional properties of thrombospondin," *Blood*, vol. 67, no. 5, pp. 1197–1209, 1986.

[121] J. Lawler, L. H. Derick, J. E. Connolly, J. H. Chen, and F. C. Chao, "The structure of human platelet thrombospondin," *Journal of Biological Chemistry*, vol. 260, no. 6, pp. 3762–3772, 1985.

[122] J. Lawler, H. S. Slayter, and J. E. Coligan, "Isolation and characterization of a high molecular weight glycoprotein from human blood platelets," *Journal of Biological Chemistry*, vol. 253, no. 23, pp. 8609–8616, 1978.

[123] J. Lawler, F. C. Chao, and P. H. Fang, "Observation of a high molecular weight platelet protein released by thrombin," *Thrombosis and Haemostasis*, vol. 37, no. 2, pp. 355–357, 1977.

[124] P. Bornstein, "Thrombospondins: structure and regulation of expression," *FASEB Journal*, vol. 6, no. 14, pp. 3290–3299, 1992.

[125] J. E. Coligan and H. S. Slayter, "Structure of thrombospondin," *Journal of Biological Chemistry*, vol. 259, no. 6, pp. 3944–3948, 1984.

[126] J. Lawler and R. O. Hynes, "The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins," *Journal of Cell Biology*, vol. 103, no. 5, pp. 1635–1648, 1986.

[127] J. Lawler, M. Duquette, L. Urry, K. McHenry, and T. F. Smith, "The evolution of the thrombospondin gene family," *Journal of Molecular Evolution*, vol. 36, no. 6, pp. 509–516, 1993.

[128] J. C. Adams, "Thrombospondins: multifunctional regulators of cell interactions," *Annual Review of Cell and Developmental Biology*, vol. 17, pp. 25–51, 2001.

[129] P. Bornstein and E. H. Sage, "Thrombospondins," *Methods in Enzymology*, vol. 245, pp. 62–85, 1994.

[130] J. Adams and J. Lawler, "The thrombospondin family," *Current Biology*, vol. 3, no. 3, pp. 188–190, 1993.

[131] G. Taraboletti, D. Roberts, L. A. Liotta, and R. Giavazzi, "Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor," *Journal of Cell Biology*, vol. 111, no. 2, pp. 765–772, 1990.

[132] R. A. Majack, L. V. Goodman, and V. M. Dixit, "Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation," *Journal of Cell Biology*, vol. 106, no. 2, pp. 415–422, 1988.

[133] G. Taraboletti, D. D. Roberts, and L. A. Liotta, "Thrombodspondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains," *Journal of Cell Biology*, vol. 105, no. 5, pp. 2409–2415, 1987.

[134] T. Vogel, N. Guo, H. C. Krutzsch et al., "Modulation of endothelial cell proliferation, adhesion, and motility by recombinant heparin-binding domain and synthetic peptides from the type I repeats of thrombospondin," *Journal of Cellular Biochemistry*, vol. 53, no. 1, pp. 74–84, 1993.

[135] N. V. Ketis, J. Lawler, R. L. Hoover, and M. J. Karnosvky, "Effects of heat shock on the expression of thrombospondin by endothelial cells in culture," *Journal of Cell Biology*, vol. 106, no. 3, pp. 893–904, 1988.

[136] N. V. Ketis and J. Lawler, "Effects of thrombospondin antibody on the recovery of endothelial cells from hyperthermia," *Journal of Cell Science*, vol. 96, no. 2, pp. 263–270, 1990.

[137] C. Kreis, M. La Fleur, C. Menard, R. Paquin, and A. D. Beaulieu, "Thrombospondin and fibronectin are synthesized by neutrophils in human inflammatory joint disease and in a rabbit model of in vivo neutrophil activation," *Journal of Immunology*, vol. 143, no. 6, pp. 1961–1968, 1989.

[138] E. A. Jaffe, J. T. Ruggiero, and D. J. Falcone, "Monocytes and macrophages synthesize and secrete thrombospondin," *Blood*, vol. 65, no. 1, pp. 79–84, 1985.

[139] K. S. O'Shea, L.-H. J. Liu, L. H. Kinnunen, and V. M. Dixit, "Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo," *Journal of Cell Biology*, vol. 111, no. 6, pp. 2713–2723, 1990.

[140] P. Bornstein, A. Agah, and T. R. Kyriakides, "The role of thrombospondins 1 and 2 in the regulation of cell-matrix interactions, collagen fibril formation, and the response to injury," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 6, pp. 1115–1125, 2004.

[141] B. Ren, K. O. Yee, J. Lawler, and R. Khosravi-Far, "Regulation of tumor angiogenesis by thrombospondin-1," *Biochimica et Biophysica Acta*, vol. 1765, no. 2, pp. 178–188, 2006.

[142] H. Chen, M. E. Herndon, and J. Lawler, "The cell biology of thrombospondin-1," *Matrix Biology*, vol. 19, no. 7, pp. 597–614, 2000.

[143] J. Lawler, "Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth," *Journal of Cellular and Molecular Medicine*, vol. 6, no. 1, pp. 1–12, 2002.

[144] V. Zabrenetzky, C. C. Harris, P. S. Steeg, and D. D. Roberts, "Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma," *International Journal of Cancer*, vol. 59, no. 2, pp. 191–195, 1994.

[145] D. L. Weinstat-Saslow, V. S. Zabrenetzky, K. VanHoutte, W. A. Frazier, D. D. Roberts, and P. S. Steeg, "Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis," *Cancer Research*, vol. 54, no. 24, pp. 6504–6511, 1994.

[146] N. Sheibani and W. A. Frazier, "Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 15, pp. 6788–6792, 1995.

[147] K. Bleuel, S. Popp, N. E. Fusenig, E. J. Stanbridge, and P. Boukamp, "Tumor suppression in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 5, pp. 2065–2070, 1999.

[148] S. C. Hsu, O. V. Volpert, P. A. Steck et al., "Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1," *Cancer Research*, vol. 56, no. 24, pp. 5684–5691, 1996.

[149] M. Streit, P. Velasco, L. F. Brown et al., "Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas," *American Journal of Pathology*, vol. 155, no. 2, pp. 441–452, 1999.

[150] A. Fontana, S. Filleur, J. Guglielmi et al., "Human breast tumors override the antiangiogenic effect of stromal thrombospondin-1 in vivo," *International Journal of Cancer*, vol. 116, no. 5, pp. 686–691, 2005.

[151] G. D. Grossfeld, D. A. Ginsberg, J. P. Stein et al., "Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression," *Journal of the National Cancer Institute*, vol. 89, no. 3, pp. 219–227, 1997.

[152] A. A. Alvarez, J. R. Axelrod, R. S. Whitaker et al., "Thrombospondin-1 expression in epithelial ovarian carcinoma: association with p53 status, tumor angiogenesis, and survival in platinum-treated patients," *Gynecologic Oncology*, vol. 82, no. 2, pp. 273–278, 2001.

[153] L. Yao, Y.-L. Zhao, S. Itoh, S. Wada, L. Yue, and I. Furuta, "Thrombospondin-1 expression in oral squamous cell carcinomas: correlations with tumor vascularity, clinicopathological features and survival," *Oral Oncology*, vol. 36, no. 6, pp. 539–544, 2000.

[154] K. Tanaka, H. Sonoo, J. Kurebayashi et al., "Inhibition of infiltration and angiogenesis by thrombospondin-1 in papillary thyroid carcinoma," *Clinical Cancer Research*, vol. 8, no. 5, pp. 1125–1131, 2002.

[155] J. Kodama, I. Hashimoto, N. Seki et al., "Thrombospondin-1 and -2 messenger RNA expression in invasive cervical cancer: correlation with angiogenesis and prognosis," *Clinical Cancer Research*, vol. 7, no. 9, pp. 2826–2831, 2001.

[156] N. Kawahara, M. Ono, K.-I. Taguhi et al., "Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma," *Hepatology*, vol. 28, no. 6, pp. 1512–1517, 1998.

[157] J. Kodama, I. Hashimoto, N. Seki et al., "Thrombospondin-1 and -2 messenger RNA expression in epithelial ovarian tumor," *Anticancer Research*, vol. 21, no. 4, pp. 2983–2987, 2001.

[158] J. Folkman, "Anti-angiogenesis: new concept for therapy of solid tumors," *Annals of Surgery*, vol. 175, no. 3, pp. 409–416, 1972.

[159] B. A. Teicher, "A systems approach to cancer therapy," *Cancer and Metastasis Reviews*, vol. 15, no. 2, pp. 247–272, 1996.

[160] D. Fukumura and R. K. Jain, "Imaging angiogenesis and the microenvironment," *APMIS*, vol. 116, no. 7-8, pp. 695–715, 2008.

[161] C. Lu, A. A. Kamat, Y. G. Lin et al., "Dual targeting of endothelial cells and pericytes in antivascular therapy for ovarian carcinoma," *Clinical Cancer Research*, vol. 13, no. 14, pp. 4209–4217, 2007.

[162] G. M. Tozer, C. Kanthou, G. Lewis, V. E. Prise, B. Vojnovic, and S. A. Hill, "Tumour vascular disrupting agents: combating treatment resistance," *British Journal of Radiology*, vol. 81, pp. S12–S20, 2008.

[163] D. Fukumura and R. K. Jain, "Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization," *Microvascular Research*, vol. 74, no. 2-3, pp. 72–84, 2007.

[164] R. K. Jain, "Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy," *Nature Medicine*, vol. 7, no. 9, pp. 987–989, 2001.

[165] P. V. Dickson, J. B. Hamner, T. L. Sims et al., "Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy," *Clinical Cancer Research*, vol. 13, no. 13, pp. 3942–3950, 2007.

[166] R. Yap, D. Veliceasa, U. Emmenegger et al., "Metronomic low-dose chemotherapy boosts CD95-dependent antiangiogenic effect of the thrombospondin peptide ABT-510: a complementation antiangiogenic strategy," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6678–6685, 2005.

[167] J. Greenaway, J. Lawler, R. Moorehead, P. Bornstein, J. Lamarre, and J. Petrik, "Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1)," *Journal of Cellular Physiology*, vol. 210, no. 3, pp. 807–818, 2007.

[168] J. Greenaway, J. Henkin, J. Lawler, R. Moorehead, and J. Petrik, "ABT-510 induces tumor cell apoptosis and inhibits ovarian tumor growth in an orthotopic, syngeneic model of epithelial ovarian cancer," *Molecular Cancer Therapeutics*, vol. 8, no. 1, pp. 64–74, 2009.

[169] K. Bein and M. Simons, "Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity," *Journal of Biological Chemistry*, vol. 275, no. 41, pp. 32167–32173, 2000.

[170] X. Qian, V. L. Rothman, R. F. Nicosia, and G. P. Tuszynski, "Expression of thrombospondin-1 in human pancreatic adenocarcinomas: role in matrix metalloproteinase-9 production," *Pathology and Oncology Research*, vol. 7, no. 4, pp. 251–259, 2001.

[171] A. Agah, T. R. Kyriakides, J. Lawler, and P. Bornstein, "The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice," *American Journal of Pathology*, vol. 161, no. 3, pp. 831–839, 2002.

[172] M. E. Herndon, C. S. Stipp, and A. D. Lander, "Interactions of neural glycosaminoglycans and proteoglycans with protein ligands: assessment of selectivity, heterogeneity and the participation of core proteins in binding," *Glycobiology*, vol. 9, no. 2, pp. 143–155, 1999.

[173] C. A. Elzie and J. E. Murphy-Ullrich, "The N-terminus of thrombospondin: the domain stands apart," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 6, pp. 1090–1101, 2004.

[174] M. A. Ferrari do Outeiro-Bernstein, S. S. Nunes, A. C. M. Andrade, T. R. Alves, C. Legrand, and V. Morandi, "A recombinant NH2-terminal heparin-binding domain of the adhesive glycoprotein, thrombospondin-1, promotes endothelial tube formation and cell survival: a possible role for syndecan-4 proteoglycan," *Matrix Biology*, vol. 21, no. 4, pp. 311–324, 2002.

[175] P. K. Anonick, J. K. Yoo, D. J. Webb, and S. L. Gonias, "Characterization of the antiplasmin activity of human thrombospondin-1 in solution," *Biochemical Journal*, vol. 289, no. 3, pp. 903–909, 1993.

[176] P. J. Hogg, "Thrombospondin 1 as an enzyme inhibitor," *Thrombosis and Haemostasis*, vol. 72, no. 6, pp. 787–792, 1994.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 135285, 12 pages
doi:10.1155/2010/135285

Review Article

Eph Receptors and Ephrin Ligands: Important Players in Angiogenesis and Tumor Angiogenesis

Birgit Mosch, Bettina Reissenweber, Christin Neuber, and Jens Pietzsch

Department of Radiopharmaceutical Biology, Institute of Radiopharmacy, Research Center Dresden-Rossendorf, 01328 Dresden, Germany

Correspondence should be addressed to Birgit Mosch, b.mosch@fzd.de

Received 2 December 2009; Accepted 5 January 2010

Academic Editor: Arkadiusz Dudek

Eph receptors and their ephrin ligands were identified in the late 1980's. Subsequently, they were linked to different physiological and pathophysiological processes like embryonic development, angiogenesis, and tumorigenesis. In this regard, recent work focused on the distribution and effects of Eph receptors and ephrins on tumor cells and tumor microenvironment. The purpose of this review is to outline the role of these molecules in physiological angiogenesis and pathophysiological tumor angiogenesis. Furthermore, novel therapeutical approaches are discussed as Eph receptors and ephrins represent attractive targets for antiangiogenic therapy.

1. Eph Receptors and Ephrins

1.1. Structure and Signaling. Eph receptors were identified in the late 1980's [1] and are known as largest family of receptor tyrosine kinases. They consist of a glycosylated extracellular domain with the immunoglobulin-like ligand-binding site, followed by a cysteine-rich region and two fibronectin type III repeats (Figure 1). Connected via a single transmembrane spanning domain, the intracellular region contains a juxtamembrane domain, a tyrosine kinase domain, a sterile alpha motif, and a PDZ-(Postsynaptic density 95-Discs large-Zonula occludentes-1) binding motif [1, 2]. Eph receptors bind membrane bound ligands, the ephrins, and both, receptors and ligands, are divided into two subclasses A or B based on binding properties and structural homologies. Class A ephrins are membrane-bound via a glycosylphosphatidylinositol anchor and class B ephrins contain a transmembrane domain and a short cytoplasmic region with conserved tyrosine residues and a PDZ-binding motif. Class A Eph receptors preferentially bind all A-type ephrins and class B Eph receptors bind all B-type ligands. However, there are some exceptions, as EphA1 primarily binds ephrinA1, EphA4 binds both, A- and B-type ligands, and ephrinA5 binds EphA receptors as well as EphB2 (Figure 2) [3–6]. Until today, 15 different receptors and 9 ligands are known (https://eph-nomenclature.med.harvard.edu/table_1.html).

In contrast to other receptor tyrosine kinases, Eph receptors/ephrins show unique properties in their activation and signaling. For the activation of the receptors not only dimerization as in most receptor tyrosine kinases is required but also multimerization of the ligands [7]. Multimer-induced signaling seems to be different from signals of normal dimers in so far as the degree of multimerization of the ephrins accounts for the kind and strength of biological effects [8]. As Eph receptors bind ligands which are also membrane bound, cell-cell contact is needed for Eph receptor activation. On the other hand, recent work demonstrated that at least A-type ephrins can be released from the cell surface [9, 10]. These soluble proteins were shown to be functionally active and possibly represent an additional signaling mechanism without mandatory cell contact. Nevertheless, Eph receptor/ephrin signaling can also proceed bidirectionally, "forward" and "reverse" [11–13]. "Forward signaling" involves binding of ephrins by the appropriate Eph receptor. This leads to autophosphorylation of intracellular tyrosine residues of the Eph receptor and further to activation of different downstream signal transduction cascades [14, 15]. In the case of B-type ephrins, signaling can also take place "reverse", if the cytoplasmic

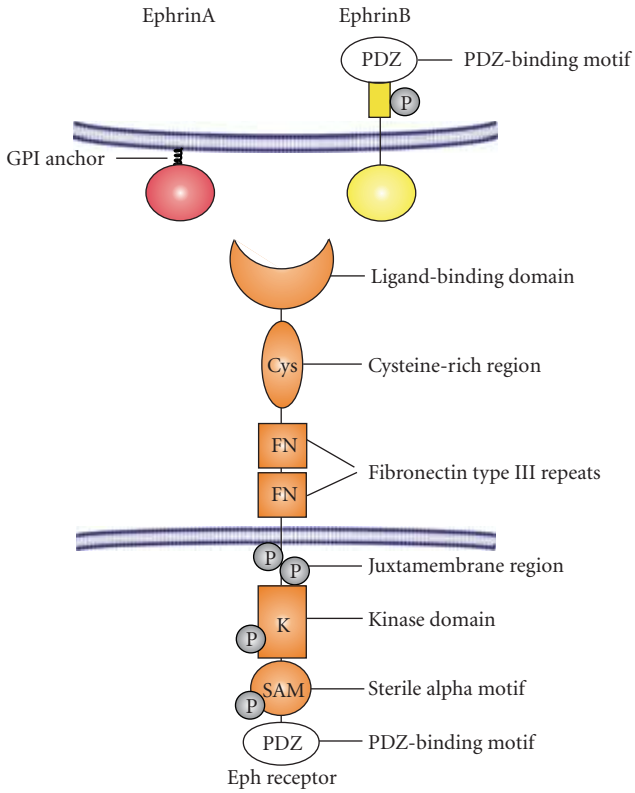


Figure 1: Structure of Eph receptors and ephrin ligands. PDZ: Postsynaptic density 95-Discs large-Zonula occludentes-1-protein, GPI: glycosylphosphatidylinositol.

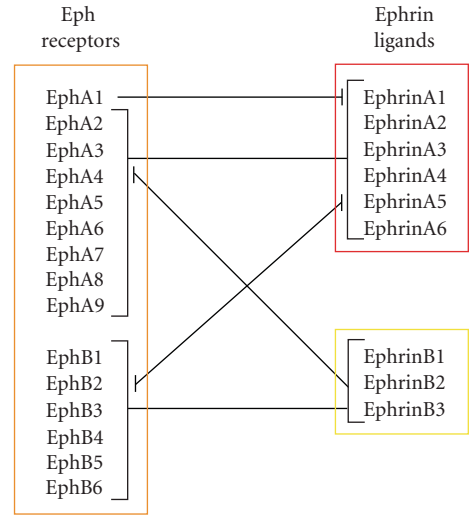


Figure 2: Major interactions of Eph receptors and ephrin ligands.

tail of the ephrin is phosphorylated which also results in activation of different signaling cascades. Moreover, it should be noted that ephrinA ligands might also have the potency to reverse signaling (overview in [4]). Many studies of the last decade indicate a complex cross-talk between Eph receptors/ephrins and other signaling pathways which is necessary for consistent biological functions. The interactions between Eph receptors/ephrins and different cell surface receptors, adhesion molecules, channels, pores, and cell surface proteases are reviewed in [16].

Taken together, Eph receptors and their appropriate ephrin ligands represent an essential communication system that directs cell motility, repulsion and adhesion, cell-cell and cell-matrix contacts in a number of biological processes. Due to the focused topic of this article, only two of them, angiogenesis and tumor angiogenesis, should be elucidated in detail, while other processes will be outlined in brief.

1.2. Embryonic and Neural Development. Eph receptor/ephrin signaling plays a crucial role in embryonic development [17]. As an example, it has been shown that altered expression of EphA3 and ephrinA5 leads to defects in gastrulation and somite development [18]. Furthermore, together with integrin-α5 and fibronectin, Eph receptors/ephrins are discussed to mediate mesenchymal-to-epithelial transition and, hence, formation of somite boundaries [19].

A further role of Eph receptor/ephrin signaling is suggested in the developing and adult vertebrate brain. Due to their complementary expression pattern, EphA4, EphB2, EphB3, and their B-type ligands are implicated in the formation of rhombomere boundaries. Thereby, bidirectional signaling seems to be required for the restriction of cell intermingling between neighboring rhombomeres [20, 21]. Furthermore, many studies analyzed the involvement of Eph receptor/ephrin signaling in neuronal growth cone collapse, leading to axon guidance by inhibition [17]. For instance, studies on EphA4- and ephrinB3-null mice indicated that both proteins are required for normal formation of the corticospinal tract fibres, whereby Eph receptor forward signaling is mandatory [22, 23]. The importance of proper ephrin ligand expression for correct outgrowth of retinal ganglion cell axons was analyzed by Hornberger and colleagues. They demonstrated that unscheduled overexpression of ephrinA2 in temporal axons leads to insensitivity of guiding outgrowing axons of the caudate tectum by repulsion [24]. In the development of the visual system it was shown that EphB2 and EphB3 receptors and B-type ephrins are involved in axon pathfinding of retinal ganglion cells to the optic disc and that deletion of both EphB2 and EphB3 leads to increased frequency of axon guidance errors in this model [25]. Furthermore, the EphB2 receptor is also involved in synaptic functions (synaptic plasticity) in the adult mammalian central nervous system [26, 27]. In this regard, Henderson and colleagues found that mice lacking the EphB2 receptor show reduced synaptic *N*-methyl-*D*-aspartate-mediated current and reduced long-term potentiation in hippocampal and dentate gyrus synapses [28].

1.3. Tumorigenesis. Eph receptor/ephrin signaling plays not only a role in physiological processes, but also in pathophysiological processes such as tumorigenesis [7, 29]. Thus, many ephrins and Eph receptors were found to be upregulated in several human carcinomas such as breast, colon, liver, prostate, and melanoma and are often associated with tumor

progression and metastasis (for overview see [7, 29–31]). On the other hand, also downregulation of Eph receptors can lead to increased metastasis and carcinogenesis as shown for EphA1 in colorectal cancer, EphA7 in prostate carcinomas, and EphB6 in melanoma [32–34]. Thereby, Eph receptors do not operate like classical oncogenic growth factor receptors, because their activation does only in exceptional cases influence proliferation of the tumor cells [35, 36]. Rather dysregulation of Eph receptor activity seems to effect cell-matrix attachment, cell-cell attachment, organization of the cytoskeleton, and modification of tumor cell survival, which could result in increased cellular motility, tumor cell invasion, and metastasis. Cell-matrix attachment can be influenced by Eph receptors via modulating the integrin activity. For instance, EphA2 stimulation with ephrinA1 leads to decreased focal adhesion kinase (FAK) phosphorylation which further results in inactive conformation of integrins and, finally, inhibition of integrin-mediated adhesion, cell spreading, and migration [37]. It is assumed that also small GTPases of the Ras and Rho family could be linked to decreased integrin activation and cellular adhesion [38]. However, the modification of cell attachment is probably dependent on the Eph receptor/ephrin ligand ratio. A high expression of Eph receptor and low expression of ephrin ligand could represent an "advantage" for tumor growth and metastasis [29]. A possible cause for imbalanced Eph receptor/ephrin ratio was recently analyzed by Winter and colleagues who identified binding sites of multiple mRNA-stabilizing and destabilizing factors at the 3'UTR sequences of Eph/ephrin transcripts. They found that binding of HuR protein (a member of the embryonic lethal abnormal vision family of RNA-binding proteins [39]) to these regions destabilized Eph/ephrin transcripts in tumor cell lines [40].

The interaction of Eph receptors and ephrins with other adhesion molecules such as E-cadherin could influence cell-cell attachment. Thereby, it is assumed that E-cadherin can influence the expression and cellular localization of Eph receptors and vice versa [41–43]. The modification of the cytoskeleton is another important prerequisite for enhanced cellular motility and invasion, respectively, and there exists evidence of involvement of Eph receptor/ephrin signaling. For instance, EphA3/ephrinA5 signaling induces growth cone collapse in retinal ganglion cells and cell rounding, blebbing, and detachment in EphA3-expressing human kidney epithelial cells and melanoma cell lines [44, 45]. In both studies it could be shown that Rho kinase is involved in the observed effects. This was further confirmed by Clifford and colleagues, who demonstrated that EphA3 receptor suppresses motility through regulation of Rho GTPases in rhabdomyosarcoma cell lines [46]. Moreover, Eph/ephrin signaling can influence cell survival as shown recently by Feng and colleagues. They demonstrated that overexpression of ephrinA2 in hepatocellular carcinoma cells leads to enhanced tumor cell survival and proved that this is caused by resistance to tumor necrosis factor-α-(TNF-α-) induced apoptosis [47]. In this regard, Holen et al. demonstrated in Jurkat TAg cells that signaling through ephrinA induced activation of Scr and Akt kinases, resulting in inhibition of antigen receptor-induced apoptosis [48]. Finally, it should be noted that some reports describe functionally relevant Eph receptor mutations in some tumor entities. For instance, mutations have been identified in EphA3 in melanoma and glioblastoma, and EphA3, EphA4, EphA7, and EphB6 in colorectal cancers [49, 50].

2. Angiogenesis

Angiogenesis is defined as growth of new blood vessels by sprouting from existing vessels [51, 52]. The lumen of blood vessels is faced by a single-layer squamous epithelium consisting of endothelial cells (ECs) which is separated from the circumjacent outer layers by the basal membrane. In small vessels (e.g., venules) ECs are enclosed by pericytes, in larger vessels by elastin fibres, smooth muscle cells and connective tissue. On one hand, ECs participate in the generation of blood vessels during embryonic development; on the other hand, they retain their ability to proliferation and migration in adult organisms, where they renew the inner wall of existing blood vessels and rebuild new vessels, for instance, in uterus mucosa during menstruation and wound healing. At the beginning of the formation of a new capillary ECs form lateral pseudopodia which develop to a hollow tube. This new capillary sprout expands until it meets another capillary sprout for fusion, resulting in blood flow. This process is regulated by different expression of surface molecules on arterial and venous capillaries.

Angiogenesis is activated by signals from the target tissues. The reaction of the ECs spans four periods: secretion of proteases to cleave the basal membrane of the parental capillary, migration of ECs towards the signal, proliferation of ECs, and, finally, formation of tubes and differentiation of the ECs. Activating signals are soluble factors whose receptors are localized predominantly on ECs. The most important factor is vascular endothelial growth factor (VEGF) and its regulator hypoxia-inducing factor (HIF-1α), which stimulates transcription of the *VEGF* gene [51, 53]. Other important growth factors, like acidic and basic fibroblast growth factor (aFGF, bFGF), can also initiate angiogenesis, whereby they affect not only ECs but also other cell types [51]. Additional vascular ECs-specific growth factors involve four members of the angiopoetin family and at least one member of the ephrin family, whereby those factors have to operate highly coordinated to form functional vessels. Finally, factors not specific for ECs are required such as platelet-derived growth factor (PDGF) and tumor growth factor-β (TGF-β) [54]. Generally, it is assumed that VEGF functions as initiator of angiogenesis in development and adult organisms (with formation of immature vessels), followed by angiopoetin-1 and ephrinB2 function, necessary for maturation and stabilization of the vessel [54]. Moreover, angiogenesis is regulated not only by activating signals but also by inhibitors, for instance, thrombospondin-1, interferon-α, platelet factor-4, and angiostatin. To date, more than 20 inducers or inhibitors of angiogenesis have been identified [51].

2.1. Role of Eph Receptors and Ephrins in Angiogenesis. Concerning Eph/ephrin signaling in angiogenesis, the pair of EphB4/ephrinB2 seems to play a key role. They are assumed to define vascular borders due to their reciprocal distribution: ephrinB2 on arteries and EphB4 on veins already in early developmental stages [55–57]. The expression of ephrinB2 persists until late embryogenesis and adulthood, with distribution expanding from arterial ECs to surrounding smooth muscle cells and pericytes [54, 58]. Generally, interplay between ECs and perivascular supporting cells mediated by ephrinB2/Eph signaling is critical for vascular development as shown in several studies [30]. For instance, Foo and colleagues demonstrated that vascular smooth muscle cells require ephrinB2 for normal association with small-diameter blood vessels [59]. In this context, Oike et al. showed that unscheduled ubiquitous ephrinB2 expression in mice development leads to sudden death in embryonic stages due to defective recruitment of vascular smooth muscle cells to the ascending aorta [60]. Simultaneously, the authors suggest that bidirectional signaling is mandatory and that cell-to-cell repellent effects are important comparable to their role in the development of the central nervous system. In this regard, Füller et al. hypothesized that distinct propulsive and repulsive effector functions of endothelial ephrinB2 and EphB4 prevent intermingling of cells and mediate spatial position signals during angiogenesis and vessel assembly [61]. The importance of reverse signaling through ephrinB2 for vascular development is outlined by Adams et al. and analyzed in detail by Salvucci et al., who found that phosphorylation at the intracellular domain of ephrinB is dependent of Src kinases and is assumed to play a role in pericyte-to-ECs assembly into vascular structures [62, 63]. Additionally, migration and proliferation of ECs were analyzed by Steinle et al., who found that stimulation of EphB4 receptors with ephrinB2-Fc fragments leads to phosphorylation of Akt kinase and, furthermore, to increased proliferation and migration of the ECs. The authors show that this is mediated by the phosphatidylinositol 3-kinase/Akt/endothelial nitric-oxide synthase/protein kinase G/mitogen-activated protein kinase axis [64].

Beside EphB4/ephrinB2 other B-class Eph and ephrins play a role in vascularization and angiogenesis. In this regard, ephrinsB1, B3, and EphB2, B3, B4 are required for the regulation of the formation of the vascular network during cardiovascular development and for vascularization processes in the female reproductive system [65–67]. Furthermore, ephrinB1 is assumed to mediate ECs attachment on extracellular matrix by activation of integrins [68].

In the case of A-class Eph/ephrins, mainly EphA2 and ephrinA1 seem to be important for angiogenic processes. For instance ephrinA1 is expressed in vascular development during embryogenesis in murine endocardium, dorsal aorta and primary head veins and later in intersomitic vessels and the limb bud vasculature [69]. This implicates that ephrinA1 expression corresponds to regions of vasculogenesis and/or angiogenesis, and presumably enhances angiogenesis [55, 69]. Additional studies illuminated the role of involved pathways. Referring to this, the role of VEGF was analyzed by Cheng et al., who demonstrated that soluble EphA2-Fc receptors inhibited VEGF-induced survival, migration, sprouting of ECs and corneal angiogenesis [70]. The authors furthermore show that TNF-α induced ephrinA1 expression on ECs. This was found to be mediated by JNK and p38MAPK signaling pathways, leading to ECs migration and blood vessel assembly [71]. Another study showed that interaction of ephrinA1 with EphA2 induced activation of PI3 kinase and Rac1 GTPase leading to ECs aggregation and migration [72]. The role of EphA2/ephrinA1 in adult angiogenesis was further analyzed by different in vitro studies. For instance it was demonstrated that ephrinA1 enhanced assembly of human umbilical venous endothelial cells (HUVEC) in matrigel and that soluble EphA2-Fc receptors inhibited microvessel formation in a rat aortic ring assay [73, 74].

3. Tumor Angiogenesis

Angiogenesis can occur not only in physiological conditions but also in abnormal processes such as tumorigenesis. It is an early- to midstage event in many human cancers and a crucial step for the transition of a small, harmless cluster of mutated tumor cells into a large, malignant growth, capable of spreading to other organs throughout the body [75]. Without angiogenesis tumor size is restricted due to lack of nutrients, growth factors, and oxygen, resulting in counterbalance of dying and proliferating cells. Hypoxia in solid tumors occurs at a distance of $\geq 70\,\mu$m from functional blood vessels and it is generally accepted that tumors do not exceed a volume of 1-2 mm^3 without the induction of angiogenesis [51, 76]. Tumor angiogenesis starts with the appearance of proteins that promote neovascularization (angiogenesis). Such proteins are produced by tumor cells themselves or by infiltrating immune cells, such as macrophages [77]. Alternatively, angiogenic proteins can be mobilized by tumor cells from the nearby tissue. Once the process is initiated it cannot be controlled or even stopped by the malignant cells [75]. Instead, newly dividing ECs release different proteins that can stimulate the proliferation or motility of tumor cells, leading to support of metastasis.

Generally, tumor cells produce two types of protein: one kind stimulates angiogenesis the other inhibits it, which lead to the hypothesis of an angiogenic switch in tumor angiogenesis [51, 76]. The most prominent angiogenic inducers are bFGF, aFGF, and VEGF with their corresponding receptors on ECs and among inhibitors are α-Interferon, platelet factor-4, and thrombospondin-1 [51]. FGF and other angiogenic factors can be sequestered in the extracellular matrix of many cell types, for instance ECs, and is believed to be released by proteolytic degradation of the extracellular matrix [51, 78]. For inhibitors alternative storage mechanisms are described: they are assumed to be stored as cryptic parts of larger molecules that are not per se inhibitors. Among them are a 29 kDa fragment of fibronectin [79], a 16 kDa fragment of prolactin [80, 81], angiostatin as fragment of plasminogen [82], a small fragment of platelet factor-4 [83], a propeptide of type 1 collagen [84], and a peptide fragment of endothelial growth factor [85]. The balance between angiogenic inducers and inhibitors

determines whether the tumor can switch on angiogenesis, whereby tumor angiogenesis is preferentially induced by a loss or decrease in the production of inhibitors. Nevertheless, the underlying mechanisms are still poorly understood and dysregulation of transcription or the activation of different proteases are under discussion.

An alternative way to facilitate tumor perfusion independent of tumor angiogenesis is the concept of vasculogenic mimicry [86, 87]. Thereby it is assumed that tumor cells re-express endothelial and mesenchymal markers, normally appearing on embryonic cells. This is accompanied by induction of vascular structures mimicking blood vessels and thus promoting tumor growth. For instance, metastatic melanoma cells are able to constitute channels filled with blood cells. This tubules exhibit a basal lamina but no ECs and the formation seems not to be dependent of bFGF, TGF-β, VEGF, PDGF, TNF-α, hypoxia, or integrins [87, 88]. In consequence, the formation of tubular networks on one hand results in better supply with nutrients and oxygen, on the other hand it can facilitate the invasion of tumor cells into the blood flow, thus, promoting metastasis [89]. Although the underlying mechanisms are not fully understood, the involvement of receptor tyrosine kinases, especially Eph receptors, is strongly suggested. In an in vitro study Hess and colleagues showed that transient knockout of EphA2 expression in aggressive uveal melanoma tumor cells resulted in inhibition of tubular network formation [88]. Further the authors found that phosphorylation of EphA2 by ephrinA1 leads to activation of downstream signaling kinases such as FAK and PI3 kinase and, furthermore, to the formation of vessel-like networks [90].

3.1. Role of Eph Receptors and Ephrins in Tumor Angiogenesis. The first reports concerning a direct connection between Eph receptor/ephrin signaling and tumor angiogenesis appeared approximately 10 years ago. Nikolova and colleagues investigated the B-class Eph receptors and ephrins and found a spatially, temporarily, and hormonally coordinated expression of EphB4 and ephrinB2 during normal mouse mammary morphogenesis. The receptor was predominantly localized in the myoepithelial cells surrounding the ducts and alveoli whereas ligand expression was limited to the luminal epithelial cells [91]. The disruption of the balanced expression lead to onset of carcinogenesis with loss of ligand expression and shift of receptor expression from myoepithelial cells surrounding the ducts to ECs with progressive malignancy [91]. The importance of EphB4/ephrinB2 in tumor angiogenesis and tumor growth was also demonstrated in recent work on mouse models. In this regard, Kimura and colleagues found that soluble ephrinB2-Fc molecules suppressed growth of head and neck squamous cell carcinoma xenografts by inducing maturation of vessels in the tumor [92]. Other studies investigating the effects of EphB4/ephrinB2 on tumor microvasculature, tumor growth, and survival of tumor cells indicated that EphB4 could act as a survival advantage in head and neck squamous cell carcinoma and in breast cancer, respectively [93, 94]. Class A molecules were analyzed by Ogawa et al. using two xenograft models from human breast cancer and Kaposi sarcoma. They found both ephrinA1 and EphA2 expressed in tumor cells and endothelial cells in these xenografts, and also in vasculature and tumor cells of surgically removed human cancers [95]. A further study revealed EphA2, in combination with VEGF, to be overexpressed in squamous cell carcinoma of oral tongue and, therefore, implicated in malignancy [96]. Today it is known that Eph receptors and ephrins are expressed in both tumor cells and tumor vasculature of many types of cancer, often at higher levels than in endogenous tissue [30]. Thereby, Eph receptor activation (forward signaling) is important as demonstrated by different studies using soluble receptors. Blocking EphA receptor signaling using soluble EphA2-Fc and EphA3-Fc receptors decreased tumor vascular density, tumor volume and cell proliferation in vivo, suggesting that the soluble receptors inhibited blood vessel recruitment by the tumor [74, 97, 98]. Furthermore, EphA2 kinase function in the tumor microenvironment seems necessary not only for tumor angiogenesis but also for metastatic progression [99, 100].

Nevertheless, reverse signaling through ephrins is another important factor in tumor angiogenesis. Expression of truncated, soluble EphB4 receptor in breast cancer cells in a mouse xenograft model (with ephrinB2 ligand primarily expressed in the vasculature) increased tumor angiogenesis, suggesting that soluble EphB4 promotes tumor growth by stimulating angiogenesis through ephrinB2 signaling [101]. Another study showed that EphB4 and ephrinB2 are expressed by ECs of human malignant brain tumors and overexpression of different EphB4 variants in blood vessels in tumor xenografts leads to the assumption that EphB4 acts as negative regulator of blood vessel branching and vascular network formation [102]. The involvement of additional Eph receptors in the switch of dormant tumors to the fast-growing angiogenic phenotype was analyzed recently by Almog and colleagues, who found increased EphA5 plasma levels in mice and, furthermore, that mRNA levels in tumor specimens of glioma patients correlated with disease stage. Hence, among other investigated molecules, EphA5 receptor possibly could represent a novel early cancer biomarker [103].

An important question remains unanswered, concerning the initiation of the altered Eph receptor/ephrin expression in tumor cells and tumor vasculature. Until now it is not fully understood which mechanisms lead to this dysregulation, but it is hypothesized that hypoxia could play a role in this context. For instance in a mouse skin flap model of hypoxia Vihanto and colleagues showed that hypoxia upregulates not only HIF-1α and VEGF but also EphB4, ephrinB2, EphA2 and ephrinA1 both on mRNA and protein levels up to 48 hours after induction of hypoxia [30, 104]. Furthermore, transcriptional profiles of umbilical cord blood and bone marrow-derived stem and progenitor cells showed that *EphA3* gene (among many other genes) is upregulated after hypoxia [105]. Another study, using HIF-2α knockdown mice showed that also HIF-2α interacts in hypoxia-induced tumor vascularization through activation of at least ephrinA1 [106]. In contrast, in neonatal rats exposed to chronic hypoxia, among others, expression of HIF-2α and ephrinA1 was downregulated [107]. However,

it remains an important field and the identification of regulating mechanisms could provide novel targets for anti-angiogenic cancer therapies.

4. Therapeutical Interventions Targeting Eph Receptors and Ephrin Ligands

In contrast to many other therapeutic approaches, anti-angiogenic therapy does not aim to destroy tumor cells directly. Instead, it prevents tumor growth by its insufficient supply with nutrients and oxygen as a result of omitted blood vessel formation [75]. Numerous small molecule inhibitors and neutralizing antibodies targeting regulators of angiogenesis such as VEGF/VEGF receptors are recently under development and in clinical evaluation [108]. For instance, recently the Food and Drug Administration of the U.S.A. approved the anti-VEGF-A-neutralizing antibody Bevacizumab for treatment of stage III-IV colorectal cancer in combination with chemotherapy and for treatment of nonsquamous non-small cell lung cancers, as well as small molecule tyrosine kinase inhibitors for treatment of renal cell cancer (Sorafenib, Sunitinib) and hepatocellular carcinoma (Sorafenib) [109]. As Eph receptors and ephrins are also significantly involved in angiogenesis and tumor angiogenesis and, therewith, in tumor progression and metastasis, they represent important targets for cancer therapy [19, 30].

To date, there are different approaches to target Eph receptors and/or ephrins, either extracellularly by preventing receptor-ligand interactions or intracellularly through inhibition of tyrosine kinases or modification of gene transcription or translation (Figure 3). One of them is the application of monoclonal antibodies, which show high specificity and are already well established tools in tumor therapy. The first ones were directed against EphA2 and showed a significant inhibition of tumor growth in vitro [110, 111]. Furthermore, effective targeting and internalizing into antigen-positive tumors in different mouse xenograft models have been reported for EphA3 and EphB2 monoclonal antibodies [112, 113]. Although the specificity for a particular binding partner is probably limited, another approach with great potential represents blocking of the Eph receptor/ephrin signaling between tumor cells and ECs by the introduction of soluble Eph receptors. In this regard, it was demonstrated that soluble monomeric EphB4 receptor resulted in dramatically reduced tumor growth in mouse models [114, 115]. Furthermore, Scehnet and colleagues fused the extracellular domain of EphB4 with human serum albumin for blocking ephrinB2 which results in inhibited migration and invasion of Kaposi sarcoma cells in response to various growth factors [116]. In addition, the role of A-class Eph receptors was analyzed and inhibition of tumor angiogenesis and suppressed tumor growth in vivo was demonstrated for soluble EphA2-Fc and EphA3-Fc receptors [74, 97, 98]. Not only Eph receptors but also ephrins show therapeutic potency as truncated soluble forms. In this regard, soluble, monomeric ephrinA1 is a functional ligand for EphA2 in glioblastoma multiforme and modulates processes relevant to the progression of malignancy [10]. Beyond tumor pathology, soluble ephrinB2-Fc or EphB4-Fc chimeras, respectively, and soluble ephrinB2 were shown to reduce pathologic neovascularization in the retina [117, 118]. Moreover, a possible therapeutic strategy represents conjugation of ephrins to gold-coated silica nanoshells, which was used to selectively target prostate tumor cells [119]. An alternative strategy for targeting Eph receptor/ephrin signaling is the application of mimetic or antagonist peptides, which were generated so far for A-class as well as for B-class Eph receptors [120–123]. Finally, an alternative "extracellular" strategy is described by Yamaguchi and colleagues who investigated peptide-pulsed dendritic cell vaccines and found that immunization with dendritic cells pulsed with EphA2-derived peptides inhibited tumor growth in vivo in EphA2-positive murine colorectal adenocarcinomas [124].

Therapeutical strategies focusing on intracellular structures involve inhibitors, selective for a single or for multiple tyrosine kinases. In this regard, several 2,5-dimethylpyrrolyl benzoic acid derivatives have been generated as selective small molecule inhibitors for EphA4 receptors, as well as 2,4-bis-anilinopyrimidines for the inhibition of EphB4 receptors [125–127]. In addition, various *N*-substituted 3-amino-4-methylbenzamide based type II kinase inhibitors were analyzed concerning their potency to inhibit EphB2 receptor [128]. A well-characterized multiple-targeted tyrosine kinase inhibitor is dasatinib. It is a dual Src/Abl kinase inhibitor, whereby FAK, Crk-associated substrate, and EphA2 receptor are assumed as additional targets. The inhibitor shows potent anti-proliferative activity against hematologic malignancies [129] and has recently been approved for treatment of all stages of chronic myelogeneous leukemia [130]. Beneath its therapeutic effects in leukemias it was shown that dasatinib blocks migration and invasion of human melanoma cells without affecting proliferation and survival [130]. Furthermore, it was demonstrated that dasatinib blocks growth, migration and invasion of breast cancer cells [131], induced apoptosis and inhibited proliferation and invasion in different ovarian cancer cell lines [132]. Of importance, dasatinib also showed therapeutic potency to inhibit EphA2 in pancreatic cancer [133]. An additional conceivable approach for therapies directed against intracellular targets is the regulation of the gene expression using small interfering RNA or antisense oligodeoxynucleotides. In this regard, Kumar et al. demonstrated that knockdown of EphB4 expression leads to anti-tumoral effects in breast cancer in vitro and in vivo [93]. Furthermore, it was demonstrated that knockdown of EphA2 suppressed ephrinA1- and VEGF-induced endothelial cell migration and inhibited cell proliferation and induced apoptosis in human glioma cells [70, 134].

In part the pharmacological approaches against Eph receptor-/ephrin-mediated tumor angiogenesis discussed above also provide the possibility to develop strategies for imaging of tumor vascularization, for instance, by means of fluorescent- or radiolabeled-small molecule kinase inhibitors or peptide ligands.

Overall, difficulties targeting Eph receptor/ephrin signaling in cancer therapy should be kept in mind. Heterogenous expression patterns of various Eph receptors/ephrins in tumor and normal tissue complicate the discrimination of malignant cells from nonmalignant cells [135]. Furthermore,

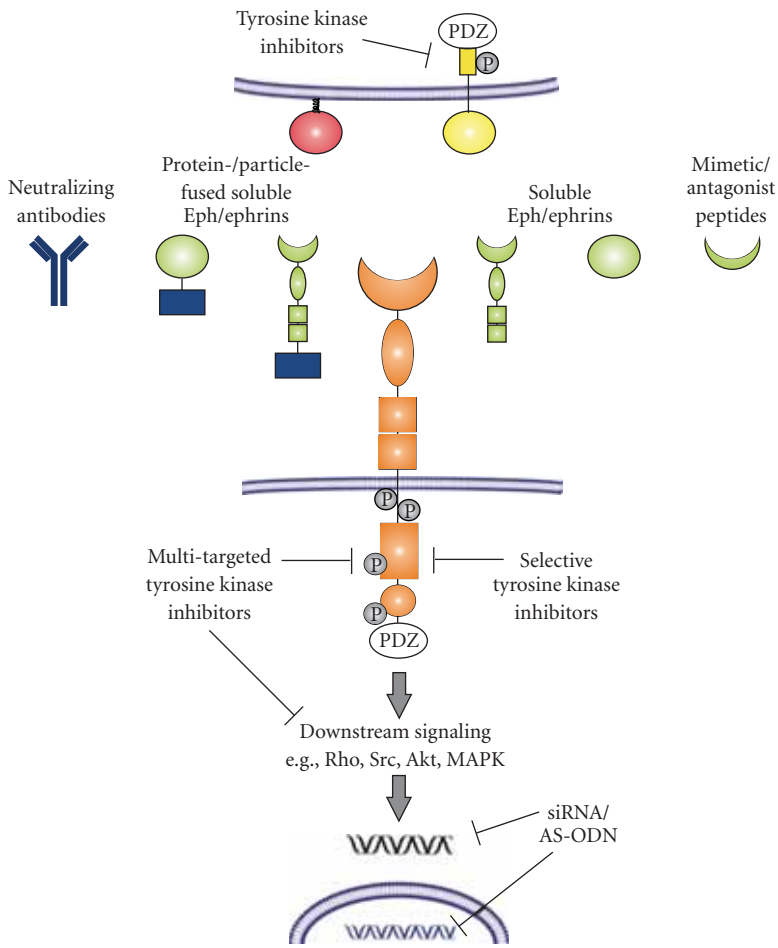


Figure 3: Potential target sites for Eph receptor/ephrin-associated antiangiogenic therapy. The illustrated strategies for intracellular inhibition of forward signaling via kinase inhibitors and gene silencing, respectively, also could be applied for reverse signaling. PDZ: Post synaptic density 95-Discs large-Zonula occludentes-1-protein, siRNA: small interfering RNA, AS-ODN: antisense oligodeoxynucleotides, Rho: Rho-GTPase Src: Src kinase, Akt: Akt kinase, MAPK: mitogen-activated protein kinase.

the effects of Eph receptor-targeting agents on normal epithelial cells are insufficiently analyzed until today [136]. Another limitation in targeting Eph receptors represents the occasional opposing effects of one Eph receptor as tumor suppressor and tumor promoter [136]. In this regard, signaling of ephrinA1 and tumor cell-specific EphA2 suppresses processes like growth and migration, whereas interaction of ephrinA1 with ECs-specific EphA2 seems to stimulate these same effects [137]. Furthermore, the efficacy of EphA2 antibody-based therapy may depend on tumor type as no suppressive effect on tumor growth was observed in a colorectal tumor model [138], whereas mice harboring ErbB2 in mammary epithelium were sensitive to therapeutic inhibition of EphA2 [139]. When targeting the Eph kinase activity, it should be noted that inhibition is useful in tumors where kinase activity promotes tumorigenesis (melanoma) but may instead be ineffective or even detrimental for the treatment of other types of cancer where Eph receptor signaling suppresses tumorigenesis [136]. In addition, the binding promiscuity of Eph receptors and ephrin ligands as well as their capability to bidirectional signaling will further complicate targeting strategies and increase the potential for adverse side effects. Therapies designed to either activate or block an Eph receptor may also alter the signaling function of the ligand in adjacent cells [136, 140]. After all, possible interactions of Eph receptor/ephrin therapeutic agents with other agents should be considered. It is assumed, that the kinase inhibitor imatinib can counteract the anti-oncogenic effects of EphB4 agonists in breast cancer [136]. On the other hand, chemotherapeutic agents that target ErbB receptors may enhance the effects of EphB4-targeted therapies [136]. Despite and due to the mentioned limitations it is necessary to understand the complex functions of Eph receptors/ephrins in homeostasis and tumor progression to avoid undesirable side effects or unintentional exacerbation of disease functions [30]. In this regard, targeting Eph receptor/ephrin signaling to inhibit tumor angiogenesis and, therewith, tumor growth represents a promising approach in fighting cancer.

5. Conclusion

Eph receptors and their ligands, the ephrins, form a complex cellular communication system. Its complexity is based on the large number of different receptor and ligand molecules, their promiscuous binding properties, the ability to bidirectional signaling, formation of multimers, and crosstalk with other signaling pathways and molecules. An intricacy, we just begin to understand. Eph receptors and ephrins are involved in embryonic development, development of the nervous system, angiogenesis and also in tumorigenesis and tumor angiogenesis, respectively. They mediate cell-cell repellent effects, cell-cell and cell-matrix attachment, they influence cell survival and cytoskeleton dynamics, affecting cell motility, which could further result in tumor progression, invasion and metastasis. In the last decade Eph receptors and ephrin ligands were put in perspective to anti-tumoral and anti-angiogenic therapy. To date, many different therapeutic strategies targeting Eph receptors or ephrins are pursued and hopefully result in improvement of cancer treatment in the near future.

Acknowledgments

The authors thank Constantin Mamat, Ph.D., and Susan Richter for stimulating discussions.

References

[1] H. Hirai, Y. Maru, K. Hagiwara, J. Nishida, and F. Takaku, "A novel putative tyrosine kinase receptor encoded by the eph gene," *Science*, vol. 238, no. 4834, pp. 1717–1720, 1987.

[2] D. Brantley-Sieders, S. Schmidt, M. Parker, and J. Chen, "Eph receptor tyrosine kinases in tumor and tumor microenvironment," *Current Pharmaceutical Design*, vol. 10, no. 27, pp. 3431–3442, 2004.

[3] R. Zhou, "The Eph family receptors and ligands," *Pharmacology and Therapeutics*, vol. 77, no. 3, pp. 151–181, 1998.

[4] K. Kullander and R. Klein, "Mechanisms and functions of Eph and ephrin signalling," *Nature Reviews Molecular Cell Biology*, vol. 3, no. 7, pp. 475–486, 2002.

[5] J.-P. Himanen, M. J. Chumley, M. Lackmann, et al., "Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling," *Nature Neuroscience*, vol. 7, no. 5, pp. 501–509, 2004.

[6] E. B. Pasquale, "Eph-ephrin promiscuity is now crystal clear," *Nature Neuroscience*, vol. 7, no. 5, pp. 417–418, 2004.

[7] H. Surawska, P. C. Ma, and R. Salgia, "The role of ephrins and Eph receptors in cancer," *Cytokine and Growth Factor Reviews*, vol. 15, no. 6, pp. 419–433, 2004.

[8] E. Stein, A. A. Lane, D. P. Cerretti, et al., "Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses," *Genes and Development*, vol. 12, no. 5, pp. 667–678, 1998.

[9] S. C. Alford, J. Bazowski, H. Lorimer, S. Elowe, and P. L. Howard, "Tissue transglutaminase clusters soluble A-type ephrins into functionally active high molecular weight oligomers," *Experimental Cell Research*, vol. 313, no. 20, pp. 4170–4179, 2007.

[10] J. Wykosky, E. Palma, D. M. Gibo, S. Ringler, C. P. Turner, and W. Debinski, "Soluble monomeric EphrinA1 is released from tumor cells and is a functional ligand for the EphA2 receptor," *Oncogene*, vol. 27, no. 58, pp. 7260–7273, 2008.

[11] K. Brückner, E. B. Pasquale, and R. Klein, "Tyrosine phosphorylation of transmembrane ligands for Eph receptors," *Science*, vol. 275, no. 5306, pp. 1640–1643, 1997.

[12] S. J. Holland, N. W. Gale, G. Mbamalu, G. D. Yancopoulos, M. Henkemeyer, and T. Pawson, "Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands," *Nature*, vol. 383, no. 6602, pp. 722–725, 1996.

[13] E. B. Pasquale, "Eph-ephrin bidirectional signaling in physiology and disease," *Cell*, vol. 133, no. 1, pp. 38–52, 2008.

[14] K. L. Binns, P. P. Taylor, F. Sicheri, T. Pawson, and S. J. Holland, "Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors," *Molecular and Cellular Biology*, vol. 20, no. 13, pp. 4791–4805, 2000.

[15] A. H. Zisch, C. Pazzagli, A. L. Freeman, et al., "Replacing two conserved tyrosines of the EphB2 receptor with glutamic acid prevents binding of SH2 domains without abrogating kinase activity and biological responses," *Oncogene*, vol. 19, no. 2, pp. 177–187, 2000.

[16] D. Arvanitis and A. Davy, "Eph/ephrin signaling: networks," *Genes and Development*, vol. 22, no. 4, pp. 416–429, 2008.

[17] N. Holder and R. Klein, "Eph receptors and ephrins: effectors of morphogenesis," *Development*, vol. 126, no. 10, pp. 2033–2044, 1999.

[18] A. C. Oates, M. Lackmann, M.-A. Power, et al., "An early developmental role for Eph-ephrin interaction during vertebrate gastrulation," *Mechanisms of Development*, vol. 83, no. 1-2, pp. 77–94, 1999.

[19] P. W. Janes, S. Adikari, and M. Lackmann, "Eph/ephrin signalling and function in oncogenesis: lessons from embryonic development," *Current Cancer Drug Targets*, vol. 8, no. 6, pp. 473–489, 2008.

[20] R. Klein, "Neural development: bidirectional signals establish boundaries," *Current Biology*, vol. 9, no. 18, pp. R691–R694, 1999.

[21] G. Mellitzer, Q. Xu, and D. G. Wilkinson, "Eph receptors and ephrins restrict cell intermingling and communication," *Nature*, vol. 400, no. 6739, pp. 77–81, 1999.

[22] M. Dottori, L. Hartley, M. Galea, et al., "EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 22, pp. 13248–13253, 1998.

[23] K. Kullander, N. K. Mather, F. Diella, M. Dottori, A. W. Boyd, and R. Klein, "Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo," *Neuron*, vol. 29, no. 1, pp. 73–84, 2001.

[24] M. R. Hornberger, D. Dütting, T. Ciossek, et al., "Modulation of EphA receptor function by coexpressed EphrinA ligands on retinal ganglion cell axons," *Neuron*, vol. 22, no. 4, pp. 731–742, 1999.

[25] E. Birgbauer, C. A. Cowan, D. W. Sretavan, and M. Henkemeyer, "Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina," *Development*, vol. 127, no. 6, pp. 1231–1241, 2000.

[26] Y. Chen, A. K. Y. Fu, and N. Y. Ip, "Bidirectional signaling of ErbB and Eph receptors at synapses," *Neuron Glia Biology*, vol. 4, no. 3, pp. 211–221, 2008.

[27] R. Klein, "Bidirectional modulation of synaptic functions by Eph/ephrin signaling," *Nature Neuroscience*, vol. 12, no. 1, pp. 15–20, 2009.

[28] J. T. Henderson, J. Georgiou, Z. Jia, et al., "The receptor tyrosine kinase EphB2 regulates NMDA-dependent synaptic function," *Neuron*, vol. 32, no. 6, pp. 1041–1056, 2001.

[29] V. C. Dodelet and E. B. Pasquale, "Eph receptors and ephrin ligands: embryogenesis to tumorigenesis," *Oncogene*, vol. 19, no. 49, pp. 5614–5619, 2000.

[30] L. Frieden and J. Chen, "Ephrins and Eph receptors in cancer," in *Recent Progress in Cancer Research*, C. K. Tang, Ed., pp. 11–35, 2007.

[31] S. H. Wimmer-Kleikamp and M. Lackmann, "Eph-modulated cell morphology, adhesion and motility in carcinogenesis," *IUBMB Life*, vol. 57, no. 6, pp. 421–431, 2005.

[32] Y. Dong, J. Wang, Z. Sheng, et al., "Downregulation of EphA1 in colorectal carcinomas correlates with invasion and metastasis," *Modern Pathology*, vol. 22, no. 1, pp. 151–160, 2009.

[33] M. Guan, C. Xu, F. Zhang, and C. Ye, "Aberrant methylation of EphA7 in human prostate cancer and its relation to clinicopathologic features," *International Journal of Cancer*, vol. 124, no. 1, pp. 88–94, 2009.

[34] C. Hafner, F. Bataille, S. Meyer, et al., "Loss of EphB6 expression in metastatic melanoma," *International Journal of Oncology*, vol. 23, no. 6, pp. 1553–1559, 2003.

[35] Y. Maru, H. Hirai, and F. Takaku, "Overexpression confers an oncogenic potential upon the eph gene," *Oncogene*, vol. 5, no. 3, pp. 445–447, 1990.

[36] H. Miao, B.-R. Wei, D. M. Peehl, et al., "Activation of EphA receptor tyrosine kinase inhibits the Ras/MAPK pathway," *Nature Cell Biology*, vol. 3, no. 5, pp. 527–530, 2001.

[37] H. Miao, E. Burnett, M. Kinch, E. Simon, and B. Wang, "Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation," *Nature Cell Biology*, vol. 2, no. 2, pp. 62–69, 2000.

[38] J. X. Zou, B. Wang, M. S. Kalo, A. H. Zisch, E. B. Pasquale, and E. Ruoslahti, "An Eph receptor regulates integrin activity through R-Ras," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 24, pp. 13813–13818, 1999.

[39] I.-E. Gallouzi, C. M. Brennan, M. G. Stenberg, et al., "HuR binding to cytoplasmic mRNA is perturbed by heat shock," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 7, pp. 3073–3078, 2000.

[40] J. Winter, S. Roepcke, S. Krause, et al., "Comparative 3′UTR analysis allows identification of regulatory clusters that drive Eph/ephrin expression in cancer cell lines," *PLoS ONE*, vol. 3, no. 7, article e2780, 2008.

[41] S.-T. Chiu, K.-J. Chang, C.-H. Ting, H.-C. Shen, H. Li, and F.-J. Hsieh, "Over-expression of EphB3 enhances cell-cell contacts and suppresses tumor growth in HT-29 human colon cancer cells," *Carcinogenesis*, vol. 30, no. 9, pp. 1475–1486, 2009.

[42] C. Cortina, S. Palomo-Ponce, M. Iglesias, et al., "EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells," *Nature Genetics*, vol. 39, no. 11, pp. 1376–1383, 2007.

[43] N. D. Zantek, M. Azimi, M. Fedor-Chaiken, B. Wang, R. Brackenbury, and M. S. Kinch, "E-cadherin regulates the function of the EphA2 receptor tyrosine kinase," *Cell Growth and Differentiation*, vol. 10, no. 9, pp. 629–638, 1999.

[44] I. D. Lawrenson, S. H. Wimmer-Kleikamp, P. Lock, et al., "Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293T and melanoma cells by CrkII and Rho-mediated signalling," *Journal of Cell Science*, vol. 115, no. 5, pp. 1059–1072, 2002.

[45] S. Wahl, H. Barth, T. Ciossek, K. Aktories, and B. K. Mueller, "Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase," *Journal of Cell Biology*, vol. 149, no. 2, pp. 263–270, 2000.

[46] N. Clifford, L. M. Smith, J. Powell, S. Gattenlöhner, A. Marx, and R. O'Connor, "The EphA3 receptor is expressed in a subset of rhabdomyosarcoma cell lines and suppresses cell adhesion and migration," *Journal of Cellular Biochemistry*, vol. 105, no. 5, pp. 1250–1259, 2008.

[47] Y.-X. Feng, J.-S. Zhao, J.-J. Li, et al., "Liver cancer: EphrinA2 promotes tumorigenicity through Rac1/Akt/NF-κB signaling pathway," *Hepatology*, vol. 51, no. 2, pp. 535–544, 2010.

[48] H. L. Holen, M. Shadidi, K. Narvhus, O. Kjøsnes, A. Tierens, and H.-C. Aasheim, "Signaling through ephrin-A ligand leads to activation of Src-family kinases, Akt phosphorylation, and inhibition of antigen receptor-induced apoptosis," *Journal of Leukocyte Biology*, vol. 84, no. 4, pp. 1183–1191, 2008.

[49] A. Balakrishnan, F. E. Bleeker, S. Lamba, et al., "Novel somatic and germline mutations in cancer candidate genes in glioblastoma, melanoma, and pancreatic carcinoma," *Cancer Research*, vol. 67, no. 8, pp. 3545–3550, 2007.

[50] J. Lin, C. M. Gan, X. Zhang, et al., "A multidimensional analysis of genes mutated in breast and colorectal cancers," *Genome Research*, vol. 17, no. 9, pp. 1304–1318, 2007.

[51] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[52] M. L. Iruela-Arispe and H. F. Dvorak, "Angiogenesis: a dynamic balance of stimulators and inhibitors," *Thrombosis and Haemostasis*, vol. 78, no. 1, pp. 672–677, 1997.

[53] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[54] G. D. Yancopoulos, S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash, "Vascular-specific growth factors and blood vessel formation," *Nature*, vol. 407, no. 6801, pp. 242–248, 2000.

[55] D. M. Brantley-Sieders and J. Chen, "Eph receptor tyrosine kinases in angiogenesis: from development to disease," *Angiogenesis*, vol. 7, no. 1, pp. 17–28, 2004.

[56] Y. H. Kim, H. Hu, S. Guevara-Gallardo, M. T. Y. Lam, S.-Y. Fong, and R. A. Wang, "Artery and vein size is balanced by Notch and ephrin B2/EphB4 during angiogenesis," *Development*, vol. 135, no. 22, pp. 3755–3764, 2008.

[57] H. U. Wang, Z.-F. Chen, and D. J. Anderson, "Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4," *Cell*, vol. 93, no. 5, pp. 741–753, 1998.

[58] N. W. Gale, P. Baluk, L. Pan, et al., "Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells," *Developmental Biology*, vol. 230, no. 2, pp. 151–160, 2001.

[59] S. S. Foo, C. J. Turner, S. Adams, et al., "Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly," *Cell*, vol. 124, no. 1, pp. 161–173, 2006.

[60] Y. Oike, Y. Ito, K. Hamada, et al., "Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells," *Blood*, vol. 100, no. 4, pp. 1326–1333, 2002.

[61] T. Füller, T. Korff, A. Kilian, G. Dandekar, and H. G. Augustin, "Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells," *Journal of Cell Science*, vol. 116, no. 12, pp. 2461–2470, 2003.

[62] R. H. Adams, F. Diella, S. Hennig, F. Helmbacher, U. Deutsch, and R. Klein, "The cytoplasmic domain of the ligand EphrinB2 is required for vascular morphogenesis but not cranial neural crest migration," *Cell*, vol. 104, no. 1, pp. 57–69, 2001.

[63] O. Salvucci, D. Maric, M. Economopoulou, et al., "EphrinB reverse signaling contributes to endothelial and mural cell assembly into vascular structures," *Blood*, vol. 114, no. 8, pp. 1707–1716, 2009.

[64] J. J. Steinle, C. J. Meininger, R. Forough, G. Wu, M. H. Wu, and H. J. Granger, "Eph B4 receptor signaling mediates endothelial cell migration and proliferation via the phosphatidylinositol 3-kinase pathway," *Journal of Biological Chemistry*, vol. 277, no. 46, pp. 43830–43835, 2002.

[65] R. H. Adams, G. A. Wilkinson, C. Weiss, et al., "Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis," *Genes and Development*, vol. 13, no. 3, pp. 295–306, 1999.

[66] J. Cross, M. Hemberger, Y. Lu, et al., "Trophoblast functions, angiogenesis and remodeling of the maternal vasculature in the placenta," *Molecular and Cellular Endocrinology*, vol. 187, no. 1-2, pp. 207–212, 2002.

[67] M. Egawa, S. Yoshioka, T. Higuchi, et al., "Ephrin B1 is expressed on human luteinizing granulosa cells in corpora lutea of the early luteal phase: the possible involvement of the B class Eph-ephrin system during corpus luteum formation," *Journal of Clinical Endocrinology and Metabolism*, vol. 88, no. 9, pp. 4384–4392, 2003.

[68] U. Huynh-Do, E. Stein, A. A. Lane, H. Liu, D. P. Cerretti, and T. O. Daniel, "Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins," *EMBO Journal*, vol. 18, no. 8, pp. 2165–2173, 1999.

[69] J. L. McBride and J. C. Ruiz, "Ephrin-A1 is expressed at sites of vascular development in the mouse," *Mechanisms of Development*, vol. 77, no. 2, pp. 201–204, 1998.

[70] N. Cheng, D. M. Brantley, H. Liu, et al., "Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis," *Molecular Cancer Research*, vol. 1, no. 1, pp. 2–11, 2002.

[71] N. Cheng, D. M. Brantley, and J. Chen, "The ephrins and Eph receptors in angiogenesis," *Cytokine and Growth Factor Reviews*, vol. 13, no. 1, pp. 75–85, 2002.

[72] D. M. Brantley-Sieders, J. Caughron, D. Hicks, A. Pozzi, J. C. Ruiz, and J. Chen, "EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation," *Journal of Cell Science*, vol. 117, no. 10, pp. 2037–2049, 2004.

[73] T. O. Daniel, E. Stein, D. P. Cerretti, P. L. St. John, B. Robert, and D. R. Abrahamson, "ELK and LERK-2 in developing kidney and microvascular endothelial assembly," *Kidney International*, vol. 50, no. 57, pp. S73–S81, 1996.

[74] P. Dobrzanski, K. Hunter, S. Jones-Bolin, et al., "Antiangiogenic and antitumor efficacy of EphA2 receptor antagonist," *Cancer Research*, vol. 64, no. 3, pp. 910–919, 2004.

[75] J. Folkman, "Fighting cancer by attacking its blood supply," *Scientific American*, vol. 275, no. 3, pp. 150–154, 1996.

[76] D. Ribatti, B. Nico, E. Crivellato, A. M. Roccaro, and A. Vacca, "The history of the angiogenic switch concept," *Leukemia*, vol. 21, no. 1, pp. 44–52, 2007.

[77] C. Liss, M. J. Fekete, R. Hasina, and M. W. Lingen, "Retinoic acid modulates the ability of macrophages to participate in the induction of the angiogenic phenotype in head and neck squamous cell carcinoma," *International Journal of Cancer*, vol. 100, no. 3, pp. 283–289, 2002.

[78] A. Baird and N. Ling, "Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response," *Biochemical and Biophysical Research Communications*, vol. 142, no. 2, pp. 428–435, 1987.

[79] G. A. Homandberg, J. E. Williams, and D. Grant, "Heparin-binding fragments of fibronectin are potent inhibitors of endothelial cell growth," *American Journal of Pathology*, vol. 120, no. 3, pp. 327–332, 1985.

[80] C. Clapp, J. A. Martial, R. C. Guzman, F. Rentier-Delrue, and R. I. Weiner, "The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis," *Endocrinology*, vol. 133, no. 3, pp. 1292–1299, 1993.

[81] N. Ferrara, C. Clapp, and R. Weiner, "The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells," *Endocrinology*, vol. 129, no. 2, pp. 896–900, 1991.

[82] M. S. O'Reilly, L. Holmgren, Y. Shing, et al., "Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma," *Cell*, vol. 79, no. 2, pp. 315–328, 1994.

[83] S. K. Gupta, T. Hassel, and J. P. Singh, "A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 17, pp. 7799–7803, 1995.

[84] S. S. Tolsma, O. V. Volpert, D. J. Good, W. A. Frazier, P. J. Polverini, and N. Bouck, "Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity," *Journal of Cell Biology*, vol. 122, no. 2, pp. 497–511, 1993.

[85] J. Nelson, W. E. Allen, W. N. Scott, et al., "Murine epidermal growth factor (EGF) fragment (33–42) inhibits both EGF- and laminin-dependent endothelial cell motility and angiogenesis," *Cancer Research*, vol. 55, no. 17, pp. 3772–3776, 1995.

[86] M. J. Bissell, "Tumor plasticity allows vasculogenic mimicry, a novel form of angiogenic switch: a rose by any other name?" *American Journal of Pathology*, vol. 155, no. 3, pp. 675–679, 1999.

[87] A. J. Maniotis, R. Folberg, A. Hess, et al., "Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry," *American Journal of Pathology*, vol. 155, no. 3, pp. 739–752, 1999.

[88] A. R. Hess, E. A. Seftor, L. M. G. Gardner, et al., "Molecular regulation of tumor cell vasculogenic mimicry by tyrosine phosphorylation: role of epithelial cell kinase (Eck/EphA2)," *Cancer Research*, vol. 61, no. 8, pp. 3250–3255, 2001.

[89] S. Meyer, C. Hafner, and T. Vogt, "Role of receptor tyrosine kinase in the angiogenesis," *Hautarzt*, vol. 53, no. 9, pp. 629–642, 2002.

[90] A. R. Hess, E. A. Seftor, L. M. Gruman, M. S. Kinch, R. E. B. Seftor, and M. J. C. Hendrix, "VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry," *Cancer Biology and Therapy*, vol. 5, no. 2, pp. 228–233, 2006.

[91] Z. Nikolova, V. Djonov, G. Zuercher, A.-C. Andres, and A. Ziemiecki, "Cell-type specific and estrogen dependent expression of the receptor tyrosine kinase EphB4 and its ligand ephrin-B2 during mammary gland morphogenesis," *Journal of Cell Science*, vol. 111, no. 18, pp. 2741–2751, 1998.

[92] M. Kimura, Y. Kato, D. Sano, et al., "Soluble form of ephrinB2 inhibits xenograft growth of squamous cell carcinoma of the Head and neck," *International Journal of Oncology*, vol. 34, no. 2, pp. 321–327, 2009.

[93] S. R. Kumar, J. Singh, G. Xia, et al., "Receptor tyrosine kinase EphB4 is a survival factor in breast cancer," *American Journal of Pathology*, vol. 169, no. 1, pp. 279–293, 2006.

[94] R. Masood, S. Ram Kumar, U. K. Sinha, et al., "EphB4 provides survival advantage to squamous cell carcinoma of the head and neck," *International Journal of Cancer*, vol. 119, no. 6, pp. 1236–1248, 2006.

[95] K. Ogawa, R. Pasqualini, R. A. Lindberg, R. Kain, A. L. Freeman, and E. B. Pasquale, "The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization," *Oncogene*, vol. 19, no. 52, pp. 6043–6052, 2000.

[96] Z. Shao, W.-F. Zhang, X.-M. Chen, and Z.-J. Shang, "Expression of EphA2 and VEGF in squamous cell carcinoma of the tongue: correlation with the angiogenesis and clinical outcome," *Oral Oncology*, vol. 44, no. 12, pp. 1110–1117, 2008.

[97] D. M. Brantley, N. Cheng, E. J. Thompson, et al., "Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo," *Oncogene*, vol. 21, no. 46, pp. 7011–7026, 2002.

[98] N. Cheng, D. Brantley, W. B. Fang, et al., "Inhibition of VEGF-dependent multistage carcinogenesis by soluble EphA receptors," *Neoplasia*, vol. 5, no. 5, pp. 445–456, 2003.

[99] D. M. Brantley-Sieders, W. B. Fang, D. J. Hicks, G. Zhuang, Y. Shyr, and J. Chen, "Impaired tumor microenvironment in EphA2-deficient mice inhibits tumor angiogenesis and metastatic progression," *FASEB Journal*, vol. 19, no. 13, pp. 1884–1886, 2005.

[100] C. Lu, M. M. K. Shahzad, H. Wang, et al., "EphA2 overexpression promotes ovarian cancer growth," *Cancer Biology and Therapy*, vol. 7, no. 7, pp. 1098–1103, 2008.

[101] N. K. Noren, M. Lu, A. L. Freeman, M. Koolpe, and E. B. Pasquale, "Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 15, pp. 5583–5588, 2004.

[102] R. Erber, U. Eichelsbacher, V. Powajbo, et al., "EphB4 controls blood vascular morphogenesis during postnatal angiogenesis," *EMBO Journal*, vol. 25, no. 3, pp. 628–641, 2006.

[103] N. Almog, L. Ma, R. Raychowdhury, et al., "Transcriptional switch of dormant tumors to fast-growing angiogenic phenotype," *Cancer Research*, vol. 69, no. 3, pp. 836–844, 2009.

[104] M. M. Vihanto, J. Plock, D. Erni, B. M. Frey, F. J. Frey, and U. Huynh-Do, "Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin," *FASEB Journal*, vol. 19, no. 12, pp. 1689–1691, 2005.

[105] E. Martin-Rendon, S. J. M. Hale, D. Ryan, et al., "Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia," *Stem Cells*, vol. 25, no. 4, pp. 1003–1012, 2007.

[106] T. Yamashita, K. Ohneda, M. Nagano, et al., "Hypoxia-inducible transcription factor-2α in endothelial cells regulates tumor neovascularization through activation of ephrin A1," *Journal of Biological Chemistry*, vol. 283, no. 27, pp. 18926–18936, 2008.

[107] W. E. Truog, D. Xu, I. I. Ekekezie, et al., "Chronic hypoxia and rat lung development: analysis by morphometry and directed microarray," *Pediatric Research*, vol. 64, no. 1, pp. 56–62, 2008.

[108] D. Marme, "The impact of anti-angiogenic agents on cancer therapy," *Journal of Cancer Research and Clinical Oncology*, vol. 129, no. 11, pp. 607–620, 2003.

[109] M. Shibuya, "Vascular endothelial growth factor-dependent and -independent regulation of angiogenesis," *Journal of Biochemistry and Molecular Biology*, vol. 41, no. 4, pp. 278–286, 2008.

[110] K. Carles-Kinch, K. E. Kilpatrick, J. C. Stewart, and M. S. Kinch, "Antibody targeting of the EphA2 tyrosine kinase inhibits malignant cell behavior," *Cancer Research*, vol. 62, no. 10, pp. 2840–2847, 2002.

[111] K. T. Coffman, M. Hu, K. Carles-Kinch, et al., "Differential EphA2 epitope display on normal versus malignant cells," *Cancer Research*, vol. 63, no. 22, pp. 7907–7912, 2003.

[112] W. Mao, E. Luis, S. Ross, et al., "EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer," *Cancer Research*, vol. 64, no. 3, pp. 781–788, 2004.

[113] C. Vearing, F.-T. Lee, S. Wimmer-Kleikamp, et al., "Concurrent binding of anti-EphA3 antibody and ephrin-A5 amplifies EphA3 signaling and downstream responses: potential as EphA3-specific tumor-targeting reagents," *Cancer Research*, vol. 65, no. 15, pp. 6745–6754, 2005.

[114] N. Kertesz, V. Krasnoperov, R. Reddy, et al., "The soluble extracellular domain of EphB4 (sEphB4) antagonizes EphB4-EphrinB2 interaction, modulates angiogenesis, and inhibits tumor growth," *Blood*, vol. 107, no. 6, pp. 2330–2338, 2006.

[115] G. Martiny-Baron, T. Korff, F. Schaffner, et al., "Inhibition of tumor growth and angiogenesis by soluble EphB4," *Neoplasia*, vol. 6, no. 3, pp. 248–257, 2004.

[116] J. S. Scehnet, E. J. Ley, V. Krasnoperov, et al., "The role of Ephs, Ephrins, and growth factors in Kaposi sarcoma and implications of EphrinB2 blockade," *Blood*, vol. 113, no. 1, pp. 254–263, 2009.

[117] M. H. Davies, D. O. Zamora, J. R. Smith, and M. R. Powers, "Soluble ephrin-B2 mediates apoptosis in retinal neovascularization and in endothelial cells," *Microvascular Research*, vol. 77, no. 3, pp. 382–386, 2009.

[118] D. O. Zamora, M. H. Davies, S. R. Planck, J. T. Rosenbaum, and M. R. Powers, "Soluble forms of EphrinB2 and EphB4 reduce retinal neovascularization in a model of proliferative retinopathy," *Investigative Ophthalmology and Visual Science*, vol. 46, no. 6, pp. 2175–2182, 2005.

[119] A. M. Gobin, J. J. Moon, and J. L. West, "Ephrin A1-targeted nanoshells for photothermal ablation of prostate cancer cells," *International Journal of Nanomedicine*, vol. 3, no. 3, pp. 351–358, 2008.

[120] J. E. Chrencik, A. Brooun, M. I. Recht, et al., "Structure and thermodynamic characterization of the EphB4/Ephrin-B2 antagonist peptide complex reveals the determinants for receptor specificity," *Structure*, vol. 14, no. 2, pp. 321–330, 2006.

[121] J. E. Chrencik, A. Brooun, M. I. Recht, et al., "Three-dimensional structure of the EphB2 receptor in complex with an antagonistic peptide reveals a novel mode of inhibition," *Journal of Biological Chemistry*, vol. 282, no. 50, pp. 36505–36513, 2007.

[122] M. Koolpe, R. Burgess, M. Dail, and E. B. Pasquale, "EphB receptor-binding peptides identified by phage display enable design of an antagonist with ephrin-like affinity," *Journal of Biological Chemistry*, vol. 280, no. 17, pp. 17301–17311, 2005.

[123] M. Koolpe, M. Dail, and E. B. Pasquale, "An ephrin mimetic peptide that selectively targets the EphA2 receptor," *Journal of Biological Chemistry*, vol. 277, no. 49, pp. 46974–46979, 2002.

[124] S. Yamaguchi, T. Tatsumi, T. Takehara, et al., "Immunotherapy of murine colon cancer using receptor tyrosine kinase EphA2-derived peptide-pulsed dendritic cell vaccines," *Cancer*, vol. 110, no. 7, pp. 1469–1477, 2007.

[125] C. Bardelle, T. Coleman, D. Cross, et al., "Inhibitors of the tyrosine kinase EphB4. Part 2: structure-based discovery and optimisation of 3,5-bis substituted anilinopyrimidines," *Bioorganic and Medicinal Chemistry Letters*, vol. 18, no. 21, pp. 5717–5721, 2008.

[126] C. Bardelle, D. Cross, S. Davenport, et al., "Inhibitors of the tyrosine kinase EphB4. Part 1: structure-based design and optimization of a series of 2,4-bis-anilinopyrimidines," *Bioorganic and Medicinal Chemistry Letters*, vol. 18, no. 9, pp. 2776–2780, 2008.

[127] R. Noberini, M. Koolpe, S. Peddibhotla, et al., "Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors," *Journal of Biological Chemistry*, vol. 283, no. 43, pp. 29461–29472, 2008.

[128] Y. Choi, F. Syeda, J. R. Walker, et al., "Discovery and structural analysis of Eph receptor tyrosine kinase inhibitors," *Bioorganic and Medicinal Chemistry Letters*, vol. 19, no. 15, pp. 4467–4470, 2009.

[129] S. J. Keam, "Dasatinib: in chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia," *BioDrugs*, vol. 22, no. 1, pp. 59–69, 2008.

[130] R. Buettner, T. Mesa, A. Vultur, F. Lee, and R. Jove, "Inhibition of Src family kinases with dasatinib blocks migration and invasion of human melanoma cells," *Molecular Cancer Research*, vol. 6, no. 11, pp. 1766–1774, 2008.

[131] C. S. Pichot, S. M. Hartig, L. Xia, et al., "Dasatinib synergizes with doxorubicin to block growth, migration, and invasion of breast cancer cells," *British Journal of Cancer*, vol. 101, no. 1, pp. 38–47, 2009.

[132] G. E. Konecny, R. Glas, J. Dering, et al., "Activity of the multikinase inhibitor dasatinib against ovarian cancer cells," *British Journal of Cancer*, vol. 101, no. 10, pp. 1699–1708, 2009.

[133] Q. Chang, C. Jorgensen, T. Pawson, and D. W. Hedley, "Effects of dasatinib on EphA2 receptor tyrosine kinase activity and downstream signalling in pancreatic cancer," *British Journal of Cancer*, vol. 99, no. 7, pp. 1074–1082, 2008.

[134] Z. Zhou, X. Yuan, Z. Li, et al., "RNA interference targeting EphA2 inhibits proliferation, induces apoptosis, and cooperates with cytotoxic drugs in human glioma cells," *Surgical Neurology*, vol. 70, no. 6, pp. 562–568, 2008.

[135] D. Vaught, D. M. Brantley-Sieders, and J. Chen, "Eph receptors in breast cancer: roles in tumor promotion and tumor suppression," *Breast Cancer Research*, vol. 10, no. 6, p. 217, 2008.

[136] N. K. Noren and E. B. Pasquale, "Paradoxes of the EphB4 receptor in cancer," *Cancer Research*, vol. 67, no. 9, pp. 3994–3997, 2007.

[137] J. Wykosky and W. Debinski, "The EphA2 receptor and EphrinA1 ligand in solid tumors: function and therapeutic targeting," *Molecular Cancer Research*, vol. 6, no. 12, pp. 1795–1806, 2008.

[138] D. Kiewlich, J. Zhang, C. Gross, et al., "Anti-EphA2 antibodies decrease EphA2 protein levels in murine CT26 colorectal and human MDA-231 breast tumors but do not inhibit tumor growth," *Neoplasia*, vol. 8, no. 1, pp. 18–30, 2006.

[139] D. M. Brantley-Sieders, G. Zhuang, D. Hicks, et al., "The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling," *Journal of Clinical Investigation*, vol. 118, no. 1, pp. 64–78, 2008.

[140] J. Chen, G. Zhuang, L. Frieden, and W. Debinski, "Eph receptors and ephrins in cancer: common themes and controversies," *Cancer Research*, vol. 68, no. 24, pp. 10031–10033, 2008.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 201026, 10 pages
doi:10.1155/2010/201026

Review Article

Myeloid Cells in the Tumor Microenvironment: Modulation of Tumor Angiogenesis and Tumor Inflammation

Michael C. Schmid and Judith A. Varner

Moores UCSD Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, La Jolla, CA 92093-0912, USA

Correspondence should be addressed to Judith A. Varner, jvarner@ucsd.edu

Received 31 October 2009; Revised 9 February 2010; Accepted 2 March 2010

Academic Editor: Debabrata Mukhopadhyay

Myeloid cells are a heterogeneous population of bone marrow-derived cells that play a critical role during growth and metastasis of malignant tumors. Tumors exhibit significant myeloid cell infiltrates, which are actively recruited to the tumor microenvironment. Myeloid cells promote tumor growth by stimulating tumor angiogenesis, suppressing tumor immunity, and promoting metastasis to distinct sites. In this review, we discuss the role of myeloid cells in promoting tumor angiogenesis. Furthermore, we describe a subset of myeloid cells with immunosuppressive activity (known as myeloid-derived suppressor cells). Finally, we will comment on the mechanisms regulating myeloid cell recruitment to the tumor microenvironment and on the potential of myeloid cells as new targets for cancer therapy.

1. Introduction

Angiogenesis, the growth of new blood vessels, occurs at different stages during embryonic development, physiological processes such as wound healing and reproduction, and numerous diseases, including inflammation, tumor progression, and metastasis [1]. The human immune system is composed of an innate and an adaptive branch. They both play a key role in maintaining homeostasis within our organism. The innate immune system is mainly composed of myeloid lineage cells, such as macrophages, neutrophils, and mast cells [2]. Under nontumor conditions, these cells provide the first line of protection against pathogens. Importantly, during tumor progression, myeloid cells are implicated in promoting tumor angiogenesis, causing resistance against antiangiogenic therapies in cancer, and suppressing the immune response during cancer [3–5].

2. Angiogenesis and Vasculogenesis during Tumor Growth

2.1. Angiogenesis. Neovascularization, the formation of new blood vessels, plays important roles in development, inflammation, and wound repair. Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 μm of blood vessels, the diffusion limit of oxygen. In 1971, Dr. Judah Folkman observed that neovascularization occurs around tumors and proposed that new blood vessel growth is necessary to supply nutrients and oxygen to tumor cells during exponential tumor growth [6]. These observations stimulated an intensive search for the mechanisms regulating tumor angiogenesis. It is now known that new blood vessels originate from preexisting vessels by activation, proliferation and migration of endothelial cells through a process named "angiogenesis" [4]. Specific growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), stimulate the proliferation and migration of naturally quiescent endothelial cells, resulting in the formation of new vessel structures during embryonic development and tumor growth [7]. Although tumor cells were first thought to drive the cellular events underpinning tumor angiogenesis and growth, considerable evidence has now emerged for the central role of tumor infiltrating myeloid cells such as monocytes, macrophages, and neutrophils in this phenomenon [8–12].

2.2. Vasculogenesis. Vasculogenesis is the coalescence of new blood vessels from individual endothelial cells or progenitor cells. Until recently, vasculogenesis was thought to be restricted to the formation of the initial vascular tree during embryonic vascular development. In 1997, Asahara et al., [13] isolated mononuclear cells from human peripheral blood that were enriched for expression of the hematopoietic stem cell marker CD34 [13]. Upon culture in endothelial growth media, these cells expressed endothelial lineage markers, such as CD31, Tie2, and VEGF receptor 2 (VEGFR2), and incorporated into blood vessels in ischemic tissues. These cells were therefore described as bone marrow derived endothelial progenitor cells (EPCs). Subsequent studies described a VEGFR2 and AC133 expressing subpopulation of these CD34 positive circulating cells that could form endothelial colonies in vitro [14, 15]. This suggested that EPCs are able to differentiate into endothelial cells and that such cells are incorporated into sites of active angiogenesis including ischemia, tumor angiogenesis, and metastasis in adult organisms [16, 17]. Since then, the study of circulating EPCs has generated considerable interest and controversy. Different markers, methods, and different kinds of cancer models used to identify EPC probably contributed to the widely divergent reports of the level of incorporation of these cells into newly formed tumor blood vessels [5]. These levels ranged from highs of 20% to 50% to lows of 5% or even less, the lower levels being more common [18–20].

3. M1 and M2 Tumor Associated Macrophages

3.1. Classical and Alternative Activation. Monocytes and macrophages belong to the myeloid cell lineage and derive from myeloid progenitor cells. These precursor cells are located in the bone marrow; upon maturation, monocytes are released into the bloodstream. Circulating blood monocytes migrate into tissues where they differentiate into resident tissue macrophages.

Macrophages are activated in response to environmental signals, including microbial products and cytokines. Activated macrophages can be divided into M1 (classical activated) and M2 (alternative activated) phenotype (Figure 1) [21]. Classical activation occurs in response to bacterial moieties such as lipopolysacharide (LPS) and immune stimuli such as interferon-γ (IFN-γ). M1 macrophages mediate resistance against intracellular parasites and tumors and elicit tissue disruptive reactions by secreting tumoricidal agents such as tumor necrosis factor α (TNF-α), interleukin-12 (IL-12), reactive nitrogen (iNOS), and oxygen intermediates (ROS). In addition, M1 macrophages promote T-helper-1 (Th1) responses. By contrast, M2 activated macrophages come in different varieties depending on the eliciting signals, which include IL-4, IL-13, IL-10, and glucocorticoid hormones. In general, M2 macrophages have an immune suppressive phenotype and release cytokines including IL-10 that promote a Th2 immune response [22–24]. Macrophages in tumors—usually termed tumor-associated macrophages (TAMs)—often express the M2 phenotype. However, recent evidence suggested that the phenotype of TAM varies with the stage of tumor progression. M1 macrophages are often abundant in chronic inflammatory sites, where tumors are initiated and start to develop. Then the macrophages switch to an M2-like phenotype as the tumor begins to invade, vascularize, and develop [25].

3.2. Proangiogenic Phenotype. M2-like TAMs release a number of potent proangiogenic cytokines, such as VEGF-A, VEGF-C, TNF-α, IL-8, and bFGF [26, 27]. Additionally, these TAMs also express a broad array of proteases known to play roles in the angiogenic process. These proteases include urokinase-type plasminogen activator (uPA), the matrix metalloproteinases MMP-2, MMP-7, MMP-9, and MMP-12, and elastase [28, 29]. uPA and MMP support angiogenesis by remodeling and breaking down the extracellular matrix (ECM). Degradation of ECM leads to the mobilization of growth factors and facilitates the migration of vascular cells into new environments [30–32]. Strong correlations are observed between TAM densities and vascular densities in many human tumor types, suggesting that TAMs regulate neovascularization. Importantly, high TAM densities are indicative of poor prognoses in breast, prostate, ovarian, and cervical cancers [33–35].

4. Myeloid Derived Suppressor Cells

4.1. Heterogeneous Family. Besides promoting angiogenesis, a subset of myeloid cells can facilitate tumor growth by their ability to downregulate the immune response against cancer cells. These so-called myeloid derived suppressor cells (MDSCs) are a heterogeneous population of cells that consist of myeloid progenitor cells and immature myeloid cells (IMCs). In healthy individuals, IMCs are generated in the bone marrow. They quickly differentiate into mature granulocytes, macrophages, or dendritic cells (DCs). In contrast, in pathological conditions such as cancer, a partial block in the differentiation of IMCs into mature myeloid cells occurs, which results in the expansion of the MDSC population. MDSCs can be found in the bone marrow (BM), spleen, and tumor sites and have been identified in most patients and in experimental mice with tumors based on their ability to suppress T cell activation [36].

MDSCs lack the expression of cell surface markers that are specifically expressed on monocytes, macrophages, or DC. In mice MDSCs are uniformly characterized by the expression of the cell surface molecules detected by antibodies to Gr1 and CD11b. Gr1 includes the macrophage and neutrophil markers Ly6C and Ly6G, respectively, whereas CD11b (also known as integrin αM) is characteristic for the myeloid-cell lineage. In recent years, several other surface molecules have been used to identify additional subset of suppressive MDSC, including CD80 [37], CD115 (also known as macrophage colony-stimulating factor (M-CSF) receptor, and CD124 (IL-4 receptor alpha chain (IL-4Ra)) [38].

In addition, nuclear morphology has also been used to characterize mouse MDSC. MDSCs that are mononuclear are considered "monocytic" and are typically $CD11b^{+}\,Ly6G^{+/-}\,Ly6C^{high}$, whereas those with

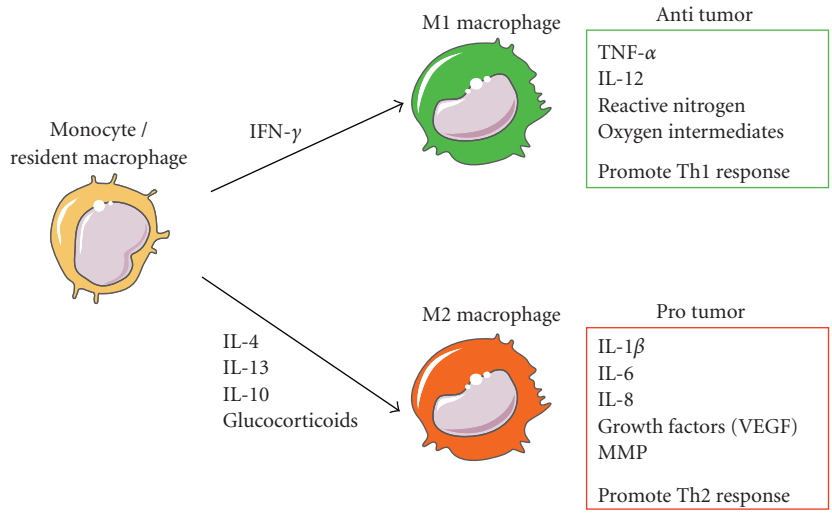


Figure 1: Cytokines produced in the tumor microenvironment can give rise to macrophages with distinct physiologies. Classical activated macrophages (M1) arise in response to interferon γ (IFN-γ). M1 macrophages elicit tissue disruptive reactions by producing tumor necrosis factor α (TNF-α), interleukin 12 (IL-12), reactive nitrogen, and oxygen intermediates. M1-activated macrophages are part of the polarized Th1 response. M2 macrophages are generated in response to various stimuli, including IL-4, IL-13, IL-10, and glucocorticoids. Tumor-associated macrophages have properties of M2-activated cells. They express many proangiogenic and angiogenic modulatory factors such as IL-1β, IL-6, IL-8, vascular endothelial growth factors (VEGFs), and matrix metalloproteinases (MMPs). M2 macrophages are part of the Th2 response.

multilobed nuclei are "granulocytic/neutrophil-like" and are $CD11b^+Ly6G^+Ly6C^-$ [39, 40].

In cancer patients MDSCs are typically defined as $CD11b^+CD14^{neg}$, cells that express the common myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells, and of the MHC class II molecule HLA-DR [41, 42]. In addition MDSCs have also been identified within a $CD15^+$ population in human peripheral blood [43].

4.2. Mechanism of Immune Suppression by MDSC. Several mechanisms have been associated with the immunosuppressive effects of myeloid cells, including secretion of immunosuppressive cytokines, upregulation of nitric oxide (NO), generation of ROS, and increased activity of L-arginase [44].

Arginase, iNOS, ROS, and COX2: L-arginine plays a critical role in the immunosuppressive activity of MDSC. T-cell proliferation and activation depends on the availability of L-arginine. L-arginine is a nonessential amino acid and is a substrate for two enzymes, inducible NO synthase (iNOS or NOS2) and arginase 1. MDSCs express both enzymes at high levels [36]. Recent data suggests that the increased activity of arginase 1 and iNOS in MDSC leads to enhanced L-arginine catabolism, which results in a reduction or depletion of L-arginine in the microenvironment. The lack of L-arginine results in inhibition of T-cell function [41, 45].

MDSC-produced ROS inhibits $CD8^+$ T cell by catalyzing the nitration of the TCR and thereby preventing T cell peptide-MHC interactions [46]. In addition, several known tumor-derived factors, such as TGF-β, IL-3, IL-6, IL-10, platelet-derived growth factor (PDGF), and granulocyte macrophage colony stimulating factor (GM-CSF), can induce the production of ROS by MDSC [36, 47].

Cyclooxygenase-2 (COX2) is a key factor in the activation of MDSC, because it regulates the expression of arginase 1, iNOS and prostaglandin E2 (PGE2). PGE2 and COX2 are produced by many tumors and are major contributors to the inflammatory milieu [48]. PGE2 was also shown to upregulate $CD11b^+CD14^-CD15^+$ MDSC in patients with renal cancer [41]. Therefore, elevated PGE2 levels were associated with higher levels and more suppressive MDSC. COX2 inhibitors have proven clinical applications for the treatment of colon cancer and intestinal polyposis [49].

Cytokines: MDSC-derived cytokines can suppress antitumor immunity. Secretion of the type 2 cytokine IL-10 downregulates the production of the type 1 cytokine IL-12 in macrophages. In addition, IL-10 and VEGF inhibit the maturation of DC [50]. TGF-β has also been associated with MDSC immune suppressive functions. In fibrosarcoma and colon carcinoma tumor models, MDSC produced TGF-β in response to IL-13 stimulation, which resulted in decreased tumor immunosurveillance of cytotoxic T-cells [51, 52].

5. Various Protumorigenic Myeloid Subpopulations

In recent times, most studies have analyzed the role that TAM and MDSC have on tumor angiogenesis and progression. However, there is now increasing evidence to show that various other myeloid subpopulations, such as Tie2 expressing monocytes, neutrophils, eosinophils, mast

cells, and dendritic cells, also actively participate in these processes. In this paragraph we briefly discuss the likely mechanisms by which these cells driving tumor angiogenesis and progression.

5.1. Tie2 Expressing Monocytes. De Palma et al., [53] recently identified a distinct lineage of myeloid cells that can be distinguished from other monocytes by their expression of the angiopoietin receptor Tie2 [53]. Although Tie2 is broadly expressed on vascular endothelial cells and generally regarded as an EC specific marker, Tie2 expressing monocytes (TEMs) are distinct from ECs and do not incorporate in the tumor endothelium. TEMs are a small monocyte subset that circulate in the mouse and human peripheral blood and appear to be preferentially recruited to tumors and other sites of angiogenesis [53]. In mouse blood, TEMs express CD45, the pan leukocyte marker, and CD11b, but do not express Gr1 (Ly6G/C), which is detected on granulocytes, DC, and MDSC. TEMs are a subset of tumor infiltrating F4/80$^+$ macrophages. In distinct tumor areas, TEM may account up to 30% of the total F4/80 macrophages [54, 55]. The close proximity of some TEMs to the tumor vasculature suggested to De Palma and colleagues that these cells might contribute to the regulation of tumor angiogenesis. The specific elimination of TEM by suicide gene strategy in mouse tumor models inhibited tumor angiogenesis. Interestingly, ablation of TEM did not affect the recruitment of TAM or neutrophils into these tumors, suggesting that, rather than being precursors of TAM, TEMs comprise a distinct monocyte subpopulation with potent proangiogenic activity. However, it is not clear whether TEM and TAM derive from a common monocytic precursor, or whether tumor microenvironmental factors can induce TAM to acquire a "TEM phenotype" or vice versa. It was suggested that TEMs stimulate angiogenesis by expressing the potent proangiogenic molecule bFGF (although the actual release of this growth factors has yet to be demonstrated) [53].

5.2. Neutrophils. Neutrophils are phagocytic, polymorphonuclear cells and are the most abundant subpopulation of leukocytes in the blood and are principally involved in acute inflammatory response to invading microorganisms. Increases levels of neutrophils have been observed in patients with gastric, colon, and lung cancer [56, 57]. In humans, neutrohils can be identified by the cell surface marker CD66b (also known as CEACAM8), or by the cytoplasmic marker myeloperoxidase (MPO) coupled with cell morphology. In murine tumors, Gr1$^+$ cells are usually considered to be neutrophils or cells derived from neutrophil precursors. However, it should be noted that murine MDSCs also express Gr1$^+$ [36].

The mechanism by which tumor-associated neutrophils mediate or modulate tumor angiogenesis has not been fully elucidated. Tumor-associated neutrophils are a major source of MMP9 (along with macrophages and mast cells) in various murine tumor models and so could promote angiogensis by releasing potent angiogenic factors such as VEGF that are usually sequestered in the ECM [58]. In addition, TNFα-stimulated neutrophils undergo degranulation and thereby releasing their intracellular VEGF storage, which subsequently induces endothelial cell proliferation and tube formation in vitro [59]. Recently Fridlender et al. [11] described that tumor-associated neutrophils (TANs) can be polarized in the tumor microenvironment into N1 and N2 phenotype similar as described previously for tumor-associated macrophages. Thereby, within the tumor microenvironment, TGF-β induced and maintained a population of TAN with an N2 tumor-promoting phenotype.

5.3. Eosinophils. Eosinophils are characterized by the expression of CCR3 and CD125. They are multifunctional leukocytes implicated in the pathogenesis of numerous inflammatory processes including parasitic helminths infections and allergic diseases [60]. Increased numbers of eosinophils have been reported for several human tumors including oral squamous cell carcinoma, gastrointestinal tumors, Hodgkin lymphoma, and nasopharyngeal carcinoma [61–64]. The highly potent and selective eosinophil chemoattractant CCL11 (eotaxin), which binds to CCR3, was described to mediate the recruitment of eosinophils to the tumor microenvironment [65]. The role of eosinophils in the tumor microenvironment remains unclear. Accumulation of eosinophils in the necrotic region suggests that eosinophils may promote necrosis and might have antitumor activity [66]. Alternatively, there is evidence to suggest that eosinophils recruited to tumor sites can influence angiogenesis. Eosinophils contain VEGF in their secretory granules, which are rapidly secreted upon activation with IL-15 [67]. In addition, TNFα-stimulated eosinophils release proangiogenic factors like bFGF, IL-6, IL-8, PDGF, and MMP9 [68]. However, the release of proangiogenic factors of IL-15 and TNFα-stimulated eosinophils has only been observed in vitro and has yet to be confirmed in tumors.

5.4. Mast Cells. Mature mast cells (MCs) populate most tissues but are found in highest numbers in the skin, airways, and digestive tract, where they are thought to act as a first line of defense against infiltrating pathogens and parasites. MCs also have an important role in generating and maintaining innate and adaptive immune responses as well as the development of autoimmune disorders and tolerance. MCs are usually identified by basic Giemsa or toluidine blue staining, expression of cell surface markers such as C-kit receptor (CD117) in human and CD34 in mice, or stored cytoplasmic molecules including tryptase and chymase [69]. MC originate from the bone marrow as immature cells and migrate to peripheral tissues where they mature in situ. Mast cells are now recognized as an early and persistent infiltrating cell type in many tumors, often entering before significant tumor growth and angiogenesis occurred. Mast cells accumulate at the boundary between healthy tissues and malignancies and are often found in close association with blood vessels within the tumor microenvironment. They express many proangiogenic compounds such as VEGF, bFGF, MMP9, TGF-β, TNFα, and IL-8. In several human tumors increased MC density positively correlates with

increased microvessel density and in some cases, with poor prognosis [70].

5.5. Dendritic Cells. Dendritic cells (DCs) are specialized antigen presenting cells that acquire, process, and present tumor-associated antigens to T-cells for the induction of antigen-specific tumor immune response. Two distinct populations of DC exit in mouse and human tissues: (i) myeloid DC (MDC) and (ii) plasmacytoid DC (PDC). MDCs express CD11c and CD33 and lack CD45R, CD123, and Lin, whereas PDCs are $CD123^+$, $CD45R^+$, $CD4^+$, $CD11c^-$, $ILT3^+$, $ILT1^-$, and Lin^- [71]. MDCs originate in the bone marrow as immature cells (iDC) that lack the classical mature DC markers, CD1a, CD83, CD40, and CD86. Once they process foreign antigen, they become activated, undergo maturation, and migrate to lymphoid tissue where they initiate activation of antigen-specific T cells [72].

By their potential capacity to activate tumor-specific T-cell responses, DC play an important role in cancer immunosurveillance. Interestingly, circulating and tumor-infiltrating DCs from cancer patients appear to be phenotypically and functionally defective. Several tumor-derived factors have been shown to be responsible for systemic and local DC defects [73]. Beside the vast majority of reports of MDC in cancer focusing on their suppressed immunoregulatory function, it has become apparent that iDCs also promote tumor neovascularization. For example, Conejo-Garcia and colleagues [74] described a mechanism of tumor vasculogensis mediated by DC precursors. β-defensin mediated recruited of DC precursors to tumors enhanced tumor vascularization and growth in the presence of increased VEGF-A expression. Thereby VEGF-A induced the simultaneous expression of both, endothelial and DC markers, on DC precursors and the DC precursors underwent endothelial-like specialization. These cells were termed vascular leukocytes (VLCs) and are highly present in human ovarian carcinomas. Depending on the milieu, VLCs can assemble into functional blood vessels or act as antigen-presenting cells [75].

A recent report underlined the important role of immature DC during tumor vascularization [76]. In this study only tumor cells implanted with immature DC, but not with mature DC, revealed increased neovascularization and growth. In addition, complete depletion of DC in a transgenic $CD11c^+$DTR-Tg mice model abrogated angiogenesis in bFGF loaded Matrigels and inhibited the growth of intraperitoneally injected B16 melanoma cells (although the tumor model used in this study is uncommon).

Beside the role of immature DC/VLC in vasculogenesis, immature DC might also promote angiogenesis. A recent report showed that human iDCs upregulate proangiogenic cytokines such as VEGF and IL-8 on exposure to severe hypoxia in vitro [77]. Beside their proangiogenic role, VEGF and IL-8 are also immunosuppressive cytokines capable to inhibit DC maturation and so might act in an autocrine as well as a proangiogenic manner if released by immature DC in hypoxic tumor sites.

6. Myeloid Cell Mediate Resistance to Antiangiogenic Drugs

Recently, Shojaei et al. [78] reported that accumulation of $CD11b^+Gr1^+$ cells in tumors renders their refractory to anti-angiogenic blockage by VEGF antibodies. Different murine tumor cell lines were tested for their responsiveness to anti-VEGF antibody treatment. Refractory tumors were associated with significant increase in the frequency of tumor infiltrating $CD11b^+Gr1^+$ cells compared to sensitive tumors. Moreover, when normally sensitive tumor cells were mixed with these cells that are resistant to anti-VEGF antibodies and transplanted into other mice, the transplanted tumors resist anti-VEGF antibodies. In contrast, $CD11b^+Gr1^+$ cells isolated from sensitive tumors were unable to mediate refractoriness to anti-VEGF treatment, indicating that the tumor directly modulates $CD11b^+Gr1^+$ cells to promote angiogenesis independent of VEGF. Gene array analysis revealed an upregulation of G-CSF and monocyte chemotactic protein 1 (MCP-1) in resistant tumors, both factors known to be involved in the mobilization of bone marrow derived myeloid cells to the peripheral blood. In addition, proinflammatory factors such as macrophage inflammatory protein 2 (MIP-2), IL-1 inducible protein, and IL-1β were also upregulated in resistant tumors, while resistant-mediating $CD11b^+Gr1^+$ cells revealed increased expression of proinflammatory cytokine receptors such as IL-1, IL-4, IL-11, and IL-13. Taken together, these findings suggest that inflammation is an important aspect of tumor refractoriness in response to anti-VEGF antibody treatment.

Fischer et al., [79] described the use of neutralizing murine antiplacental growth factor (PlGF) monoclonal antibody [79]. Anti-PlGF antibody inhibited growth and metastasis of various tumors, including those resistant to VEGF-receptor 2 (VEGFR2) inhibitors. In contrast to anti-VEGFR2 treatment, anti-PlGF prevented infiltration of angiogenic macrophages and severe tumor hypoxia and, thus, did not switch on the "angiogenic rescue program" which is considered to be responsible for the resistance to anti-VEGFR2 treatment.

7. Mobilization and Recruitment of Myeloid Cells into Tumors

Substantial evidence indicates that myeloid cells and their precursors promote neovascularization in tumors and inflammatory tissues. These cells are actively recruited to the tumor microenvironment from the bloodstream. Immune cell trafficking in vivo is regulated by chemokines and by members of the integrin, immunoglobulin superfamily, and selectin adhesion molecule families [80, 81]. Hypoxia, as well as chemokines and their receptors, stimulates homing of circulating myeloid cells to tissues. When tumors encounter low oxygen tension, they adapt by promoting expression of genes associated with angiogenesis, metastasis, and invasion. This transcriptional response pathway is mediated to a large extent by the dimeric transcription factor complexes of hypoxia-inducible factors (HIFs) [82]. HIF1 activity

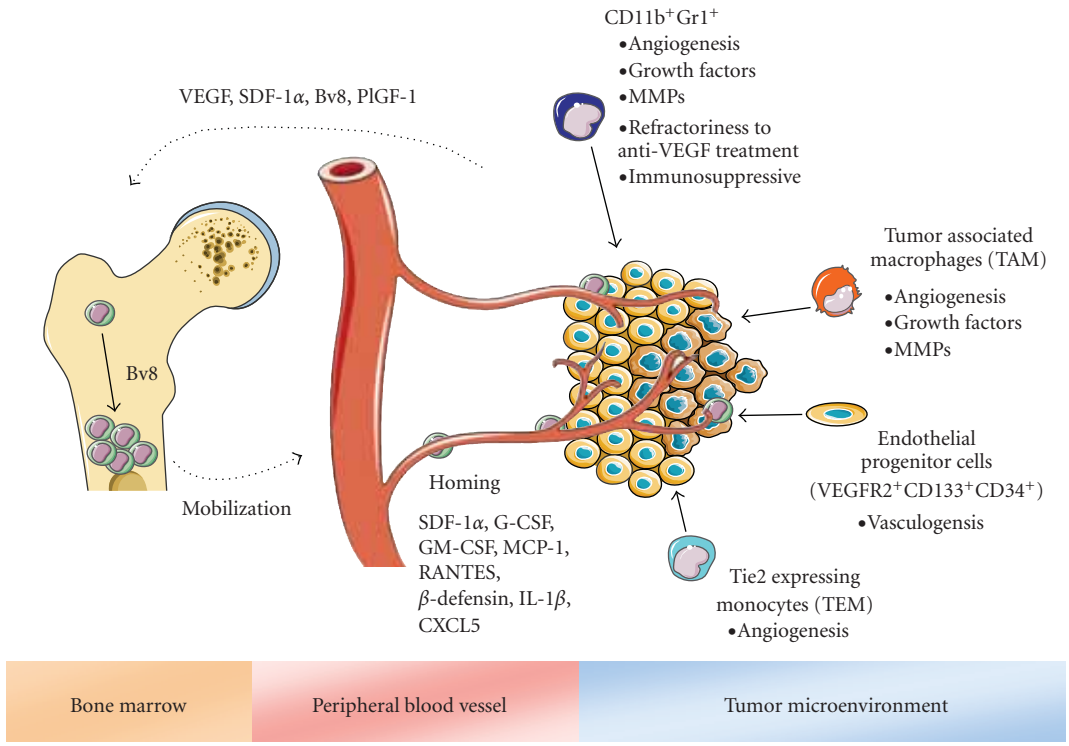


Figure 2: Recruitment of diverse bone marrow-derived cell populations to the tumor microenvironment and their effects on tumor progression. Tumor and stromal cells mobilize various subpopulations of tumor promoting bone marrow-derived cells to the peripheral blood through secretion of cytokines and chemokines. Diverse chemoattractant factors promote the recruitment and infiltration of these cells to the tumor microenvironment where they suppress the antitumor immunity or promote tumor angiogenesis and vasculogenesis or raise refractoriness against anti-VEGF therapy.

promotes neovascularization by the induction of variety of proangiogenic factors like VEGF-A, VEGFR1, PDGF-B, bFGF, and angiopoietins that stimulate new blood vessel formation within hypoxic areas. In addition, HIF activity also regulates the expression of several chemoattractant factors, including MCP-1, CSF-1, VEGF-A, TNFα, and SDF-1α, each of them capable to attract myeloid cells to invade hypoxic tissues [83].

MCP-1 (or CCL2) and RANTES (or CCL5) increased the infiltration of TAM into primary tumors, including breast and ovarian carcinomas, melanoma, and glioblastoma [84–87]. Furthermore, MCP-1 and RANTES stimulate the secretion of matrix-degrading enzymes, such as MMP9 and MMP12 by macrophages.

IL-8 also serves as a monocyte chemoattractant. This chemokine is also a proangiogenic factor and an autocrine growth factor for several human tumor cell types [88]. IL-8 stimulates the adhesion of monocytes, which express low levels of the IL-8 receptors CXCR1 and CXCR2, to vascular endothelium under flow conditions. These studies indicate that IL-8 and CXCR-1/2 interactions play roles in monocyte recruitment. Several cytokines and growth factors, including colony stimulating factor-1 (CSF-1), VEGF, and PDGF, have been implicated in the recruitment of monocytes into tumors [89–91]. CSF-1 is produced by various types of human tumors and is a potent chemoattractant for macrophages. Coordinated expression of CSF-1 in macrophages and epidermal growth factor (EGF) in mammary tumor cells resulted in increased myeloid cell invasion into mammary tumors [91].

IL-1β, another myeloid cell cytokine, increased infiltration of neutrophils and macrophages in a mouse model of corneal neovascularization. In contrast, deletion of monocytes by genetic approaches or by use of toxins significantly suppressed IL-1β induced angiogenesis [92].

β-defensin may also serve as a recruitment factor for myeloid lineage cells. β-defensin is a chemoattractant factor for DC. Conjeo-Garcia and colleagues found that the recruitment of dendritic precursor cells into tumors required the presence of β-defensin [74]. Depletion of β-defensin or inhibition of its receptor CCR6 using function-blocking antibodies abolished the infiltration of dendritic precursor cells into tumors. These studies indicate that the ligand/receptor pair β-defensin/CCR6 is essential for dendritic precursor cell recruitment.

A key role for SDF-1α in progenitor cell recruitment was recently described [93]. Syngeneic tumors transplanted into thrombocytopenic mice (such as Thpo-/- and Mpl-/- mice) exhibited impaired neovascularization and reduced release of the chemokine SDF-1α. Further studies demonstrated that hematopoietic cytokines including soluble Kit-ligand and thrombopoietin trigger the release of SDF-1α from

platelets, which results in the mobilization of unique subset of hemangiogenic progenitor cells (CXCR4+ VEGFR1+) to neoangiogenic niches.

Du et al., [9] recently reported that HIF-1α, the direct effector of hypoxia, induces recruitment of diverse bone marrow derived subpopulations, containing Tie2$^+$, VEGFR1$^+$, CD11b$^+$, and F4/80$^+$, as well as EPC and pericyte progenitors to promote neovascularization in glioblastoma. HIF-1α contributed to the induction of SDF-1α in glioblastoma cells, which in turn promoted tumor progression by recruiting MMP9$^+$ vascular modulatory bone marrow cells [9].

Recently Bv8, also known as prokineticin-2, was identified as a critical regulator for CD11b$^+$Gr1$^+$-mediated angiogenesis. Bv8 and the related EG-VEGF were also characterized as mitogens for specific endothelial cell types [94]. Both Bv8 and EG-VEGF bind two highly homologous G-protein-coupled receptors termed PKR-1 and PKR2. Bv8 expression was reported to be upregulated in CD11b$^+$Gr1$^+$ cells after tumor implantation [8]. Bv8 was shown to mobilize hematopoietic cells such as CD11b$^+$Gr1$^+$ cells to the blood and also stimulated the production of granulocytic and monocytic colonies in vitro [95]. Notably, anti-Bv8 treatment of mice implanted with human tumors resulted in a significant reduction in tumor growth and tumor angiogenesis. This effect was associated with a reduction in CD11b$^+$Gr1$^+$ mobilization from the bone marrow. Interestingly G-CSF dramatically upregulates Bv8 expression [8, 96]. Hence, G-CSF produced by the tumor cells or tumor associated fibroblast may result in an upregulation of Bv8 in the BM, which, in turn, results in the induction of differentiation of myeloid progenitors and their mobilization to the peripheral blood.

The immune suppressive molecule TGF-β has also been implicated in myeloid cell functions. Experiments with a transplanted and spontaneous mammary carcinoma demonstrated increased levels of TGF-β in the tumor microenvironment if the tumor cells were deficient for the type II TGF-β receptor (*Tgfbr2* KO). These authors demonstrated that a deficiency in the receptor resulted in an increase in CXCL5 (ENA-78) and SDF-1α in the tumor microenvironment. Further analysis targeting the CXCL5 receptor CXCR2 with antagonist decreased the recruitment of CD11b$^+$Gr1$^+$ cells to orthotopic transplanted *Tgfbr2* KO breast adenocarcinomas [97].

These studies demonstrate that a variety of inflammatory stimuli can recruit diverse subsets of myeloid cells to invade tumor tissue.

8. Conclusions

Links between chronic inflammation and cancer have been recognized for several decades. Studies to understand the recruitment of proangiogenic myeloid cells populations and immunsuppressive MDSC and their contributions to angiogenesis are ongoing. All these studies suggest that several myeloid subpopulations may play roles during neovascularization of tumors, mediating refractoriness to anti-angiogenic therapies, or the escape from immune surveillance (Figure 2). Much progress is needed with regards to the characterization of markers to identify cells subsets that have specific regulatory roles. This might further help to understand why so many different characterized cell types appear to have overlapping functions.

Myeloid cells represent novel targets for therapeutic strategies. The mobilization and recruitment of myeloid cells by the tumor defines myeloid cells as a potential delivery system to target the tumor microenvironment. One such approach was recently shown using TEM. Mice transplanted with TEM expressing interferon α (IFN-α), a potent cytokine with angiostatic and anti-proliferative activity [98] under the *Tie2* promotor, inhibited tumor progression in several tumor models [99]. Targeting cytokines and cytotoxic proteins to tumors by means of gene-modified myeloid cells thus represents a promising strategy to treat cancer [100].

In contrast, the tumor promoting properties of myeloid cells define these cells as putative targets for anticancer therapies. Anti-angiogenic agents were already described to be the most efficacious when combined with cytotoxic agents and/or therapies targeted towards ablating myeloid cells [5, 101]. Furthermore, suppression of myeloid cell recruitment to the tumor microenvironment offers a new strategy to inhibit tumor neovascularization, while stimulation of homing may promote tissue recovery from ischemia.

References

[1] P. Carmeliet, "Angiogenesis in life, disease and medicine," *Nature*, vol. 438, no. 7070, pp. 932–936, 2005.

[2] L. M. Coussens and Z. Werb, "Inflammation and cancer," *Nature*, vol. 420, no. 6917, pp. 860–867, 2002.

[3] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[4] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[5] R. S. Kerbel, "Tumor angiogenesis," *The New England Journal of Medicine*, vol. 358, no. 19, pp. 2039–2049, 2008.

[6] J. Folkman, E. Merler, C. Abernathy, and G. Williams, "Isolation of a tumor factor responsible or angiogenesis," *Journal of Experimental Medicine*, vol. 133, no. 2, pp. 275–288, 1971.

[7] P. Carmeliet and R. K. Jain, "Angiogenesis in cancer and other diseases," *Nature*, vol. 407, no. 6801, pp. 249–257, 2000.

[8] F. Shojaei, X. Wu, C. Zhong, et al., "Bv8 regulates myeloid-cell-dependent tumour angiogenesis," *Nature*, vol. 450, no. 7171, pp. 825–831, 2007.

[9] R. Du, K. V. Lu, C. Petritsch, et al., "HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion," *Cancer Cell*, vol. 13, no. 3, pp. 206–220, 2008.

[10] D. G. DeNardo, J. B. Barreto, P. Andreu, et al., "CD4$^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages," *Cancer Cell*, vol. 16, no. 2, pp. 91–102, 2009.

[11] Z. G. Fridlender, J. Sun, S. Kim, et al., "Polarization of tumor-associated neutrophil phenotype by TGF-β: "N1" versus "N2" TAN," *Cancer Cell*, vol. 16, no. 3, pp. 183–194, 2009.

[12] M. C. Schmid and J. A. Varner, "Myeloid cell trafficking and tumor angiogenesis," *Cancer Letters*, vol. 250, no. 1, pp. 1–8, 2007.

[13] T. Asahara, T. Murohara, A. Sullivan, et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[14] M. Peichev, A. J. Naiyer, D. Pereira, et al., "Expression of VEGFR-2 and AC133 by circulating human $CD34^+$ cells identifies a population of functional endothelial precursors," *Blood*, vol. 95, no. 3, pp. 952–958, 2000.

[15] M. Gill, S. Dias, K. Hattori, et al., "Vascular trauma induces rapid but transient mobilization of $VEGFR2^+AC133^+$ endothelial precursor cells," *Circulation Research*, vol. 88, no. 2, pp. 167–174, 2001.

[16] D. Gao, D. Nolan, K. McDonnell, et al., "Bone marrow-derived endothelial progenitor cells contribute to the angiogenic switch in tumor growth and metastatic progression," *Biochimica et Biophysica Acta*, vol. 1796, no. 1, pp. 33–40, 2009.

[17] D. Gao, D. J. Nolan, A. S. Mellick, K. Bambino, K. McDonnell, and V. Mittal, "Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis," *Science*, vol. 319, no. 5860, pp. 195–198, 2008.

[18] M. Garcia-Barros, F. Paris, C. Cordon-Cardo, et al., "Tumor response to radiotherapy regulated by endothelial cell apoptosis," *Science*, vol. 300, no. 5622, pp. 1155–1159, 2003.

[19] H. Spring, T. Schuler, B. Arnold, G. J. Hammerling, and R. Ganss, "Chemokines direct endothelial progenitors into tumor neovessels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18111–18116, 2005.

[20] B. A. Peters, L. A. Diaz Jr., K. Polyak, et al., "Contribution of bone marrow-derived endothelial cells to human tumor vasculature," *Nature Medicine*, vol. 11, no. 3, pp. 261–262, 2005.

[21] A. Mantovani, S. Sozzani, M. Locati, P. Allavena, and A. Sica, "Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes," *Trends in Immunology*, vol. 23, no. 11, pp. 549–555, 2002.

[22] A. Mantovani, T. Schioppa, C. Porta, P. Allavena, and A. Sica, "Role of tumor-associated macrophages in tumor progression and invasion," *Cancer and Metastasis Reviews*, vol. 25, no. 3, pp. 315–322, 2006.

[23] F. O. Martinez, L. Helming, and S. Gordon, "Alternative activation of macrophages: an immunologic functional perspective," *Annual Review of Immunology*, vol. 27, pp. 451–483, 2009.

[24] F. O. Martinez, A. Sica, A. Mantovani, and M. Locati, "Macrophage activation and polarization," *Frontiers in Bioscience*, vol. 13, no. 2, pp. 453–461, 2008.

[25] S. K. Biswas, A. Sica, and C. E. Lewis, "Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms," *Journal of Immunology*, vol. 180, no. 4, pp. 2011–2017, 2008.

[26] J. S. Lewis, R. J. Landers, J. C. E. Underwood, A. L. Harris, and C. E. Lewis, "Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas," *Journal of Pathology*, vol. 192, no. 2, pp. 150–158, 2000.

[27] C. Sunderkotter, K. Steinbrink, M. Goebeler, R. Bhardwaj, and C. Sorg, "Macrophages and angiogenesis," *Journal of Leukocyte Biology*, vol. 55, no. 3, pp. 410–422, 1994.

[28] E. Giraudo, M. Inoue, and D. Hanahan, "An amino-bisphosphonate targets MMP-9—expressing macrophages and angiogenesis to impair cervical carcinogenesis," *Journal of Clinical Investigation*, vol. 114, no. 5, pp. 623–633, 2004.

[29] R. Hildenbrand, I. Dilger, A. Horlin, and H. J. Stutte, "Urokinase and macrophages in tumour angiogenesis," *British Journal of Cancer*, vol. 72, no. 4, pp. 818–823, 1995.

[30] F. Balkwill and A. Mantovani, "Inflammation and cancer: back to Virchow?" *The Lancet*, vol. 357, no. 9255, pp. 539–545, 2001.

[31] S. Huang, M. Van Arsdall, S. Tedjarati, et al., "Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice," *Journal of the National Cancer Institute*, vol. 94, no. 15, pp. 1134–1142, 2002.

[32] I. Esposito, M. Menicagli, N. Funel, et al., "Inflammatory cells contribute to the generation of an angiogenic phenotype in pancreatic ductal adenocarcinoma," *Journal of Clinical Pathology*, vol. 57, no. 6, pp. 630–636, 2004.

[33] P. J. Polverini and S. J. Leibovich, "Effect of macrophage depletion on growth and neovascularization of hamster buccal pouch carcinomas," *Journal of Oral Pathology*, vol. 16, no. 9, pp. 436–441, 1987.

[34] R. D. Leek and A. L. Harris, "Tumor-associated macrophages in breast cancer," *Journal of Mammary Gland Biology and Neoplasia*, vol. 7, no. 2, pp. 177–189, 2002.

[35] A. Nishie, M. Ono, T. Shono, et al., "Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas," *Clinical Cancer Research*, vol. 5, no. 5, pp. 1107–1113, 1999.

[36] D. I. Gabrilovich and S. Nagaraj, "Myeloid-derived suppressor cells as regulators of the immune system," *Nature Reviews Immunology*, vol. 9, no. 3, pp. 162–174, 2009.

[37] R. Yang, Z. Cai, Y. Zhang, W. H. Yutzy IV, K. F. Roby, and R. B. S. Roden, "CD80 in immune suppression by mouse ovarian carcinoma-associated $Gr\text{-}1^+CD11b^+$ myeloid cells," *Cancer Research*, vol. 66, no. 13, pp. 6807–6815, 2006.

[38] G. Gallina, L. Dolcetti, P. Serafini, et al., "Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on $CD8^+$ T cells," *Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2777–2790, 2006.

[39] J.-I. Youn, S. Nagaraj, M. Collazo, and D. I. Gabrilovich, "Subsets of myeloid-derived suppressor cells in tumor-bearing mice," *Journal of Immunology*, vol. 181, no. 8, pp. 5791–5802, 2008.

[40] Y. Sawanobori, S. Ueha, M. Kurachi, et al., "Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice," *Blood*, vol. 111, no. 12, pp. 5457–5466, 2008.

[41] A. C. Ochoa, A. H. Zea, C. Hernandez, and P. C. Rodriguez, "Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma," *Clinical Cancer Research*, vol. 13, no. 2, pp. 721s–725s, 2007.

[42] B. Almand, J. I. Clark, E. Nikitina, et al., "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer," *Journal of Immunology*, vol. 166, no. 1, pp. 678–689, 2001.

[43] J. Schmielau and O. J. Finn, "Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients," *Cancer Research*, vol. 61, no. 12, pp. 4756–4760, 2001.

[44] S. Kusmartsev and D. I. Gabrilovich, "Role of immature myeloid cells in mechanisms of immune evasion in cancer," *Cancer Immunology, Immunotherapy*, vol. 55, no. 3, pp. 237–245, 2006.

[45] P. C. Rodriguez, M. S. Ernstoff, C. Hernandez, et al., "Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes," *Cancer Research*, vol. 69, no. 4, pp. 1553–1560, 2009.

[46] S. Nagaraj, K. Gupta, V. Pisarev, et al., "Altered recognition of antigen is a mechanism of $CD8^+$ T cell tolerance in cancer," *Nature Medicine*, vol. 13, no. 7, pp. 828–835, 2007.

[47] H. Sauer, M. Wartenberg, and J. Hescheler, "Reactive oxygen species as intracellular messengers during cell growth and differentiation," *Cellular Physiology and Biochemistry*, vol. 11, no. 4, pp. 173–186, 2001.

[48] M. M. Taketo, "Cyclooxygenase-2 inhibitors in tumorigenesis (part I)," *Journal of the National Cancer Institute*, vol. 90, no. 20, pp. 1529–1536, 1998.

[49] M. M. Bertagnolli, "Chemoprevention of colorectal cancer with cyclooxygenase-2 inhibitors: two steps forward, one step back," *The Lancet Oncology*, vol. 8, no. 5, pp. 439–443, 2007.

[50] S. Ostrand-Rosenberg and P. Sinha, "Myeloid-derived suppressor cells: linking inflammation and cancer," *Journal of Immunology*, vol. 182, no. 8, pp. 4499–4506, 2009.

[51] S. Fichtner-Feigl, M. Terabe, A. Kitani, et al., "Restoration of tumor immunosurveillance via targeting of interleukin-13 receptor-α2," *Cancer Research*, vol. 68, no. 9, pp. 3467–3475, 2008.

[52] M. Terabe, S. Matsui, J.-M. Park, et al., "Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence," *Journal of Experimental Medicine*, vol. 198, no. 11, pp. 1741–1752, 2003.

[53] M. De Palma, M. A. Venneri, R. Galli, et al., "Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors," *Cancer Cell*, vol. 8, no. 3, pp. 211–226, 2005.

[54] M. De Palma, M. A. Venneri, and L. Naldini, "In vivo targeting of tumor endothelial cells by systemic delivery of lentiviral vectors," *Human Gene Therapy*, vol. 14, no. 12, pp. 1193–1206, 2003.

[55] M. De Palma and L. Naldini, "Tie2-expressing monocytes (TEMs): novel targets and vehicles of anticancer therapy?" *Biochimica et Biophysica Acta*, vol. 1796, no. 1, pp. 5–10, 2009.

[56] M. Eck, B. Schmausser, K. Scheller, S. Brandlein, and H. K. Muller-Hermelink, "Pleiotropic effects of CXC chemokines in gastric carcinoma: differences in CXCL8 and CXCL1 expression between diffuse and intestinal types of gastric carcinoma," *Clinical and Experimental Immunology*, vol. 134, no. 3, pp. 508–515, 2003.

[57] A. Bellocq, M. Antoine, A. Flahault, et al., "Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome," *American Journal of Pathology*, vol. 152, no. 1, pp. 83–92, 1998.

[58] G. Bergers, R. Brekken, G. McMahon, et al., "Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis," *Nature Cell Biology*, vol. 2, no. 10, pp. 737–744, 2000.

[59] M. McCourt, J. H. Wang, S. Sookhai, and H. P. Redmond, "Proinflammatory mediators stimulate neutrophil-directed angiogenesis," *Archives of Surgery*, vol. 134, no. 12, pp. 1325–1332, 1999.

[60] M. E. Rothenberg and S. P. Hogan, "The eosinophil," *Annual Review of Immunology*, vol. 24, pp. 147–174, 2006.

[61] R. G. Dorta, G. Landman, L. P. Kowalski, J. R. P. Lauris, M. R. Latorre, and D. T. Oliveira, "Tumour-associated tissue eosinophilia as a prognostic factor in oral squamous cell carcinomas," *Histopathology*, vol. 41, no. 2, pp. 152–157, 2002.

[62] L.-M. Looi, "Tumor-associated tissue eosinophilia in nasopharyngeal carcinoma. A pathologic study of 422 primary and 138 metastatic tumors," *Cancer*, vol. 59, no. 3, pp. 466–470, 1987.

[63] J. Teruya-Feldstein, E. S. Jaffe, P. R. Burd, D. W. Kingma, J. E. Setsuda, and G. Tosato, "Differential chemokine expression in tissues involved by Hodgkin's disease: direct correlation of eotaxin expression and tissue eosinophilia," *Blood*, vol. 93, no. 8, pp. 2463–2470, 1999.

[64] H. J. Nielsen, U. Hansen, I. J. Christensen, C. M. Reimert, N. Brunner, and F. Moesgaard, "Independent prognostic value of eosinophil and mast cell infiltration in colorectal cancer tissue," *Journal of Pathology*, vol. 189, no. 4, pp. 487–495, 1999.

[65] S. C. M. Lorena, D. T. Oliveira, R. G. Dorta, G. Landman, and L. P. Kowalski, "Eotaxin expression in oral squamous cell carcinomas with and without tumour associated tissue eosinophilia," *Oral Diseases*, vol. 9, no. 6, pp. 279–283, 2003.

[66] J. Mattes, M. Hulett, W. Xie, et al., "Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process," *Journal of Experimental Medicine*, vol. 197, no. 3, pp. 387–393, 2003.

[67] T. Horiuchi and P. F. Weller, "Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony-stimulating factor and interleukin-5," *American Journal of Respiratory Cell and Molecular Biology*, vol. 17, no. 1, pp. 70–77, 1997.

[68] S. A. Cormier, A. G. Taranova, C. Bedient, et al., "Pivotal advance: eosinophil infiltration of solid tumors is an early and persistent inflammatory host response," *Journal of Leukocyte Biology*, vol. 79, no. 6, pp. 1131–1139, 2006.

[69] D. Ribatti and E. Crivellato, "The controversial role of mast cells in tumor growth," *International Review of Cell and Molecular Biology*, vol. 275, pp. 89–131, 2009.

[70] E. Crivellato, B. Nico, and D. Ribatti, "Mast cells and tumour angiogenesis: new insight from experimental carcinogenesis," *Cancer Letters*, vol. 269, no. 1, pp. 1–6, 2008.

[71] M. Colonna, G. Trinchieri, and Y.-J. Liu, "Plasmacytoid dendritic cells in immunity," *Nature Immunology*, vol. 5, no. 12, pp. 1219–1226, 2004.

[72] E. Gottfried, M. Kreutz, and A. Mackensen, "Tumor-induced modulation of dendritic cell function," *Cytokine and Growth Factor Reviews*, vol. 19, no. 1, pp. 65–77, 2008.

[73] I. Fricke and D. I. Gabrilovich, "Dendritic cells and tumor microenvironment: a dangerous liaison," *Immunological Investigations*, vol. 35, no. 3-4, pp. 459–483, 2006.

[74] J. R. Conejo-Garcia, F. Benencia, M.-C. Courreges, et al., "Tumor-infiltrating dendritic cell precursors recruited by a β-defensin contribute to vasculogenesis under the influence of Vegf-A," *Nature Medicine*, vol. 10, no. 9, pp. 950–958, 2004.

[75] G. Coukos, J. R. Conejo-Garcia, R. Buckanovich, and F. Benencia, "Vascular leukocytes: a population with angiogenic and immunossuppressive properties highly represented in ovarian cancer," *Advances in Experimental Medicine and Biology*, vol. 590, pp. 185–193, 2007.

[76] O. Fainaru, N. Almog, C. W. Yung, et al., "Tumor growth and angiogenesis are dependent on the presence of immature dendritic cells," *The FASEB Journal*, vol. 24, no. 5, pp. 1411–1418, 2010.

[77] A. Ricciardi, A. R. Elia, P. Cappello, et al., "Transcriptome of hypoxic immature dendritic cells: modulation of chemokine/receptor expression," *Molecular Cancer Research*, vol. 6, no. 2, pp. 175–185, 2008.

[78] F. Shojaei, X. Wu, A. K. Malik, et al., "Tumor refractoriness to anti-VEGF treatment is mediated by $CD11b^+Gr1^+$ myeloid cells," *Nature Biotechnology*, vol. 25, no. 8, pp. 911–920, 2007.

[79] C. Fischer, B. Jonckx, M. Mazzone, et al., "Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels," *Cell*, vol. 131, no. 3, pp. 463–475, 2007.

[80] C. Weber and R. R. Koenen, "Fine-tuning leukocyte responses: towards a chemokine 'interactome'," *Trends in Immunology*, vol. 27, no. 6, pp. 268–273, 2006.

[81] A. D. Luster, R. Alon, and U. H. von Andrian, "Immune cell migration in inflammation: present and future therapeutic targets," *Nature Immunology*, vol. 6, no. 12, pp. 1182–1190, 2005.

[82] A. J. Giaccia, M. C. Simon, and R. Johnson, "The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease," *Genes and Development*, vol. 18, no. 18, pp. 2183–2194, 2004.

[83] D. Liao and R. S. Johnson, "Hypoxia: a key regulator of angiogenesis in cancer," *Cancer and Metastasis Reviews*, vol. 26, no. 2, pp. 281–290, 2007.

[84] T. Ueno, M. Toi, H. Saji, et al., "Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer," *Clinical Cancer Research*, vol. 6, no. 8, pp. 3282–3289, 2000.

[85] Y. Niwa, H. Akamatsu, H. Niwa, H. Sumi, Y. Ozaki, and A. Abe, "Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer," *Clinical Cancer Research*, vol. 7, no. 2, pp. 285–289, 2001.

[86] E. Y. Lin, A. V. Nguyen, R. G. Russell, and J. W. Pollard, "Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy," *Journal of Experimental Medicine*, vol. 193, no. 6, pp. 727–739, 2001.

[87] C. Murdoch, A. Giannoudis, and C. E. Lewis, "Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues," *Blood*, vol. 104, no. 8, pp. 2224–2234, 2004.

[88] Y. M. Zhu, S. J. Webster, D. Flower, and P. J. Woll, "Interleukin-8/CXCL8 is a growth factor for human lung cancer cells," *British Journal of Cancer*, vol. 91, no. 11, pp. 1970–1976, 2004.

[89] M. Uutela, M. Wirzenius, K. Paavonen, et al., "PDGF-D induces macrophage recruitment, increased interstitial pressure, and blood vessel maturation during angiogenesis," *Blood*, vol. 104, no. 10, pp. 3198–3204, 2004.

[90] B. Barleon, S. Sozzani, D. Zhou, H. A. Weich, A. Mantovani, and D. Marme, "Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1," *Blood*, vol. 87, no. 8, pp. 3336–3343, 1996.

[91] S. Goswami, E. Sahai, J. B. Wyckoff, et al., "Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop," *Cancer Research*, vol. 65, no. 12, pp. 5278–5283, 2005.

[92] S. Nakao, T. Kuwano, C. Tsutsumi-Miyahara, et al., "Infiltration of COX-2-expressing macrophages is a prerequisite for IL-1β-induced neovascularization and tumor growth," *Journal of Clinical Investigation*, vol. 115, no. 11, pp. 2979–2991, 2005.

[93] D. K. Jin, K. Shido, H.-G. Kopp, et al., "Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of $CXCR4^+$ hemangiocytes," *Nature Medicine*, vol. 12, no. 5, pp. 557–567, 2006.

[94] J. LeCouter, J. Kowalski, J. Foster, et al., "Identification of an angiogenic mitogen selective for endocrine gland endothelium," *Nature*, vol. 412, no. 6850, pp. 877–884, 2001.

[95] J. LeCouter, C. Zlot, M. Tejada, F. Peale, and N. Ferrara, "Bv8 and endocrine gland-derived vascular endothelial growth factor stimulate hematopoiesis and hematopoietic cell mobilization," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 48, pp. 16813–16818, 2004.

[96] F. Shojaei, X. Wu, X. Qu, et al., "G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 16, pp. 6742–6747, 2009.

[97] L. Yang, J. Huang, X. Ren, et al., "Abrogation of TGFβ signaling in mammary carcinomas recruits $Gr\text{-}1^+CD11b^+$ myeloid cells that promote metastasis," *Cancer Cell*, vol. 13, no. 1, pp. 23–35, 2008.

[98] G. R. Stark, I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber, "How cells respond to interferons," *Annual Review of Biochemistry*, vol. 67, pp. 227–264, 1998.

[99] M. De Palma, R. Mazzieri, L. S. Politi, et al., "Tumor-targeted interferon-α delivery by Tie2-expressing monocytes inhibits tumor growth and metastasis," *Cancer Cell*, vol. 14, no. 4, pp. 299–311, 2008.

[100] K. S. Aboody, J. Najbauer, and M. K. Danks, "Stem and progenitor cell-mediated tumor selective gene therapy," *Gene Therapy*, vol. 15, no. 10, pp. 739–752, 2008.

[101] N. Ferrara and R. S. Kerbel, "Angiogenesis as a therapeutic target," *Nature*, vol. 438, no. 7070, pp. 967–974, 2005.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 539706, 6 pages
doi:10.1155/2010/539706

Review Article

Sympathetic Neurotransmitters and Tumor Angiogenesis—Link between Stress and Cancer Progression

Jason Tilan and Joanna Kitlinska

Department of Physiology & Biophysics, Georgetown University, Basic Science Building 231A, 3900 Reservoir Rd., NW, Washington, DC 20007, USA

Correspondence should be addressed to Joanna Kitlinska, jbk4@georgetown.edu

Received 16 November 2009; Accepted 18 March 2010

Academic Editor: Sundaram Ramakrishnan

Recent evidence supports a longstanding hypothesis that chronic stress can influence tumor growth and progression. It has been shown that sympathetic neurotransmitters, such as catecholamines and neuropeptides, can affect both cancer cell growth and tumor vascularization. Depending on neurotransmitter and type of tumor, these effects can be both stimulatory and inhibitory. Norepinephrine (NE) and epinephrine (E) are potent stimulators of vascularization, acting both by inducing the release of angiogenic factors from tumor cells and directly on endothelial cell (EC) functions. As a result, activation of the adrenergic system increases growth of various types of tumors and has been shown to mediate stress-induced augmentation of tumor progression. Dopamine (DA), on the other hand, interferes with VEGF signaling in endothelial cells, blocks its angiogenic functions and inhibits tumor growth. Another sympathetic neurotransmitter coreleased with NE, neuropeptide Y (NPY), directly stimulates angiogenesis. However, proangiogenic actions of NPY can be altered by its direct effect on tumor cell proliferation and survival. In consequence, NPY can either stimulate or inhibit tumor growth, depending on tumor type. Hence, sympathetic neurotransmitters are powerful modulators of tumor growth and can become new targets in cancer therapy.

1. Introduction

Stress is an inevitable element of our lives. Stressful events activate the sympathetic nervous system and hypothalamic-pituitary-adrenal axis, which lead to the release of biochemical mediators of stress, such as cortisol, catecholamines, and neuropeptides [1, 2]. The elevated levels of these factors are used as clinical markers of stress. These stress mediators trigger a variety of physiological changes meant to improve the performance of the organism, such as increasing blood pressure and heart rate and enhancing the immune response. Thus, a short, acute stress has been shown to exert various beneficial effects. However, when stress becomes chronic, the prolonged exposure to the same stress mediators, which were beneficial in acute stress, often triggers pathological processes and contributes to the development or exacerbation of various diseases, including cancer [3].

Chronic stress has been implicated in the stimulation of tumor development and progression by both clinical and animal studies [4–6]. Initially, stress-induced suppression of the immune response was suggested as the major mechanism of this phenomenon [7]. As opposed to acute stress, which enhances immunity and has been shown to increase resistance to cancer, chronic stress impairs immune responses and in this way facilitates tumor growth [8, 9]. However, there is also growing evidence indicating that stress mediators, such as glucocorticoids and sympathetic neurotransmitters, can directly affect tumor cell proliferation and survival as well as tumor angiogenesis. The direct effects on tumor cells vary significantly between different stress mediators and types of tumors [10–13]. In contrast, their actions on tumor vascularization involve interactions with common angiogenic factors, such as vascular endothelial growth factor (VEGF), and seem to be universal between different tumor types [5, 14–16]. Thus, stress mediators and their receptors can become novel targets in antiangiogenic tumor therapy. This review will focus on sympathetic neurotransmitters and their effects on tumor vascularization.

2. Norepinephrine and Epinephrine

Norepinephrine (NE) and epinephrine (E) belong to a family of catecholamines and are one of the best characterized stress neurohormones. NE is released primarily from the sympathetic nerves, while E is mainly secreted from the adrenal medulla. As the sympatho-adrenomedullary system is responsible for the body's fight-or-flight stress response, circulating levels of both catecholamines are increased during stress [17]. NE and E activate the same α and β adrenoreceptors (AR), which are widely distributed in all tissues.

Recently, NE and E have been implicated in stress-induced augmentation of tumor growth and progression. In an orthotopic model of ovarian carcinoma, the growth-promoting effect of stress was mimicked by a β-AR agonist, isoproternol, and blocked by its antagonist, propranolol [5, 6]. Similarly, activation of β-AR resulted in an increase in metastases in animal models of lung and breast cancer [18, 19]. In all of the above models, the growth-promoting effects of stress, as well as direct activation of β-ARs, was associated with a significant increase in tumor vascularization, while β-AR blockers reduced vessel density [5, 6]. Moreover, tumors derived from stressed animals had elevated levels of VEGF and other angiogenic factors, and the growth promoting actions of β-AR activation was reduced by blocking the VEGF pathway [5]. Thus, an increase in angiogenesis appears to be the main mechanism of growth-promoting effects of NE and E. Indeed, in various cancer cell types, such as ovarian cancer, colon cancer, melanoma, pharyngeal carcinoma, and multiple myeloma, activation of β-ARs present on tumor cells led to a dramatic increase in synthesis and release of angiogenic factors—VEGF, IL-8, and IL-6 [5, 16, 20–23]. These effects were mediated primarily via a β-AR-dependent increase in cAMP levels, which resulted in the activation of protein kinase A (PKA) and Src [5, 22]. Adrenergic stimulation has also been shown to increase the secretion of metalloproteases, MMP-2 and MMP-9, which further augment angiogenic and metastatic processes [21]. Interestingly, catecholamine-induced release of angiogenic factors from tumor cells can be further enhanced by its secretion from stromal cells, such as β-AR-positive tumor-associated macrophages [24, 25].

Although the stimulatory effects of NE and E on the release of angiogenic factors seem to be the major mechanism of their tumor-promoting actions, these neurotransmitters can also exert direct trophic effects on endothelial cells (ECs) through α-ARs. Phenylepinephrine, a non-vasoconstrictive α-AR agonist, has been shown to induce EC proliferation and migration as well as promote capillary formation. Interestingly, these effects were potentiated by hypoxia [26]. Since tissue ischemia is known to stimulate NE release from the sympathetic nerves [27], the direct angiogenic effect of NE can be significantly enhanced in hypoxic areas of tumors.

Thus far, the results of experimental studies have confirmed that AR agonists exert strong stimulatory effects on tumor growth and agree that the release of angiogenic factors is the main mechanism of these actions. These discoveries open new possibilities of treatment with well-known drugs, such as antagonists of ARs. Some clinical data indicating decreased incidence of prostate cancer among cardiovascular patients treated with β-blockers corroborated the above findings [28, 29]. However, it is important to remember that the indirect, pro-angiogenic effect of AR agonists mediated by other angiogenic factors depends on the presence of these receptors on tumor cells, thus it can be tumor-specific. Moreover, the angiogenic actions of NE and E can be further modified by their direct effect on tumor cell proliferation and invasiveness, which in turn may differ among various tumors. In many cancer cell types, such as colon, ovarian, and prostate, these effects are stimulatory [11, 12]. However, adrenergic stimulation can also inhibit proliferation of some tumor cells, as shown in melanoma and neuroblastoma [30, 31]. In breast cancer, on the other hand, the reports are contradictory. The adrenergic agonists seem to increase motility of cancer cells but at the same time inhibit their proliferation [13, 32]. In agreement with these data, another clinical study indicated no effect of treatment with β-blockers on the risk of breast cancer among cardiovascular patients [33, 34]. Thus, the success of potential cancer therapy targeting ARs will depend on the type of tumor, its receptor expression pattern, and environmental factors, such as stress, which augment NE and E effects.

3. Dopamine

Dopamine (DA) is not only a precursor of NE and E but is also an important neurotransmitter in the brain acting via two types of receptors—D1 and D2. In the periphery, DA is synthesized in mesenteric organs as well as released from sympathetic neurons and adrenal medulla [17]. Levels of DA are elevated during stress, but rather than mediating the fight-or-flight response, as NE and E do, its role involves coping with stress [35]. DA also seems to have opposite than NE and E effects on tumor growth. It has been shown that administration of DA inhibits the growth of various tumors, such as stomach, breast, and colon cancers [14, 36]. Consistently, in mice lacking the DA transporter, which is normally responsible for uptake of this neurotransmitter, the elevated DA levels were associated with reduced growth of Lewis lung carcinoma [37]. In gastric cancer, the endogenous levels of DA were significantly lower than those in surrounding healthy tissue, indicating that the neurotransmitter acts as an endogenous tumor suppressant that needs to be inactivated to allow tumor progression [36].

The main mechanism of these growth-inhibitory actions of DA involves its direct antiangiogenic effect on ECs. In all animal models, treatment with DA led to a significant reduction in tumor vascularization [14, 36, 37]. DA has also been shown to block VEGF-induced EC proliferation, migration, and vascular permeability. Further studies revealed that DA, acting through its D2 receptors, enhances endocytosis of VEGF-R2 and decreases its membrane expression. This activity of DA interferes with VEGF signaling by reducing VEGF-induced phosphorylation of its VEGF-R2 and preventing the

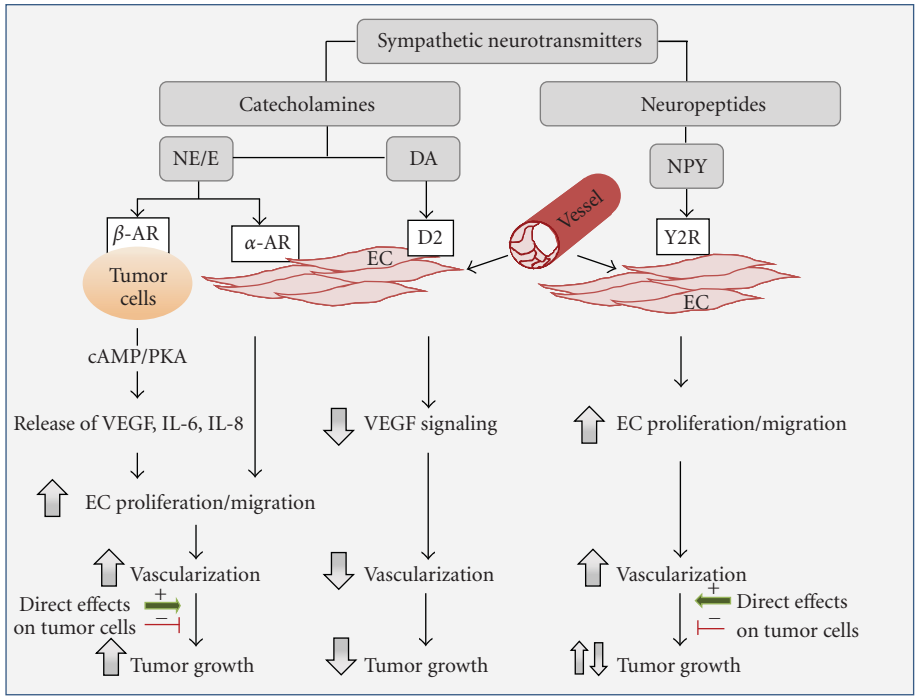


Figure 1: Activation of sympathetic neurons results in release of various neurotransmitters—catecholamines and neuropeptides. Norepinephrine (NE) and Epinephrine (E), belonging to a family of catecholamines, activate their β-adrenoreceptors (ARs) expressed on tumor cells and stimulate release of angiogenic factors, such as vascular endothelial growth factor (VEGF) and interleukins. Moreover, NE/E can directly induce endothelial cell (EC) proliferation and migration via their α-AR. Both of these processes lead to an increase in tumor vascularization. Adrenergic stimulation can also affect proliferation, survival, and invasiveness of cancer cells. This effect may be stimulatory or inhibitory, depending on tumor type. However, the proangiogenic actions of NE/E prevail over its direct effect on tumor cells. In consequence, adrenergic activation leads to an increase in tumor growth in most of the investigated tumor types. Another catecholamine, dopamine (DA), acts on its D2 receptors present on EC and interferes with VEGF signaling. As a result, dopamine reduces tumor vascularization and inhibits tumor growth. Neuropeptide Y (NPY), coreleased with NE from sympathetic nerves, directly stimulates EC proliferation and migration via its Y2Rs and increases tumor vascularization. However, NPY can also significantly alter the proliferation and survival of tumor cells. These direct actions of NPY on tumor cells are powerful enough to overcome its angiogenic activities. In consequence, the net effect of NPY varies in different types of tumors.

activation of downstream kinases—FAK and p42/44 MAPK [38, 39].

In addition to its effect on mature ECs, DA has also been shown to block VEGF signaling in endothelial progenitor cells (EPCs). As a consequence, DA not only inhibits trophic functions of VEGF in these cells but also blocks their recruitment from bone marrow. It has been shown that DA levels are decreased in the bone marrow of tumor-bearing mice, which facilitates EPC mobilization [40]. Since recent data strongly support a role for EPCs in the tumor vascularization, DA effect on EPC function may significantly contribute to its growth-inhibitory effect.

The role of DA in stress-induced changes in tumor growth and progression has not been characterized. It seems that DA is an endogenous inhibitory factor which requires inactivation for tumor growth, rather than sympathetic activation. However, in contrast to NE and E acting on specific tumors, DA effects appear to be more universal, influencing various tumor types, via its direct actions on ECs and EPCs. Thus, DA receptor agonists may become attractive antiangiogenic drugs in cancer therapy.

4. Neuropeptide Y

Neuropeptide Y (NPY) is a 36-amino-acid peptide coreleased with NE from sympathetic nerves. The actions of the peptide are mediated by multiple receptors-designated Y1–Y5 [41]. NPY is mainly known due to its anxiolytic effect in the brain and central regulation of food intake. In the periphery, NPY inhibits the release of NE after sympathetic stimulation and acts as a vasoconstrictor [41]. There is also a growing number of evidences that NPY is a growth factor for variety of cells. The peptide has been shown to stimulate proliferation of vascular smooth muscle cells and neuronal precursors, while the trophic effect of NPY on ECs revealed its angiogenic properties [42–47].

The main mechanism of NPY-induced angiogenesis involves its direct effect on ECs. The peptide stimulates proliferation and migration of ECs and promotes capillary tube formation, while in vivo, endogenous NPY facilitates vascularization of ischemic tissues [43, 46, 47]. These actions are dependent on endothelial nitric oxide synthase (eNOS) activation and, partially, on the VEGF pathway [46]. The angiogenic activities of NPY are mediated mainly by its

Y2Rs, since NPY-induced angiogenesis is severely impaired in Y2R −/− mice [48, 49].

Due to its angiogenic properties, NPY has been implicated in various pathological conditions associated with a deregulation of tissue vascularization, such as retinopathy, wound healing, atherosclerosis, and obesity [48, 50–52]. Recently, its role in tumor angiogenesis has also been shown. In malignancies originating from neuroendocrine tissues, such as neuroblastoma and Ewing's sarcoma, NPY released from tumor cells seems to be an essential factor involved in their vascularization. Antagonists to NPY receptors blocked the effect of both neuroblastoma and Ewing's sarcoma-conditioned media on EC proliferation. Consequently, treatment with exogenous NPY significantly increased vascularization of subcutaneous xenografts derived from both tumor cell types [10].

As in the case of NE and E, the angiogenesis-related growth-stimulatory actions of NPY are further modified by its direct effect on tumor cell growth and survival. For example, in neuroblastoma, the peptide stimulates proliferation of tumor cells via the same angiogenic Y2Rs, thereby further augmenting the growth of neuroblastoma xenografts. In contrast, in Ewing's sarcoma, NPY induces tumor cell apoptosis via Y1 and Y5Rs. As a result, exogenous NPY inhibits growth of Ewing's sarcoma xenografts in vivo, despite increase in their vascularization [10].

Although neuroendocrine tumors, which synthesize and release endogenous NPY, seem the most susceptible to tumor growth regulation by this peptide, NPY and its receptors have also been implicated in nonneuronal types of tumors. For example, peptide YY (PYY), which belongs to the same family of peptides and acts through the same receptors as NPY, has been shown to inhibit proliferation of breast and prostate cancer cells via Y4Rs and pancreatic cancer cells via Y2Rs [53–56]. Thus, these direct effects on tumor cell proliferation and survival are an important aspect of NPY actions in tumors and are often potent enough to overcome its angiogenesis-mediated growth-promoting effect.

Thus far, most of the studies addressing the role of stress in promoting cancer growth focus on the best known stress mediators—catecholamines and glucocorticoids. There are no studies directly linking NPY with stress-induced tumor growth and progression. However, systemic NPY levels are also upregulated during stress, particularly those intensive and prolonged in nature. Moreover, NPY is more stable than both NE and glucocorticoids. Hence, once stimulated, the elevated levels of NPY persist for a longer period of time [57]. The physiological role of NPY is to help cope with stress due to its central, anxiolytic effects [58, 59]. However, it has been shown that elevated peripheral circulating levels of NPY induced by intensive chronic stress can result in significant deleterious effects, such as enhanced atherosclerosis and diet-induced obesity, both of which are diseases associated with intensive tissue growth and upregulated angiogenesis [52, 60]. Thus, while high levels of NPY in the brain improve stress coping, chronically elevated levels of the peptide in the circulation can result in a variety of side effects. Whether enhanced tumor growth is one of them remains to be investigated.

5. Summary

As summarized above, the discoveries of recent years provided a significant body of evidence confirming an important role of sympathetic neurotransmitters and, consequently, chronic stress in regulating of tumor vascularization (Figure 1). This research opens new avenues for developing novel therapeutics, as well as using already existing and well-characterized drugs, such as β-blockers and DA receptor agonists, in new clinical settings. This seems to be particularly important, since cancer diagnosis per se is usually a stressful event for the patient. However, careful consideration needs to be given to other actions of stress mediators, such as cancer-specific effects on tumor cells themselves, as well as changes in immune system, which can indirectly affect tumor development and progression. Finally, since patterns of neuro-hormonal activation vary with different types of stress [17], tumor exposure to particular stress mediators would vary, too. Thus, potential therapeutic value of modifying particular stress pathways may be dependent on a variety of factors.

Acknowledgments

This work was supported by Grant no. 1R01CA123211-01 from the National Institutes of Health, grant no. CA093337 from the Department of Defense, and funding from Children's Cancer Foundation (Baltimore, MD) to Joanna Kitlinska.

References

[1] G. P. Chrousos and P. W. Gold, "The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis," *Journal of the American Medical Association*, vol. 267, no. 9, pp. 1244–1252, 1992.

[2] T. E. Seeman, B. H. Singer, J. W. Rowe, R. I. Horwitz, and B. S. McEwen, "Price of adaptation—allostatic load and its health consequences: MacArthur studies of successful aging," *Archives of Internal Medicine*, vol. 157, no. 19, pp. 2259–2268, 1997.

[3] B. S. McEwen and E. Stellar, "Stress and the individual. Mechanisms leading to disease," *Archives of Internal Medicine*, vol. 153, no. 18, pp. 2093–2101, 1993.

[4] M. H. Antoni, S. K. Lutgendorf, S. W. Cole, et al., "The influence of bio-behavioural factors on tumour biology: pathways and mechanisms," *Nature Reviews Cancer*, vol. 6, no. 3, pp. 240–248, 2006.

[5] P. H. Thaker, L. Y. Han, A. A. Kamat, et al., "Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma," *Nature Medicine*, vol. 12, no. 8, pp. 939–944, 2006.

[6] J.-W. Lee, M. M. K. Shahzad, Y. G. Lin, et al., "Surgical stress promotes tumor growth in ovarian carcinoma," *Clinical Cancer Research*, vol. 15, no. 8, pp. 2695–2702, 2009.

[7] J. P. Godbout and R. Glaser, "Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer," *Journal of Neuroimmune Pharmacology*, vol. 1, no. 4, pp. 421–427, 2006.

[8] F. S. Dhabhar, A. N. Saul, C. Daugherty, T. H. Holmes, D. M. Bouley, and T. M. Oberyszyn, "Short-term stress enhances cellular immunity and increases early resistance to squamous cell carcinoma," *Brain, Behavior, and Immunity*, vol. 24, no. 1, pp. 127–137, 2010.

[9] F. S. Dhabhar, "Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology," *NeuroImmunoModulation*, vol. 16, no. 5, pp. 300–317, 2009.

[10] J. Kitlinska, K. Abe, L. Kuo, et al., "Differential effects of neuropeptide Y on the growth and vascularization of neural crest-derived tumors," *Cancer Research*, vol. 65, no. 5, pp. 1719–1728, 2005.

[11] D. Palm, K. Lang, B. Niggemann, et al., "The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by β-blockers," *International Journal of Cancer*, vol. 118, no. 11, pp. 2744–2749, 2006.

[12] A. K. Sood, R. Bhatty, A. A. Kamat, et al., "Stress hormone-mediated invasion of ovarian cancer cells," *Clinical Cancer Research*, vol. 12, no. 2, pp. 369–375, 2006.

[13] A. E. Carie and S. M. Sebti, "A chemical biology approach identifies a β-2 adrenergic receptor agonist that causes human tumor regression by blocking the Raf-1/Mek-1/Erk1/2 pathway," *Oncogene*, vol. 26, no. 26, pp. 3777–3788, 2007.

[14] C. Sarkar, D. Chakroborty, U. R. Chowdhury, P. S. Dasgupta, and S. Basu, "Dopamine increases the efficacy of anticancer drugs in breast and colon cancer preclinical models," *Clinical Cancer Research*, vol. 14, no. 8, pp. 2502–2510, 2008.

[15] D. Chakroborty, C. Sarkar, B. Basu, P. S. Dasgupta, and S. Basu, "Catecholamines regulate tumor angiogenesis," *Cancer Research*, vol. 69, no. 9, pp. 3727–3730, 2009.

[16] E. V. Yang, S.-J. Kim, E. L. Donovan, et al., "Norepinephrine upregulates VEGF, IL-8, and IL-6 expression in human melanoma tumor cell lines: implications for stress-related enhancement of tumor progression," *Brain, Behavior, and Immunity*, vol. 23, no. 2, pp. 267–275, 2009.

[17] D. S. Goldstein, "Catecholamines and stress," *Endocrine Regulations*, vol. 37, no. 2, pp. 69–80, 2003.

[18] S. Ben-Eliyahu, R. Yirmiya, J. C. Liebeskind, A. N. Taylor, and R. P. Gale, "Stress increases metastatic spread of a mammary tumor in rats: evidence for mediation by the immune system," *Brain, Behavior, and Immunity*, vol. 5, no. 2, pp. 193–205, 1991.

[19] R. Melamed, E. Rosenne, K. Shakhar, Y. Schwartz, N. Abudarham, and S. Ben-Eliyahu, "Marginating pulmonary-NK activity and resistance to experimental tumor metastasis: suppression by surgery and the prophylactic use of a β-adrenergic antagonist and a prostaglandin synthesis inhibitor," *Brain, Behavior, and Immunity*, vol. 19, no. 2, pp. 114–126, 2005.

[20] Z. R. Yang and K.-C. Chou, "Bio-support vector machines for computational proteomics," *Bioinformatics*, vol. 20, no. 5, pp. 735–741, 2004.

[21] E. V. Yang, A. K. Sood, M. Chen, et al., "Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells," *Cancer Research*, vol. 66, no. 21, pp. 10357–10364, 2006.

[22] M. B. Nilsson, G. Armaiz-Pena, R. Takahashi, et al., "Stress hormones regulate interleukin-6 expression by human ovarian carcinoma cells through a Src-dependent mechanism," *The Journal of Biological Chemistry*, vol. 282, no. 41, pp. 29919–29926, 2007.

[23] H. P. S. Wong, L. Yu, E. K. Y. Lam, E. K. K. Tai, W. K. K. Wu, and C.-H. Cho, "Nicotine promotes colon tumor growth and angiogenesis through β-adrenergic activation," *Toxicological Sciences*, vol. 97, no. 2, pp. 279–287, 2007.

[24] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[25] S. K. Lutgendorf, D. M. Lamkin, N. B. Jennings, et al., "Biobehavioral influences on matrix metalloproteinase expression in ovarian carcinoma," *Clinical Cancer Research*, vol. 14, no. 21, pp. 6839–6846, 2008.

[26] M. C. Vinci, L. Bellik, S. Filippi, et al., "Trophic effects induced by α_{1D}-adrenoceptors on endothelial cells are potentiated by hypoxia," *American Journal of Physiology*, vol. 293, no. 4, pp. H2140–H2147, 2007.

[27] V. Borovsky, M. Herman, G. Dunphy, et al., "CO_2 asphyxia increases plasma norepinephrine in rats via sympathetic nerves," *American Journal of Physiology*, vol. 274, no. 1, part 2, pp. R19–R22, 1998.

[28] L. Perron, I. Bairati, F. Harel, and F. Meyer, "Antihypertensive drug use and the risk of prostate cancer (Canada)," *Cancer Causes and Control*, vol. 15, no. 6, pp. 535–541, 2004.

[29] C. Rodriguez, E. J. Jacobs, A. Deka, et al., "Use of blood-pressure-lowering medication and risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort," *Cancer Causes and Control*, vol. 20, no. 5, pp. 671–679, 2009.

[30] C. Pifl, J. Zezula, A. Spittler, et al., "Antiproliferative action of dopamine and norepinephrine in neuroblastoma cells expressing the human dopamine transporter," *The FASEB Journal*, vol. 15, no. 9, pp. 1607–1609, 2001.

[31] A. C. Scarparo, D. H. Sumida, M. T. C. C. Patrão, M. C. W. Avellar, M. A. Visconti, and A. M. D. L. Castrucci, "Catecholamine effects on human melanoma cells evoked by α_1-adrenoceptors," *Archives of Dermatological Research*, vol. 296, no. 3, pp. 112–119, 2004.

[32] T. L. Drell IV, J. Joseph, K. Lang, B. Niggemann, K. S. Zaenker, and F. Entschladen, "Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells," *Breast Cancer Research and Treatment*, vol. 80, no. 1, pp. 63–70, 2003.

[33] C. R. Meier, L. E. Derby, S. S. Jick, and H. Jick, "Angiotensin-converting enzyme inhibitors, calcium channel blockers, and breast cancer," *Archives of Internal Medicine*, vol. 160, no. 3, pp. 349–353, 2000.

[34] C. I. Li, K. E. Malone, N. S. Weiss, D. M. Boudreau, K. L. Cushing-Haugen, and J. R. Daling, "Relation between use of antihypertensive medications and risk of breast carcinoma among women ages 65–79 years," *Cancer*, vol. 98, no. 7, pp. 1504–1513, 2003.

[35] S. R. Snider and O. Kuchel, "Dopamine: an important neurohormone of the sympathoadrenal system. Significance of increased peripheral dopamine release for the human stress response and hypertension," *Endocrine Reviews*, vol. 4, no. 3, pp. 291–309, 1983.

[36] D. Chakroborty, C. Sarkar, R. B. Mitra, S. Banerjee, P. S. Dasgupta, and S. Basu, "Depleted dopamine in gastric cancer tissues: dopamine treatment retards growth of gastric cancer by inhibiting angiogenesis," *Clinical Cancer Research*, vol. 10, no. 13, pp. 4349–4356, 2004.

[37] M. Asada, S. Ebihara, Y. Numachi, et al., "Reduced tumor growth in a mouse model of schizophrenia, lacking the dopamine transporter," *International Journal of Cancer*, vol. 123, no. 3, pp. 511–518, 2008.

[38] S. Basu, J. A. Nagy, S. Pal, et al., "The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor/vascular endothelial growth factor," *Nature Medicine*, vol. 7, no. 5, pp. 569–574, 2001.

[39] C. Sarkar, D. Chakroborty, R. B. Mitra, S. Banerjee, P. S. Dasgupta, and S. Basu, "Dopamine in vivo inhibits VEGF-induced phosphorylation of VEGFR-2, MAPK, and focal adhesion kinase in endothelial cells," *American Journal of Physiology*, vol. 287, no. 4, pp. H1554–H1560, 2004.

[40] D. Chakroborty, U. R. Chowdhury, C. Sarkar, R. Baral, P. S. Dasgupta, and S. Basu, "Dopamine regulates endothelial progenitor cell mobilization from mouse bone marrow in tumor vascularization," *Journal of Clinical Investigation*, vol. 118, no. 4, pp. 1380–1389, 2008.

[41] W. F. Colmers and C. Wahlestedt, *The Biology of Neuropeptide Y and Related Peptides*, Humana Press, Totowa, NJ, USA, 1993.

[42] Z. Zukowska-Grojec, P. Pruszczyk, C. Colton, et al., "Mitogenic effect of neuropeptide Y in rat vascular smooth muscle cells," *Peptides*, vol. 14, no. 2, pp. 263–268, 1993.

[43] Z. Zukowska-Grojec, E. Karwatowska-Prokopczuk, W. Rose, et al., "Neuropeptide Y a novel angiogenic factor from the sympathetic nerves and endothelium," *Circulation Research*, vol. 83, no. 2, pp. 187–195, 1998.

[44] D. E. Hansel, B. A. Eipper, and G. V. Ronnett, "Neuropeptide Y functions as a neuroproliferative factor," *Nature*, vol. 410, no. 6831, pp. 940–944, 2001.

[45] J. Pons, J. Kitlinska, H. Ji, et al., "Bimodal mitogenic action of neuropeptide Y in vascular smooth muscle cells: dimerization of the receptors?" *Molecular Biology of the Cell*, vol. 13, supplement, article 80a, 2002.

[46] E. W. Lee, M. Michalkiewicz, J. Kitlinska, et al., "Neuropeptide Y induces ischemic angiogenesis and restores function of ischemic skeletal muscles," *Journal of Clinical Investigation*, vol. 111, no. 12, pp. 1853–1862, 2003.

[47] S. Movafagh, J. P. Hobson, S. Spiegel, H. K. Kleinman, and Z. Zukowska, "Neuropeptide Y induces migration, proliferation, and tube formation of endothelial cells bimodally via Y1, Y2, and Y5 receptors," *The FASEB Journal*, vol. 20, no. 11, pp. 1924–1926, 2006.

[48] A. J. Ekstrand, R. Cao, M. Björndahl, et al., "Deletion of neuropeptide Y $(NPY)_2$ receptor in mice results in blockage of NPY-induced angiogenesis and delayed wound healing," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 6033–6038, 2003.

[49] E. W. Lee, D. S. Grant, S. Movafagh, and Z. Zukowska, "Impaired angiogenesis in neuropeptide Y (NPY)-Y2 receptor knockout mice," *Peptides*, vol. 24, no. 1, pp. 99–106, 2003.

[50] L. Li, E. W. Lee, H. Ji, and Z. Zukowska, "Neuropeptide Y-induced acceleration of postangioplasty occlusion of rat carotid artery," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 23, no. 7, pp. 1204–1210, 2003.

[51] M. Koulu, S. Movafagh, J. Tuohimaa, et al., "Neuropeptide Y and Y2-receptor are involved in development of diabetic retinopathy and retinal neovascularization," *Annals of Medicine*, vol. 36, no. 3, pp. 232–240, 2004.

[52] L. E. Kuo, J. B. Kitlinska, J. U. Tilan, et al., "Neuropeptide Y acts directly in the periphery on fat tissue and mediates stress-induced obesity and metabolic syndrome," *Nature Medicine*, vol. 13, no. 7, pp. 803–811, 2007.

[53] C. D. Liu, A. Balasubramaniam, R. E. Saxton, M. Paiva, and D. W. McFadden, "Human pancreatic cancer growth is inhibited by peptide YY and BIM-43004-1," *Journal of Surgical Research*, vol. 58, no. 6, pp. 707–712, 1995.

[54] K. R. Grise, A. J. Rongione, E. C. Laird, and D. W. McFadden, "Peptide YY inhibits growth of human breast cancer in vitro and in vivo," *Journal of Surgical Research*, vol. 82, no. 2, pp. 151–155, 1999.

[55] T. Heisler, S. Towfigh, N. Simon, and D. W. McFadden, "Peptide YY and vitamin E inhibit hormone-sensitive and -insensitive breast cancer cells," *Journal of Surgical Research*, vol. 91, no. 1, pp. 9–14, 2000.

[56] A. Yu, P. Somasundar, A. Balsubramaniam, A. T. Rose, L. Vona-Davis, and D. W. McFadden, "Vitamin E and the Y4 agonist BA-129 decrease prostate cancer growth and production of vascular endothelial growth factor," *Journal of Surgical Research*, vol. 105, no. 1, pp. 65–68, 2002.

[57] Z. Zukowska-Grojec, "Neuropeptide Y. A novel sympathetic stress hormone and more," *Annals of the New York Academy of Sciences*, vol. 771, pp. 219–233, 1995.

[58] A. Thorsell, M. Michalkiewicz, Y. Dumont, et al., "Behavioral insensitivity to restraint stress, absent fear suppression of behavior and impaired spatial learning in transgenic rats with hippocampal neuropeptide Y overexpression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 23, pp. 12852–12857, 2000.

[59] C. C. Carvajal, F. Vercauteren, Y. Dumont, M. Michalkiewicz, and R. Quirion, "Aged neuropeptide Y transgenic rats are resistant to acute stress but maintain spatial and non-spatial learning," *Behavioural Brain Research*, vol. 153, no. 2, pp. 471–480, 2004.

[60] L. Li, A.-C. Jönsson-Rylander, K. Abe, and Z. Zukowska, "Chronic stress induces rapid occlusion of angioplasty-injured rat carotid artery by activating neuropeptide Y and its Y1 receptors," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 10, pp. 2075–2080, 2005.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 817375, 5 pages
doi:10.1155/2010/817375

Research Article

IL-17B Can Impact on Endothelial Cellular Traits Linked to Tumour Angiogenesis

Andrew J. Sanders,[1] Xiaoxia Guo,[1] Malcolm D. Mason,[2] and Wen G. Jiang[1]

[1] *Metastasis and Angiogenesis Research Group, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK*
[2] *Department of Clinical Oncology, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK*

Correspondence should be addressed to Andrew J. Sanders, sandersaj1@cf.ac.uk

Received 7 December 2009; Revised 16 February 2010; Accepted 6 March 2010

Academic Editor: Arkadiusz Dudek

IL-17B is a member of the IL-17 cytokine family which have been implicated in inflammatory response and autoimmune diseases such as rheumatoid arthritis. The founding member of this family, IL-17 (or IL-17A), has also been implicated in promoting tumour angiogenesis through the induction of other proangiogenic factors. Here we examine the potential of recombinant human IL-17B to contribute to the angiogenic process. In vitro rhIL-17B was able to inhibit HECV endothelial cell-matrix adhesion and cellular migration and also, at higher concentrations, could substantially reduce tubule formation compared to untreated HECV cells in a Matrigel tubule formation assay. This data suggests that IL-17B may act in an antiangiogenic manner.

1. Introduction

IL-17, initially termed CTLA-8, was identified in 1993 through subtractive hybridisation of a rodent cDNA library and was found to share homology to the ORF13 gene of the Herpesvirus saimiri. Subsequent studies identified both the receptor for IL-17/CTLA-8 cytokine and the human version of IL-17. IL-17 has been shown to induce IL-6 and IL-8 production together with enhanced expression of ICAM-1 and NF-κB activity [1–3]. Since their initial discovery, research into the IL-17 cytokine family has identified six members, IL-17 (IL-17A) -IL-17F, which signal through a family of IL-17 receptors, IL-17RA–IL-17RE (reviewed in full in [4, 5]).

IL-17 can cause the induction of proinflammatory factors, contributing to the immune response. However this cytokine has also been shown to play a role in autoimmune disorders such as rheumatoid arthritis [6–9]. Various studies have also investigated the role of IL-17 in cancer progression, discovering that IL-17 possesses both anti- and pro tumour roles. IL-17-transfected haematopoietic tumours grafted onto syngeneic immunocompetent mice grew at a significantly reduced rate to mock transfected control tumours and this reduction was associated with the enhanced generation of CTLs [10]. Another study suggests that endogenous IL-17 may be involved in tumour immunity and show that MC38 colon cancer cells inoculated subcutaneously developed significantly faster in IL-17-deficient mice than wild-type mice and developed significantly more metastatic foci than wild-type mice following intravenous injection of MC38 [11]. The protumorigenic effects of IL-17 seem largely due to its ability to contribute to the inflammatory response and enhance angiogenesis. IL-17-transfected tumour cells have been shown to enhance in vivo growth and show significantly enhanced tumour vascularity compared to control cells, whilst in vitro IL-17 treatment stimulated vascular endothelial cell migration and cord formation and caused upregulation of a number of proangiogenic factors in fibroblasts and tumour cells [12]. Subsequent work has shown that IL-17 could enhance HGF-, VEGF-, and bFGF-induced vascular endothelial cell growth and thus may help to mediate angiogenesis promoted through these growth factors [13]. Elevated levels of serum IL-17 have been detected in multiple myeloma patients, with levels being significantly higher in stage II and III patients than in stage I patients and in these patients serum IL-17 levels were found to correlate positively with levels of VEGF, TNFα, and micro vessel density [14].

Currently there are few studies focusing on the IL-17B member of the IL-17 cytokine family in cancer progression and angiogenesis. IL-17B was initially cloned and characterised in 2000 [15]. IL-17B shares approximately 27% amino acid identity with IL-17, has a wider expression pattern with transcripts being demonstrated in human adult pancreas, small intestine, stomach, and testis, and can stimulate the release of tumour necrosis factor alpha (TNFα) and IL-1β in the THP-1 monocytic cell line [15]. IL-17B has also been suggested to play an important role in inflammatory arthritis [16]. In the current study we use rhIL-17B to assess the role of IL-17B in the human HECV endothelial cell line and its potential to impact on traits, such as cellular migration and tubule formation, associated with the angiogenic process.

2. Methodology

2.1. Reagents, Cell Lines, and Culture Conditions. The human HECV endothelial cell line was purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Cells were routinely subcultured in Modified Eagle Medium (DMEM) (PAA Laboratories Ltd., Somerset, UK) supplemented with 10% Fetal Bovine Serum (PAA Laboratories Ltd, Somerset, UK), penicillin, and streptomycin. The cells were maintained in an incubator at 37°C and 5% CO_2 humidity. Recombinant human IL-17B (rhIL-17B) was purchased from R&D systems (Abingdon, UK).

2.2. Cell Function Assays

2.2.1. In Vitro Cell Growth Assay. The impact of rhIL-17B on HECV cell growth was assessed using an in vitro cell growth assay. Cells were seeded into a 96-well plate at a seeding density of 3,000 cells/well and treated with a range of rhIL-17B concentrations (0 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, and 250 ng/mL). Triplicate plates were set up and incubated for periods of overnight, 3 days or 5 days. Following incubation, the medium was removed; cells were fixed in 4% (v/v) formalin for 5 minutes, stained in 0.5% (w/v) crystal violet for 5 minutes, and rinsed. The crystal violet stain taken up by the cells was then extracted in a 10% (v/v) acetic acid solution and cell growth was determined spectrophotometrically using a Bio-Tek ELx800 multiplate reader (Bio-Tek Instruments Inc., Vermont, USA).

2.2.2. In Vitro Matrigel Adhesion Assay. The impact of rhIL-17B on HECV cell-matrix adhesion was assessed using an in vitro Matrigel adhesion assay previously described [17]. In brief, wells of a 96-well plate were precoated with 5 μg of Matrigel (BD Biosciences, Oxford, UK) before seeding 30,000 HECV cells in medium containing a range of rhIL-17B concentrations (0 ng/mL control, 10 ng/mL, 50 ng/mL, and 100 ng/mL). Cells were incubated for 45 minutes before being subjected to intense washing to remove nonadherent cells, fixed in 4% (v/v) formalin, and stained with 0.5% (w/v) crystal violet. Adherent cells were subsequently observed under a microscope and the number of adherent cells per field were calculated in a number of random fields.

2.2.3. In Vitro Migration/Wounding Assay. A migration/wounding assay was used to assess the impact of rhIL-17B on HECV cellular migration. This protocol was modified from a previously described method [18]. Briefly, cells were cultured in a 24-well plate until they reached a near confluent monolayer. This monolayer was subsequently scratched with a 21G needle and the medium was replaced with fresh medium containing a range of concentrations of rhIL-17B (0 ng/mL control, 10 ng/mL, 50 ng/mL, and 100 ng/mL). Cellular migration from the two wound fronts was tracked and recorded over a 90-minute period using a time-lapse video recorder (Panasonic, Japan). Migration rates were those calculated at 15 minute-time intervals within the 90-minute period using Optimas 6 motion analysis software.

2.2.4. In Vitro Matrigel Tubule Formation Assay. Matrigel endothelial cell tube formation assays were set up to assess any impact on angiogenic effect following treatment with rhIL-17B at a range of concentrations (0 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, and 250 ng/mL). The protocol used was modified from that previously reported [19]. Briefly, 250 μg of Matrigel was seeded into a 96-well plate in serum-free medium and left to gel in an incubator for a minimum of 40 minutes. Once the Matrigel had set, 30,000 HECV cells were seeded onto the Matrigel layer and incubated for 4-5 hours to allow tubule formation to occur. Following incubation, any tubules that had formed were visualized under low magnification and images captured. Total tubule perimeter/field in these images were later quantified using ImageJ software.

2.3. Statistical Analysis. All experimental procedures were repeated a minimum of three independent times. Data was analysed using Minitab 14 statistical package using a two-sample two-tailed t-test to compare treated samples to that of untreated controls. Values of $P < .05$ were taken as being statistically significant.

3. Results and Discussion

3.1. rhIL-17B Had Little Impact on HECV Cell Growth over a Range of Concentrations. rhIL-17B was added to the cells at concentrations of 10 ng/mL, 50 ng/mL, 100 ng/mL, and 250 ng/mL and the growth rates were compared to those an untreated control in order to determine the toxicity of rhIL-17B. Treatment of HECV cells with rhIL-17B seemed to have little effect on the growth rate of this cell line over the incubation period and concentrations tested (Figure 1). Following 3-day incubation at the various treatments, there is very little variation between the growth rates of the treated cells and the untreated control cells. Following a longer 5-day treatment of HECV cells with rhIL-17B, a noticeable drop in growth can be seen in the highest tested concentration of 250 ng/mL; however all other treatment concentrations show little variability in growth rates compared to untreated control HECV cells. No significant difference was observed at any concentration at any specific incubation period when compared to the untreated control of the same incubation

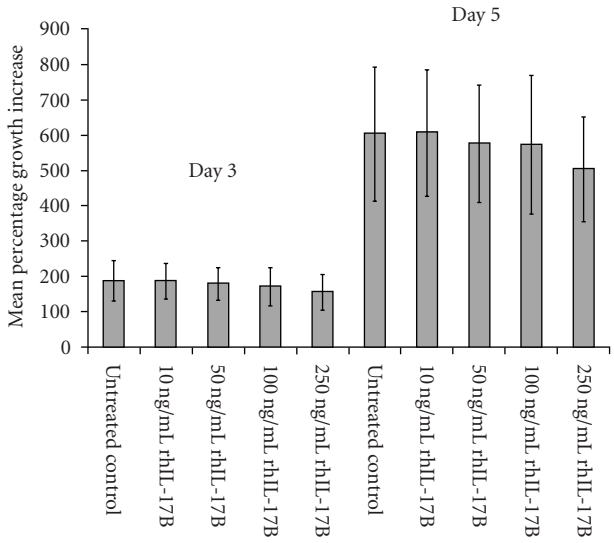


Figure 1: HECV growth was not affected by rhIL-17B treatment. No significant difference in HECV growth rate was observed following 3- or 5-day incubation periods between the untreated (0 ng/mL) control and any other rhIL-17B treatment concentration (10 ng/mL, 50 ng/mL, 100 ng/mL, and 250 ng/mL).

period. This suggests that IL-17B, at the tested concentrations, does not play a significant role in the regulation of cell growth in the HECV human endothelial cell line.

3.2. rhIL-17B Negatively Impacted on HECV Cell-Matrix Adhesion and Migration. Treatment of HECV endothelial cells with rhIL-17B could influence both cell-matrix adhesion (Figure 2(a)) and cellular migration (Figure 2(b)). Lower concentrations of rhIL-17B (10 ng/mL and 50 ng/mL) had minimal effect on these two traits; however at the higher concentration of 100 ng/mL a significant reduction in both cell-matrix adhesion and cellular migration in comparison to untreated control HECV cells was noticed. Treatment of HECV cells with 100 ng/mL rhIL-17B reduced the capacity of the cells to adhere to an artificial Matrigel basement membrane ($P = .004$). Similarly, at this concentration, rhIL-17B significantly reduced cell migration over a 90-minute period where the migration rates of cells treated with 100 ng/ml rhIL-17B were significantly lower than those of the untreated control cells after 75 minutes ($P = .048$) and at the experimental endpoint of 90 minutes ($P = .027$). Thus, it appears that IL-17B, at higher concentrations, may play a role in regulating HECV cell-matrix adhesion and cell migration. A previous study has investigated the role of IL-17 in endothelial cell migration in vitro, using a modified Boyden chamber assay system, and demonstrated a promotional effect of IL-17 on endothelial cell migration [12]. Our study examined the impact of rhIL-17B on endothelial cell migration using a wounding cell migration system, and whilst differing methodology makes it difficult to draw direct comparisons between IL-17 and IL-17B, our results indicate a potential role for IL-17B in the migration of HECV endothelial cells.

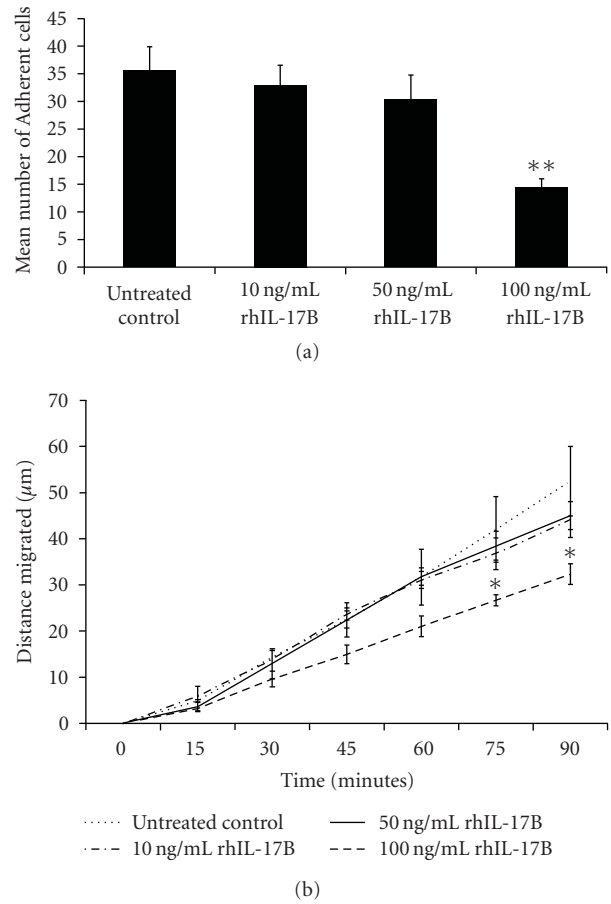


Figure 2: (a) Effect of rhIL-17B on HECV cell-matrix adhesion. A significant reduction in cell-matrix adhesion was observed following treatment with 100 ng/mL rhIL-17B. (b) Effect of rhIL-17B on HECV cell migration. Similarly, 100 ng/mL rhIL-17B significantly inhibited HECV cell migration and a significant difference compared to the untreated control in migration was observed following 75- and 90-minute periods. $^{*}P < .05$; $^{**}P < .01$.

3.3. rhIL-17B Can Reduce HECV Tubule Formation. Untreated HECV endothelial cells formed tubule-like structures when seeded onto Matrigel demonstrating their angiogenic potential. Treatment of these cells with rhIL-17B adversely affected the capability of this cell line to form these tubule structures (Figure 3). This adverse effect was most notable at the highest concentration of 250 ng/mL where, following quantification of tubule perimeter, a significant decrease in tubules was observed in comparison to untreated control cells ($P = .023$). Whilst a significant reduction in tubule formation was only observed at the 250 ng/mL concentration, there does appear some marginal effects, following quantification, of rhIL-17B on HECV tubule formation at 100 ng/mL and possibly even at 50 ng/mL, where mean total perimeter length/field was generally reduced in comparison to the untreated control, though both of these treatments did not reach significance. Thus, this data suggests that IL-17B may negatively impact on the angiogenesis process through inhibiting formation of new vessels. This role for IL-17B

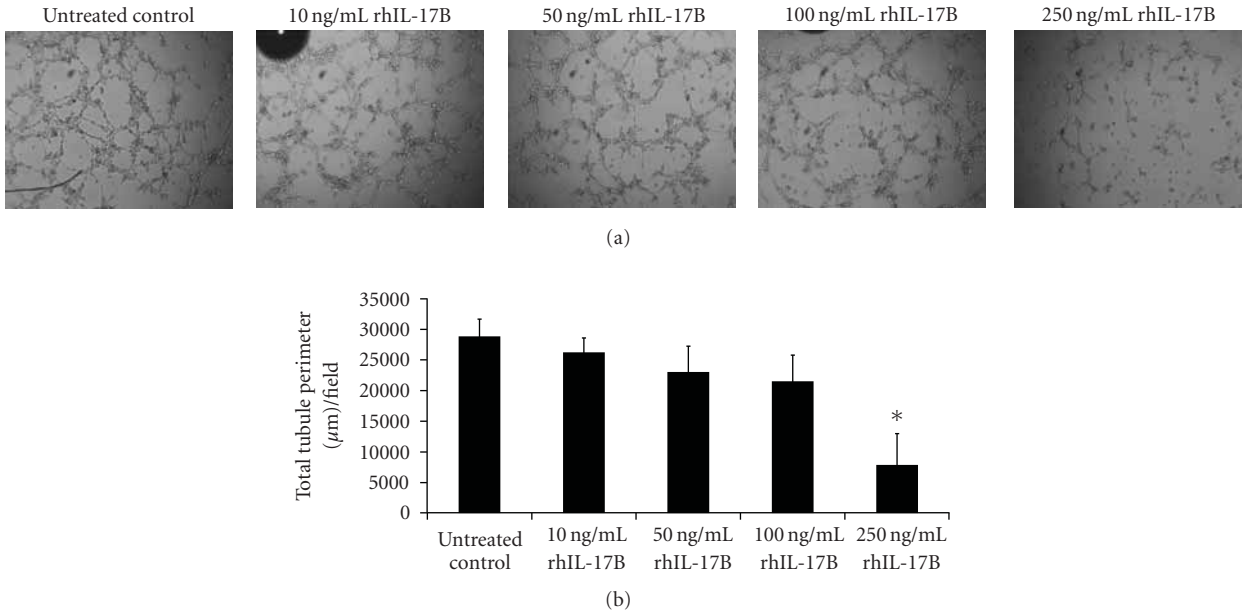


Figure 3: rhIL-17B inhibited HECV endothelial cell tubule formation. (a) Representative pictures of tubule formation following incubation with varying concentrations of rhIL-17B. (b) Histogram representing mean quantified total tubule length per field. A significant reduction was observed following treatment with 250 ng/mL rhIL-17B. $^*P < .05$.

seems to be in contrast with the proangiogenic effect of IL-17 observed by Numasaki et al., whose study demonstrated the capacity of IL-17 to enhance vascular endothelial cell cord formation over a range of concentrations in vitro and promote increased tumour vascularity in in vivo models [12]. In this study Numasaki et al. showed an enhancement of microvessel development at 50–500 ng/mL IL-17 concentrations. Whilst our study also tested a range of rhIL-17B concentrations, significant result were only observed at the higher dose (250 ng/mL) and little effect was observed at our lowest tested concentration of 10 ng/mL. However, IL-17B and its anti-angiogenic actions are not alone in the family. Other members of the IL-17 family have also been suggested to have anti-angiogenic influences at higher concentrations and an anti-angiogenic role for IL-17F was seen in a study by Starnes et al., which demonstrated the capacity of IL-17F to inhibit capillary tubule formation in an in vitro endothelial cell capillary tubule formation assay at concentrations of 100, 375, and 750 ng/mL [20]. Exerting biological functions at a higher concentration appears to be a common feature for the IL-17 family members. Another example is IL-17D, which has significant effects at a concentration range of 500–1000 ng/mL [21]. Thus, our data implies that IL-17B may function differently to IL-17 in angiogenesis or may itself not be acting as an independent angiogenic factor. Additionally, the high concentrations needed to bring about significant effects in vitro may limit the usefulness of IL-17B in vivo where physiological concentrations of this cytokine are likely to be substantially lower. Further investigation into both the mode of action of IL-17B together with additional studies examining the efficacy and toxicity of IL-17B over a range of concentration in vivo are required to fully realise the potential of this cytokine.

4. Conclusions

The data presented suggests that IL-17B may negatively impact on the angiogenic process through its ability to inhibit endothelial cell migration and tubule formation whilst having little effect on endothelial cell growth. Thus, it appears as though IL-17B could play an opposite role to that of IL-17 in the angiogenic process which, whilst also having little effect on endothelial cell proliferation, promoted endothelial cell migration and tubule formation [12], though direct comparisons between these two cytokines using similar methodologies are required to identify any contrasting roles. As far as the authors are aware, this data is the first implicating a role for IL-17B in the angiogenesis process. Additional work is required to fully investigate and establish the effects, either direct or indirect, of IL-17B in endothelial cells and to examine the efficacy of this cytokine to impact on angiogenesis using in vivo models. Thus, initial data suggests that IL-17B may hold potential as an anti-angiogenic therapeutic; however, the high concentrations needed to bring about any inhibition and how these would be tolerated in vivo must also be considered in future studies.

Acknowledgment

The authors wish to acknowledge and thank Cancer Research Wales for supporting this work.

References

[1] E. Rouvier, M.-F. Luciani, M.-G. Mattei, F. Denizot, and P. Golstein, "CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene," *Journal of Immunology*, vol. 150, no. 12, pp. 5445–5456, 1993.

[2] Z. Yao, S. L. Painter, W. C. Fanslow, et al., "Human IL-17: a novel cytokine derived from T cells," *Journal of Immunology*, vol. 155, no. 12, pp. 5483–5486, 1995.

[3] Z. Yao, W. C. Fanslow, M. F. Seldin, et al., "Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor," *Immunity*, vol. 3, no. 6, pp. 811–821, 1995.

[4] S. L. Gaffen, "Structure and signalling in the IL-17 receptor family," *Nature Reviews Immunology*, vol. 9, no. 8, pp. 556–567, 2009.

[5] T. A. Moseley, D. R. Haudenschild, L. Rose, and A. H. Reddi, "Interleukin-17 family and IL-17 receptors," *Cytokine and Growth Factor Reviews*, vol. 14, no. 2, pp. 155–174, 2003.

[6] M. Chabaud, J. M. Durand, N. Buchs, et al., "Human interleukin-17: a T cell-derived proinflammatory cytokine produced by the rheumatoid synovium," *Arthritis and Rheumatism*, vol. 42, no. 5, pp. 963–970, 1999.

[7] M. Chabaud, P. Garnero, J.-M. Dayer, P.-A. Guerne, F. Fossiez, and P. Miossec, "Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis," *Cytokine*, vol. 12, no. 7, pp. 1092–1099, 2000.

[8] M. Ziolkowska, A. Koc, G. Luszczykiewicz, et al., "High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism," *Journal of Immunology*, vol. 164, no. 5, pp. 2832–2838, 2000.

[9] E. Lubberts, L. A. B. Joosten, B. Oppers, et al., "IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis," *Journal of Immunology*, vol. 167, no. 2, pp. 1004–1013, 2001.

[10] F. Benchetrit, A. Ciree, V. Vives, et al., "Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism," *Blood*, vol. 99, no. 6, pp. 2114–2121, 2002.

[11] I. Kryczek, S. Wei, W. Szeliga, L. Vatan, and W. Zou, "Endogenous IL-17 contributes to reduced tumor growth and metastasis," *Blood*, vol. 114, no. 2, pp. 357–359, 2009.

[12] M. Numasaki, J.-I. Fukushi, M. Ono, et al., "Interleukin-17 promotes angiogenesis and tumor growth," *Blood*, vol. 101, no. 7, pp. 2620–2627, 2003.

[13] H. Takahashi, M. Numasaki, M. T. Lotze, and H. Sasaki, "Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells," *Immunology Letters*, vol. 98, no. 2, pp. 189–193, 2005.

[14] M. G. Alexandrakis, C. A. Pappa, S. Miyakis, et al., "Serum interleukin-17 and its relationship to angiogenic factors in multiple myeloma," *European Journal of Internal Medicine*, vol. 17, no. 6, pp. 412–416, 2006.

[15] H. Li, J. Chen, A. Huang, et al., "Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 2, pp. 773–778, 2000.

[16] Y. Yamaguchi, K. Fujio, H. Shoda, et al., "IL-17B and IL-17C are associated with TNF-α production and contribute to the exacerbation of inflammatory arthritis," *Journal of Immunology*, vol. 179, no. 10, pp. 7128–7136, 2007.

[17] W. G. Jiang, S. Hiscox, M. B. Hallett, C. Scott, D. F. Horrobin, and M. C. A. Puntis, "Inhibition of hepatocyte growth factor-induced motility and in vitro invasion of human colon cancer cells by gamma-linolenic acid," *British Journal of Cancer*, vol. 71, no. 4, pp. 744–752, 1995.

[18] W. G. Jiang, S. E. Hiscox, C. Parr, et al., "Antagonistic effect of NK4, a novel hepatocyte growth factor variant, on in vitro angiogenesis of human vascular endothelial cells," *Clinical Cancer Research*, vol. 5, no. 11, pp. 3695–3703, 1999.

[19] D. S. Grant, K.-I. Tashiro, B. Segui-Real, Y. Yamada, G. R. Martin, and H. K. Kleinman, "Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro," *Cell*, vol. 58, no. 5, pp. 933–943, 1989.

[20] T. Starnes, M. J. Robertson, G. Sledge, et al., "Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production," *Journal of Immunology*, vol. 167, no. 8, pp. 4137–4140, 2001.

[21] T. Starnes, H. E. Broxmeyer, M. J. Robertson, and R. Hromas, "Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis," *Journal of Immunology*, vol. 169, no. 2, pp. 642–646, 2002.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 509329, 10 pages
doi:10.1155/2010/509329

Research Article

An Active Form of Sphingosine Kinase-1 Is Released in the Extracellular Medium as Component of Membrane Vesicles Shed by Two Human Tumor Cell Lines

Salvatrice Rigogliuso,[1] Chiara Donati,[2] Donata Cassarà,[1] Simona Taverna,[1,3] Monica Salamone,[1,4] Paola Bruni,[2] and Maria Letizia Vittorelli[1]

[1] *Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Viale delle, Scienze ed. 16, 90128 Palermo, Italy*
[2] *Dipartimento di Scienze Biochimiche, Università di Firenze, Viale G.B. Morgagni n. 50, 50134 Florence, Italy*
[3] *Dipartimento Biopatologia e Metodologie Biomediche, Università di Palermo, Via Divisi 83, 90133 Palermo, Italy*
[4] *IAMC-CNR, U.O. Capo Granitola, Mazara del Vallo, 91026 Trapani, Italy*

Correspondence should be addressed to Maria Letizia Vittorelli, mlvitt@unipa.it

Received 16 November 2009; Accepted 8 March 2010

Academic Editor: Kalpna Gupta

Expression of sphingosine kinase-1 (SphK-1) correlates with a poor survival rate of tumor patients. This effect is probably due to the ability of SphK-1 to be released into the extracellular medium where it catalyzes the biosynthesis of sphingosine-1-phosphate (S1P), a signaling molecule endowed with profound proangiogenic effects. SphK-1 is a leaderless protein which is secreted by an unconventional mechanism. In this paper, we will show that in human hepatocarcinoma Sk-Hep1 cells, extracellular signaling is followed by targeting the enzyme to the cell surface and parallels targeting of FGF-2 to the budding vesicles. We will also show that SphK-1 is present in a catalitycally active form in vesicles shed by SK-Hep1 and human breast carcinoma 8701-BC cells. The enzyme substrate sphingosine is present in shed vesicles where it is produced by neutral ceramidase. Shed vesicles are therefore a site for S1P production in the extracellular medium and conceivably also within host cell following vesicle endocytosis.

1. Introduction

Malignant tumors have the remarkable ability to adapt their stromal environment to their benefit. They alter the surrounding extracellular matrix and modify normal cell behavior to facilitate tumor cell growth, invasion, immune evasion, and angiogenesis [1].

Most of these effects are mediated by the release of small vesicles from the tumor cells into the extracellular medium. Shed vesicles are known to facilitate tumor invasion [2–4], mainly by proteolytic enzymes associated with their membrane [5–9]. Indeed, the vesicle membranes are selectively enriched in some components including MMP-9 [7] and other proteolytic enzymes [4, 6], together with β1 Integrin and class I HLA molecules [7]. Enrichment of ganglioside G_{D3} and caveolin-1 has also been reported [10]. Moreover, vesicles use several mechanisms to contribute to tumor escape from immune reactions [11–16].

Notably, vesicles carry many proangiogenic growth factors, expressed differently depending on the vesicle origin, and that act on endothelial cells to promote angiogenesis. Indeed, FGF-2 was detected in vesicles shed by human hepatocarcinoma Sk-Hep1 cells [17, 18]; VEGF was found to be present in vesicles shed by human ovarian carcinoma cells [19] and in vesicles shed by neurons and astrocytes [20, 21]; angiogenin, IL-6, IL-8, VEGF, and TIMPs were found in vesicles shed by glioblastoma tumor cells [22]. Additionally, the sphingolipid fraction of vesicles shed by HT1080 fibrosarcoma and DU-145 human prostate carcinoma cells also showed proangiogenic activity [23]. Sphingomyelin is a normal component of plasma membranes where it is largely clustered in the outer membrane leaflet. It is subjected to intense metabolism which is responsible for the formation of a number of bioactive metabolites including ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1- phosphate (S1P) [24].

Ceramide, generated by sphingomyelinase (SMase) action on spingomyelin, appears to be a critical regulator of cell growth arrest, differentiation, and apoptosis [25, 26]. Sphingosine is formed by ceramide deacylation catalyzed by at least three different isoforms of ceramidase, which differ in optimal pH, primary structure, and cellular localization [27]. The enzyme sphingosine kinase (SphK) catalyzes the formation of S1P from sphingosine and ATP [28]. Two distinct SphK isoforms, SphK-1 and SphK-2, have been cloned [29, 30]. SphK-1, the more intensely researched isoform, is primarily localized in the cytosol, but, following ERK dependent phosphorylation elicited by various stimuli, it becomes translocated to the plasma membrane [31]. SphK-1 has been shown to regulate a wide variety of cellular processes, including the promotion of cell proliferation, survival, and motility [32] and, just as importantly, it possesses oncogenic potential [33]. Previous studies have established that SphK-1, like FGF-2 and several other proteins, can be released in the extracellular environment although it lacks a conventional secretory signal sequence. The mechanism of SphK-1 secretion is unconventional and likely involves a nonstandard pathway independent of the endoplasmic reticulum/Golgi system; the SphK-1 secretion mechanism is only known to require functional actin dynamics [34]. Notably, the SphK product S1P, among multiple biological activities, exerts a strong proangiogenic effect which is known to act synergistically with growth factors such as FGF-2 [35, 36] and VEGF [35].

In this study we investigated whether vesicles shed by hepatocarcinoma and carcinoma cultured cells contain S1P-generating enzymes. The data from this research demonstrates that neutral ceramidase (nCDase) and SphK-1 are localized in vesicles, supporting the view that S1P participates in the proangiogenic activity exerted by these particles.

2. Materials and Methods

2.1. Cells and Culture Media. Human SK-Hep1 hepatocarcinoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS; Euroclone, Celbio). Human breast carcinoma 8701-BC cells, kindly provided by Profcessor Minafra [37], were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Euroclone, Celbio). Bovine GM7373 fetal aortic endothelial cells were grown in Eagle's minimal essential medium (Euroclone, Celbio) supplemented with 10% FCS, vitamins, and essential and nonessential amino acids.

2.2. Cell Extraction. Cells were removed from plate by a scraper and centrifuged at 2000 g for 5 minutes; pelleted cells were then resuspended in 300 μL of Triton X100 (1%) on phosphate buffer saline (PBS). After 10- minute incubation at room temperature, the cell extract was centrifuged at 800 g for 10 minutes The amount of protein extracted from cells was determined using the Bradford microassay method (Bio-Rad, Segrate, Milan, Italy) employing bovine serum albumin (Sigma-Aldrich) as a standard.

2.3. Vesicle Purification from Conditioned Medium. Vesicles were purified from the conditioned medium as described above [38]. Briefly, the medium was conditioned by culturing subconfluent healthy cells for 3 or 24 hours and were centrifuged at 2000 g for 10 minutes and at 4000 g for 15 minutes The supernatant was ultracentrifuged at 105,000 g in a Ti-60 Rotor (Backman) for 90 minutes Pelleted vesicles were resuspended in PBS. The amount of isolated vesicles was determined by measuring the protein concentration using the Bradford microassay method (Bio-Rad, Segrate, Milan, Italy) using bovine serum albumin (Sigma-Aldrich) as a standard.

2.4. Western Blotting. After SDS-PAGE electrophoresis were cast in 7.5% gels, proteins was blotted onto a nitrocellulose membrane (Hybond; Amersham Biosciences) that was saturated with 3% nonfat dry milk in Tris Buffer Saline 50 mM pH 7.9/Tween 0.05% (TBS-T). After 5 washes in TBS-T for 5 minutes each, the nitrocellulose membranes were incubated overnight at 4°C, with mouse monoclonal anti-nCDase antibody 1 : 200 (kindly donated by Professor Ito, Fukuoka, Japan) [39]. The primary antibody was followed by peroxidase-conjugated anti-mouse antibodies (1 : 10000) (Amersham Biosciences) for 1 hour at room temperature. Immunocomplexes were visualized with the ECL Western blotting kit (Amersham Biosciences) using Hyperfilm.

2.5. Confocal Immunofluorescence. Cells, seeded at low density (2.000 cells/well) onto microscope cover slips in 12-well culture plates (Nunc), were grown overnight in the complete medium and, when needed, for 3 more days in a serum-free medium with three medium changes. SphK-1 and SphK-2 were detected by using as primary antibodies, rabbit polyclonal anti-SphK-1 antibody (kindly donated by Prof. Obeid, Charleston, SC, USA) [40] 1 : 100, and rabbit polyclonal anti-SphK-2 antibodies 1 : 100 (kindly provided by Dr. Nakamura, Kobe, Japan), respectively, [41]. Secondary antibodies used were antirabbit TRITC-conjugated antibodies (1 : 200 Sigma); β_1 Integrin was detected using C27 anti-β_1 Integrin rat primary monoclonal antibody 1 : 150 [42] and antirat TRITC conjugated secondary antibody (1 : 320, Sigma). FGF-2 was detected using mouse monoclonal anti-FGF-2 antibody (0.5 mg/mL 1 : 200, Upstate Biotechnology type II) and Texas Redconjugated antimouse antibody (1 : 200, Amersham Biosciences).

In order to stain nuclei, cells were fixed in 3.7% formaldehyde and then stained for 10 minutes with propidium iodide (Sigma).

Immunostained cells were analyzed by confocal microscopy (Olympus 1X70 with Melles Griot laser system).

2.6. Staining of Vesicle Lipids. Vesicle lipids were stained with the lipophilic styryl compound FM4-64 (Molecular Probes). Purified vesicles (180 μg) were resuspended in 1 ml PBS and stained with FM4-64 dissolved in PBS without calcium and magnesium. FM4-64 was added at a final concentration of 5 μg/ml; samples were incubated at room temperature for 15 minutes. Stained vesicles were collected by centrifugation at 50,000 g for 1 hour, resuspended in 50 μl PBS and added to GM7373 cells to monitor vesicle targeting.

2.7. Transient Cell Transfection. SK-Hep1 cells were plated in six-well culture plates at 3×10^5 cells/well and maintained overnight in high-glucose DMEM containing 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The next day, cells were transfected with SKpeGFP plasmid encoding for SphK-GFP chimera (kindly donated by Professor Spiegel, Richmond, VA, USA) [43]. Transfection was carried out using Lipofectamine Reagent (GIBCO Life Technologies), according to the manufacturer's instructions.

2.8. Sphingosine Kinase Assay. SphK activity was assayed in isolated vesicles or serum-free conditioned medium as described by Olivera et al. [28]. Briefly, 50 μg vesicle proteins were resuspended in 100 μl of the reaction mixture which contained 20 mM Tris-HCl, pH 7.4, 20% (v/v) glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM sodium fluoride, protease inhibitors (10 mg/ml leupeptin,10 mg/ml aprotinin, and 1 mM PMSF), and 0.5 mM 4-deoxypyridoxine.

The serum-free conditioned medium was concentrated approximately 40-fold before being employed for enzymatic activity measurement.

The enzymatic reaction was initiated by adding 50 μM sphingosine and 1 mM [γ^{32}P] ATP. In some cases, assays were performed which omitted sphingosine to evaluate the availability of endogenous sphingosine. After 30- minutes incubation at 37°C, the reaction was terminated by adding 20 μl 1 N HCl and 900 μl of chloroform/methanol/HCl (100 : 200 : 1, v/v). Lipids were then extracted, separated by TLC, labeled SIP and quantified by liquid scintillation essentially as previously described [44]. Specific activity of SphK was expressed as pmol of S1P produced/min/mg of protein.

2.9. Neutral Ceramidase Activity Assay. NCDase activity was determined using C12-NBD-ceramide as a substrate as previously described [45]. Briefly, 100 pmol of C12-NBD-ceramide (NBD-C12:0, d18:1) was incubated for 2 h at 37°C with an appropriate amount of proteins in 20 μl of 25 mM Tris-HCl buffer pH 7.5 and 0.25% (w/v) Triton X-100. Samples were then applied to a TLC plate, which was developed with chloroform, methanol, and 25% ammonia (90 : 20: 0.5, v/v). Spots corresponding to NBD-dodecanoic acid and C12-NBD-ceramide were scraped, incubated with methanol at 37°C to extract the compounds from the silica, and their fluorescence at 470/525 nm excitation/emission wavelengths was measured using a Shimadzu 9000 spectrophotofluorimeter. The compounds were quantified using a standard curve of known amounts of C12-NBD-ceramide and NBD-dodecanoic acid.

3. Results

3.1. Immunolocalization of SphK-1 and Sphk-2 in 8701-BC and Sk-Hep-1 Cells. In a first group of experiments, expression and localization of SphK-1 and SphK-2 were analyzed by immunofluorescence in 8701 BC carcinoma cells and in Sk-Hep1 hepatocarcinoma cells (Figure 1).

Moreover, since it had been previously demonstrated that β_1 integrin is clustered in shed vesicles [7, 17], distribution of the two proteins was compared with distribution of β_1 integrin.

In both cell lines, the distribution of SphK-2 was quite different from the distribution of β_1 integrin (Figures 1(a) and 1(b) line a). SphK-2 was indeed clustered in the cell nucleus. However, it was absent in the cell membrane where instead β_1 integrin was located. In contrast, SphK-1 and β_1 integrin appear to colocalize at the plasma membrane (Figures 1(a) and 1(b) line b). Moreover, as can more clearly be seen in cells transiently transfected with GFP-SphK-1 (Figure 2), both SphK-1 and β_1 integrin seem to be more dense in specific areas of the plasma membrane, and clustering appears to occur in areas of the cell membrane from which vesicles are released (Figure 2(d)).

3.2. Effects of Serum Addition on SphK-1 Trafficking toward the Cell Periphery. In a previous study, we observed that vesicles shed by Sk-Hep1 cells mediate FGF-2 release and that vesicle shedding and release of FGF-2 were simultaneously induced by the addition of serum to starved cells [17]. By monitoring intracellular movements of the growth factor subsequent to serum addition, we showed that within one hour FGF-2 was targeted to the cell periphery and to the cell nucleus and nucleolus. FGF-2 movements toward the cell periphery required actin filament integrity [18].

Similarly to FGF-2, SphK-1 is a leaderless protein secreted by unconventional mechanisms [29, 34] whose movement toward the cell periphery is mediated by actin filaments [34]. We therefore theorized that the two proteins could share a similar export mechanism and we analyzed whether intracellular movements of SphK-1 were influenced by serum addition and whether the enzyme colocalized with FGF-2.

Intracellular distribution of SphK-1 was therefore analyzed by immunolocalization in starved cells as well as at time intervals after serum addition and compared with FGF-2 distribution. As shown in Figure 3, in starved cells the two proteins did not colocalize. In SphK-1 they were partially localized in small granules and in FGF-2 they were totally dispersed.

Thirty minutes after the serum was added both proteins were clustered in granules and showed a clear colocalization. One hour after the serum was added large granules containing both proteins were present near the cell membrane. In contrast, cell nuclei were exclusively stained by anti-FGF-2 antibodies.

These results suggest that FGF-2 and SphK-1 share a similar transport mechanism toward the cell periphery and that the two proteins are both likely to be targeted to the budding vesicles.

3.3. Detection of an Active Form of SphK-1 in Shed Vesicles. In order to establish if an active form of SphK-1 is shed as a component of membrane vesicles, we ascertained SphK activity in vesicles or cell-conditioned media in some experiments. Results reported in Table 1 show that SphK-1

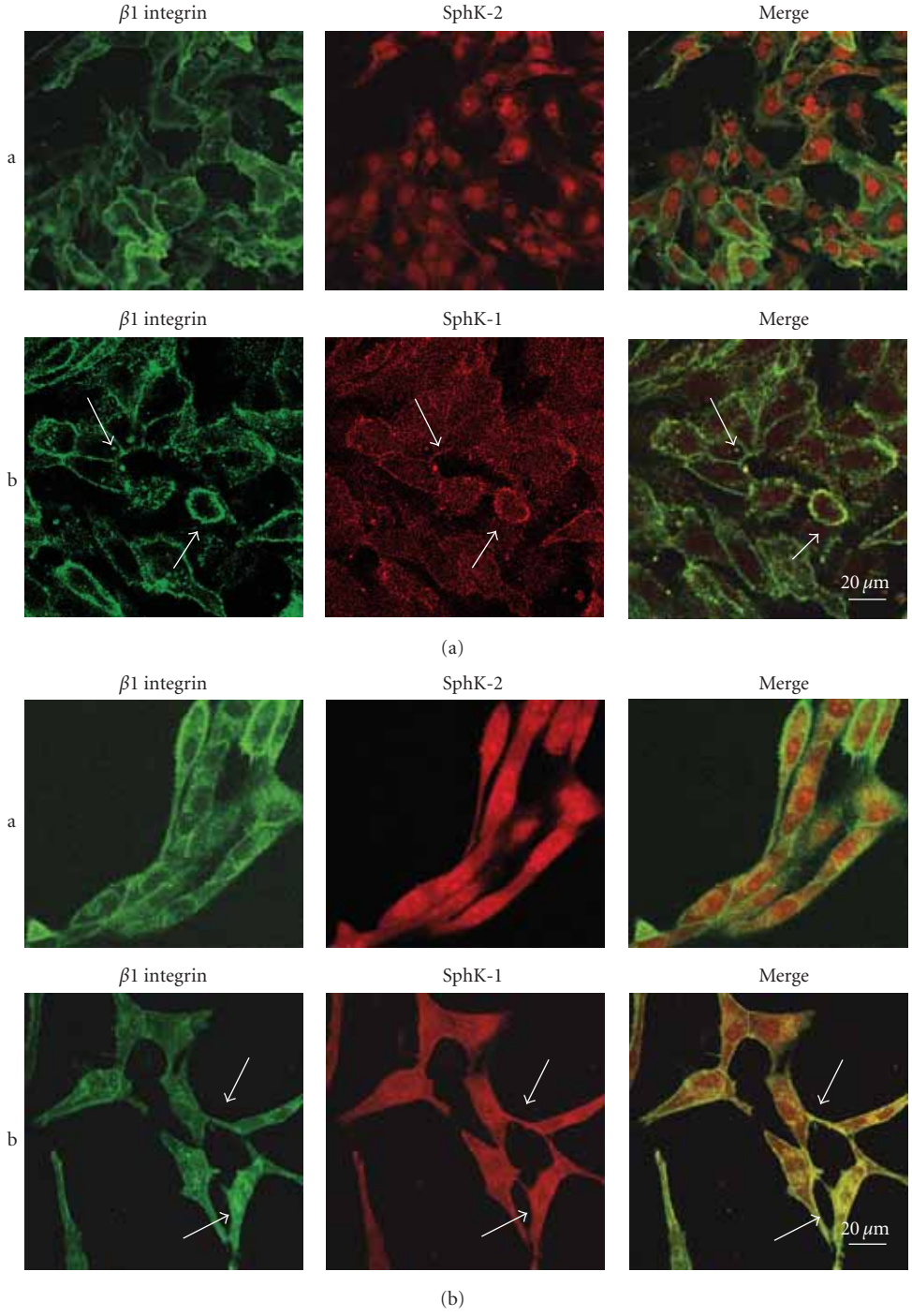


Figure 1: Comparative analysis of β1 Integrin, SphK-2, and SphK-1 immunolocalization. (a) Localization in 8701-BC cells. (b) Localization in Sk-Hep1 cells. Line a: Immunolocalization of β1 Integrin and SphK-2 showing a different distribution of the two molecules. Line b: Immunolocalization of β1 Integrin and SphK-1. Arrows indicate colocalization areas. β1 Integrin was detected using FITC-conjugated secondary antibodies and SphK-2 and SphK-1 using Texas red-conjugated secondary antibodies. Arrows indicate colocalization areas.

was clearly detectable in vesicles shed by Sk-Hep1 and 8701-BC cells, although the activity was found to be greater in vesicles shed by Sk-Hep1 cells.

SphK-1 activity was also tested in vesicle-deprived conditioned media. No enzymatic activity could be detected in serum-containing media, even when it was concentrated. Instead, it was possible to detect a low SphK activity in 40-fold-concentrated serum-free medium that had been conditioned by maintaining cells in culture for 24 h. As shown in Table 1, when vesicles were collected from a medium to which 2M NaCl had been added in order to solubilize proteoglycans and other molecules unspecifically

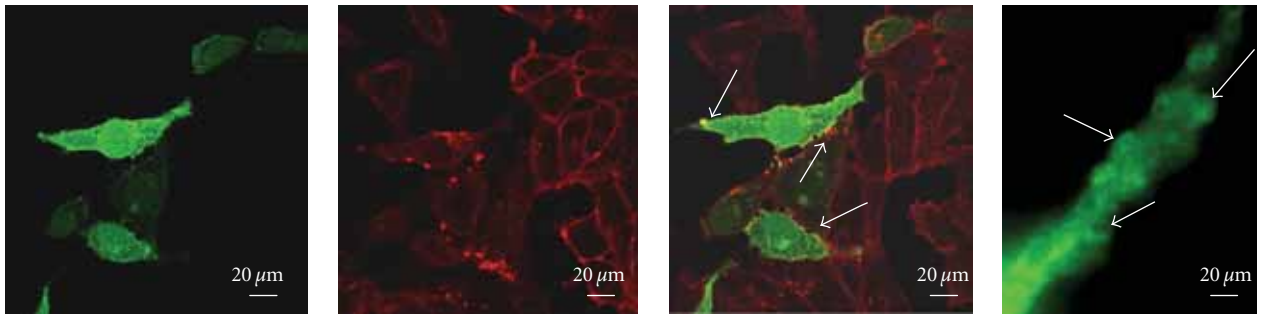


Figure 2: Comparative analysis of SphK-1 and of β1 Integrin localization in transfected Sk-Hep1 cells. (a) GFP-bound SphK-1 localization in transfected cells. The protein is localized in cell membranes where it shows uneven clustering in small spots. (b) Immunolocalization of β1 Integrin detected using Texas red-conjugated secondary antibodies. β1 Integrin is seen in cell membranes of both transfected and non-transfected cells. Like SphK-1, β1 Integrin shows uneven clustering in small spots. (c) Double staining shows colocalization of the two proteins in some areas of the plasma membrane (indicated by arrows). (d) Enlargement of a cell protrusion showing budding areas (indicated by arrows) in which SphK-1 appears to have a preferential localization.

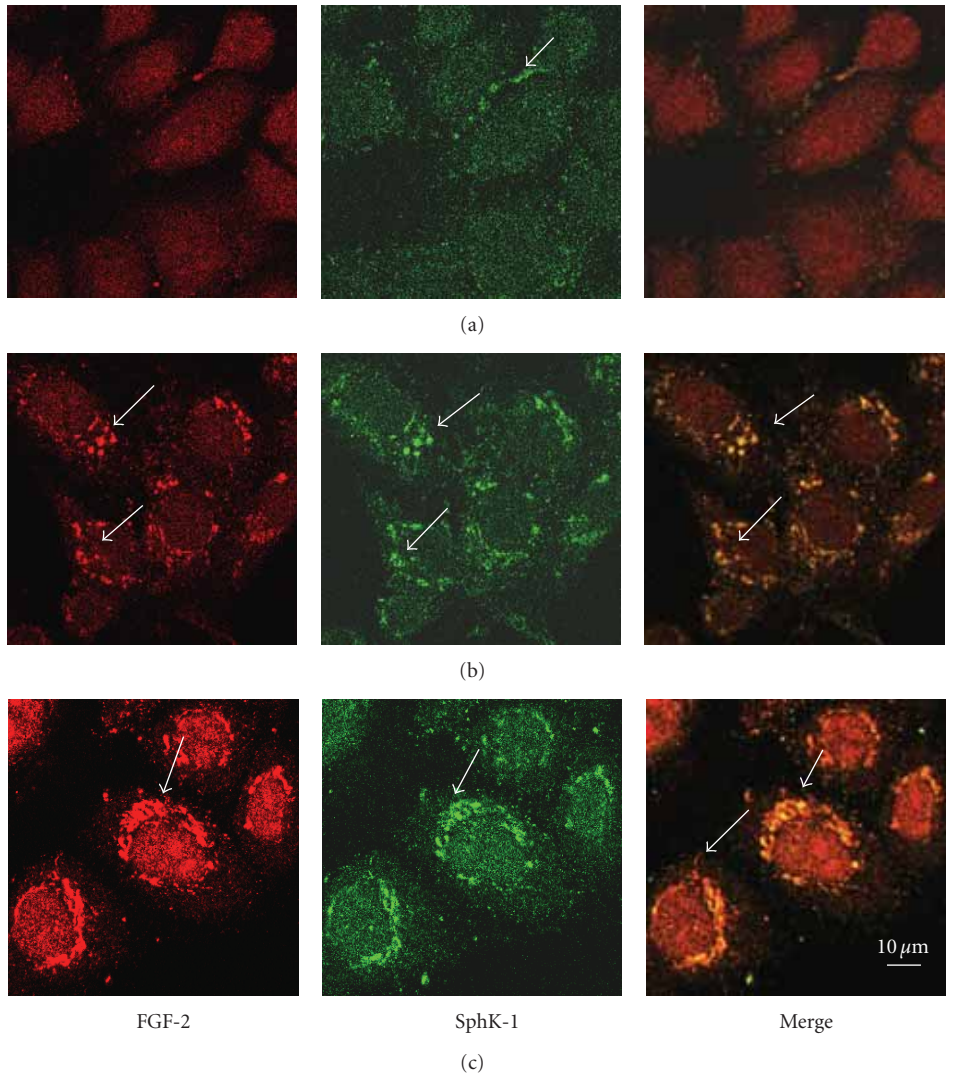


Figure 3: Course of endogenous SphK-1 and endogenous FGF-2 targeting to the cell periphery over time, observed by immunolocalization experiments. FGF-2 and SphK-1 immunolocalization at 0, 30, and 60 minutes after serum addition (lines (a), (b), (c), resp.) Sections 3 μm from surface. FGF-2 was detected using Texas red-conjugated secondary antibodies; SphK-1 was detected using FITC-conjugated antibodies. Arrows indicate granules of protein localization.

Table 1: Enzimatic assay of SphK-1 activity.

Sample	Incubation mixture with exogenous sphingosine	Incubation mixture without exogenous sphingosine
SK-Hep1 vesicles	43.93 pmol/min/mg of protein	26.70 pmol/min/mg of protein
SK-Hep1 vesicles*	43.59 pmol/min/mg of protein	
SK-Hep1 C.M.	2.23 pmol/min/mg of protein	
8701 BC vesicles	14.44 pmol/min/mg of protein	12.61 pmol/min/mg of protein
8701 BC vesicles*	16.70 pmol/min/mg of protein	
8701 BC CM	2.16 pmol/min/mg of protein	

* Vesicles recovered from medium in which 2 M NaCl had been added.

Table 2

Sample	Enzimatic activity of nCDase
8701 BC cells	2,08 pmol/min/mg of protein
8701 BC vesicles	5,25 pmol/min/mg of protein

bound to the vesicle membrane, the enzymatic activity was not affected.

The addition of sphingosine increased labeled S1P production; however, the catalytic activity of the enzyme was also observed when this substrate was not added. This result indicates that sphingosine is already present in shed components of membrane vesicles.

Sphingosine is likely produced by nCDase, which is known to be localized in plasma membranes and also found in the extracellular medium [46, 47]. An enzymatically active form of nCDase was actually found to be present in vesicles shed by 8701 BC (Figure 4 and Table 2). Moreover, in Sk-Hep1 cells, we observed colocalization of nCDase and β_1 integrin (data not shown), indicating that it is also likely that nCDase is present in vesicles shed by this cell line.

3.4. Fate of Shed Vesicles. Since S1P can be produced in the membrane of shed vesicles, the molecule could remain, at least in part, within the vesicle. Vesicles could adhere to the plasma membrane of cells surrounding the tumor and S1P could exert its effects by interacting with receptors localized at the cell surface. On the other hand, vesicles could be internalized by the host cell and consequently release S1P inside cells where it could act as intracellular messenger. In order to verify these hypotheses, we analyzed the targets of shed vesicles after adding them to GM7373 cells, an immortalized line of embryonic bovine aortic endothelial cells.

For this purpose, vesicles released by Sk-Hep1 cells were labeled for 15 minutes with the lipid marker FM4-64. Labeled vesicles were then added to in vitro cultured GM7373 cells which in turn had been labeled with antibodies against β_1 integrin. As shown in Figure 5, at 10 minutes after incubation vesicles were observed to be bound to the cell membrane, while after 20-minutes incubation most vesicles were internalized and visible in the cytoplasm. At 30 minutes, the signal borne by lipid marker FM4-64 was no longer visible, indicating that the lipids of the vesicle membranes had degraded. In principle therefore, vesicle-associated S1P could act on both membrane receptors and intracellular targets.

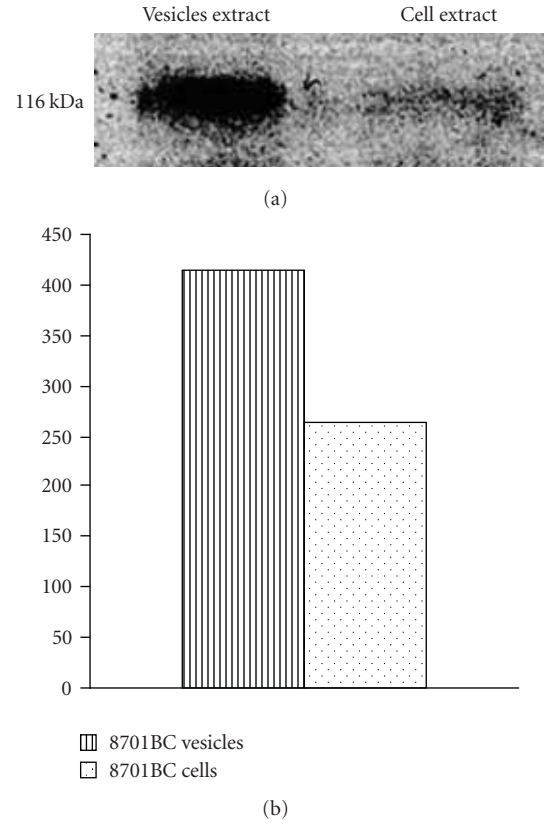
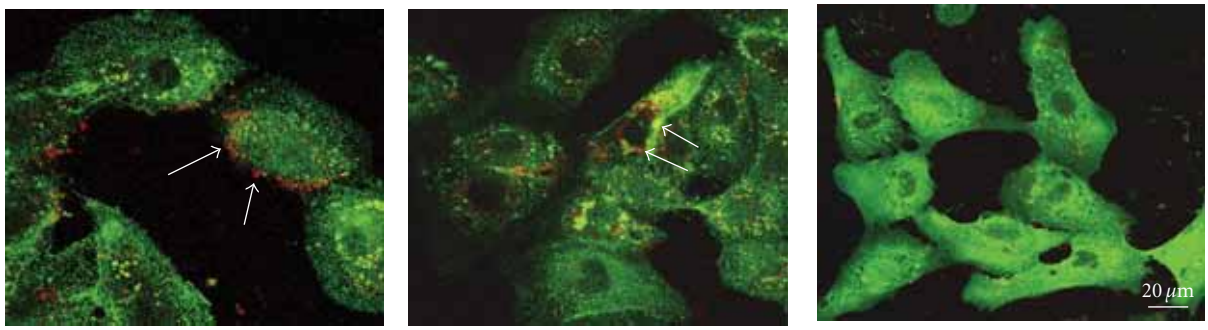


Figure 4: Western Blot analysis for nCDase (a) and its densitometric analisys (b) on 8701BC vesicles and cell extracts.

4. Conclusions

Membrane vesicles shed by tumor cells appear to exert a variety of effects on the surrounding cells. Vesicles are rich in enzymatic activities able to modify extracellular medium composition, thus facilitating tumor cell migration and angiogenesis. Depending on their origin, they also convey different signaling molecules which exert their effects on lymphocytes, mesenchymal cells, and endothelial cells. Shed vesicles have been shown to induce angiogenesis using a variety of mechanisms including the action of proteins such



(a) (b) (c)

Figure 5: Interactions of shed vesicles with endothelial cells. Vesicles shed by SK-Hep1 cells, labelled with lipid styryl dye FM4-64 (red fluorescence), were added to GM7373 endothelial cells in which β1 Integrin was stained using FITC-conjugated secondary antibodies (green fluorescence). Cells were incubated with vesicles, respectively, for (a) 10 minutes, (b) 20 minutes, and (c) 30 minutes. The arrows indicate vesicle localization.

as FGF-2, VEGF, angiogenin, IL-6, IL-8, and TIMPs, and lipid molecules such as sphingomyelin.

Based on the present results, nCDase and SphK-1 can now be included among the signaling molecules transferred by shed membrane vesicles, suggesting that S1P formed at level of the vesicle membranes plays a role in the biological processes regulated by these particles.

Interestingly, nCDase, here identified as a component of shed vesicles, was previously identified in various subcellular compartments such as endosomes, mitochondria, and microdomains of the plasma membrane [39, 48] but was also found to be involved in extracellular sphingolipid metabolism [47]. In this regard it was demonstrated that although nCDase is localized at the plasma membrane as a type II integral membrane protein, the enzyme is released in the extracellular medium after the proteolytic action of secretases [49, 50]. Moreover, in agreement with the present results, nCDase, together with acid SMase, was identified as a component of a complex in the cell membrane domain which is subjected to budding as well as in conditioned medium associated with caveolin-1, a key structural protein of caveole [51] which was also detected in shed vesicles [52]. SphK-1 is a secreted leaderless protein, and the shedding of membrane vesicles appears to represent a mechanism which accounts for its secretion. The presence of the enzymatic protein in shed vesicles does not per se exclude the face that other mechanisms may also participate in the release of SphK-1. Indeed it was reported that, in FGF-1 overexpressing NIH 3T3 cells, SphK-1 is secreted together with FGF-1 as a component of a high molecular weight complex [53, 54]. However, SphK-1 is also secreted by cells which do not express FGF-1, and in the absence of stress signaling which induces FGF-1 secretion. Here we have demonstrated that at least in some instances SphK-1 is secreted as a component of shed vesicles. Since shed vesicles also contain nCDase, which provides the rate-limiting substrate for S1P production by catalyzing sphingosine generation, it is likely that these particles cause sustained S1P production.

SphK-1 and S1P produced by its enzymatic activity are able to mediate a network of paracrine signaling. It is well known that acting on the two membrane receptors SIP_1 and SIP_3, S1P induces morphogenesis in HUVEC cells [35]. Moreover, since vesicles carry several other molecules able to affect angiogenesis, the overall effects of vesicles on surrounding endothelial cells will be amplified and differently modulated depending on the specific composition of the vesicles.

The exact mechanism by which the S1P message borne by shed vesicles is delivered to the host cell remains to be explored. Indeed, an attractive hypothesis is that after interacting with the recipient cell plasma membrane, vesicles are internalized via endocytosis. SphK-1 and SIP would therefore be delivered into the cytoplasm of the receiving cell, where, as already known, SIP could exert its intracellular effects, regulating various processes among which cell survival is prominent [55, 56]. Indeed vesicles were shown to convey molecules, such as mRNA and iRNA, to the cytoplasm of surrounding cells, and it was recently reported that mRNA included in shed vesicles can be translated in recipient cells following endocytosis [22].

Alternatively, because SphK-1 association with cell membranes is strengthened by interaction with phosphatidylserine following its phosphorylation [57] and since exposure of phosphatidylserine on the outer leaflet is a hallmark of shed membrane vesicles [58, 59], it can also be speculated that SphK-1, localized in these particles, can generate S1P in the extracellular environment. If this is the case, the bioactive lipid generated outside the endothelial cells could determine key effects on development and proliferation of endothelial cells, acting as a ligand of $S1P_1$ and/or $S1P_3$ receptors [60, 61]. Independent of the action mechanism, the ability of shed vesicles to carry on key enzymes for S1P production which this study brought to light illuminates a novel aspect of their biochemical properties which is relevant to a complete understanding of their proangiogenic activity.

Acknowledgments

This work was supported by the Italian Ministry of University and Scientific and Technological Research (to M.L.V. and

P.B.) and by grants from University of Florence and "Ente Cassa di Risparmio di Firenze" (to P.B.). they would like to thank Salvatore Agnello for his technical assistance with the confocal microscopy. They would also like to thank Professor Salvatore Minafra for providing them with 8701-BC cells.

References

[1] C. F. Singer, D. Gschwantler-Kaulich, A. Fink-Retter, et al., "Differential gene expression profile in breast cancer-derived stromal fibroblasts," *Breast Cancer Research and Treatment*, vol. 110, no. 2, pp. 273–281, 2008.

[2] G. Poste and G. L. Nicolson, "Arrest and metastasis of blood-borne tumor cells are modified by fusion of plasma membrane vesicles from highly metastatic cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 1, pp. 399–403, 1980.

[3] D. D. Taylor, C. G. Taylor, C. G. Jiang, and P. H. Black, "Characterization of plasma membrane shedding from murine melanoma cells," *International Journal of Cancer*, vol. 41, no. 4, pp. 629–635, 1988.

[4] A. Ginestra, M. D. La Placa, F. Saladino, D. Cassara, H. Nagase, and M. L. Vittorelli, "The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness," *Anticancer Research*, vol. 18, no. 5, pp. 3433–3437, 1998.

[5] S. Zucker, J. M. Wieman, R. M. Lysik, D. P. Wilkie, N. Ramamurthy, and B. Lane, "Metastatic mouse melanoma cells release collagen-gelatin degrading metalloproteinases as components of shed membrane vesicles," *Biochimica et Biophysica Acta*, vol. 924, no. 1, pp. 225–237, 1987.

[6] A. Ginestra, S. Monea, G. Seghezzi, et al., "Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells," *Journal of Biological Chemistry*, vol. 272, no. 27, pp. 17216–17222, 1997.

[7] V. Dolo, A. Ginestra, D. Cassara, et al., "Selective localization of matrix metalloproteinase 9, β1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells," *Cancer Research*, vol. 58, no. 19, pp. 4468–4474, 1998.

[8] G. Taraboletti, S. D'Ascenzo, P. Borsotti, R. Giavazzi, A. Pavan, and V. Dolo, "Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells," *American Journal of Pathology*, vol. 160, no. 2, pp. 673–680, 2002.

[9] L. E. Graves, E. V. Ariztia, J. R. Navari, H. J. Matzel, M. S. Stack, and D. A. Fishman, "Proinvasive properties of ovarian cancer ascites-derived membrane vesicles," *Cancer Research*, vol. 64, no. 19, pp. 7045–7049, 2004.

[10] V. Dolo, R. Li, M. Dillinger, et al., "Enrichment and localization of ganglioside G_{D3} and caveolin-1 in shed tumor cell membrane vesicles," *Biochimica et Biophysica Acta*, vol. 1486, no. 2-3, pp. 265–274, 2000.

[11] P. Alexander, "Escape from immune destruction by the host through shedding of surface antigens: is this a characteristic shared by malignant and embryonic cells?" *Cancer Research*, vol. 34, no. 8, pp. 2077–2082, 1974.

[12] D. D. Poutsiaka, E. W. Schroder, D. D. Taylor, E. M. Levy, and P. H. Black, "Membrane vesicles shed by murine melanoma cells selectively inhibit the expression of Ia antigen by macrophages," *Journal of Immunology*, vol. 134, no. 1, pp. 138–144, 1985.

[13] V. Dolo, E. Adobati, S. Canevari, M. A. Picone, and M. L. Vittorelli, "Membrane vesicles shed into the extracellular medium by human breast carcinoma cells carry tumor-associated surface antigens," *Clinical and Experimental Metastasis*, vol. 13, no. 4, pp. 277–286, 1995.

[14] E. Zuccato, E. J. Blott, O. Holt, et al., "Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation," *Journal of Cell Science*, vol. 120, no. 1, pp. 191–199, 2007.

[15] A. Clayton, J. P. Mitchell, J. Court, M. D. Mason, and Z. Tabi, "Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2," *Cancer Research*, vol. 67, no. 15, pp. 7458–7466, 2007.

[16] D. I. Gabrilovich, "Molecular mechanisms and therapeutic reversal of immune suppression in cancer," *Current Cancer Drug Targets*, vol. 7, no. 1, p. 1, 2007.

[17] S. Taverna, G. Ghersi, A. Ginestra, et al., "Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells," *Journal of Biological Chemistry*, vol. 278, no. 51, pp. 51911–51919, 2003.

[18] S. Taverna, S. Rigogliuso, M. Salamone, and M. L. Vittorelli, "Intracellular trafficking of endogenous fibroblast growth factor-2," *FEBS Journal*, vol. 275, no. 7, pp. 1579–1592, 2008.

[19] G. Taraboletti, S. D'Ascenzo, I. Giusti, et al., "Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH," *Neoplasia*, vol. 8, no. 2, pp. 96–103, 2006.

[20] G. Schiera, P. Proia, C. Alberti, M. Mineo, G. Savettieri, and I. Di Liegro, "Neurons produce FGF2 and VEGF and secrete them at least in part by shedding extracellular vesicles," *Journal of Cellular and Molecular Medicine*, vol. 11, no. 6, pp. 1384–1394, 2007.

[21] P. Proia, G. Schiera, M. Mineo, et al., "Astrocytes shed extracellular vesicles that contain fibroblast growth factor-2 and vascular endothelial growth factor," *International Journal of Molecular Medicine*, vol. 21, no. 1, pp. 63–67, 2008.

[22] J. Skog, T. Wurdinger, S. van Rijn, et al., "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers," *Nature Cell Biology*, vol. 10, no. 12, pp. 1470–1476, 2008.

[23] C. W. Kim, H. M. Lee, T. H. Lee, C. Kang, H. K. Kleinman, and Y. S. Gho, "Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin," *Cancer Research*, vol. 62, no. 21, pp. 6312–6317, 2002.

[24] S. Pyne and N. J. Pyne, "Sphingosine 1-phosphate signalling in mammalian cells," *Biochemical Journal*, vol. 349, no. 2, pp. 385–402, 2000.

[25] Y. A. Hannun, "Functions of ceramide in coordinating cellular responses to stress," *Science*, vol. 274, no. 5294, pp. 1855–1859, 1996.

[26] R. N. Kolesnick and M. Kronke, "Regulation of ceramide production and apoptosis," *Annual Review of Physiology*, vol. 60, pp. 643–665, 1998.

[27] M. Ito, N. Okino, M. Tani, S. Mitsutake, and K. Mori, "Molecular evolution of neutral ceramidase: signalling molecule and virulence factor," *Tanpakushitsu Kakusan Koso*, vol. 47, pp. 455–462, 2002.

[28] A. Olivera, T. Kohama, Z. Tu, S. Milstien, and S. Spiegel, "Purification and characterization of rat kidney sphingosine kinase," *Journal of Biological Chemistry*, vol. 273, no. 20, pp. 12576–12583, 1998.

[29] T. Kohama, A. Olivera, L. Edsall, M. M. Nagiec, R. Dickson, and S. Spiegel, "Molecular cloning and functional characterization of murine sphingosine kinase," *Journal of Biological Chemistry*, vol. 273, no. 37, pp. 23722–23728, 1998.

[30] H. Liu, M. Sugiura, V. E. Nava, et al., "Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform," *Journal of Biological Chemistry*, vol. 275, no. 26, pp. 19513–19520, 2000.

[31] S. M. Pitson, P. A. B. Moretti, J. R. Zebol, et al., "Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation," *EMBO Journal*, vol. 22, no. 20, pp. 5491–5500, 2003.

[32] S. Spiegel and S. Milstien, "Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways," *Biochemical Society Transactions*, vol. 31, no. 6, pp. 1216–1219, 2003.

[33] S. M. Pitson, P. Xia, T. M. Leclercq, et al., "Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling," *Journal of Experimental Medicine*, vol. 201, no. 1, pp. 49–54, 2005.

[34] N. Ancellin, C. Colmont, J. Su, et al., "Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation," *Journal of Biological Chemistry*, vol. 277, no. 8, pp. 6667–6675, 2002.

[35] M.-J. Lee, S. Thangada, K. P. Claffey, et al., "Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate," *Cell*, vol. 99, no. 3, pp. 301–312, 1999.

[36] K. Harvey, R. A. Siddiqui, D. Sliva, J. G. N. Garcia, and D. English, "Serum factors involved in human microvascular endothelial cell morphogenesis," *Journal of Laboratory and Clinical Medicine*, vol. 140, no. 3, pp. 188–198, 2002.

[37] S. Minafra, V. Morello, F. Glorioso, et al., "A new cell line (8701-BC) from primary ductal infiltrating carcinoma of human breast," *British Journal of Cancer*, vol. 60, no. 2, pp. 185–192, 1989.

[38] V. Dolo, A. Ginestra, G. Ghersi, H. Nagase, and M. L. Vittorelli, "Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities," *Journal of Submicroscopic Cytology and Pathology*, vol. 26, no. 2, pp. 173–180, 1994.

[39] S. Mitsutake, M. Tani, N. Okino, et al., "Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 26249–26259, 2001.

[40] K. R. Johnson, K. P. Becker, M. M. Facchinetti, Y. A. Hannun, and L. M. Obeid, "PKC-dependent activation of sphingosine kinase 1 and translocation to the plasma membrane. Extracellular release of sphingosine-1-phosphate induced by phorbol 12-myristate 13-acetate (PMA)," *Journal of Biological Chemistry*, vol. 277, no. 38, pp. 35257–35262, 2002.

[41] N. Igarashi, T. Okada, S. Hayashi, T. Fujita, S. Jahangeer, and S.-I. Nakamura, "Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis," *Journal of Biological Chemistry*, vol. 278, no. 47, pp. 46832–46839, 2003.

[42] W. L. Monsky, C.-Y. Lin, A. Aoyama, et al., "A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells," *Cancer Research*, vol. 54, no. 21, pp. 5702–5710, 1994.

[43] A. Olivera, H. M. Rosenfeldt, M. Bektas, et al., "Sphingosine kinase type 1 induces G12/13-mediated stress fiber formation, yet promotes growth and survival independent of G protein-coupled receptors," *Journal of Biological Chemistry*, vol. 278, no. 47, pp. 46452–46460, 2003.

[44] C. Donati, F. Cencetti, C. De Palma, et al., "TGFβ protects mesoangioblasts from apoptosis via sphingosine kinase-1 regulation," *Cellular Signalling*, vol. 21, no. 2, pp. 228–236, 2009.

[45] E. Romiti, E. Meacci, M. Tani, et al., "Neutral/alkaline and acid ceramidase activities are actively released by murine endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 275, no. 3, pp. 746–751, 2000.

[46] Y.-H. Hwang, M. Tani, T. Nakagawa, N. Okino, and M. Ito, "Subcellular localization of human neutral ceramidase expressed in HEK293 cells," *Biochemical and Biophysical Research Communications*, vol. 331, no. 1, pp. 37–42, 2005.

[47] M. Tani, M. Ito, and Y. Igarashi, "Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space," *Cellular Signalling*, vol. 19, no. 2, pp. 229–237, 2007.

[48] M. Tani, N. Okino, K. Mori, T. Tanigawa, H. Izu, and M. Ito, "Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase. A novel but highly conserved gene family of neutral/alkaline ceramidases," *Journal of Biological Chemistry*, vol. 275, no. 15, pp. 11229–11234, 2000.

[49] M. Tani, H. Iida, and M. Ito, "O-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein," *Journal of Biological Chemistry*, vol. 278, no. 12, pp. 10523–10530, 2003.

[50] Y. Yoshimura, M. Tani, N. Okino, H. Iida, and M. Ito, "Molecular cloning and functional analysis of zebrafish neutral ceramidase," *Journal of Biological Chemistry*, vol. 279, no. 42, pp. 44012–44022, 2004.

[51] E. Romiti, E. Meacci, C. Donati, et al., "Neutral ceramidase secreted by endothelial cells is released in part associated with caveolin-1," *Archives of Biochemistry and Biophysics*, vol. 417, no. 1, pp. 27–33, 2003.

[52] V. Dolo, S. D'Ascenzo, M. Sorice, et al., "New approaches to the study of sphingolipid enriched membrane domains: the use of electron microscopic autoradiography to reveal metabolically tritium labeled sphingolipids in cell cultures," *Glycoconjugate Journal*, vol. 17, no. 3-4, pp. 261–268, 2000.

[53] I. Prudovsky, A. Mandinova, R. Soldi, et al., "The non-classical export routes: FGF1 and IL-1α point the way," *Journal of Cell Science*, vol. 116, no. 24, pp. 4871–4881, 2003.

[54] R. Soldi, A. Mandinova, K. Venkataraman, et al., "Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway," *Experimental Cell Research*, vol. 313, no. 15, pp. 3308–3318, 2007.

[55] J. D. Saba and T. Hla, "Point-counterpoint of sphingosine 1-phosphate metabolism," *Circulation Research*, vol. 94, no. 6, pp. 724–734, 2004.

[56] R. Bassi, V. Anelli, P. Giussani, G. Tettamanti, P. Viani, and L. Riboni, "Sphingosine-1-phosphate is released by cerebellar astrocytes in response to bFGF and induces astrocyte proliferation through Gi-protein-coupled receptors," *Glia*, vol. 53, no. 6, pp. 621–630, 2006.

[57] R. V. Stahelin, J. H. Hwang, J.-H. Kim, et al., "The mechanism of membrane targeting of human sphingosine kinase 1," *Journal of Biological Chemistry*, vol. 280, no. 52, pp. 43030–43038, 2005.

[58] H. F. G. Heijnen, A. E. Schiel, R. Fijnheer, H. J. Geuze, and J. J. Sixma, "Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and α-granules," *Blood*, vol. 94, no. 11, pp. 3791–3799, 1999.

[59] A. MacKenzie, H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North, and A. Surprenant, "Rapid secretion of interleukin-1β by microvesicle shedding," *Immunity*, vol. 15, no. 5, pp. 825–835, 2001.

[60] S. Pyne and N. J. Pyne, "Sphingosine 1 phosphate signaling via the endothelial differentation gene family of G protein coupled receptors," *Pharmacology and Therapeutics*, pp. 115–131, 2000.

[61] Y. Liu, R. Wada, T. Yamashita, et al., "Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation," *Journal of Clinical Investigation*, vol. 106, no. 8, pp. 951–961, 2000.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 412985, 17 pages
doi:10.1155/2010/412985

Review Article

Hsp90 as a Gatekeeper of Tumor Angiogenesis: Clinical Promise and Potential Pitfalls

J. E. Bohonowych, U. Gopal, and J. S. Isaacs

Department of Cell & Molecular Pharmacology, Hollings Cancer Center, Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425, USA

Correspondence should be addressed to J. S. Isaacs, isaacsj@musc.edu

Received 25 December 2009; Accepted 12 April 2010

Academic Editor: Sundaram Ramakrishnan

Tumor vascularization is an essential modulator of early tumor growth, progression, and therapeutic outcome. Although antiangiogenic treatments appear promising, intrinsic and acquired tumor resistance contributes to treatment failure. Clinical inhibition of the molecular chaperone heat shock protein 90 (Hsp90) provides an opportunity to target multiple aspects of this signaling resiliency, which may elicit more robust and enduring tumor repression relative to effects elicited by specifically targeted agents. This review highlights several primary effectors of angiogenesis modulated by Hsp90 and describes the clinical challenges posed by the redundant circuitry of these pathways. The four main topics addressed include (1) Hsp90-mediated regulation of HIF/VEGF signaling, (2) chaperone-dependent regulation of HIF-independent VEGF-mediated angiogenesis, (3) Hsp90-dependent targeting of key proangiogenic receptor tyrosine kinases and modulation of drug resistance, and (4) consideration of factors such as tumor microenvironment that pose several challenges for the clinical efficacy of anti-angiogenic therapy and Hsp90-targeted strategies.

1. Introduction

The concept of antiangiogenic targeting as a means to suppress malignancy came to the forefront of cancer therapeutics in the early 1970s, based upon the pioneering work of Folkman [1]. Tumor vascularization is a critical component of cancer progression, malignancy, and metastasis. As rapidly dividing cancer cells reach a critical tumor size, the mass outgrows its supply of blood, oxygen, and nutrients. Continued tumor growth depends upon the ability of microenvironmental stressors to trigger the activation of a complex and intricately interconnected signaling network that culminates in vascularization of the growing tumor. This activation process is essential for initiation of the "angiogenic switch," a rate limiting event of tumor progression. Subsequent tumor vascularization culminates in either enhanced angiogenesis, the sprouting from local vessels, or vasculogenesis, the formation of neovessels through bone marrow-derived cell (BMDC) recruitment. Realization that the angiogenic switch may represent a universal Achilles heel for all tumors, coupled with elucidation of druggable targets within this network, has invigorated the field of antiangiogenic therapy, resulting in a rich pipeline of therapeutic compounds [2].

2. Emergence of Hsp90 Inhibitors as Antiangiogenic Chemotherapeutic Agents

Although a number of antiangiogenic therapies are presently in clinical use, the vast majority of these target a specific molecule or receptor family [3]. Despite a relatively high degree of specificity, the clinical efficacy of these therapies as curative agents remains poor. Though initial responsiveness may occur, the ultimate outcome is treatment resistance due to drug-dependent selection of intrinsic and adaptive resistance mechanisms. Therefore, attention has turned to chemotherapeutics targeting heat shock protein 90 (Hsp90), which simultaneously target multiple proangiogenic regulators, and may thus weaken the signaling resiliency characteristic of tumor cells. The prototypic Hsp90 inhibitor geldanamycin (GA) demonstrates potent antiangiogenic and antitumorigenic properties [4, 5]. At the molecular level, Hsp90 plays a critical role in the proper folding of its

client or substrate proteins [6] and also serves as a scaffold protein to facilitate interactions between several receptor tyrosine kinases (RTKs) and their substrates. Therefore, Hsp90 inhibitors are unique as antiangiogenic agents in that they regulate the activity of hundreds of proteins, many of which support cancer growth [7–9]. In addition, Hsp90 expression is increased in many cancers, allowing sustained activation of cancer-specific dysregulated pathways and the buffering of stress conditions characteristic of the tumor microenvironment [10]. As a result, the evaluation of emergent Hsp90 inhibitors is a current focus of drug discovery efforts across multiple cancers [8, 9, 11].

The first discovered Hsp90 inhibitor, GA, is a naturally occurring benzoquinone ansamycin [12] that acts as a nucleotide mimetic to inhibit ATP-dependent Hsp90 chaperone activity. Although useful as a research tool, the pharmacological liabilities of GA prohibit its clinical use [13] leading to the development of subsequent generations of Hsp90 inhibitors including the GA analog 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) and its water soluble version 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), both of which are being evaluated in multiple Phase I, II, and III clinical trials [14–16]. Alternatively, Hsp90 function and angiogenesis may be impaired by histone deacetylase (HDAC) inhibitors, that act upon the chaperone in a manner distinct from compounds that target its N-terminal ATP-binding pocket. Currently, the HDAC inhibitor LBH589/Panobinostat is under evaluation in Phase I and II trials [17]. The development of Hsp90 inhibitors, although in its infancy a decade ago, is now coming to the forefront of cancer therapy, with over 13 new entities being tested in a variety of preclinical models and human trials [18]. The antiangiogenic and antitumor effects of these agents will be discussed herein.

2.1. Rationale for Hsp90-Dependent Targeting of HIF. Hypoxia inducible factor (HIF) is perhaps one of the most potent proangiogenic proteins regulated by Hsp90. Of the three known HIF isoforms, HIF-1 and HIF-2 contribute to cancer progression and their widespread overexpression in cancers correlates with increased mortality [19]. During tumor growth, HIF transactivates genes to favor survival under conditions of decreasing oxygen and nutrient availability [20]. A substantial number of these genes, such as vascular endothelial growth factor (VEGF) (Figure 1), fall within the category of proangiogenic cytokines, which collectively tip the scales in favor of angiogenesis and neovascularization as part of the angiogenic switch. Activation of this HIF-driven angiogenic switch releases growth constraints upon the tumor and sustains subsequent progression. Tumor cells have evolved multiple mechanisms for upregulating HIF expression and activity, most commonly via modulation of the HIF-α subunit, normally a tightly regulated labile protein subject to proteasomal degradation [21–23]. In general, these mechanisms block oxygen-dependent prolyl hydroxylase (PHD) enzymes from tagging HIF for degradation, ultimately preventing its proteasomal degradation via the von Hippel Lindau (VHL) ubiquitin ligase. VHL can also be inactivated via genetic mutation, genetic loss, or epigenetic suppression, all events most commonly associated with hereditary and sporadic clear cell renal cell carcinoma (CCRCC) [24, 25]. HIF overexpression correlates with highly vascularized tumors, resistance to chemo- and radiotherapy, and overall poor prognosis [26]. The essential role of HIF in tumor angiogenesis has been functionally validated in many models [27, 28], highlighting its importance and validity as a clinical target.

Although no specific anti-HIF targeting strategies have been approved, a number of currently utilized antiangiogenic agents have suppressive effects upon HIF activity or synthesis and diminish HIF-mediated VEGF expression [19]. Several of these agents exhibit antiangiogenic and antitumorigenic effects in preclinical models [29–32] and two specific HIF inhibitors are being evaluated in Phase I trials, the small molecule PX-478 [33], and the antisense oligonucleotide EZN-2968 [34]. The use of Hsp90 inhibitors as a strategy to target HIF emerged shortly after HIF was first identified as an Hsp90 client protein [35]. Many of the antivascular effects of Hsp90 inhibitors are likely due to the ability of this class of inhibitors to downregulate HIF activity. We and others have shown that both the HIF-1α and HIF-2α subunits are client proteins for Hsp90 and that chaperone activity is required for HIF stability and function [35–39]. Importantly, Hsp90 inhibitors, such as GA and its clinical derivative 17-AAG, promote proteasomal degradation HIF-α in a PHD/VHL and oxygen-independent manner, instead of utilizing the ubiquitin ligase RACK1 [37, 40–42]. Thus, Hsp90 inhibitors abrogate HIF signaling even in the absence of a functional PHD/VHL system, suggesting that these pharmacological agents may be able to dampen the constitutive HIF signaling associated with most solid tumors. This notion is supported by the ability of Hsp90 inhibitors to decrease VEGF secretion from cancer cells, impair endothelial cell tubule formation *in vitro*, and reduce *in vivo* tumor size and vascularization [43–45].

2.2. Hsp90 as an Effector of VEGF Expression and Angiogenic Activity. Although HIF is a main effector of VEGF-mediated signaling, HIF-independent proangiogenic processes also contribute to increased VEGF-dependent proangiogenic signaling [46]. NFκB potently induces tumor vascularization, in part through its ability to upregulate VEGF expression through an IL-8/NFκB signaling axis [47] (Figure 1). Secretion of IL-8, a potent proangiogenic factor, is mediated through a variety of mechanisms including other cytokines, cellular stress, and hypoxia (Figure 1) [48]. A positive feedback loop has also been reported whereby NFκB can induce IL-8 and angiogenin secretion to promote tumor neovascularization through the recruitment of bone marrow derived cells [49]. Blockade of NFκB signaling in an orthotopic model of ovarian cancer inhibited tumor growth, reduced tumor angiogenesis, and suppressed VEGF and IL-8 expression [50]. In addition to its role in HIF-independent upregulation of VEGF, NFκB has also been shown to transcriptionally induce the physiological expression of HIF-1 [51]; however, it is not known whether this pathway may also exist in transformed cells. Although NFκB itself is not a validated Hsp90 client protein, multiple direct

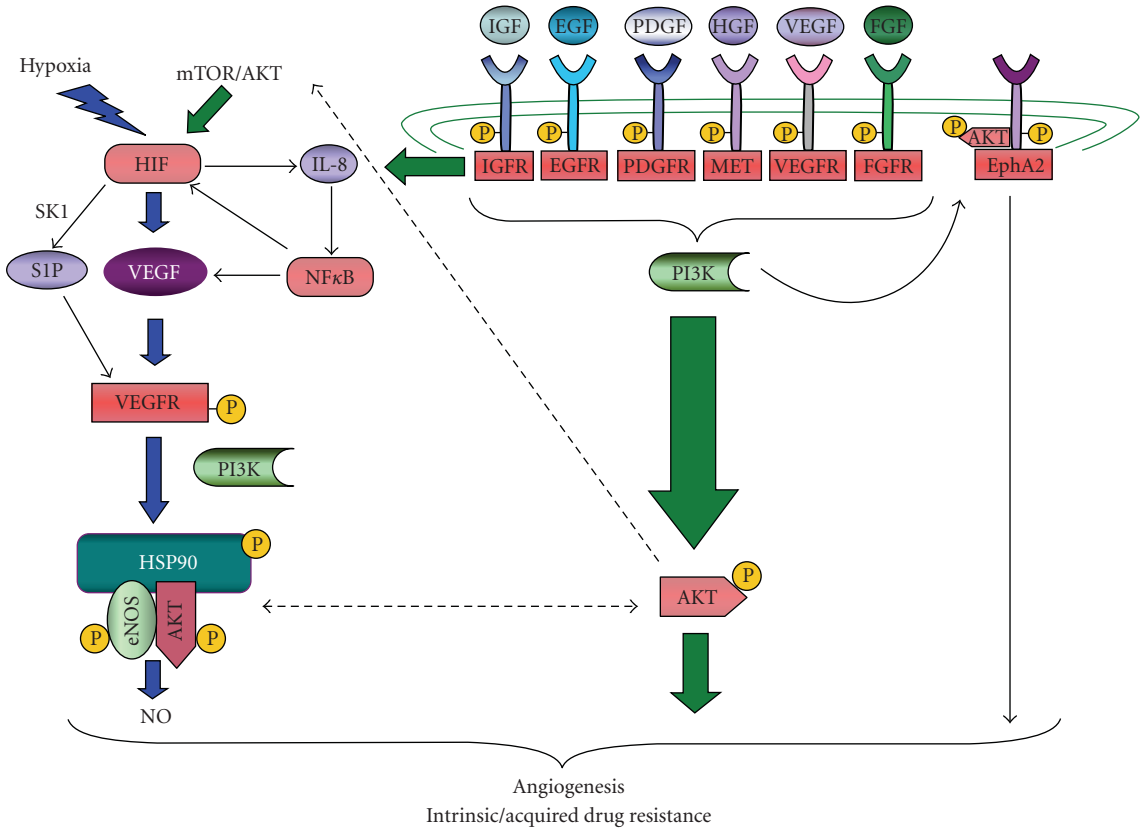


Figure 1: *Hsp90-dependent modulation of proangiogenic signaling pathways in cancer*. Hsp90 regulates multiple arms of angiogenic signaling in cancer. Key signaling molecules that are either direct clients or indirectly modulated by Hsp90 are shaded in red. One pathway that is commonly upregulated during tumorigenesis is the HIF/VEGF signaling axis. Tumor hypoxia and other stimuli induce HIF expression and subsequent activity, leading to a cascade of events that reinforce VEGF expression and angiogenesis. Importantly, several key mediators of this pathway, including HIF and VEGFR, are dependent upon Hsp90 for their function. As indicated, RTK activation also potently upregulates HIF via AKT/mTOR -mediated translation. RTKs additionally transactivate EphA2, a recently identified Hsp90 client protein known to participate in tumor vascularization. Providing another level of complexity, HIF also upregulates the expression of several RTK ligands (e.g., HGF and TGF-alpha), as well as RTK receptors (EGFR, IGFR), thereby reinforcing these signaling networks. Hsp90 additionally plays a role in NFκB-dependent VEGF expression and regulates downstream effectors of VEGF signaling, including AKT-mediated eNOS phosphorylation. Given the intertwining levels among Hsp90 and angiogenic signaling cascades, Hsp90 intervention is predicted to impair signaling at many levels within these redundant pathways, with the overall effect of suppressing tumor angiogenesis.

modulators of NFκB activation are subject to regulation by Hsp90, permitting Hsp90-inhibitor-mediated suppression of NFκB [52–55]. Moreover, NFκB may directly regulate Hsp90 expression through two binding sequences in the Hsp90 promoter, [56] suggesting the possibility of a feedback loop whereby Hsp90 inhibition decreases NFκB activity, which may further decrease Hsp90 levels. It remains to be seen whether these HIF-independent angiogenic regulators of VEGF are appropriately suppressed upon Hsp90 inhibitor treatment in relevant preclinical models.

In addition to its involvement in both HIF and NFκB mediated VEGF production, Hsp90 also modulates downstream effectors of VEGF-dependent signaling. VEGF mediates many of its proangiogenic effects through stimulation of the enzyme endothelial nitric oxide synthase (eNOS). In endothelial cells, activated eNOS leads to nitric oxide (NO) synthesis and release [57], which in turn promotes angiogenesis through endothelial cell proliferation and migration, as well as having effects on blood flow through modulation vascular tone and permeability [58, 59]. Mechanistically, VEGF binds to and activates the VEGF-R2 receptor, promoting phosphorylation of associated Hsp90 [60]. This phosphorylated Hsp90, in turn, serves as a scaffold to facilitate the association between eNOS and AKT, thereby promoting AKT-dependent phosphorylation and activation of eNOS (Figure 1) [61]. Microenvironmental factors such as hypoxia may facilitate angiogenesis by increasing the interaction between Hsp90 and eNOS concomitant with increased AKT activity in endothelial cells [62]. The association between eNOS and Hsp90 can be further stabilized by sphingosine-1-phosphate (S1P), a bioactive lipid induced during hypoxia in an HIF-dependent manner [63, 64], and reciprocally, S1P may stimulate HIF activity [65] and elicit chemoresistance [66]. Given that VEGFR, AKT, eNOS, and NFκB share a dependence upon Hsp90, [67, 68] (Figure 1), Hsp90 inhibitors have the potential to target

multiple steps of this pathway, as demonstrated by the potent suppression of VEGF and NO release both *in vitro* [69, 70] and in preclinical models [71, 72]. Additionally, 17-AAG directly inhibits transcription of eNOS mRNA in the *in vitro* angiogenic HUVEC (human umbilical vein endothelial cells) model, although the mechanism remains unknown [71]. Moreover, Hsp90 inhibitors decrease the expression of activated AKT and eNOS in HUVEC cells, concomitant with inhibitory effects upon tubule formation [72]. Therefore, the antiangiogenic properties of Hsp90 inhibitors are due in part to their ability to suppress HIF-dependent and independent VEGF expression and dampen the signaling potential of VEGF through modulation of VEGFR, AKT, and eNOS function. In light of these functions, Hsp90 chemotherapeutics have the potential to potently suppress tumor angiogenesis by attenuating the secretion of cancer-derived angiogenic factors as well as by blocking paracrine and autocrine signaling in the tumor-associated endothelium.

2.3. Hitting Hard: Hsp90 Inhibition as a Multifaceted Strategy to Combat Tumor Vascularity and Drug Resistance. Although VEGF plays a pivotal role in tumor angiogenesis and VEGF-targeted agents represent the cornerstone of many antiangiogenic approaches in malignant disease, the majority of these initially responsive tumors subsequently acquire drug resistance [3]. Broadly speaking, antiangiogenic tyrosine kinase inhibitors (TKIs) act by neutralizing VEGF/VEGFR signaling, VEGFR in combination with other proangiogenic RTKs, or target distinct RTKs that may not include VEGFR [3, 73, 74]. Common among all TKI-initiated strategies is a lack of durable response, an outcome that represents the biggest clinical challenge with TKI therapy. This universal property of therapeutic failure is a product of both *de novo* resistance, due to the inherent genetic complexity and heterogeneity of tumors, and to acquired resistance, a result of the plasticity and signaling redundancy characteristic of tumor cells [3, 75–77]. In the case of failure of VEGF-targeted therapy, the activation of compensatory mechanisms fosters adaptation and independence from VEGF signaling [78]. Compensatory and redundant signaling is a characteristic of several cancer types, such as glioblastoma multiforme (GBM), wherein activation of PDGF, MET, and EGFR family members collectively limits the therapeutic efficacy of specific TKI agents [79]. Importantly, the ability of angiogenic cytokines to activate their cognate RTK receptors is also a major determinant in conferring chemoresistance. Therefore, the salient finding that RTKs comprise the largest category of Hsp90 client proteins [7, 68, 80] (Figure 1) holds clinical promise for the ability of Hsp90-directed agents to suppress angiogenic signaling and overcome therapeutic resistance in diverse cancers. The following section will discuss Hsp90-modulated effectors of RTK driven angiogenesis, highlight several redundant signaling mechanisms contributing to drug resistance, and outline prospects for Hsp90 intervention and opportunities for reversal of this resistance.

The PDGF-FGF-VEGF signaling module represents a highly integrated pathway commonly activated in a number of cancers. The PDGF-α receptor participates in cell transformation, regulation of vascular permeability, and VEGF expression [81, 82]. Highlighting the complexity and interconnectedness of angiogenic signaling, PDGF also synergizes with FGF to stimulate neovascularization [83] and FGF, in turn, cooperates with VEGF to stimulate blood vessel maturation and function [84]. Clinically, several antiangiogenic TKIs are currently approved as inhibitors for these growth factor/RTK pairs, such as imatinib mesylate (gleevec), sorafenib, and others [85]. The interconnectedness of these pathways suggests a potential benefit of combining TKIs in antiangiogenic therapy, supported by the clinical observation that combination of a pan-VEGFR inhibitor (sunitinib) with a PDGF inhibitor (AGO13736) delayed tumor progression more effectively in cytokine refractory renal cancer [86]. The EGF-IGF-HGF-VEGF signaling module represents another proangiogenic molecular hub. Activation of the MET RTK receptor by its ligand, hepatocyte growth factor (HGF), which may be upregulated by a HIF-dependent pathway [87], has pleiotropic angiogenic effects including upregulation of VEGF and its receptor [88], as well as stimulation of HIF-mediated VEGF secretion [89]. MET also cooperates with epidermal growth factor receptor (EGFR) through the downstream effectors PI3K/AKT [77]. EGFR family members EGFR and HER2 are overexpressed in many cancers and are integral to tumor progression, in part due to their ability to stimulate release of angiogenic factors including VEGF [90]. Continuing the theme of cross-communication, EGFR and HER2 cooperate with IGFR through the PI3K pathway to synergistically increase vessel growth [91]. Although cancer cells harboring amplified MET are initially sensitive to MET TKIs *in vitro*, they evade this inhibition, despite durable MET inhibition, via reactivation of EGFR and downstream mediators [92]. Antiangiogenic TKIs targeting these receptors are being evaluated in Phase I and II clinical trials [3].

The targeting of EGFR with gefitinib provides a well-characterized scenario illustrating the pleiotropic mechanisms at play in drug evasion. One common mechanism for therapeutic escape is conferred by secondary mutations in RTKs that impair the ability of targeted therapeutics to recognize and block receptor function. For example, although at least three EGFR TKIs are in clinical trials (imatinib, gefitinib, and erlotinib), resistance occurs with a frequency of 70% in lung cancer patients, due to the acquisition of secondary mutations in the kinase domain [76, 93]. Another caveat is that gefitinib treatment results in the activation of signaling pathways that collectively serve to limit its antitumor activity and allow acquired resistance to emerge. A number of these resistance mechanisms have been identified in both preclinical and clinical studies and include activation of oncogenic signaling, HGF, VEGF, and PI3K/AKT activation, the latter of which plays a role in *de novo* resistance and sustenance of EGFR activation in a number of cancers [77, 85, 94–98]. A number of studies implicate the contribution of IGF-1R and FGFR as participants in both intrinsic and acquired resistance to EGFR TKIs [98]. These findings strongly advocate the use of combination therapy as a means to overcome this signaling redundancy and subsequent drug resistance. In

support of this notion, it was demonstrated that coadministration of gefitinib and an IGF-1R inhibitor (NVP-AEW541) in a gefitinib resistant xenograft model reversed drug resistance [99]. Other reports also document the importance of cotargeting downstream molecules, including the Hsp90 regulated effectors PI3K/mTOR and MEK, in regaining drug responsiveness in EGFR mutant cancers [98]. These examples highlight the large scope of compensatory mechanisms utilized by tumor cells and the need for broad acting agents and/or combinatorial approaches such as those represented by Hsp90-directed chemotherapeutics.

The inherent ability of Hsp90-targeted agents to suppress multiple proangiogenic receptors and effector proteins bodes well for their prospects in overcoming the redundancy of signaling required for adaptive responses. In support of this premise, geldanamycins (GAs) destabilize MET, inhibit its angiogenic function [100], and suppress coordinate signaling effectors that elude receptor-specific targeting strategies [92]. Blocking Hsp90 also inhibits IGFR and PDGFR function and reduces their angiogenic potential *in vivo* [45, 101]. Similarly, GAs suppress EGFR/HER2 signaling, impair multiple pathways associated with receptor upregulation, and reduce tumor growth and vascularization *in vivo* [45, 102, 103]. Importantly, TKI resistant EGFR mutant receptors are exquisitely sensitive to Hsp90 inhibitors and these agents synergize with EGFR inhibitors [104]. Interestingly, clinical administration of 17-AAG led to tumor regression in HER-2 positive metastatic breast cancer patients [105], demonstrating the oncogene-addicted nature of this tumor. The basis for the poor response in HER-2 negative subtypes remains unknown, given that Hsp90 inhibition also cotargets pivotal downstream effectors of RTK signaling, such as FAK, MAPK, and AKT [67, 106, 107], which would be expected to amplify their suppressive effects and thwart adaptive responses. This ability of Hsp90 inhibition to fundamentally alter network circuitry in tumor cells bodes well for their ability to potentiate the efficacy of targeted or cytotoxic drugs administered in tandem, a notion supported by its synergistic antitumor and antivascular in numerous *in vitro* and preclinical models [104, 108–112].

3. EphA2 Receptor as a Conduit for RTK Activation and a Driver of Angiogenesis

The Eph RTK superfamily is of recent interest in relation to proangiogenic proteins targeted by Hsp90-directed therapy. Several of the 16 Eph RTKs demonstrate functions pertaining to vessel development during embryogenesis and in cancer [113]. In particular, the EphA2 receptor is emerging as a pivotal regulator of physiological and pathological angiogenesis. EphA2 plays an essential role in developmental angiogenesis, as null endothelial cells fail to undergo cell migration and vascular assembly both *in vitro* and *in vivo* [114]. A definitive role for EphA2 in malignancy is illustrated by diminished tumor growth, angiogenesis, and metastasis in EphA2 deficient mice [115]. Furthermore, EphA2 is commonly detected in the tumor associated vasculature, where it facilitates angiogenesis [116–118]. EphA2 is overexpressed in a number of human malignancies, particularly in highly vascular GBM tumors, where it serves as a prognostic factor [119]. Unlike most other ligand/RTK interactions, the association of ephrinA1 ligand with the EphA2 receptor is inhibitory, due to subsequent internalization and proteasomal degradation of the receptor [113, 120]. In cancers, ligand expression is downregulated during the malignant process [119], leading to constitutive EphA2 signaling. In terms of therapeutic options, no clinical TKIs against EphA2 have yet been developed, nor have any already approved clinical agents been shown to target EphA2. However, targeting of EphA2 signaling by either siRNA-mediated suppression of receptor expression [121] or administration of a selective antibody [122] are approaches that have demonstrated efficacy in preclinical models. Highlighting an alternative therapeutic approach, we found that EphA2 receptor activity requires Hsp90 function [123] and, further, that Hsp90-targeting agents interfere with EphA2-mediated signaling and promote receptor destabilization. Therefore, Hsp90 inhibition may represent a therapeutic approach to neutralize receptor function and reduce the aggressiveness of EphA2-driven cancers.

In keeping with the theme of cross-communication, EphA2 is earning its place as an essential member of an expanding network of protumorigenic effectors. First, EGFR activation drives MAPK signaling, which leads to its upregulation in a number of aggressive cancers [124]. Secondly, EGFR family members co-opt EphA2 to promote cell motility and proliferation [115, 125]. Third, EphA2 also cooperates with VEGFR, as demonstrated by its requirement for VEGF-induced endothelial cell migration and tubule formation [126]. Finally, EphA2 function is transduced by its ability to complex with AKT, the latter of which is activated by diverse RTK ligands (EGF, FGF, HGF, and PDGF) [120]. This multifaceted mechanism for EphA2 activation reinforces the theme of the previous section in that the effective blockade of EphA2 signaling will require the simultaneous cotargeting of multiple RTKs. Growth factor-mediated AKT phosphorylation in turn leads to AKT-dependent EphA2 phosphorylation, which facilitates EphA2/AKT complex formation (Figure 1). Formation of this signaling unit is critical for tumor cell migration and invasiveness. Although it remains unclear how this pathway mechanistically translates to angiogenic potential, AKT is a known mediator of angiogenic processes [127] and AKT-mediated EphA2 phosphorylation within GBM-associated tumor vasculature increases with malignancy [120]. Given that AKT is a validated Hsp90 target protein [128] activated in a number of malignancies, and that AKT acts as a conduit for EphA2 signaling, Hsp90-targeting strategies should be effective in blocking EphA2/AKT-dependent angiogenesis. Interestingly, most of the aforementioned angiogenic cytokines are also reported to stimulate VEGF secretion, due in part to their ability to stimulate HIF translation via mTOR, a downstream effector for AKT. As recently reviewed [19], many of the aforementioned TKIs also downregulate HIF translation. Collectively, cytokine-mediated activation of RTKs, and subsequently of AKT, stimulates HIF translation, thereby potentiating VEGF-dependent signaling and sustained angiogenesis (Figure 1). In a recent twist,

it was shown that HIF-2 regulates both the expression and activation of multiple RTKs [129]. Furthermore, the suppression of HIF dramatically improves tumor responses to Sunitinib in colon cancer cells and strikingly prolonged complete responses in half of the tumor bearing mice [130]. This demonstrated ability of HIF to regulate RTK signaling and potentiate the effects of antivascular agents further emphasizes the complexity and cross-pollination of these signaling pathways and supports the rationale for utilizing Hsp90-targeted agents as a strategy to cotarget HIF proteins and proangiogenic RTKs.

4. Murky Waters: Clinical Challenges of Hsp90 Inhibition

Although Hsp90 sustains a multitude of angiogenic processes critical for cancer progression, and Hsp90-targeted agents demonstrate favorable responses across multiple cancers in preclinical models, they have fared less well in the clinic. Recent clinical failures include the use of 17-AAG in advanced prostate cancer and CCRCC [131, 132], the latter a particularly surprising outcome given the putative HIF-dependent and angiogenic nature of this tumor. However, these patients had already presented with advanced metastatic disease, and it is therefore unclear whether improved responses might have been observed with earlier intervention. This section will evaluate some of the more complex issues and potential pitfalls that may offer insight into the variable clinical response of these inhibitors, including pharmacologic considerations, undesired effects of Hsp90-directed therapy, and role of the tumor microenvironment.

4.1. Toxicity, Metabolism, and Delivery. Discordance between Hsp90-targeted efficacy in preclinical models compared with less favorable clinical outcomes may be due to a number of pharmacologic factors, independent of the ability of these agents to target the appropriate protumorigenic pathways. One issue may pertain to drug formulation, as 17-AAG/Tanespimycin is a substrate for the multidrug resistance (MDR) transporter P-glycoprotein, and the related MRP efflux pump [133]. Acquired resistance of cells via this mechanism has been observed in cell culture [134]. Another caveat is that drug potency requires reduction by NAD(P)H:Quinone Oxidoreductase I (NQO1) [133, 135, 136]; yet information is lacking on NQO1 expression profiles in treated patients. A new generation of purine-based Hsp90 inhibitors was subsequently developed [137] whose activity does not depend upon this reduction event [134]. Purine-based compounds are currently at the forefront of Hsp90 inhibitor advancement with several derivatives exhibiting increased potency and decreased toxicity when compared to 17-AAG [138]. Furthermore, these agents are not subject to metabolism by NQO1/DT-diaphorase enzymes nor to efflux by P-glycoprotein, and tumor cells in culture that have acquired resistance to 17-AAG remain susceptible to these newer agents [134, 139]. Therefore, despite disappointing early clinical results with 17-AAG, clinical enthusiasm for the next generation of Hsp90 inhibitors remains high.

4.2. Molecular Caveats of Hsp90 Inhibition. In addition to drug formulation challenges, inhibition of cellular Hsp90 initiates a heat shock response [140] that triggers activation of heat shock factor 1 (HSF-1) [140] and corresponding upregulation of prosurvival chaperones Hsp27, Hsp70, and Hsp90. This heat shock response antagonizes drug potency *in vitro* and *in vivo* [140–143]. Furthermore, HSF-1 expression is essential in supporting malignant transformation [144]. A heat shock response is similarly initiated with the newer purine derivatives [139, 142] and therefore represents a characteristic associated with this general class of Hsp90 targeting agents. However, it may be possible to address this undesired effect through combination treatments. For example, cisplatin suppresses 17-AAG-mediated HSF-1 activation and synergistically promotes tumor cell death *in vitro* [145]. It remains to be seen whether clinical modulation of the HSF-1-mediated heat shock response may enhance the clinical efficacy of Hsp90-directed therapy.

Another less well-understood consideration involves molecular factors that may alter the efficiency of Hsp90-mediated client destabilization. Many Hsp90 client proteins are destabilized via the concerted action of ubiquitin ligases and the proteasomal pathway; however little is known about the molecular effectors involved in client destabilization following Hsp90 inhibition. Taking HIF as an example, we have previously demonstrated that the association of HIF with its dimerization partner ARNT promotes dissociation of Hsp90 from HIF, with resultant protection from Hsp90 inhibiting agents [40]. Since that report, others have documented additional HIF binding or accessory proteins that may share a similar propensity to modulate the efficiency of Hsp90 directed HIF degradation [42, 146, 147], highlighting the previously unappreciated molecular complexity associated with Hsp90-targeted therapy. Recently, a group demonstrated that expression of Cullin5, a ubiquitin ligase, was required for optimal degradation of HIF and ErbB2 [148]. Importantly, Cullin5 expression is decreased in an overwhelming majority of breast cancers [149], suggesting that tumor cells may acquire the ability to evade or limit the destabilization effects of Hsp90-dependent chemotherapy upon subsets of clients. The identification of these modifiers and an analysis of their expression in human cancers may be required to gauge the clinical potential of Hsp90-directed therapy against specific clients or signaling pathways.

4.3. Fighting a Tiger: Contribution of Stroma to Tumor Angiogenesis and Malignant Progression. Another factor in the disconnect between preclinical results and efficacy in patients is likely attributed to the influence of the tumor-adjacent stromal tissue. The tumor stroma, which influences the microenvironment surrounding the neoplastic lesion, is a driving force of both tumor growth and vascularization. This heterogenous stroma is comprised of a variety of cell types, including fibroblasts/myofibroblasts, smooth muscle cells and endothelial cells, immune cells, and bone marrow progenitor cells (Figure 2) [150], all of which collaborate to spur tumor growth. Clinically, an abundant fraction of the tumor reactive stroma is composed of cancer-associated fibroblasts (CAFs), or myofibroblasts, whose presence is

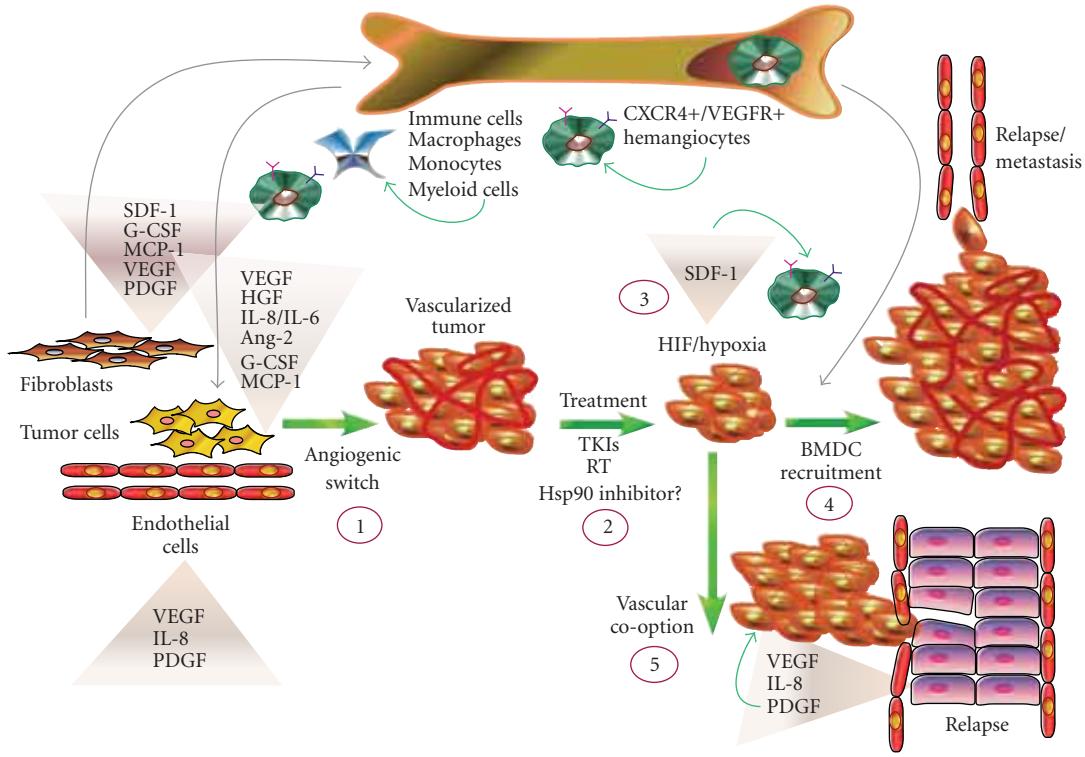


FIGURE 2: *Potential for Hsp90 intervention strategies in curtailing angiogenic processes.* Tumor stromal cells, such as cancer-associated fibroblasts (CAFs) and endothelial cells (ECs), communicate with tumor cells via their secretion of cytokines and thus contribute to the angiogenic switch (1). Cytokines from recruited BMDC progenitors contribute to this milieu to further stimulate tumor vascularization (1). Hsp90 inhibition may prevent HIF-driven cytokine release from tumor and stromal cells (i.e., SDF-1, VEGF, HGF, etc.), as well as HIF-mediated CXCR4+ expression in BMDCs, with the potential effect of curtailing recruitment of CXCR4+ progenitors to the tumor. Hsp90 inhibition also attenuates cytokine signaling via RTK inhibition (i.e., VEGFR, PDGFR), which may collectively prevent or delay the angiogenic switch. Therapeutic approaches utilizing radiotherapy (RT), tyrosine kinase inhibitors (TKIs), or Hsp90-targeted agents suppress tumor vascularization and growth (2). This initial reduction in vascularity may promote tumor hypoxia (3), subsequent HIF activation, and SDF-1 secretion, the latter of which may further stimulate BMDC recruitment (4). Hsp90 inhibitors are similarly predicted to suppress BMDC recruitment and HIF-driven cytokine secretion as in (1). Alternatively, when challenged with reduced HIF expression and decreased BMDC recruitment, tumor cells may coopt the vasculature of normal tissue (5). In this scenario, Hsp90 suppression is predicted to reduce the efficiency of EC-derived factors that support this process. The overall efficacy of Hsp90 inhibition upon tumor vascularization will depend upon the balance of Hsp90-dependent and Hsp90-independent signaling effectors driving the angiogenic process.

recognized as a risk factor for neoplastic transformation. This tumor supporting function is due to their ability to upregulate ECM proteins and secrete growth factors and cytokines, (i.e., fibroblast growth factor (FGF), connective tissue growth factor (CTGF), stromal-derived factor (SDF-1) and VEGF), thereby creating conditions that constitute an optimal milieu for tumor development [151, 152]. CAFs robustly stimulate tumor angiogenesis and cancer progression in a number of xenograft models [153–157]. Myofibroblasts are also a component of nontumorigenic fibrotic tissue, such as in gastric, pancreatic, and hepatic stellate cells, which similarly secrete angiogenic cytokines and support paracrine signaling in adjacent epithelial cells [158]. Hypoxic signaling further drives fibrogenesis [159], and the hypoxia-mediated upregulation of VEGF and angiogenic cytokines from stellate cells perpetuates the fibrotic and hypoxic cycle [158, 160].

Given that myofibrolasts support and sustain cancer progression, there is heightened interest in agents that may target the CAF-rich stroma [161]. Although Hsp90-targeted strategies are predicted to interfere with the VEGFR, PDGFR, and TGF beta receptor signaling that participates in tumor-stromal communication, reports examining the ability of either TKIs or Hsp90 inhibitors to attenuate CAF/stellate signaling are limited. Multi-TKIs reverse properties associated with the activated stellate phenotype *in vitro* and *in vivo* [162, 163], concomitant with reduced tumor vascularity [164], and Imatinib inhibits PDGF/AKT signaling and ECM production in breast stromal fibroblasts [165]. Hsp90 inhibitors exhibit cytotoxicity against CAFs derived from gastric cancer [44], which are potent mediators of tumor angiogenesis [166]. Interestingly, Hsp90 inhibition suppresses the reactive stromal phenotype in hepatic stellate cells, prior to initiating apoptosis [167], suggesting that Hsp90-targeted agents may be effective in reducing stromal support of tumorigenic progression.

Given that CAFs promote tumor vascularization in part via their ability to stimulate endothelial cell function,

attention has now shifted to the ability of TKIs to cotarget the tumor endothelium. The stromal vasculature is comprised of endothelial cells and perivascular pericytes, the latter of which provide both survival signals and structural support to facilitate a mature and functional vasculature [168]. The pathways crucial to EC-pericyte communication and vascular stabilization include PDGF/PDGFR and VEGF/VEGFR signaling. Secretion of PDGF from cancer cells stimulates VEGF upregulation in pericytes via a PI3K/AKT mechanism, which protects ECs from apoptosis [169]. Furthermore, PDGF signaling is essential for tumor vascularization in a preclinical model of pancreatic cancer [170] and upregulated PDGF signaling is a characteristic of the tumor stroma in a preclinical model of cervical cancer [171]. In the latter example, expression of the PDGF receptor was primarily localized to stromal fibroblasts and pericytes, and inhibition of PDGFR signaling with Imatinib decreased pericyte coverage of tumor endothelial cells, concomitant with suppression of tumor vascularity and growth.

Hsp90 inhibitors are well characterized in their ability to suppress tumor vasculature and growth in multiple preclinical models; yet their specific effects upon endothelial cells and pericytes remain largely unexplored. A limited number of reports demonstrate that Hsp90 inhibition reduces proliferation, differentiation, motility, and angiogenic signaling in normal human endothelial cells [72, 139] and may elicit death of normal vascular smooth muscle cells (representative of pericytes) at high concentrations [44], but the molecular mechanisms for these effects remain obscure. The precise molecular effects of Hsp90 inhibition within the tumor-associated vasculature have not been clarified, and use of normal cells that resemble components of the tumor vasculature may not accurately reflect the tumor endothelium. Further, Hsp90 inhibitors may differentially destabilize proteins in tumor and vascular cells [101], illustrating the potential for differential tumor and stromal responses to therapeutics. Importantly, Hsp90 therapeutics may exhibit preferential uptake and selectivity for tumor tissue, proportional to the cancer specific hyperactivity of the Hsp90 chaperone [172, 173], inviting the question of whether these agents will effectively target components of the nontumorigenic stroma.

4.4. The Tumor Stroma as an Enabler of Therapeutic Resistance. Clinically, chemoresistance may be due to a failure to target the proangiogenic signaling derived from tumor stroma, a notion supported by the ability of CAFs to mediate drug sensitivity [174, 175]. Endothelial cells also contribute to chemoresistance, as demonstrated by the use of temozolomide in the treatment glioblastoma multiforme (GBM). While temozolomide exhibits toxicity against GBM cells in culture, glioma-derived endothelial cells are refractory to treatment, thus offering a possible explanation for the clinical failure of these agents [176]. Similarly, the ability of pericytes to protect against EC apoptosis [169] implicates their potential role in attenuating the efficacy of chemotherapeutics. In support of this notion, dual inhibition of PDGFR and VEGFR signaling is synergistic in reducing pericyte coverage of tumor endothelium, and this combination is maximally effective in suppressing tumor growth over either agent alone in a mouse model of pancreatic islet cancer [177]. In addition to its role in chemoresistance, the tumor stroma also plays a major role in mediating the response to radiotherapy (RT). It is established that RT induces the expression and secretion of VEGF and proangiogenic cytokines in cancer cells, stromal endothelial cells, and fibroblasts [178–180]. This cytokine upregulation stimulated by the initiation of vascular repair mechanisms and HIF-dependent signaling [181, 182] represents a major barrier to chemosensitivity [183–185].

Antiangiogenic TKIs have met with limited success in combating radioresistance, despite their impairment of proangiogenic RTK signaling in both tumor and stromal cells [186]. Although not yet validated clinically, we, and others, have shown that Hsp90 inhibition imparts radiosensitivity to various cancer cell types in culture and *in vivo* [187–189]. While the basis for this enhancement is not well defined, Hsp90-dependent HIF targeting is one likely mechanism, supported by the finding that blockade of HIF/VEGF signaling, coupled with RT, potently destroys tumor vasculature in a xenograft model [190]. In further support of the ability of Hsp90 inhibition to impair this signaling axis, Hsp90-directed therapy overrides the protective effects of VEGF-mediated signaling in endothelial cells, in part through inhibition of AKT [191]. Since many of the RT-induced wound repair response pathways depend upon a number of Hsp90 clients, including HIF, AKT, MEK/ERK, PDGFR, and VEGFR, Hsp90 inhibition represents an alternate approach to impair multiple radioresponse regulatory proteins and improve outcome. In fact, it remains a possible, though unconfirmed notion, that the ability of Hsp90 inhibitors to destabilize interactions between ECs and pericytes may underlie their ability to suppress tumor vascularity and potentiate chemo- and radiosensitivity. However, given that optimal responses to the combination of anti-VEGF therapy with RT occur during the "normalization window" characterized by stabilization of the tumor vasculature subsequent to enhanced pericyte coverage [192], the use of Hsp90 inhibitors will have to be judiciously applied for optimal therapeutic benefit.

Similar to RT, vascular targeting agents exacerbate tumor hypoxia and activate HIF signaling, events antagonistic to treatment objectives. HIF activation stimulates cytokine secretion and recruitment of circulating BMDCs (Figure 2), which may replenish vascular components such as pericytes, CAFs, and myeloid cells. Mobilization of BMDCs to hypoxic areas promotes neovascularization and contributes to cancer aggressiveness and drug resistance [2, 193, 194]. Although factors regulating BMDC recruitment are not well defined, tumor hypoxia and the HIF-regulated cytokines VEGF and SDF-1 are established as major effectors in the recruitment of VEGFR and CXCR4 expressing BMDCs [49, 195, 196] (Figure 2). In a well-validated preclinical model, hypoxic or irradiated glioma cells each secreted factors that stimulated the homing of hematopoietic progenitors in a HIF-dependent manner [197]. The enhancement of tumor hypoxia via genetic or pharmacologic interference with VEGF signaling similarly recruited BMDCs and fueled aggressiveness [198, 199] (Figure 2). It remains to be seen

whether Hsp90-directed therapy may effectively suppress HIF-mediated recruitment of BMDCs and whether attenuated recruitment may be sufficient for tumor suppression. Indeed, BMDC recruitment was diminished in a HIF-1 knockout mouse model of glioma; yet tumor cells became invasive and continued to thrive via cooption of the host vasculature [200]. In this example, the host cells were still competent to respond to tumor-mediated HIF/VEGF signaling, leaving open the possibility that Hsp90 inhibition, which would target HIF signaling in conjunction with VEGF mediated responses, may thwart this undesired adaptive event (Figure 2).

Another level of complexity is due to the nature of angiogenic compensation, in that HIF-independent compensatory mechanisms, such as NFκB-dependent cytokine release, can also promote BMDC recruitment [49]. Hsp90 inhibitors have the potential to impair a subset of HIF-independent mechanisms, for example, by virtue of their ability to suppress effectors such as NFκB. Although the role of Hsp90-dependent signaling within the context of BMDC-mediated neovascularization has not been explored, the ability of Hsp90 inhibitors to suppress BMDC stimulatory properties upon multiple myeloma cells in a coculture model [72] suggests that these agents may indeed have utility in preventing some aspects of BMDC-derived neovascularization. However, these agents are likely to incur a number limitations, given the recent identification of a subset of myeloid cells that are refractory to VEGF-dependent therapy, due to expression of G-CSF and Bv8, the latter of which drives local angiogenesis, as well as BMDC recruitment [199, 201]. As these ligands activate G-coupled protein receptors (GPCRs) that may not rely upon Hsp90 function, it is possible that this alternative angiogenic pathway will be immune to Hsp90-directed therapy. It therefore remains to be determined whether the lack of durable clinical response of Hsp90-directed therapy is due to an inability to suppress BMDC recruitment, to inhibit vascular cooption, or to suppress these compensatory angiogenic pathways. A further characterization of these pathways may pave the way to more successful regimens that employ rationally designed combination therapies.

4.5. The Road to Nirvana: Improving Preclinical Models, Drug Analyses, and Clinical Correlates. With the overarching goal of understanding the basis for clinical responses to Hsp90-targeted therapy, a continuum of models must be evaluated, each with its inherent strengths and weaknesses. Although cell-based *in vitro* models are necessary to decipher defined molecular signaling events, these systems do not recapitulate the complexity of the tumor microenvironment, such as dimensionality, matrix incorporation, or stromal communication, all factors that influence antiangiogenic responses [202, 203]. Xenograft models, which embody much of the complexity of the tumor microenvironment, are a necessary prerequisite for evaluating the utility of antiangiogenic agents in distinct cancers. However, the majority of these models utilize immunocompromised mice, which lack the full complement of immune components that may contribute to stromal dependent angiogenesis. Some of these differences invariably account for the ability of Hsp90 inhibitors, and other chemotherapeutics, to exhibit more potent tumor efficacy in preclinical models compared with their clinical responses. Immune competent genetic models of progressive cancer that more closely mimic the complexity of human malignancy represent valuable tools for dissection of stepwise genetic events in this process and may also help pinpoint when antiangiogenic agents would be most efficacious.

Another caveat to deciphering optimal treatment regimens is imparted by the molecular heterogeneity of cancers. Functionally, this is exemplified by the differential wiring of signaling networks, and the corresponding differential response to Hsp90 inhibition, a trait similarly reflected with other chemotherapeutics. These varying responses likely reflect the distinct molecular signatures driving cancer survival, each with a unique dependence upon Hsp90 function [204], combined with disparate local microenvironments from the periphery to the core. Moreover, differential client affinity in diverse cell and cancer types [205] hints at more complex molecular regulation. Further, the recent revelation that an average human tumor may have upwards of 20,000 mutations [206, 207] strongly supports the rationale for therapeutic strategies incorporating more broadly acting drugs, such as those represented by the category of Hsp90-directed agents. In addition to tumor cell heterogeneity, components of the tumor vasculature may demonstrate comparable complexity. In support of this notion, VEGF resistant CAFs may upregulate PDGF signaling [175] and promote chemoresistance. Similarly, the tumor endothelium exhibits significant heterogeneity of expressed surface molecules, as validated with use of phage display technology, based upon the principle of differential homing of peptides to the vasculature [208–210]. Interestingly, this approach identified distinct molecular signatures of the tumor endothelium that occur in a stage specific manner, with clinical implications for the variable tumor responses observed for TKIs. In a preclinical model of cervical cancer, a subset of TKI agents demonstrated preferential activity at distinct tumor stages, implicating their selective effects upon either tumor initiation or signaling events activated at later stages [171]. In this model, combination treatments (i.e., cotargeting VEGFR and PDGFR) demonstrated improved antitumor activity of progressive tumors, illuminating that optimal targeting approaches may need to consider prevention and intervention strategies as a component of the heterogeneity of the tumor-associated vasculature. Similar dynamic treatment analyses in well-defined preclinical models will further define optimal clinical usage for Hsp90 inhibitors.

In addition to allowing a dynamic evaluation of drug efficacy, preclinical models are useful tools to assess complex drug-dependent molecular changes. A subset of microRNAs was recently identified as components of the angiogenic switch in a genetic model of pancreatic cancer. Strikingly, these same targets were similarly upregulated in patients and modulated preclinically following treatment with Sunitinib [211]. Although a comparative detailed genetic analysis has not yet been performed with Hsp90 inhibitors, one group evaluated pharmacodynamic markers and resultant tumor

angiogenesis [139], while another performed a proteomic and genetic analysis to identify a number of gene and protein changes [212]. Further preclinical models that incorporate dynamic molecular analyses have great potential for evaluating the effects of emerging Hsp90 inhibitors. Similarly, clinical trial design is likely to benefit from the incorporation of increasingly sophisticated molecular endpoints. Although the evaluation of changes in tumor burden and disease progression are established components of the clinical evaluation of anticancer agents, according to the RECIST (response evaluation criteria in solid tumors) criteria [213], these endpoints may not be optimal in obtaining nuanced information about tumor responses and changes within the tumor microenvironment. For example, in trials with Hsp90-targeted agents, a pertinent endpoint would entail the monitoring of drug-dependent depletion of relevant cancer-causing client proteins. In a recent Phase II trial of 17-AAG in melanoma, differential drug responses were observed for client proteins from patient tumors [214], which may have implications for clinical outcome. Therefore, the ability to dynamically image Hsp90-dependent client depletion *in vivo* would be a clinically valuable addition to these trials. At least two reports demonstrate the feasibility of noninvasively viewing the dynamic expression of Hsp90 client proteins in real time in preclinical models [215, 216], suggesting that incorporation of these approaches into clinical trial design may be feasible.

More recently, a number of clinical trials have included increasingly sophisticated molecular endpoints to gauge drug responses. In a phase II trial of 17-AAG in metastatic prostate cancer, the relation between drug treatment and PSA was monitored, as were a limited number of serum derived cytokines and markers from peripheral blood [131]. In a carefully designed trial of a pan-VEGF inhibitor in GBM, MRI imaging of tumor vascularity discerned a window of vascular normalization [217]. Importantly, this group also identified several vascular modulators that correlated with tumor progression, such as circulating endothelial cells (CECs) and a subset of cytokines, such as SDF-1. In another detailed study by this group, a comprehensive genetic analysis of angiogenic markers revealed that anti-VEGF therapy induced SDF-1 and inflammatory pathways in rectal cancer [218]. This integrative analysis of stage specific molecular and genetic alterations, coupled with the evaluation of surrogate biomarkers**,** represents a promising approach to better understand and possibly predict fluctuations in the clinical response to Hsp90 inhibitors. Looking forward, it must be determined whether and how Hsp90 inhibitors modulate signaling, angiogenic genes/microRNAs, cytokines, circulating endothelial and progenitor cells, tumor stroma, and which of these alterations are prognostic for tumor response or relapse. Whether the expanded repertoire of proteins targeted by Hsp90 inhibitors, compared with the relatively specific subset of proteins targeted by clinically approved antiangiogenic TKIs, will be an asset or a liability in the clinic remains to be determined. A more comprehensive understanding of the mode of action of Hsp90 inhibitors will position us to harness the power of these agents and design more effective strategies to diminish cancer lethality.

Acknowledgments

The authors wish to thank Linda Metheny-Barlow, Luke Whitesell, Bob Gemmill, and David Rowley for their support and critical review of this manuscript.

References

[1] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[2] M. Seandel, J. Butler, D. Lyden, and S. Raf, "A catalytic role for proangiogenic marrow-derived cells in tumor neovascularization," *Cancer Cell*, vol. 13, no. 3, pp. 181–183, 2008.

[3] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[4] S. Sanderson, M. Valenti, S. Gowan et al., "Benzoquinone ansamycin heat shock protein 90 inhibitors modulate multiple functions required for tumor angiogenesis," *Molecular Cancer Therapeutics*, vol. 5, no. 3, pp. 522–532, 2006.

[5] L. Whitesell, S. D. Shifrin, G. Schwab, and L. M. Neckers, "Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition," *Cancer Research*, vol. 52, no. 7, pp. 1721–1728, 1992.

[6] L. H. Pearl and C. Prodromou, "Structure and mechanism of the Hsp90 molecular chaperone machinery," *Annual Review of Biochemistry*, vol. 75, pp. 271–294, 2006.

[7] J. S. Isaacs, W. Xu, and L. Neckers, "Heat shock protein 90 as a molecular target for cancer therapeutics," *Cancer Cell*, vol. 3, no. 3, pp. 213–217, 2003.

[8] J. S. Isaacs, "Heat-shock protein 90 inhibitors in antineoplastic therapy: is it all wrapped up?" *Expert Opinion on Investigational Drugs*, vol. 14, no. 6, pp. 569–589, 2005.

[9] M. V. Powers and P. Workman, "Targeting of multiple signalling pathways by heat shock protein 90 molecular chaperone inhibitors," *Endocrine-Related Cancer*, vol. 13, supplement 1, pp. 125–135, 2006.

[10] L. Whitesell, R. Bagatell, and R. Falsey, "The stress response: implications for the clinical development of Hsp90 inhibitors," *Current Cancer Drug Targets*, vol. 3, no. 5, pp. 349–358, 2003.

[11] L. Neckers, "Hsp90 inhibitors as novel cancer chemotherapeutic agents," *Trends in Molecular Medicine*, vol. 8, no. 4, pp. S55–S61, 2002.

[12] S. M. Roe, C. Prodromou, R. O'Brien, J. E. Ladbury, P. W. Piper, and L. H. Pearl, "Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin," *Journal of Medicinal Chemistry*, vol. 42, no. 2, pp. 260–266, 1999.

[13] J. G. Supko, R. L. Hickman, M. R. Grever, and L. Malspeis, "Preclinical pharmacologic evaluation of geldanamycin as an antitumor agent," *Cancer Chemotherapy and Pharmacology*, vol. 36, no. 4, pp. 305–315, 1995.

[14] T. W. Schulte and L. M. Neckers, "The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin," *Cancer Chemotherapy and Pharmacology*, vol. 42, no. 4, pp. 273–279, 1998.

[15] Z.-Q. Tian, Y. Liu, D. Zhang et al., "Synthesis and biological activities of novel 17-aminogeldanamycin derivatives," *Bioorganic and Medicinal Chemistry*, vol. 12, no. 20, pp. 5317–5329, 2004.

[16] S. Z. Usmani, R. Bona, and Z. Li, "17 AAG for HSP90 inhibition in cancer—from bench to bedside," *Current Molecular Medicine*, vol. 9, no. 5, pp. 654–664, 2009.

[17] H. M. Prince, M. J. Bishton, and R. W. Johnstone, "Panobinostat (LBH589): a potent pan-deacetylase inhibitor with promising activity against hematologic and solid tumors," *Future Oncology*, vol. 5, no. 5, pp. 601–612, 2009.

[18] Y. S. Kim, S. V. Alarcon, S. Lee et al., "Update on Hsp90 inhibitors in clinical trial," *Current Topics in Medicinal Chemistry*, vol. 9, no. 15, pp. 1479–1492, 2009.

[19] G. L. Semenza, "Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics," *Oncogene*, vol. 29, no. 5, pp. 625–634, 2010.

[20] G. L. Semenza, "Involvement of hypoxia-inducible factor 1 in human cancer," *Internal Medicine*, vol. 41, no. 2, pp. 79–83, 2002.

[21] G. L. Semenza, "HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus," *Cell*, vol. 107, no. 1, pp. 1–3, 2001.

[22] M. Y. Koh and G. Powis, "HAF: the new player in oxygen-independent HIF-1α degradation," *Cell Cycle*, vol. 8, no. 9, pp. 1359–1366, 2009.

[23] Y. S. Chun, M. S. Kim, and J. W. Park, "Oxygen-dependent and -independent regulation of HIF-1alpha," *Journal of Korean Medical Science*, vol. 17, no. 5, pp. 581–588, 2002.

[24] K. Kondo, J. Klco, E. Nakamura, M. Lechpammer, and W. G. Kaelin Jr., "Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein," *Cancer Cell*, vol. 1, no. 3, pp. 237–246, 2002.

[25] W. G. Kaelin Jr., "The von Hippel-Lindau tumor suppressor protein and clear cell renal carcinoma," *Clinical Cancer Research*, vol. 13, no. 2, pp. 680S–684S, 2007.

[26] E. B. Rankin and A. J. Giaccia, "The role of hypoxia-inducible factors in tumorigenesis," *Cell Death and Differentiation*, vol. 15, no. 4, pp. 678–685, 2008.

[27] O. Iliopoulos, A. P. Levy, C. Jiang, W. G. Kaelin Jr., and M. A. Goldberg, "Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 20, pp. 10595–10599, 1996.

[28] D. Liao and R. S. Johnson, "Hypoxia: a key regulator of angiogenesis in cancer," *Cancer and Metastasis Reviews*, vol. 26, no. 2, pp. 281–290, 2007.

[29] W. Wang, W.-D. Jia, G.-L. Xu et al., "Antitumoral activity of rapamycin mediated through inhibition of HIF-1alpha and VEGF in hepatocellular carcinoma," *Digestive Diseases and Sciences*, vol. 54, no. 10, pp. 2128–2136, 2009.

[30] D. Del Bufalo, L. Ciuffreda, D. Trisciuoglio et al., "Antiangiogenic potential of the mammalian target of rapamycin inhibitor temsirolimus," *Cancer Research*, vol. 66, no. 11, pp. 5549–5554, 2006.

[31] M. Puppo, F. Battaglia, C. Ottaviano et al., "Topotecan inhibits vascular endothelial growth factor production and angiogenic activity induced by hypoxia in human neuroblastoma by targeting hypoxia-inducible factor-1α and -2α," *Molecular Cancer Therapeutics*, vol. 7, no. 7, pp. 1974–1984, 2008.

[32] A. Rapisarda, B. Uranchimeg, O. Sordet, Y. Pommier, R. H. Shoemaker, and G. Melillo, "Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications," *Cancer Research*, vol. 64, no. 4, pp. 1475–1482, 2004.

[33] M. Y. Koh, T. Spivak-Kroizman, S. Venturini et al., "Molecular mechanisms for the activity of PX-478, an antitumor inhibitor of the hypoxia-inducible factor-1α," *Molecular Cancer Therapeutics*, vol. 7, no. 1, pp. 90–100, 2008.

[34] L. M. Greenberger, I. D. Horak, D. Filpula et al., "A RNA antagonist of hypoxia-inducible factor-1α, EZN-2968, inhibits tumor cell growth," *Molecular Cancer Therapeutics*, vol. 7, no. 11, pp. 3598–3608, 2008.

[35] E. Minet, D. Mottet, G. Michel et al., "Hypoxia-induced activation of HIF-1: role of HIF-1α-Hsp90 interaction," *FEBS Letters*, vol. 460, no. 2, pp. 251–256, 1999.

[36] D. M. Katschinski, L. Le, S. G. Schindler, T. Thomas, A. K. Voss, and R. H. Wenger, "Interaction of the PAS B domain with HSP90 accelerates hypoxia-inducible factor-1α stabilization," *Cellular Physiology and Biochemistry*, vol. 14, no. 4-6, pp. 351–360, 2004.

[37] J. S. Isaacs, Y. J. Jung, E. G. Mimnaugh, A. Martinez, F. Cuttitta, and L. M. Neckers, "Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway," *Journal of Biological Chemistry*, vol. 277, no. 33, pp. 29936–29944, 2002.

[38] J. Yang, L. Zhang, P. J. A. Erbel et al., "Functions of the Per/ARNT/Sim domains of the hypoxia-inducible factor," *Journal of Biological Chemistry*, vol. 280, no. 43, pp. 36047–36054, 2005.

[39] M. Milkiewicz, J. L. Doyle, T. Fudalewski, E. Ispanovic, M. Aghasi, and T. L. Haas, "HIF-1α and HIF-2α play a central role in stretch-induced but not shear-stress-induced angiogenesis in rat skeletal muscle," *Journal of Physiology*, vol. 583, no. 2, pp. 753–766, 2007.

[40] J. S. Isaacs, Y.-J. Jung, and L. Neckers, "Aryl hydrocarbon nuclear translocator (ARNT) promotes oxygen-independent stabilization of hypoxia-inducible factor-1α by modulating an Hsp90-dependent regulatory pathway," *Journal of Biological Chemistry*, vol. 279, no. 16, pp. 16128–16135, 2004.

[41] Y. V. Liu, J. H. Baek, H. Zhang, R. Diez, R. N. Cole, and G. L. Semenza, "RACK1 competes with HSP90 for binding to HIF-1α and is required for O2-independent and HSP90 inhibitor-induced degradation of HIF-1α," *Molecular Cell*, vol. 25, no. 2, pp. 207–217, 2007.

[42] S. Amir, R. Wang, J. W. Simons, and N. J. Mabjeesh, "SEPT9_v1 up-regulates hypoxia-inducible factor 1 by preventing its RACK1-mediated degradation," *Journal of Biological Chemistry*, vol. 284, no. 17, pp. 11142–11151, 2009.

[43] O. Alqawi, M. Moghaddas, and G. Singh, "Effects of geldanamycin on HIF-1α mediated angiogenesis and invasion in prostate cancer cells," *Prostate Cancer and Prostatic Diseases*, vol. 9, no. 2, pp. 126–135, 2006.

[44] S. A. Lang, D. Klein, C. Moser et al., "Inhibition of heat shock protein 90 impairs epidermal growth factor-mediated signaling in gastric cancer cells and reduces tumor growth and vascularization in vivo," *Molecular Cancer Therapeutics*, vol. 6, no. 3, pp. 1123–1132, 2007.

[45] S. A. Lang, C. Moser, A. Gaumann et al., "Targeting heat shock protein 90 in pancreatic cancer impairs insulin-like growth factor-I receptor signaling, disrupts an interleukin-6/signal-transducer and activator of transcription 3/hypoxia-inducible factor-1α autocrine loop, and reduces orthotopic tumor growth," *Clinical Cancer Research*, vol. 13, no. 21, pp. 6459–6468, 2007.

[46] Y. Mizukami, Y. Kohgo, and D. C. Chung, "Hypoxia inducible factor-1-independent pathways in tumor angiogenesis," *Clinical Cancer Research*, vol. 13, no. 19, pp. 5670–5674, 2007.

[47] D. Martin, R. Galisteo, and Gutkind J. S., "CXCL8/IL8 stimulates vascular endothelial growth factor (VEGF) expression and the autocrine activation of VEGFR2 in endothelial cells by activating NFkappaB through the CBM (Carma3/Bcl10/Malt1) complex," *The Journal of Biological Chemistry* , vol. 284, no. 10, pp. 6038–6042, 2009.

[48] D. J. Brat, A. C. Bellail, and E. G. Van Meir, "The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis," *Neuro-Oncology*, vol. 7, no. 2, pp. 122–133, 2005.

[49] D. A. Chan, T. L.A. Kawahara, P. D. Sutphin, H. Y. Chang, J.-T. Chi, and A. J. Giaccia, "Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment," *Cancer Cell*, vol. 15, no. 6, pp. 527–538, 2009.

[50] S. Huang, J. B. Robinson, A. DeGuzman, C. D. Bucana, and I. J. Fidler, "Blockade of nuclear factor-κb signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8," *Cancer Research*, vol. 60, no. 19, pp. 5334–5339, 2000.

[51] J. Rius, M. Guma, C. Schachtrup et al., "NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α," *Nature*, vol. 453, no. 7196, pp. 807–811, 2008.

[52] M. Broemer, D. Krappmann, and C. Scheidereit, "Requirement of Hsp90 activity for IκB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-κB activation," *Oncogene*, vol. 23, no. 31, pp. 5378–5386, 2004.

[53] J. Crèvecoeur, M.-P. Merville, J. Piette, and G. Gloire, "Geldanamycin inhibits tyrosine phosphorylation-dependent NF-κB activation," *Biochemical Pharmacology*, vol. 75, no. 11, pp. 2183–2191, 2008.

[54] G. Qing, P. Yan, Z. Qu, H. Liu, and G. Xiao, "Hsp90 regulates processing of NF-κB2 p100 involving protection of NF-κB-inducing kinase (NIK) from autophagy-mediated degradation," *Cell Research*, vol. 17, no. 6, pp. 520–530, 2007.

[55] J. Lewis, A. Devin, A. Miller et al., "Disruption of Hsp96 function results in degradation of the death domain kinase, receptor-interacting protein (RIP), and blockage of tumor necrosis factor-induced nuclear factor-κB activation," *Journal of Biological Chemistry*, vol. 275, no. 14, pp. 10519–10526, 2000.

[56] M. Ammirante, A. Rosati, A. Gentilella et al., "The activity of hsp90α promoter is regulated by NF-κB transcription factors," *Oncogene*, vol. 27, no. 8, pp. 1175–1178, 2008.

[57] A. Papapetropoulos, G. García-Cardeña, J. A. Madri, and W. C. Sessa, "Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells," *Journal of Clinical Investigation*, vol. 100, no. 12, pp. 3131–3139, 1997.

[58] J. P. Cooke, "NO and angiogenesis," *Atherosclerosis Supplements*, vol. 4, no. 4, pp. 53–60, 2003.

[59] G. Van Buren II, E. R. Camp, A. D. Yang et al., "The role of nitric oxide in mediating tumour blood flow," *Expert Opinion on Therapeutic Targets*, vol. 10, no. 5, pp. 689–701, 2006.

[60] M. Duval, F. Le Bœuf, J. Huot, and J.-P. Gratton, "Src-mediated phosphorylation of Hsp90 in response to vascular endothelial growth factor (VEGF) is required for VEGF receptor-2 signaling to endothelial NO synthase," *Molecular Biology of the Cell*, vol. 18, no. 11, pp. 4659–4668, 2007.

[61] J. Fontana, D. Fulton, Y. Chen et al., "Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release," *Circulation Research*, vol. 90, no. 8, pp. 866–873, 2002.

[62] J.-X. Chen and B. Meyrick, "Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium," *Laboratory Investigation*, vol. 84, no. 2, pp. 182–190, 2004.

[63] F. Roviezzo, M. Bucci, C. Delisle et al., "Essential requirement for sphingosine kinase activity in eNOS-dependent NO release and vasorelaxation," *FASEB Journal*, vol. 20, no. 2, pp. 340–342, 2006.

[64] V. Anelli, C. R. Gault, A. B. Cheng, and L. M. Obeid, "Sphingosine kinase 1 is up-regulated during hypoxia in U87MG glioma cells: Role of hypoxia-inducible factors 1 and 2," *Journal of Biological Chemistry*, vol. 283, no. 6, pp. 3365–3375, 2008.

[65] M. D. Michaud, G. A. Robitaille, J.-P. Gratton, and D. E. Richard, "Sphingosine-1-phosphate: a novel nonhypoxic activator of hypoxia-inducible factor-1 in vascular cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 29, no. 6, pp. 902–908, 2009.

[66] S. E. Schnitzer, A. Weigert, J. Zhou, and B. Brüne, "Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine-1-phosphate-mediated chemoresistance in A549 lung cancer cells," *Molecular Cancer Research*, vol. 7, no. 3, pp. 393–401, 2009.

[67] S. Sato, N. Fujita, and T. Tsuruo, "Modulation of Akt kinase activity by binding to Hsp90," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 20, pp. 10832–10837, 2000.

[68] A. Citri, D. Harari, G. Shohat et al., "Hsp90 recognizes a common surface on client kinases," *Journal of Biological Chemistry*, vol. 281, no. 20, pp. 14361–14369, 2006.

[69] K. A. Pritchard Jr., A. W. Ackerman, E. R. Gross et al., "Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase," *Journal of Biological Chemistry*, vol. 276, no. 21, pp. 17621–17624, 2001.

[70] G. García-Cardeña, R. Fan, V. Shah et al., "Dynamic activation of endothelial nitric oxide synthase by Hsp90," *Nature*, vol. 392, no. 6678, pp. 821–824, 1998.

[71] J. Sun and J. K. Liao, "Induction of angiogenesis by heat shock protein 90 mediated by protein kinase Akt and endothelial nitric oxide synthase," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 12, pp. 2238–2244, 2004.

[72] Y. Okawa, T. Hideshima, P. Steed et al., "SNX-2112, a selective Hsp90 inhibitor, potently inhibits tumor cell growth, angiogenesis, and osteoclastogenesis in multiple myeloma and other hematologic tumors by abrogating signaling via Akt and ERK," *Blood*, vol. 113, no. 4, pp. 846–855, 2009.

[73] J. Y. Hsu and H. A. Wakelee, "Monoclonal antibodies targeting vascular endothelial growth factor: current status and future challenges in cancer therapy," *BioDrugs*, vol. 23, no. 5, pp. 289–304, 2009.

[74] L. M. Ellis and D. J. Hicklin, "VEGF-targeted therapy: mechanisms of anti-tumour activity," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 579–591, 2008.

[75] M. C. Heinrich, C. L. Corless, G. D. Demetri et al., "Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor," *Journal of Clinical Oncology*, vol. 21, no. 23, pp. 4342–4349, 2003.

[76] J. A. Engelman and J. Settleman, "Acquired resistance to tyrosine kinase inhibitors during cancer therapy," *Current Opinion in Genetics and Development*, vol. 18, no. 1, pp. 73–79, 2008.

[77] S. Yano, W. Wang, Q. Li et al., "Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations," *Cancer Research*, vol. 68, no. 22, pp. 9479–9487, 2008.

[78] L. Ellis and D. J. Hicklin, "Pathways mediating resistance to vascular endothelial growth factor-targeted therapy," *Clinical Cancer Research*, vol. 14, no. 20, pp. 6371–6375, 2008.

[79] J. M. Stommel, A. C. Kimmelman, H. Ying et al., "Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies," *Science*, vol. 318, no. 5848, pp. 287–290, 2007.

[80] L. Whitesell and S. L. Lindquist, "HSP90 and the chaperoning of cancer," *Nature Reviews Cancer*, vol. 5, no. 10, pp. 761–772, 2005.

[81] D. George, "Platelet-derived growth factor receptors: a therapeutic target in solid tumors," *Seminars in Oncology*, vol. 28, no. 5, pp. 27–33, 2001.

[82] D. Wang, H.-J. S. Huang, A. Kazlauskas, and W. K. Cavenee, "Induction of vascular endothelial growth factor expression in endothelial cells by platelet-derived growth factor through the activation of phosphatidylinositol 3-kinase," *Cancer Research*, vol. 59, no. 7, pp. 1464–1472, 1999.

[83] L. J. Nissen, R. Cao, E.-M. Hedlund et al., "Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis," *Journal of Clinical Investigation*, vol. 117, no. 10, pp. 2766–2777, 2007.

[84] R. Cao, A. Eriksson, H. Kubo, K. Alitalo, Y. Cao, and J. Thyberg, "Comparative evaluation of FGF-2-, VEGF-A-, and VEGF-C-induced angiogenesis lymphangiogenesis, vascular fenestrations, and permeability," *Circulation Research*, vol. 94, no. 5, pp. 664–670, 2004.

[85] P. A. Jänne, N. Gray, and J. Settleman, "Factors underlying sensitivity of cancers to small-molecule kinase inhibitors," *Nature Reviews Drug Discovery*, vol. 8, no. 9, pp. 709–723, 2009.

[86] D. J. George, "Phase 2 studies of sunitinib and AG013736 in patients with cytokine-refractory renal cell carcinoma," *Clinical Cancer Research*, vol. 13, no. 2, part 2, pp. 753–757, 2007.

[87] S. Pennacchietti, P. Michieli, M. Galluzzo, M. Mazzone, S. Giordano, and P. M. Comoglio, "Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene," *Cancer Cell*, vol. 3, no. 4, pp. 347–361, 2003.

[88] J. Wojta, C. Kaun, J. M. Breuss et al., "Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells," *Laboratory Investigation*, vol. 79, no. 4, pp. 427–438, 1999.

[89] F. Koga, S. Tsutsumi, and L. M. Neckers, "Low dose geldanamycin inhibits hepatocyte growth factor and hypoxia-stimulated invasion of cancer cells," *Cell Cycle*, vol. 6, no. 11, pp. 1393–1402, 2007.

[90] A. M. Petit, J. Rak, M.-C. Hung et al., "Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors," *American Journal of Pathology*, vol. 151, no. 6, pp. 1523–1530, 1997.

[91] Y. M. Lee, M. H. Bae, O. H. Lee et al., "Synergistic induction of in vivo angiogenesis by the combination of insulin-like growth factor-II and epidermal growth factor," *Oncology Reports*, vol. 12, no. 4, pp. 843–848, 2004.

[92] S. Wang, I. Pashtan, S. Tsutsumi, W. Xu, and L. Neckers, "Cancer cells harboring MET gene amplification activate alternative signaling pathways to escape MET inhibition but remain sensitive to Hsp90 inhibitors," *Cell Cycle*, vol. 8, no. 13, pp. 2050–2056, 2009.

[93] R. Sordella, D. W. Bell, D. A. Haber, and J. Settleman, "Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways," *Science*, vol. 305, no. 5687, pp. 1163–1167, 2004.

[94] J. G. Paez, P. A. Jänne, J. C. Lee et al., "EGFR mutations in lung, cancer: Correlation with clinical response to gefitinib therapy," *Science*, vol. 304, no. 5676, pp. 1497–1500, 2004.

[95] E. R. Camp, J. Summy, T. W. Bauer, W. Liu, G. E. Gallick, and L. M. Ellis, "Molecular mechanisms of resistance to therapies targeting the epidermal growth factor receptor," *Clinical Cancer Research*, vol. 11, no. 1, pp. 397–405, 2005.

[96] I. R. Hutcheson, J. M. Knowlden, H. E. Jones et al., "Inductive mechanisms limiting response to anti-epidermal growth factor receptor therapy," *Endocrine-Related Cancer*, vol. 13, supplement 1, pp. 89–97, 2006.

[97] M. Guix, A. C. Faber, S. E. Wang et al., "Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins," *Journal of Clinical Investigation*, vol. 118, no. 7, pp. 2609–2619, 2008.

[98] A. C. Faber, D. Li, Y. Song et al., "Differential induction of apoptosis in HER2 and EGFR addicted cancers following PI3K inhibition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 46, pp. 19503–19508, 2010.

[99] C. Moser, P. Schachtschneider, S. A. Lang et al., "Inhibition of insulin-like growth factor-I receptor (IGF-IR) using NVP-AEW541, a small molecule kinase inhibitor, reduces orthotopic pancreatic cancer growth and angiogenesis," *European Journal of Cancer*, vol. 44, no. 11, pp. 1577–1586, 2008.

[100] C. P. Webb, C. D. Hose, S. Koochekpour et al., "The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network," *Cancer Research*, vol. 60, no. 2, pp. 342–349, 2000.

[101] D. Matei, M. Satpathy, L. Cao, Y.-C. Lai, H. Nakshatri, and D. B. Donner, "The platelet-derived growth factor receptor α is destabilized by geldanamycins in cancer cells," *Journal of Biological Chemistry*, vol. 282, no. 1, pp. 445–453, 2007.

[102] F. F. Zheng, S. D. Kuduk, G. Chiosis et al., "Identification of a geldanamycin dimer that induces the selective degradation of HER-family tyrosine kinases," *Cancer Research*, vol. 60, no. 8, pp. 2090–2094, 2000.

[103] S. J. Lavictoire, D. A. E. Parolin, A. C. Klimowicz, J. F. Kelly, and I. A. J. Lorimer, "Interaction of Hsp90 with the nascent form of the mutant epidermal growth factor receptor EGFRvIII," *Journal of Biological Chemistry*, vol. 278, no. 7, pp. 5292–5299, 2003.

[104] T. Shimamura, A. M. Lowell, J. A. Engelman, and G. I. Shapiro, "Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins," *Cancer Research*, vol. 65, no. 14, pp. 6401–6408, 2005.

[105] S. Modi, A. T. Stopeck, M. S. Gordon et al., "Combination of trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2-overexpressing breast cancer: a phase I dose-escalation study," *Journal of Clinical Oncology*, vol. 25, no. 34, pp. 5410–5417, 2007.

[106] H.-J. Ochel, T. W. Schulte, P. Nguyen, J. Trepel, and L. Neckers, "The benzoquinone ansamycin geldanamycin stimulates proteolytic degradation of focal adhesion kinase," *Molecular Genetics and Metabolism*, vol. 66, no. 1, pp. 24–30, 1999.

[107] T. W. Schulte, M. V. Blagosklonny, L. Romanova et al., "Destabilization of Raf-1 by geldanamycin leads to disruption of the Raf- 1-MEK-mitogen-activated protein kinase signalling pathway," *Molecular and Cellular Biology*, vol. 16, no. 10, pp. 5839–5845, 1996.

[108] D. B. Solit, A. D. Basso, A. B. Olshen, H. I. Scher, and N. Rosen, "Inhibition of heat shock protein 90 function downregulates Akt kinase and sensitizes tumors to Taxol," *Cancer Research*, vol. 63, no. 9, pp. 2139–2144, 2003.

[109] L. K. Francis, Y. Alsayed, X. Leleu et al., "Combination mammalian target of rapamycin inhibitor rapamycin and HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin has synergistic activity in multiple myeloma," *Clinical Cancer Research*, vol. 12, no. 22, pp. 6826–6835, 2006.

[110] D. R. Premkumar, B. Arnold, and I. F. Pollack, "Cooperative inhibitory effect of ZD1839 (Iressa) in combination with 17-AAG on glioma cell growth," *Molecular Carcinogenesis*, vol. 45, no. 5, pp. 288–301, 2006.

[111] M. A. Park, G. Zhang, C. Mitchell et al., "Mitogen-activated protein kinase kinase 1/2 inhibitors and 17-allylamino-17-demethoxygeldanamycin synergize to kill human gastrointestinal tumor cells in vitro via suppression of c-FLIP-s levels and activation of CD95," *Molecular Cancer Therapeutics*, vol. 7, no. 9, pp. 2633–2648, 2008.

[112] J. S. Abramson, W. Chen, P. Juszczynski et al., "The heat shock protein 90 inhibitor IPI-504 induces apoptosis of AKT-dependent diffuse large B-cell lymphomas," *British Journal of Haematology*, vol. 144, no. 3, pp. 358–366, 2009.

[113] E. B. Pasquale, "Eph-ephrin bidirectional signaling in physiology and disease," *Cell*, vol. 133, no. 1, pp. 38–52, 2008.

[114] D. M. Brantley-Sieders, J. Caughron, D. Hicks, A. Pozzi, J. C. Ruiz, and J. Chen, "EphA2 receptor tyrosine kinase regulates endothelial cell migration and vascular assembly through phosphoinositide 3-kinase-mediated Rac1 GTPase activation," *Journal of Cell Science*, vol. 117, no. 10, pp. 2037–2049, 2004.

[115] D. M. Brantley-Sieders, G. Zhuang, D. Hicks et al., "The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling," *Journal of Clinical Investigation*, vol. 118, no. 1, pp. 64–78, 2008.

[116] D. M. Brantley-Sieders and J. Chen, "Eph receptor tyrosine kinases in angiogenesis: from development to disease," *Angiogenesis*, vol. 7, no. 1, pp. 17–28, 2004.

[117] M. Héroult, F. Schaffner, and H. G. Augustin, "Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progression," *Experimental Cell Research*, vol. 312, no. 5, pp. 642–650, 2006.

[118] N. K. Noren and E. B. Pasquale, "Paradoxes of the EphB4 receptor in cancer," *Cancer Research*, vol. 67, no. 9, pp. 3994–3997, 2007.

[119] J. Wykosky, D. M. Gibo, C. Stanton, and W. Debinski, "EphA2 as a novel molecular marker and target in glioblastoma multiforme," *Molecular Cancer Research*, vol. 3, no. 10, pp. 541–551, 2005.

[120] H. Miao, D.-Q. Li, A. Mukherjee et al., "EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt," *Cancer Cell*, vol. 16, no. 1, pp. 9–20, 2009.

[121] C. N. Landen Jr., A. Chavez-Reyes, C. Bucana et al., "Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery," *Cancer Research*, vol. 65, no. 15, pp. 6910–6918, 2005.

[122] C. N. Landen, C. Lu, L. Y. Han et al., "Efficacy and antivascular effects of EphA2 reduction with an agonistic antibody in ovarian cancer," *Journal of the National Cancer Institute*, vol. 98, no. 21, pp. 1558–1570, 2006.

[123] B. Annamalai, X. Liu, U. Gopal, and J. S. Isaacs, "Hsp90 is an essential regulator of EphA2 receptor stability and signaling: implications for cancer cell migration and metastasis," *Molecular Cancer Research*, vol. 7, no. 7, pp. 1021–1032, 2009.

[124] M. Macrae, R. M. Neve, P. Rodriguez-Viciana et al., "A conditional feedback loop regulates Ras activity through EphA2," *Cancer Cell*, vol. 8, no. 2, pp. 111–118, 2005.

[125] A. B. Larsen, M. W. Pedersen, M.-T. Stockhausen, M. V. Grandal, B. Van Deurs, and H. S. Poulsen, "Activation of the EGFR gene target EphA2 inhibits epidermal growth factor-induced cancer cell motility," *Molecular Cancer Research*, vol. 5, no. 3, pp. 283–293, 2007.

[126] D. M. Brantley-Sieders, W. B. Fang, Y. Hwang, D. Hicks, and J. Chen, "Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism mediated by EphA receptor and vascular endothelial growth factor in mice," *Cancer Research*, vol. 66, no. 21, pp. 10315–10324, 2006.

[127] Y. R. Chin and A. Toker, "Function of Akt/PKB signaling to cell motility, invasion and the tumor stroma in cancer," *Cellular Signalling*, vol. 21, no. 4, pp. 470–476, 2009.

[128] S. Chandarlapaty, A. Sawai, Q. Ye et al., "SNX2112, a synthetic heat shock protein 90 inhibitor, has potent antitumor activity against HER kinase—dependent cancers," *Clinical Cancer Research*, vol. 14, no. 1, pp. 240–248, 2008.

[129] A. Franovic, C. E. Holterman, J. Payette, and S. Lee, "Human cancers converge at the HIF-2α oncogenic axis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 50, pp. 21306–21311, 2009.

[130] K. Burkitt, S. Y. Chun, D. T. Dang, and L. H. Dang, "Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment," *Molecular Cancer Therapeutics*, vol. 8, no. 5, pp. 1148–1156, 2009.

[131] E. I. Heath, D. W. Hillman, U. Vaishampayan et al., "A phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with hormone-refractory metastatic prostate cancer," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7940–7946, 2008.

[132] E. A. Ronnen, G. V. Kondagunta, N. Ishill et al., "A phase II trial of 17-(Allylamino)-17-demethoxygeldanamycin in patients with papillary and clear cell renal cell carcinoma," *Investigational New Drugs*, vol. 24, no. 6, pp. 543–546, 2006.

[133] L. R. Kelland, S. Y. Sharp, P. M. Rogers, T. G. Myers, and P. Workman, "DT-diaphorase expression and tumor cell sensitivity to 17-allylamino,17- demethoxygeldanamycin, an inhibitor of heat shock protein 90," *Journal of the National Cancer Institute*, vol. 91, no. 22, pp. 1940–1949, 1999.

[134] N. Gaspar, S. Y. Sharp, S. Pacey et al., "Acquired resistance to 17-allylamino-17-demethoxygeldanamycin (17-AAG, tanespimycin) in glioblastoma cells," *Cancer Research*, vol. 69, no. 5, pp. 1966–1975, 2009.

[135] W. Guo, P. Reigan, D. Siegel, J. Zirrolli, D. Gustafson, and D. Ross, "Formation of 17-allylamino-demethoxygeldanamycin (17-AAG) hydroquinone by NAD(P)H:quinone oxidoreductase 1: role of 17-AAG hydroquinone in heat shock protein 90 inhibition," *Cancer Research*, vol. 65, no. 21, pp. 10006–10015, 2005.

[136] W. Guo, P. Reigan, D. Siegel, and D. Ross, "Enzymatic reduction and glutathione conjugation of benzoquinone ansamycin heat shock protein 90 inhibitors: relevance for toxicity and mechanism of action," *Drug Metabolism and Disposition*, vol. 36, no. 10, pp. 2050–2057, 2008.

[137] G. Chiosis, B. Lucas, A. Shtil, H. Huezo, and N. Rosen, "Development of a purine-scaffold novel class of hsp90 binders that inhibit the proliferation of cancer cells and induce the degradation of Her2 tyrosine kinase," *Bioorganic and Medicinal Chemistry*, vol. 10, no. 11, pp. 3555–3564, 2002.

[138] D. B. Solit and G. Chiosis, "Development and application of Hsp90 inhibitors," *Drug Discovery Today*, vol. 13, no. 1-2, pp. 38–43, 2008.

[139] S. A. Eccles, A. Massey, F. I. Raynaud et al., "NVP-AUY922: a novel heat shock protein 90 inhibitor active against xenograft tumor growth, angiogenesis, and metastasis," *Cancer Research*, vol. 68, no. 8, pp. 2850–2860, 2008.

[140] R. Bagatell, G. D. Paine-Murrieta, C. W. Taylor et al., "Induction of a heat shock factor 1-dependent stress response alters the cytotoxic activity of Hsp90-binding agents," *Clinical Cancer Research*, vol. 6, no. 8, pp. 3312–3318, 2000.

[141] A. K. McCollum, C. J. TenEyck, B. M. Sauer, D. O. Toft, and C. Erlichman, "Up-regulation of heat shock protein 27 induces resistance to 17-allylamino-demethoxygeldanamycin through a glutathione-mediated mechanism," *Cancer Research*, vol. 66, no. 22, pp. 10967–10975, 2006.

[142] A. K. McCollum, C. J. TenEyck, B. Stensgard et al., "P-glycoprotein-mediated resistance to Hsp90-directed therapy is eclipsed by the heat shock response," *Cancer Research*, vol. 68, no. 18, pp. 7419–7427, 2008.

[143] M. V. Powers, P. A. Clarke, and P. Workman, "Dual targeting of HSC70 and HSP72 inhibits HSP90 function and induces tumor-specific apoptosis," *Cancer Cell*, vol. 14, no. 3, pp. 250–262, 2008.

[144] C. Dai, L. Whitesell, A. B. Rogers, and S. Lindquist, "Heat shock factor 1 is a powerful multifaceted modifier of carcinogenesis," *Cell*, vol. 130, no. 6, pp. 1005–1018, 2007.

[145] A. K. McCollum, K. B. Lukasiewicz, C. J. TenEyck, W. L. Lingle, D. O. Toft, and C. Erlichman, "Cisplatin abrogates the geldanamycin-induced heat shock response," *Molecular Cancer Therapeutics*, vol. 7, no. 10, pp. 3256–3264, 2008.

[146] J. H. Baek, Y. V. Liu, K. R. McDonald, J. B. Wesley, H. Zhang, and G. L. Semenza, "Spermidine/spermine N1-acetyltransferase-1 binds to hypoxia-inducible factor-1α (HIF-1α) and RACK1 and promotes ubiquitination and degradation of HIF-1α," *Journal of Biological Chemistry*, vol. 282, no. 46, pp. 33358–33366, 2007.

[147] Y. V. Liu, M. E. Hubbi, F. Pan et al., "Calcineurin promotes hypoxia-inducible factor 1α expression by dephosphorylating RACK1 and blocking RACK1 dimerization," *Journal of Biological Chemistry*, vol. 282, no. 51, pp. 37064–37073, 2007.

[148] E. S. Ehrlich, T. Wang, K. Luo et al., "Regulation of Hsp90 client proteins by a Cullin5-RING E3 ubiquitin ligase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 48, pp. 20330–20335, 2010.

[149] M. J. Fay, K. A. Longo, G. A. Karathanasis et al., "Analysis of CUL-5 expression in breast epithelial cells, breast cancer cell lines, normal tissues and tumor tissues," *Molecular Cancer*, vol. 2, p. 40, 2003.

[150] C. Morrissey and R. L. Vessella, "The role of tumor microenvironment in prostate cancer bone metastasis," *Journal of Cellular Biochemistry*, vol. 101, no. 4, pp. 873–886, 2007.

[151] A. Orimo, P. B. Gupta, D. C. Sgroi et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[152] P. A. Kenny, G. Y. Lee, and M. J. Bissell, "Targeting the tumor microenvironment," *Frontiers in Bioscience*, vol. 12, pp. 3468–3474, 2007.

[153] A. F. Olumi, P. Dazin, and T. D. Tlsty, "A novel coculture technique demonstrates that normal human prostatic fibroblasts contribute to tumor formation of LNCaP cells by retarding cell death," *Cancer Research*, vol. 58, no. 20, pp. 4525–4530, 1998.

[154] J. A. Tuxhorn, G. E. Ayala, M. J. Smith, V. C. Smith, T. D. Dang, and D. R. Rowley, "Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling," *Clinical Cancer Research*, vol. 8, no. 9, pp. 2912–2923, 2002.

[155] J. A. Tuxhorn, S. J. McAlhany, T. D. Dang, G. E. Ayala, and D. R. Rowley, "Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model," *Cancer Research*, vol. 62, no. 11, pp. 3298–3307, 2002.

[156] S. W. Hayward, Y. Wang, M. Cao et al., "Malignant transformation in a nontumorigenic human prostatic epithelial cell line," *Cancer Research*, vol. 61, no. 22, pp. 8135–8142, 2001.

[157] M. Hu, J. Yao, D. K. Carroll et al., "Regulation of in situ to invasive breast carcinoma transition," *Cancer Cell*, vol. 13, no. 5, pp. 394–406, 2008.

[158] M. Erkan, C. Reiser-Erkan, C. W. Michalski et al., "Cancer-stellate cell interactions perpetuate the hypoxia-fibrosis cycle in pancreatic ductal adenocarcinoma," *Neoplasia*, vol. 11, no. 5, pp. 497–508, 2009.

[159] Y.-F. Shi, C.-C. Fong, Q. Zhang et al., "Hypoxia induces the activation of human hepatic stellate cells LX-2 through TGF-β signaling pathway," *FEBS Letters*, vol. 581, no. 2, pp. 203–210, 2007.

[160] A. Masamune, K. Kikuta, T. Watanabe, K. Satoh, M. Hirota, and T. Shimosegawa, "Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer," *American Journal of Physiology*, vol. 295, no. 4, pp. G709–G717, 2008.

[161] J. A. Joyce, "Therapeutic targeting of the tumor microenvironment," *Cancer Cell*, vol. 7, no. 6, pp. 513–520, 2005.

[162] Y. Liu, X. M. Wen, E. L. H. Lui et al., "Therapeutic targeting of the PDGF and TGF-β-signaling pathways in hepatic stellate cells by PTK787/ZK22258," *Laboratory Investigation*, vol. 89, no. 10, pp. 1152–1160, 2009.

[163] Y. Liu, E. L. H. Lui, S. L. Friedman et al., "PTK787/ZK22258 attenuates stellate cell activation and hepatic fibrosis in vivo by inhibiting VEGF signaling," *Laboratory Investigation*, vol. 89, no. 2, pp. 209–221, 2009.

[164] S. Tugues, G. Fernandez-Varo, J. Muñoz-Luque et al., "Antiangiogenic treatment with sunitinib ameliorates inflammatory infiltrate, fibrosis, and portal pressure in cirrhotic rats," *Hepatology*, vol. 46, no. 6, pp. 1919–1926, 2007.

[165] V. Gioni, T. Karampinas, G. Voutsinas et al., "Imatinib mesylate inhibits proliferation and exerts an antifibrotic effect in human breast stroma fibroblasts," *Molecular Cancer Research*, vol. 6, no. 5, pp. 706–714, 2008.

[166] X. Guo, H. Oshima, T. Kitmura, M. M. Taketo, and M. Oshima, "Stromal fibroblasts activated by tumor cells promote angiogenesis in mouse gastric cancer," *Journal of Biological Chemistry*, vol. 283, no. 28, pp. 19864–19871, 2008.

[167] S. J. Myung, J.-H. Yoon, B. H. Kim, J.-H. Lee, E. U. Jung, and H.-S. Lee, "Heat shock protein 90 inhibitor induces apoptosis and attenuates activation of hepatic stellate cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 330, no. 1, pp. 276–282, 2009.

[168] G. Bergers and S. Song, "The role of pericytes in blood-vessel formation and maintenance," *Neuro-Oncology*, vol. 7, no. 4, pp. 452–464, 2005.

[169] N. Reinmuth, W. Liu, Y. D. Jung et al., "Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival," *FASEB Journal*, vol. 15, no. 7, pp. 1239–1241, 2001.

[170] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[171] K. Pietras, J. Pahler, G. Bergers, and D. Hanahan, "Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting," *PLoS Medicine*, vol. 5, no. 1, article e19, 2008.

[172] A. Kamal, L. Thao, J. Sensintaffar et al., "A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors," *Nature*, vol. 425, no. 6956, pp. 407–410, 2003.

[173] G. Chiosis, H. Huezo, N. Rosen, E. Mimnaugh, L. Whitesell, and L. Neckers, "17AAG: low target binding affinity and potent cell activity–finding an explanation," *Molecular Cancer Therapeutics*, vol. 2, no. 2, pp. 123–129, 2003.

[174] D. C. Radisky, "Fibroblasts act as co-conspirators for chemotherapy resistance," *Cancer Biology and Therapy*, vol. 7, no. 9, pp. 1348–1349, 2008.

[175] Y. Crawford, I. Kasman, L. Yu et al., "PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment," *Cancer Cell*, vol. 15, no. 1, pp. 21–34, 2009.

[176] J. J. Virrey, E. B. Golden, W. Sivakumar et al., "Glioma-associated endothelial cells are chemoresistant to temozolomide," *Journal of Neuro-Oncology*, vol. 95, no. 1, pp. 13–22, 2009.

[177] G. Bergers, S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan, "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1287–1295, 2003.

[178] K. E. Hovinga, L. J. Stalpers, C. van Bree, et al., "Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines–a clue to radioresistance?" *Journal of Neuro-Oncology*, vol. 74, no. 2, pp. 99–103, 2005.

[179] L. W. Chung, W. C. Huang, S. Y. Sung et al., "Stromal-epithelial interaction in prostate cancer progression," *Clinical Genitourinary Cancer*, vol. 5, no. 2, pp. 162–170, 2006.

[180] N. Kamochi, M. Nakashima, S. Aoki et al., "Irradiated fibroblast-induced bystander effects on invasive growth of squamous cell carcinoma under cancer-stromal cell interaction," *Cancer Science*, vol. 99, no. 12, pp. 2417–2427, 2008.

[181] B. J. Moeller, Y. Cao, C. Y. Li, and M. W. Dewhirst, "Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules," *Cancer Cell*, vol. 5, no. 5, pp. 429–441, 2004.

[182] B. J. Moeller, M. R. Dreher, Z. N. Rabbani et al., "Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity," *Cancer Cell*, vol. 8, no. 2, pp. 99–110, 2005.

[183] J. Brieger, J. Kattwinkel, M. Berres, J. Gosepath, and W. J. Mann, "Impact of vascular endothelial growth factor release on radiation resistance," *Oncology Reports*, vol. 18, no. 6, pp. 1597–1601, 2007.

[184] R. F. Hwang, T. Moore, T. Arumugam et al., "Cancer-associated stromal fibroblasts promote pancreatic tumor progression," *Cancer Research*, vol. 68, no. 3, pp. 918–926, 2008.

[185] K. K. C. Tsai, J. Stuart, Y.-Y.E. Chuang, J. B. Little, and Z.-M. Yuan, "Low-dose radiation-induced senescent stromal fibroblasts render nearby breast cancer cells radioresistant," *Radiation Research*, vol. 172, no. 3, pp. 306–313, 2009.

[186] M. Li, G. Ping, C. Plathow et al., "Small molecule receptor tyrosine kinase inhibitor of platelet-derived growth factor signaling (SU9518) modifies radiation response in fibroblasts and endothelial cells," *BMC Cancer*, vol. 6, article 79, 2006.

[187] K. S. Bisht, C. M. Bradbury, D. Mattson et al., "Geldanamycin and 17-allylamino-17-demethoxygeldanamycin potentiate the in vitro and in vivo radiation response of cervical tumor cells via the heat shock protein 90-mediated intracellular signaling and cytotoxicity," *Cancer Research*, vol. 63, no. 24, pp. 8984–8995, 2003.

[188] J. S. Russell, W. Burgan, K. A. Oswald, K. Camphausen, and P. J. Tofilon, "Enhanced cell killing induced by the combination of radiation and the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin: a multitarget approach to radiosensitization," *Clinical Cancer Research*, vol. 9, no. 10, pp. 3749–3755, 2003.

[189] X. Yin, H. Zhang, K. Lundgren, L. Wilson, F. Burrows, and C. G. Shores, "BIIB021, a novel Hsp90 inhibitor, sensitizes head and neck squamous cell carcinoma to radiotherapy," *International Journal of Cancer*, vol. 126, no. 5, pp. 1216–1225, 2010.

[190] D. L. Schwartz, G. Powis, A. Thitai-Kumar et al., "The selective hypoxia inducible factor-1 inhibitor PX-478 provides in vivo radiosensitization through tumor stromal effects," *Molecular Cancer Therapeutics*, vol. 8, no. 4, pp. 947–958, 2009.

[191] A. E. Kabakov, Y. M. Makarova, and Y. V. Malyutina, "Radiosensitization of human vascular endothelial cells through Hsp90 inhibition with 17-N-allilamino-17-demethoxygeldanamycin," *International Journal of Radiation Oncology Biology Physics*, vol. 71, no. 3, pp. 858–865, 2008.

[192] F. Winkler, S. V. Kozin, R. T. Tong et al., "Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases," *Cancer Cell*, vol. 6, no. 6, pp. 553–563, 2004.

[193] P. J. Mishra, P. J. Mishra, R. Humeniuk et al., "Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells," *Cancer Research*, vol. 68, no. 11, pp. 4331–4339, 2008.

[194] R. S. Kerbel, R. Benezra, D. C. Lyden et al., "Endothelial progenitor cells are cellular hubs essential for neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition but not adenomas," *Proceedings of the National Academy of*

Sciences of the United States of America, vol. 105, no. 34, p. E54, 2008, author reply E55.

[195] D. K. Jin, K. Shido, H.-G. Kopp et al., "Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes," *Nature Medicine*, vol. 12, no. 5, pp. 557–567, 2006.

[196] S. Rey, K. Lee, C. J. Wang et al., "Synergistic effect of HIF-1α gene therapy and HIF-1-activated bone marrow-derived angiogenic cells in a mouse model of limb ischemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 48, pp. 20399–20404, 2010.

[197] G. Tabatabai, B. Frank, R. Möhle, M. Weller, and W. Wick, "Irradiation and hypoxia promote homing of haematopoietic progenitor cells towards gliomas by TGF-β-dependent HIF-1α-mediated induction of CXCL12," *Brain*, vol. 129, no. 9, pp. 2426–2435, 2006.

[198] M. Pàez-Ribes, E. Allen, J. Hudock et al., "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[199] F. Shojaei, X. Wu, A. K. Malik et al., "Tumor refractoriness to anti-VEGF treatment is mediated by $CD11b^{+}Gr1^{+}$ myeloid cells," *Nature Biotechnology*, vol. 25, no. 8, pp. 911–920, 2007.

[200] R. Du, K. V. Lu, C. Petritsch et al., "HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion," *Cancer Cell*, vol. 13, no. 3, pp. 206–220, 2008.

[201] F. Shojaei, X. Wu, C. Zhong et al., "Bv8 regulates myeloid-cell-dependent tumour angiogenesis," *Nature*, vol. 450, no. 7171, pp. 825–831, 2007.

[202] B. Weigelt, A. T. Lo, C. C. Park, J. W. Gray, and M. J. Bissell, "HER2 signaling pathway activation and response of breast cancer cells to HER2-targeting agents is dependent strongly on the 3D microenvironment," *Breast Cancer Research and Treatment*, vol. 122, no. 1, pp. 35–43, 2010.

[203] A. Mammoto, K. M. Connor, T. Mammoto et al., "A mechanosensitive transcriptional mechanism that controls angiogenesis," *Nature*, vol. 457, no. 7233, pp. 1103–1108, 2009.

[204] O. Ayrault, M. D. Godeny, C. Dillon et al., "Inhibition of Hsp90 via 17-DMAG induces apoptosis in a p53-dependent manner to prevent medulloblastoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 40, pp. 17037–17042, 2009.

[205] E. Caldas-Lopes, L. Cerchietti, J. H. Ahn et al., "Hsp90 inhibitor PU-H71, a multimodal inhibitor of malignancy, induces complete responses in triple-negative breast cancer models," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 20, pp. 8368–8373, 2009.

[206] E. D. Pleasance, R. K. Cheetham, P. J. Stephens et al., "A comprehensive catalogue of somatic mutations from a human cancer genome," *Nature*, vol. 463, no. 7278, pp. 191–196, 2010.

[207] E. D. Pleasance, P. J. Stephens, S. O'Meara et al., "A small-cell lung cancer genome with complex signatures of tobacco exposure," *Nature*, vol. 463, no. 7278, pp. 184–190, 2010.

[208] W. Arap, M. G. Kolonin, M. Trepel et al., "Steps toward mapping the human vasculature by phage display," *Nature Medicine*, vol. 8, no. 2, pp. 121–127, 2002.

[209] J. A. Joyce, P. Laakkonen, M. Bernasconi, G. Bergers, E. Ruoslahti, and D. Hanahan, "Stage-specific vascular markers revealed by phage display in a mouse model of pancreatic islet tumorigenesis," *Cancer Cell*, vol. 4, no. 5, pp. 393–403, 2003.

[210] J. A. Hoffman, E. Giraudo, M. Singh et al., "Progressive vascular changes in a transgenic mouse model of squamous cell carcinoma," *Cancer Cell*, vol. 4, no. 5, pp. 383–391, 2003.

[211] P. Olson, J. Lu, H. Zhang et al., "MicroRNA dynamics in the stages of tumorigenesis correlate with hallmark capabilities of cancer," *Genes and Development*, vol. 23, no. 18, pp. 2152–2165, 2009.

[212] D. Song, R. Chaerkady, A. C. Tan et al., "Antitumor activity and molecular effects of the novel heat shock protein 90 inhibitor, IPI-504, in pancreatic cancer," *Molecular Cancer Therapeutics*, vol. 7, no. 10, pp. 3275–3284, 2008.

[213] E. A. Eisenhauer, P. Therasse, J. Bogaerts et al., "New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1)," *European Journal of Cancer*, vol. 45, no. 2, pp. 228–247, 2009.

[214] D. B. Solit, I. Osman, D. Polsky et al., "Phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with metastatic melanoma," *Clinical Cancer Research*, vol. 14, no. 24, pp. 8302–8307, 2008.

[215] W. Cai, A. Ebrahimnejad, K. Chen et al., "Quantitative radioimmunoPET imaging of EphA2 in tumor-bearing mice," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 34, no. 12, pp. 2024–2036, 2007.

[216] P. M. Smith-Jones, D. B. Solit, T. Akhurst, F. Afroze, N. Rosen, and S. M. Larson, "Imaging the pharmacodynamics of HER2 degradation in response to Hsp90 inhibitors," *Nature Biotechnology*, vol. 22, no. 6, pp. 701–706, 2004.

[217] T. T. Batchelor, A. G. Sorensen, E. di Tomaso et al., "AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients," *Cancer Cell*, vol. 11, no. 1, pp. 83–95, 2007.

[218] L. Xu, D. G. Duda, E. di Tomaso, et al., "Direct evidence that bevacizumab, an anti-VEGF antibody, up-regulates SDF1alpha, CXCR4, CXCL6, and neuropilin 1 in tumors from patients with rectal cancer," *Cancer Research*, vol. 69, no. 20, pp. 7905–7910, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 767384, 6 pages
doi:10.1155/2010/767384

Review Article

The Role of Ets Factors in Tumor Angiogenesis

Peter Oettgen

Division of Cardiology, and Molecular and Vascular Biology, Department of Medicine, Research North RM 270D, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02115, USA

Correspondence should be addressed to Peter Oettgen, joettgen@bidmc.harvard.edu

Received 20 October 2009; Revised 6 February 2010; Accepted 2 March 2010

Academic Editor: Debabrata Mukhopadhyay

Angiogenesis is a critical component of tumor growth. A number of growth factors, including VEGF, FGF, and HGF, have been implicated as angiogenic growth factors that promote tumor angiogenesis in different types of cancer. Ets-1 is the prototypic member of the Ets transcription factor family. Ets-1 is known to be a downstream mediator of angiogenic growth factors. Expression of Ets-1 in a variety of different tumors is associated with increased angiogenesis. A role for other selected members of the Ets transcription factor family has also been shown to be important for the development of tumor angiogenesis. Because Ets factors also express a number of other important genes involved in cell growth, they contribute not only to tumor growth, but to disease progression. Targeting Ets factors in mouse tumor models through the use of dominant-negative Ets proteins or membrane permeable peptides directed at competitively inhibiting the DNA binding domain has now demonstrated the therapeutic potential of inhibiting selected Ets transcription factors to limit tumor growth and disease progression.

Ets-1 is the prototypic member of the Ets transcription factor family [1]. Several studies have demonstrated a role for Ets transcription factors in the regulation of endothelial-specific genes including VEGF-R1, VEGF-R2, Tie1, and Tie2. Whereas, the Ets factors Ets-1 and Ets-2 potently transactivate the Flt-1 gene promoter, they do not appear to regulate the Tie1 or Tie2 gene promoters [2, 3]. Ets-1 has been shown to cooperate with HIF-2α in the setting of hypoxia to regulate the expression of the VEGF-R2 [4, 5]. In contrast, the Ets factor ELF-1 is a potent transactivator of the Tie1 and Tie2 genes [2, 3]. ELF-1 has been shown to regulate other genes involved in angiogenesis including angiopoietin-2 and endothelial nitric oxide synthase [6, 7]. Ets-2 regulates the expression of CD13/aminopeptidase N (APN) in human endothelial cells [8]. Knockdown of Ets-2 in human endothelial cells using siRNA oligonucleotides significantly reduced the ability of the endothelial cells to form tubes and capillary networks. The selectivity of the different Ets factors to transactivate different target genes also correlates with their ability to bind to specific conserved Ets binding sites within these genes. However, when the DNA binding domain is highly homologous, as is the case for Ets-1 and Ets-2, there may be a significant overlap with respect to their downstream target genes.

Ets factors can be subcategorized into subfamilies based on DNA and protein sequence homology. For example, Elf-1, Nerf-2, and Mef are highly related and belong to one subfamily. Similarly Fli1 and ERG constitute another subfamily, as do Ets1 and Ets2. The subfamily members often have overlapping functions [9]. From an evolutionary standpoint, this built-in redundancy may be protective against the effects of genetic mutations of the individual family members for critical developmental processes such as vasculogenesis. The DNA binding domain of the Ets family of proteins is a highly conserved region of approximately 85 residues that shares a strong sequence homology and three-dimensional structural scaffold that closely mimics the helix-turn-helix family of DNA binding proteins [10, 11]. Previous investigations have clearly established that the several highly conserved residues that are localized within the recognition helix, H3, are responsible for anchoring the Ets domain through requisite DNA contacts within the major groove. However, residues within the turns separating helices H2 and H3 and the first two β-strands are also involved in critical phosphate backbone contacts within the DNA minor groove.

Expressions of selected Ets factors are also enriched at sites of active blood vessel development during embryogenesis. The Ets transcription factor Fli-1 is enriched in

the developing blood vessels of zebrafish embryos [12]. ELF-1 is highly expressed in extra-embryonic and embryonic blood vessels of the developing mouse and chicken embryos [7, 13]. The Ets factor Ets-1 is also enriched in the developing blood vessels of the chicken, and antisense oligonucleotides have been shown to inhibit angiogenesis when delivered to the chicken chorioallantoic membrane [14]. Homozygous inactivation of Ets1 is associated with abnormalities in T cell function, but with no defects in vascular development or angiogenesis [15]. Similarly, while Ets2 is a critical regulator of trophoblast function during extraembryonic development it is dispensable for development of the embryo proper [16]. Mice with a homozygous hypomorphic mutant of Ets2, in which the conserved threonine 72 phosphorylation site is mutated, and Ets2$^{T72A/T72A}$ mice are viable and appear normal [17]. When both Ets-1 and Ets-2 were simultaneously targeted, this led to striking defects in vascular development [18].

In addition to their role in regulating endothelial cell restricted genes, Ets factors have also been shown to function upstream and downstream of a number of angiogenic growth factors [19]. Overexpression of Ets-1 in tissues is associated with increased expression of both vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF). Neutralizing antibodies to VEGF and HGF markedly attenuate the angiogenic effects of overexpressing Ets-1. Ets-1 regulates the expression of several downstream targets in endothelial cells that promote an angiogenic phenotype including the VEGF receptors (VEGF-R1 and VEGF-R2), urokinase, and several matrix metalloproteinase's (MMPs) including MMP-1, MMP-3, MMP-9 (Figure 1). Interestingly, HGF is also capable of inducing the expression of Ets-1, thereby creating a positive feedback loop at sites where either Ets-1 or HGF is expressed. In addition to HGF, fibroblast growth factor (FGF) and VEGF gene expression are regulated by Ets-1 [20, 21]. More recently, the chemokine CCL2 (MCP-1/JE) has been shown to promote angiogenesis and one of the main downstream effectors is Ets-1 [22]. The particular downstream targets identified in response to CCL2 in endothelial cells are the $\beta 3$ integrins. Ets-1 is also involved in the regulation of CCL2 expression [23].

Several studies support a role for Ets-1 expression in the development of tumor angiogenesis in many different types of human cancer. Increased expression of Ets-1 in tumors is often associated with a worse prognosis (summarized in Table 1). In ovarian cancer, for example, Ets-1 expression strongly correlates with the degree of angiogenesis in the primary tumor and the development of metastatic lesions [24]. The prognosis is also significantly worse in those patients with high levels of Ets-1 expression in the tissues derived from the primary cancer. The survival rate of patients with histologically demonstrated high levels of Ets-1 expression at 24 months was 30% whereas that of patients with low levels was 70% [25]. These statistics support the overall concept that Ets-1 expression in ovarian cancers contributes to tumor growth and progression that is at least in part mediated by an increase in the degree of angiogenesis. Additional factors promoting tumor growth and progression are discussed below.

Esophageal squamous cell carcinoma (ESCC) is associated with a late presentation due to limited symptoms early in the disease. However, once detected, ESCC is a highly aggressive tumor with a propensity to metastasize. Ets-1 is overexpressed in 44/55 (80%) of tumor tissues obtained from patients with ESCC [26]. VEGF expression was observed in most of the Ets-1 overexpressing tissues and was strongly correlated with lymphnode metastasis. Kaplan-Meier survival analysis for patients with concomitant tissue expression of Ets-1 and VEGF demonstrated a significantly worse disease free survival. This again supports the concept that increased angiogenesis in Ets-1 expressing tumors is associated with increased tumor progression and a worse prognosis.

VEGF and Ets-1 are also highly expressed in breast carcinoma [27]. Ets-1 is expressed in 53% of tumors of patients with newly diagnosed breast cancer. Ets-1 overexpression in breast cancer is associated with more invasive tumors and a significantly poorer prognosis [28]. At 5 years, less than 20% of patients with Ets-1 negative tumors have relapsed, compared to nearly 50% of those with Ets-1 expressing tumors. The expression of Ets-1 strongly correlated with the microvessel density within the tumors and lymph node involvement. In a study of 123 patients with primary breast cancer followed for 62 months, Ets-1 expression correlated strongly with VEGF expression and was an independent predictor of poor prognosis [28]. As a result of these findings, antiangiogenic therapy is currently being evaluated as adjunctive therapy for breast cancer.

In a study of 60 patients with uterine cancer a strong Ets-1 expression was observed in 66% of patients, and was associated with increased tumor microvessel density [34]. In patients with cervical carcinoma, high Ets-1 expression was observed in tissues obtained from 25/60 (42%) patients and was also associated with a high microvessels density [30]. Furthermore, the 24-month survival rate of patients with high ets-1 expression was significantly worse at 54% compared to 92% for those patients with low expression of Ets-1 in the tumors. These results also support a role for Ets-1 in tumor progression that is at least in part mediated by increased angiogenesis.

Ets-1 expression has also been shown to correlate with higher microvessel density and a worse prognosis in patients with gastric cancer [31]. Of the 102 patients with primary gastric carcinoma, not involving invasion into the muscularis propria or subserosa, Ets-1 expression was observed in 76% of patients. The 5-year survival rate for these patients was 67% for patients with Ets-1 positive tumors and 89% for those with Ets-1 negative tumors. Ets-1 expression has also been evaluated in patients with colorectal carcinoma [32]. High Ets-1 expression was observed in 48.4% of patients and correlated strongly with increased microvessel density and the expression of VEGF. Similar to the studies in uterine cancer, this study also supports a role for expression of Ets-1 in cancer progression that is at least in part mediated by increased angiogenesis.

The role of Ets-1 in human brain cancer has also been evaluated. Ets-1 expression was evaluated in 81 primary and 20 recurrent astrocytic tumors [33]. 65% of glioblastomas

Table 1: Summary of Ets-1 expression, microvessel density (MVD), and prognosis in human cancer.

Tumor type	Number	% Ets-1 positive*	MVD/VEGF	Prognosis**
(1) Ovarian [24]	30	66	increased MVD	2 years survival: 10% Ets-1 (+); 60% Ets-1 (−)
(2) Ovarian [25]	30	50	increased MVD	2 years survival: 30% Ets-1 (+); 70% Ets-1 (−)
(3) Esophageal [26]	55	80	increased MVD	not evaluated
(4) Breast [27]	48	71	increased MVD	not evaluated
(5) Breast [28]	123	62	not evaluated	5 years relapse free survival: 55% Ets-1 (+); 85% Ets-1 (−)
(6) Endometrial [29]	60	66	increased VEGF	not evaluated
(7) Cervical [30]	60	42	increased MVD	2 years survival: 54% Ets-1 (+); 92% Ets-1 (−)
(8) Gastric [31]	102	76	increased MVD	5 years survival: 67% Ets-1 (+); 89% Ets-1 (−)
(9) Colon [32]	95	48	increased MVD	not evaluated
(10) Brain [33]	101	32	not evaluated	not evaluated

"Number" refers to number of patients in the study.
*% refers to percent of primary tumors that express Ets-1.
**% refers to percent of patients that are relapse free at different time points with primary tumors that expressed Ets-1 (+) or did not express Ets-1 (−).
"MVD/VEGF" refers to evaluation of either VEGF expression or microvascular density (MVD) in the tumors

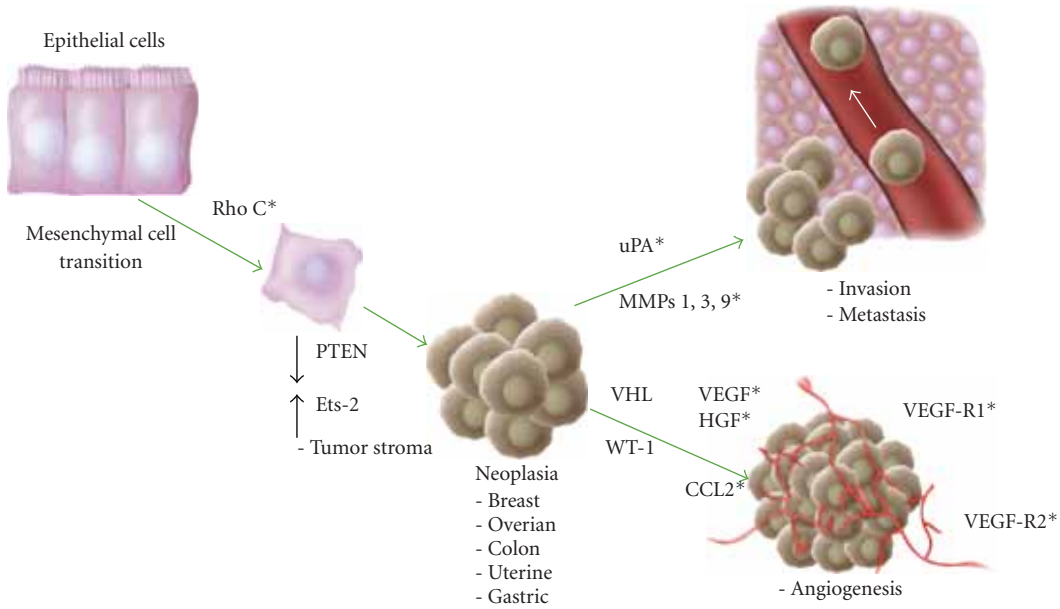


Figure 1: The role of Ets-1 in tumor development, progression, and angiogenesis. The role of Ets-1 is depicted during different stages of neoplasia from the epithelial-mesenchymal transition (EMT) to tumor growth, invasion, metastasis, and tumor angiogenesis. (∗) denotes those genes that are regulated by Ets-1.

(grade IV astrocytomas) stained for Ets-1, 25% of anaplastic astrocytomas (Grade III) were positive for Ets-1, and none of the low-grade astrocytomas (Grade II) stained positively for Ets-1. Therefore the expression of Ets-1 was significantly associated with the tumor grade. Normal brain tissue did not express significant amounts of Ets-1 protein. Ets-1 also appears to play a role in regulating tumor angiogenesis in neuroblastomas [35]. Ets-1 expression is higher in undifferentiated human neuroblastomas. Ets-1 expression is stimulated by gastrin-releasing peptide (Grp) that stimulates neuroblastoma growth, and the induction of Ets-1 in these cells promotes the expression and secretion of the angiogenic chemokine interleukin-8.

The expressions of Ets factors have also been shown to be up-regulated in a number of hematological malignancies either through translocations or through overexpression. One example of an Ets factor that is involved in several different types of hematological malignancies is Tel (ETV6).

The hematological malignancies include chronic myeloid leukemia, acute myeloid leukemia, acute lymphoblastic leukemia, and non-Hodgkin's lymphoma [36, 37]. The evidence that angiogenesis plays a pathophysiological role in leukemia has been well documented [38]. Angiogenesis in human leukemias is demonstrated by increased microvessel density (MVD) in the bone marrow, and is associated with the increased expression of hypoxia inducible factor 1α (HIF-1α), the increased expression of multiple angiogenic factors including VEGF, bFGF, and angiopoetin-2, and a reduction in the expression of endogenous angiogenesis inhibitors such as thrombospondin-1 [39]. Because ETV6 has been shown to be important for yolk sac angiogenesis, its upregulation in several hematological malignancies also suggests that it may likely play an important role in regulating angiogenic factors in hematological malignancies. Furthermore, because several of the Ets factors have been shown to function as downstream effectors of angiogenic factors, targeting ETV6 and other Ets factors in these cancers may not only limit direct tumor cell growth but also may limit the local microenvironment through inhibitory effects on angiogenesis.

With regard to tumor progression, a number of factors have been shown to promote tumor growth and/or progression. Some of these are directly regulated by Ets factors, while others act synergistically with Ets to regulate specific target genes. One of the earliest events in the development of tumors from epithelial cells is the so-called epithelial to mesenchymal transition (EMT). This process has been associated with increased expression of RhoC, loss of E-cadherin, and acquisition of mesenchymal characteristics. It has recently been shown that the expression of RhoC is regulated by Ets-1 [40]. The tumor stroma is believed to be an important contributor to some of the most malignant characteristics of epithelial tumors. It has recently been shown that targeted deletion of the tumor suppressor PTEN in stromal fibroblasts of mouse mammary glands accelerates the initiation, progression, and malignant transformation of mammary epithelial tumors [41]. More importantly, it was found that loss of PTEN in the stromal fibroblasts was associated with an increase in the expression of Ets-2. The deletion of Ets-2 and PTEN was associated with a decrease in tumor vasculature and the recruitment of macrophages, resulting in fewer and smaller tumors than deleting PTEN alone. There are other recently described links to certain Ets proteins and the development of tumor angiogenesis. For example, the Wilm's tumor suppressor WT-1 was detected in the endothelium of angiogenic blood vessels of 95% of 113 tumors of different origins. Ets-1 and WT-1 have overlapping expression patterns. It was shown that silencing of WT-1 in endothelial cells reduced cell proliferation, cell migration, and endothelial tube formation and that silencing of WT-1 diminished the expression of Ets-1 in the tumors [42]. Mutations of the tumor suppressor gene von Hippel-Lindau (VHL) are associated with highly vascularized tumors that in part have been linked to stabilization of the transcription factor hypoxia-inducible factor (HIF), which upregulates proangiogenic factors such as vascular endothelial growth factor (VEGF). Down regulation of VHL in endothelial cells has recently been linked to Ets-1 activation via increased FGF signal transduction [43]. In Kaposi's sarcoma angiogenesis is associated with the induction of angiopoeitin-2 the expression of which is regulated by the transcription factors AP-1 and Ets-1 [44]. Another important gene for the regulation of tumor angiogenesis, wound healing, and fibrosis is connective tissue growth factor (CCN2). Under pathological conditions, such as wound healing or tumor formation it has recently been shown that Ets-1 competitively inhibits the function of another Ets factor Fli-1 to promote the expression of CCN2, thereby promoting extracellular matrix deposition during cancer [45].

From a therapeutic standpoint, recent studies support important role for Ets factors as critical transcriptional regulators of tumor angiogenesis. Local administration of a dominant-negative form of Ets-1, encoding the DNA binding domain, significantly blocked tumor angiogenesis and tumor growth [46]. Similarly, in a pancreatic mouse cancer model of angiogenesis, the local administration of a dominant negative Ets-1 peptide using and adenoviral vector significantly blunted tumor growth and blood vessel density [47]. By targeting the DNA binding domain, these therapeutic agents very likely target subfamilies of Ets factors, for example, Ets-1 and Ets-2, rather than being entirely selective for a particular Ets factor [7].

Administration of dominant negative Ets proteins has not been successfully used to block tumor growth systemically. Another strategy that has been used to inhibit the function of transcription factors is the use of membrane permeable peptides. For example, a peptide with a membrane permeable sequence and the nuclear localization signal sequence of the transcription factor NF-κB can block nuclear import of this transcription factor upon activation [48]. The inhibitory function of this peptide, however, was not specific to NF-κB, and also inhibited other transcription factors such as AP-1, NFAT, and STAT1 from entering the nucleus [49]. Another membrane permeable peptide with the ability to interfere with DNA binding of a transcription factor was recently demonstrated using the Tet repressor (TetR) of *Escherichia coli* synthesized in tandem with a cell membrane transducing peptide. This peptide was able to repress the expression of a tetracycline responsive reporter unit stably transfected into the genome of HeLa cells [50]. Membrane permeable peptides consisting of the terminal portion of the DNA binding domain of the Ets factor Elf-1 in tandem with the HIV TAT peptide readily cross the cell membrane and enter the nucleus of cells where they are able to block the function of the Ets factor Elf-1. These peptides were successfully used to systemically block the growth of B16 melanoma tumor cells in vivo [7]. Furthermore, the identification of relatively short peptides of 30 to 40 amino acids long that can selectively block the DNA binding of specific transcription factors, or subsets of transcription factors, suggests that this may be a very potent novel approach toward targeting transcription factors as a therapeutic modality to inhibit tumor growth and angiogenesis. Although these peptides have been used successfully to block tumor growth in small animals, there may be certain limitations. First the cost of making these peptides may be prohibitive for use on a much larger scale in man. Second, if the peptides are

to be used repeatedly, the peptides may be immunogenic and antibodies directed against the peptides may eventually limit their use. With recent advances in drug discovery, the identification and/or development of small molecule inhibitors of subfamilies of Ets factors may be possible in the future by taking advantage of structural similarities among the subfamilies in the DNA binding domain or other functionally conserved regions of the proteins. Although transcription factors have historically been poor drug targets, the advances in computational chemistry may ultimately lead to the identification of selective inhibitors of Ets and other transcription factors.

References

[1] B. Wasylyk, S. L. Hahn, and A. Giovane, "The Ets family of transcription factors," *European Journal of Biochemistry*, vol. 211, no. 1-2, pp. 7–18, 1993.

[2] A. Dube, Y. Akbarali, T. N. Sato, T. A. Libermann, and P. Oettgen, "Role of the Ets transcription factors in the regulation of the vascular-specific Tie2 gene," *Circulation Research*, vol. 84, no. 10, pp. 1177–1185, 1999.

[3] K. Iljin, A. Dube, S. Kontusaari, et al., "Role of Ets factors in the activity and endothelial cell specificity of the mouse Tie gene promoter," *FASEB Journal*, vol. 13, no. 2, pp. 377–386, 1999.

[4] G. Elvert, A. Kappel, R. Heidenreich, et al., "Cooperative interaction of hypoxia-inducible factor-2α (HIF-2α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1)," *The Journal of Biological Chemistry*, vol. 278, no. 9, pp. 7520–7530, 2003.

[5] M. Oikawa, M. Abe, H. Kurosawa, W. Hida, K. Shirato, and Y. Sato, "Hypoxia induces transcription factor ETS-1 via the activity of hypoxia-lnducible factor-1," *Biochemical and Biophysical Research Communications*, vol. 289, no. 1, pp. 39–43, 2001.

[6] A. Hegen, S. Koidl, K. Weindel, D. Marme, H. G. Augustin, and U. Fiedler, "Expression of angiopoietin-2 in endothelial cells is controlled by positive and negative regulatory promoter elements," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 10, pp. 1803–1809, 2004.

[7] X. Huang, C. Brown, W. Ni, E. Maynard, A. C. Rigby, and P. Oettgen, "Critical role for the Ets transcription factor ELF-1 in the development of tumor angiogenesis," *Blood*, vol. 107, no. 8, pp. 3153–3160, 2006.

[8] N. Petrovic, S. V. Bhagwat, W. J. Ratzan, M. C. Ostrowski, and L. H. Shapiro, "CD13/APN transcription is induced by RAS/MAPK-mediated phosphorylation of Ets-2 in activated endothelial cells," *The Journal of Biological Chemistry*, vol. 278, no. 49, pp. 49358–49368, 2003.

[9] P. Oettgen, "Functional redundancy of Ets1 and Ets2," *Blood*, vol. 114, no. 5, pp. 934–935, 2009.

[10] C. W. Garvie, J. Hagman, and C. Wolberger, "Structural studies of Ets-1/Pax5 complex formation on DNA," *Molecular Cell*, vol. 8, no. 6, pp. 1267–1276, 2001.

[11] F. Pio, C. Z. Ni, R. S. Mitchell, et al., "Co-crystallization of an ETS domain (PU.1) in complex with DNA. Engineering the length of both protein and oligonucleotide," *The Journal of Biological Chemistry*, vol. 270, no. 41, pp. 24258–24263, 1995.

[12] L. A. Brown, A. R. F. Rodaway, T. F. Schilling, et al., "Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos," *Mechanisms of Development*, vol. 90, no. 2, pp. 237–252, 2000.

[13] A. Dube, S. Thai, J. Gaspar, et al., "ELF-1 is a transcriptional regulator of the Tie2 gene during vascular development," *Circulation Research*, vol. 88, no. 2, pp. 237–244, 2001.

[14] N. Wernert, A. Stanjek, A. Hugel, and A. Giannis, "Inhibition of angiogenesis on the chicken chorioallantoic membrane by Ets 1 antisense oligodeoxyribonucleotides," *Verhandlungen der Deutschen Gesellschaft fur Pathologie*, vol. 83, pp. 212–215, 1999.

[15] K. Barton, N. Muthusamy, C. Fischer, et al., "The Ets-1 transcription factor is required for the development of natural killer cells in mice," *Immunity*, vol. 9, no. 4, pp. 555–563, 1998.

[16] H. Yamamoto, M. L. Flannery, S. Kupriyanov, et al., "Defective trophoblast function in mice with a targeted mutation of Ets2," *Genes and Development*, vol. 12, no. 9, pp. 1315–1326, 1998.

[17] A. K. Man, L. J. T. Young, J. A. Tynan, et al., "Ets2-dependent stromal regulation of mouse mammary tumors," *Molecular and Cellular Biology*, vol. 23, no. 23, pp. 8614–8625, 2003.

[18] G. Wei, R. Srinivasan, C. Z. Cantemir-Stone, et al., "Ets1 and Ets2 are required for endothelial cell survival during embryonic angiogenesis," *Blood*, vol. 114, no. 5, pp. 1123–1130, 2009.

[19] N. Hashiya, N. Jo, M. Aoki, et al., "In vivo evidence of angiogenesis induced by transcription factor ets-1: Ets-1 is located upstream of angiogenesis cascade," *Circulation*, vol. 109, no. 24, pp. 3035–3041, 2004.

[20] G. Kitange, S. Shibata, Y. Tokunaga, et al., "Ets-1 transcription factor-mediated urokinase-type plasminogen activator expression and invasion in glioma cells stimulated by serum and basic fibroblast growth factors," *Laboratory Investigation*, vol. 79, no. 4, pp. 407–416, 1999.

[21] Z.-Q. Chen, R. J. Fisher, C. W. Riggs, J. S. Rhim, and J. A. Lautenberger, "Inhibition of vascular endothelial growth factor-induced endothelial cell migration by ETS1 antisense oligonucleotides," *Cancer Research*, vol. 57, no. 10, pp. 2013–2019, 1997.

[22] S. M. Stamatovic, R. F. Keep, M. Mostarica-Stojkovic, and A. V. Andjelkovic, "CCL2 regulates angiogenesis via activation of Ets-1 transcription factor," *Journal of Immunology*, vol. 177, no. 4, pp. 2651–2661, 2006.

[23] Y. Zhan, C. Brown, E. Maynard, et al., "Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling," *Journal of Clinical Investigation*, vol. 115, no. 9, pp. 2508–2516, 2005.

[24] J. Fujimoto, I. Aoki, H. Toyoki, et al., "Clinical implications of expression of ETS-1 related to angiogenesis in metastatic lesions of ovarian cancers," *Oncology*, vol. 66, no. 5, pp. 420–428, 2004.

[25] S. Khatun, J. Fujimoto, H. Toyoki, and T. Tamaya, "Clinical implications of expression of ETS-1 in relation to angiogenesis in ovarian cancer," *Cancer Science*, vol. 94, no. 9, pp. 769–773, 2003.

[26] T. Mukherjee, A. Kumar, M. Mathur, T. K. Chattopadhyay, and R. Ralhan, "Ets-1 and VEGF expression correlates with tumor angiogenesis, lymph node metastasis, and patient survival in esophageal squamous cell carcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 129, no. 7, pp. 430–436, 2003.

[27] H. Wu, Y. Li, L. Zhang, G. Zhu, and J. Cai, "Expression of vascular endothelial growth factor and E26 transformation-specific-1 in breast carcinoma," *Zhonghua Bing Li Xue Za Zhi*, vol. 31, pp. 222–226, 2002.

[28] P. N. Span, P. Manders, J. J. Heuvel, et al., "Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer," *Oncogene*, vol. 21, no. 55, pp. 8506–8509, 2002.

[29] J. Fujimoto, I. Aoki, H. Toyoki, S. Khatun, E. Sato, and T. Tamaya, "Expression of ETS-1 related to angiogenesis in uterine endometrium during the menstrual cycle," *Journal of Biomedical Science*, vol. 10, no. 3, pp. 320–327, 2003.

[30] J. Fujimoto, I. Aoki, H. Toyoki, S. Khatun, and T. Tamaya, "Clinical implications of expression of ETS-1 related to angiogenesis in uterine cervical cancers," *Annals of Oncology*, vol. 13, no. 10, pp. 1598–1604, 2002.

[31] S. Tsutsumi, H. Kuwano, T. Asao, K. Nagashima, T. Shimura, and E. Mochiki, "Expression of Ets-1 angiogenesis-related protein in gastric cancer," *Cancer Letters*, vol. 160, no. 1, pp. 45–50, 2000.

[32] K. Tokuhara, Y. Ogata, M. Nakagawa, and K. Shirouzu, "Ets-1 expression in vascular endothelial cells as an angiogenic and prognostic factor in colorectal carcinoma," *International Surgery*, vol. 88, no. 1, pp. 25–33, 2003.

[33] G. Kitange, M. Kishikawa, T. Nakayama, S. Naito, M. Iseki, and S. Shibata, "Expression of the Ets-1 proto-oncogene correlates with malignant potential in human astrocytic tumors," *Modern Pathology*, vol. 12, no. 6, pp. 618–626, 1999.

[34] J. Fujimoto, I. Aoki, H. Toyoki, S. Khatun, and T. Tamaya, "Clinical implications of expression of ETS-1 related to angiogenesis in uterine endometrial cancers," *Annals of Oncology*, vol. 13, no. 10, pp. 1605–1611, 2002.

[35] J. Qiao, J. H. Kang, J. Cree, B. M. Evers, and D. H. Chung, "Ets1 transcription factor mediates gastrin-releasing peptide-induced IL-8 regulation in neuroblastoma cells," *Neoplasia*, vol. 9, no. 3, pp. 184–191, 2007.

[36] I. Wlodarska, R. La Starza, M. Baens, et al., "Fluorescence in situ hybridization characterization of new translocations involving TEL (ETV6) in a wide spectrum of hematologic malignancies," *Blood*, vol. 91, no. 4, pp. 1399–1406, 1998.

[37] D. W. Sevilla, S. V. Nandula, A. I. Colovai, et al., "Diffuse large B-cell lymphoma with TEL/ETV6 translocation," *Human Pathology*, vol. 40, no. 4, pp. 588–593, 2009.

[38] T. Kessler, F. Fehrmann, R. Bieker, W. E. Berdel, and R. M. Mesters, "Vascular endothelial growth factor and its receptor as drug targets in hematological malignancies," *Current Drug Targets*, vol. 8, no. 2, pp. 257–268, 2007.

[39] W. W. Li, M. Hutnik, and G. Gehr, "Antiangiogenesis in haematological malignancies," *British Journal of Haematology*, vol. 143, no. 5, pp. 622–631, 2008.

[40] D. I. Bellovin, K. J. Simpson, T. Danilov, et al., "Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma," *Oncogene*, vol. 25, no. 52, pp. 6959–6967, 2006.

[41] A. J. Trimboli, C. Z. Cantemir-Stone, F. Li, et al., "Pten in stromal fibroblasts suppresses mammary epithelial tumours," *Nature*, vol. 461, no. 7267, pp. 1084–1091, 2009.

[42] N. Wagner, J. F. Michiels, A. Schedl, and K.-D. Wagner, "The Wilms' tumour suppressor WT1 is involved in endothelial cell proliferation and migration: expression in tumour vessels in vivo," *Oncogene*, vol. 27, no. 26, pp. 3662–3672, 2008.

[43] K. J. Champion, M. Guinea, V. Dammai, and T. Hsu, "Endothelial function of von Hippel-Lindau tumor suppressor gene: control of fibroblast growth factor receptor signaling," *Cancer Research*, vol. 68, no. 12, pp. 4649–4657, 2008.

[44] F. C. Ye, D. J. Blackbourn, M. Mengel, et al., "Kaposi's sarcoma-associated herpesvirus promotes angiogenesis by inducing angiopoietin-2 expression via AP-1 and Ets1," *Journal of Virology*, vol. 81, no. 8, pp. 3980–3991, 2007.

[45] S. S. Nakerakanti, B. Kapanadze, M. Yamasaki, M. Markiewicz, and M. Trojanowska, "Fli1 and Ets1 have distinct roles in connective tissue growth factor/CCN2 gene regulation and induction of the profibrotic gene program," *The Journal of Biological Chemistry*, vol. 281, no. 35, pp. 25259–25269, 2006.

[46] A. Pourtier-Manzanedo, C. Vercamer, E. Van Belle, V. Mattot, F. Mouquet, and B. Vandenbunder, "Expression of an Ets-1 dominant-negative mutant perturbs normal and tumor angiogenesis in a mouse ear model," *Oncogene*, vol. 22, no. 12, pp. 1795–1806, 2003.

[47] L. P. Lefter, S. Dima, M. Sunamura, et al., "Transcriptional silencing of ETS-1 efficiently suppresses angiogenesis of pancreatic cancer," *Cancer Gene Therapy*, vol. 16, no. 2, pp. 137–148, 2009.

[48] L. Zhang, T. R. Torgerson, X. Y. Liu, et al., "Preparation of functionally active cell-permeable peptides by single-step ligation of two peptide modules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9184–9189, 1998.

[49] T. R. Torgerson, A. D. Colosia, J. P. Donahue, Y. Z. Lin, and J. Hawiger, "Regulation of NF-κB, AP-1, NFAT, and STAT1 nuclear import in T lymphocytes by noninvasive delivery of peptide carrying the nuclear localization sequence of NF-κB p501," *Journal of Immunology*, vol. 161, no. 11, pp. 6084–6092, 1998.

[50] A. Mortlock, W. Low, and A. Crisanti, "Suppression of gene expression by a cell-permeable Tet repressor," *Nucleic Acids Research*, vol. 31, article e152, 2003.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 835680, 8 pages
doi:10.1155/2010/835680

Review Article

Molecular Mechanisms of Resistance to Tumour Anti-Angiogenic Strategies

Renaud Grépin and Gilles Pagès

Institute of Developmental Biology and Cancer UMR 6543, University of Nice Sophia Antipolis, CNRS, Centre Antoine Lacassagne, 06189 Nice, France

Correspondence should be addressed to Gilles Pagès, gpages@unice.fr

Received 16 November 2009; Accepted 5 January 2010

Academic Editor: Arkadiusz Dudek

Tumour angiogenesis, described by Folkman in the early seventies, is an essential, complex, and dynamic process necessary for the growth of all solid tumours. Among the angiogenic factors secreted by the tumour cells, the Vascular Endothelial Growth Factor (VEGF) is one of the most important. Most types of human cancer cells express elevated levels of this proangiogenic factor and its receptors. New molecules, called anti-angiogenic, are developed to impair VEGF pathway and tumour vasculature. Despite important results, the clinical benefits of anti-VEGF therapy are relatively modest and usually measured in weeks or months. Why following anti-angiogenic therapy do some patients respond transiently and then why does tumour grow again and disease progress and which compensatory mechanisms could explain the anti-angiogenic treatment failure?

1. Introduction

Tumour angiogenesis, as described by Folkman in the early seventies and confirmed today [1], is an essential, complex, and dynamic process necessary for the growth of all solid tumours. It stipulates that a tumour cannot grow through a defined volume if it is not vascularized. The cores of solid tumours rapidly undergo hypoxic with low oxygen levels and nutrients deficiency. Tumour cells counteract this process by producing angiogenic factors responsible for growth and migration of quiescent endothelial cells of proximal blood vessels. The consequence is the creation of a new vascular network to supply the tumour with oxygen, nutrients, growth factors, proteolytic enzymes, tumours cells, and dissemination in host [2, 3]. Tumour angiogenesis and hypoxia are considered as hallmarks of solid tumours [4, 5]. Among the angiogenic factors secreted by tumour cells, the Vascular Endothelial Growth Factor (VEGF) is one of the most important. Different stimuli are responsible of its production. Among them hypoxia was one of the first described. During the angiogenic switch, the frail existing balance between pro- and anti-angiogenic factors is broken in favour of proangiogenic factors [6]. Most types of human cancer cells express elevated levels of VEGF. They also express VEGF receptors at their surface including VEGF-R1,2,3, VEGF-R3 participating to lymphangiogenesis [7–11]. All these results have allowed the development of therapeutic tools targeting VEGF (Bevacizumab) [12] or their receptors (Tyrosine kinase inhibitors) [13].

2. Examples of Antiangiogenic Treatments Commonly Used in Metastatic Cancers

For example, Bevacizumab (Avastin, Roche), a humanized neutralizing monoclonal antibody against VEGF, is the first anti-angiogenic molecule approved by the FDA in 2004. This antibody, which is becoming an important anti-angiogenic treatment, is commonly used in association with a cytotoxic chemotherapy for late stages of colon cancer, nonsmall-cell lung cancer, breast cancer, glioblastoma, and metastatic renal cell carcinoma (mRCC). Sorafenib tosylate (Nexavar, Bayer) and Sunitinib malate (Sutent, Pfizer), two small tyrosine kinase inhibitors (TKI), target multiple signaling pathways including VEGF and platelet-derived growth factors (PDGFs). Sorafenib and Sunitinib are approved by the FDA to treat mRCC as a single agent. Sunitinib is used in

gastrointestinal stromal tumours (GISTs) and Sorafenib is used for treating patients with liver cancer. Every year a variety of new anti-angiogenic molecules are developed with more than 800 clinical trials [14].

Despite important results, the clinical benefits of anti-VEGF therapy are relatively modest and usually measured in weeks or months [15]. In some cases patients do not respond to anti-VEGF treatments. For example, Bevacizumab used as a single agent to treat colorectal and nonsmall-cell lung tumours is inefficient [12, 16]. Recently a phase III study of Bevacizumab plus chemotherapy in early-stage colon cancer did not meet its primary endpoint for lowering the risk of the cancer recurrence compared to chemotherapy alone [17]. Discontinuous TKI treatments (4 weeks on/2 weeks off) in patients with metastatic breast cancer or mRCC can carry the risk of tumour progression during drug-free break periods [18, 19]. Thereby, in a preclinical model, rapid tumour revascularisation has been reported after removal of anti-VEGF therapy [20]. Multiple angiogenesis inhibitors have been therapeutically validated in preclinical cancer models and several in clinical trials. Why following anti-angiogenic therapy do some patients respond transiently and then why does tumour growth again and disease progress? Which compensatory mechanisms could explain the anti-angiogenic treatment resistance?

The fraction of nonresponsive patients included in anti-angiogenic clinical trials such as anti-VEGF antibody treatment or tyrosine kinase inhibitors is significant [13, 15, 21]. In these cases anti-angiogenic treatment does not permet to obtain beneficial effects. Hence, there is no cessation or retardation of the tumour growth or increase of survival. Resistance to anti-angiogenic agents can be the result of intrinsic tumour resistances or acquired resistances. Different mechanisms can explain these resistances including redundant angiogenic factors with upregulation of alternative angiogenic signals, induction of hypoxia, selection of more aggressive tumour cells, recruitment of bone marrow-derived proangiogenic cells and inflammatory cells invasion, modification of vascular pericyte coverage, and vessel cooption.

2.1. The Angiogenic Redundancy and Alternative Proangiogenic Pathways. The angiogenic redundancy is the first resistance mechanism identified following anti-VEGF therapy [22, 23]. VEGF is the predominant angiogenic factor in human tumours. However, during tumour development redundant proangiogenic factors could be produced including Fibroblast Growth Factors (FGFs), Platelet Derived Growth Factors (PDGFs), Placenta Growth Factor (PlGF), and Tumour Necrosis Factor-α (TNF-α) [24–26] (Figure 1(1)). For example, in early-stage breast cancers VEGF is the major proangiogenic factor whereas in late-stage additional angiogenic molecules are produced including FGF-2 [23].

Preclinical and clinical anti-VEGF studies have shown that tumours can grow despite VEGF pathway inhibition due to angiogenic redundancy. In a preclinical model of pancreatic neuron-endocrine cancer, after few days of anti-VEGFR2 antibody (DC101) treatment, the vascular density of tumours is reduced and tumours have regions of acute hypoxia. Histological analysis shows that tumours have a wide front of tumour invasion compared to controls and tumours are more invasive after one week of treatment and this increases after four weeks of continuous treatment. Despite the disruption of VEGF angiogenic switch following long-term treatment, a phenotypic resistance to anti-VEGFR2 therapy emerges. During this phase, the tumour's revascularization is increased indicating an active tumour angiogenesis. This progression phase is linked to enhanced production of redundant angiogenic factors, such as FGF family members. If mice are treated with anti-VEGFR, then with FGF-trap just before tumour vasculature regrowth, the tumour growth and neovascularization are attenuated. This independence of VEGF pathway is associated with hypoxia-mediated induction of proangiogenic factors such as FGF-1 and 2, ephrin A1 and 2, and angiopoietin 1 [22]. A synergism between the low expression of two angiogenic factors such as FGF-2 and PDGF-bb could be sufficient to promote angiogenic response although it is expressed at low levels [27, 28]. Following anti-VEGF therapy, levels of plasmatic placental growth factor (PlGF) are increased and seem to be implicated in the angiogenic redundancy [26, 29]. In experimental studies, VEGF-sensitive and resistant tumours respond to PlGF antibody treatment, this antibody enhances the efficiency of anti-VEGFR2 therapy, and it reduces tumour angiogenesis and metastasis without inducing hypoxia [24]. Moreover, anti-PlGF prevents infiltration of angiogenic macrophages. However, clinical studies on VEGF-trap, that binds both VEGF-A and PlGF, do not show an additional benefit compared to Bevacizumab. The withdrawal of one proangiogenic factor could be counterbalanced by production of compensatory angiogenic growth factors and/or chemokines leading to angiogenic rescue program [30]. To prevent angiogenic redundancy different actors implicated in tumour angiogenesis might be targeted at the same time.

2.2. Hypoxia: A Major Inducer of Angiogenic Redundancy. Antiangiogenic therapies reduce and normalize tumour vasculature but increase intratumour hypoxia [31, 32] (Figure 1(2)). Hypoxia and overexpression of hypoxia-induced factor-1 (HIF-1) have been associated with radiation therapy and chemotherapy resistance, selection of invasive and metastatic cells, and a poor clinical prognosis of solid tumours [33]. HIF-1 is considered as the major regulator of angiogenic actors following hypoxia. It regulates a lot of genes involved in angiogenesis (VEGF, PlGF, VEGFR-1), proliferation and migration of endothelial cells (such as VEGF, PlGF, FGF2, CXCL12/CXCR4, PDGF), pericytes recruitment (PDGF, Ang-1), and modification of vascular permeability (VEGF/VEGFR-1, Ang-2) [34, 35].

The treatment of recurrent glioblastoma patients with a tyrosine kinase inhibitor targeting VEGF receptors initially leads to disease stable but resistances appear after few weeks. The development of resistance following VEGF blockade is associated with an increase of circulating levels of basic FGF, stromal cell-derived factor 1 alpha (SDF1α), two genes

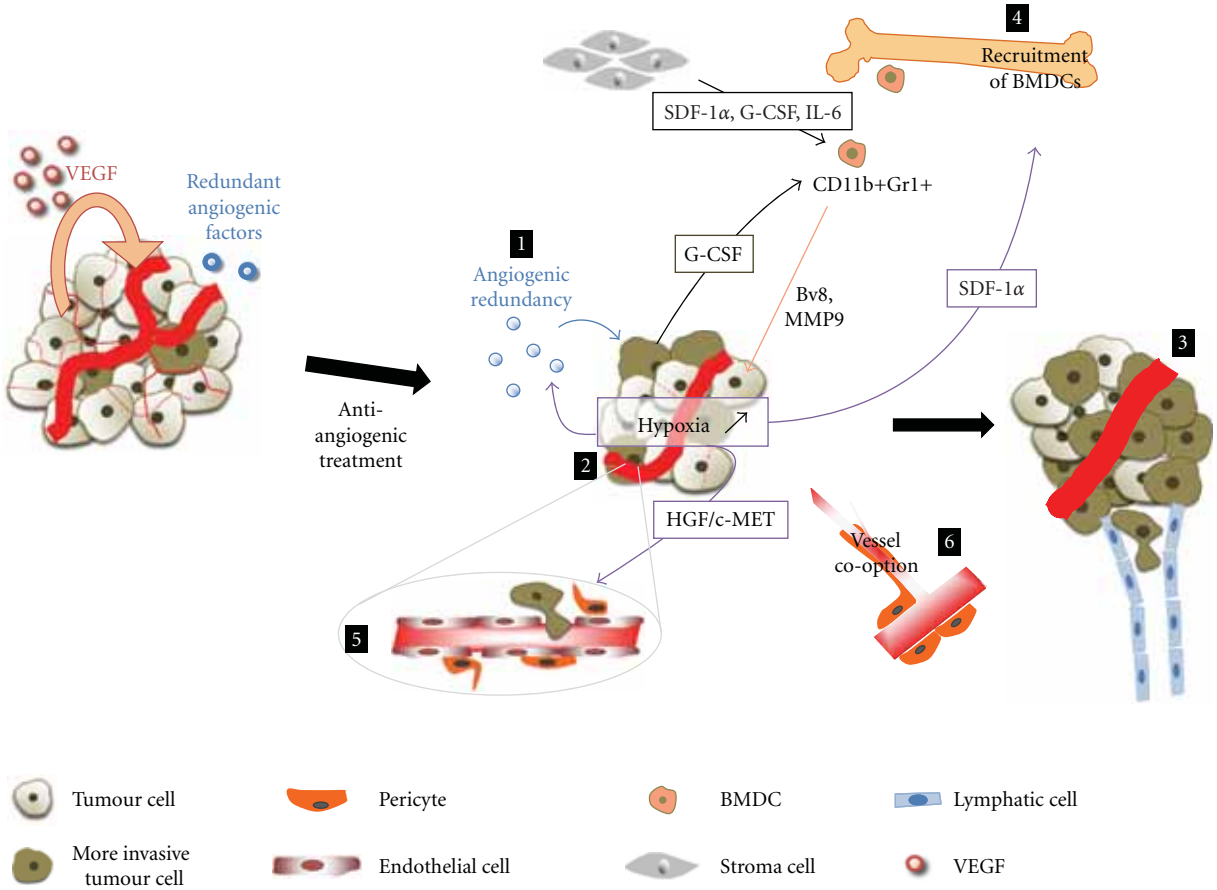


Figure 1: Molecular mechanisms involved in tumor anti-angiogenesis therapies resistance. (1) Following anti-VEGF therapies, redundant angiogenic factors are produced by tumour cells. Antiangiogenic treatments reduce and normalize tumour vasculature but increase intratumour hypoxia (2). Hypoxia induces SDF-1α which recruits BMDCs such as CD11b+Gr1+, redundant angiogenic factors (1), and activates HGF/c-MET pathway (5). BMDCs are activated by factors secreted by stroma cells (SDF-1$\propto$, G-CSF, and IL-6). Inhibition of VEGF induces endothelial cells apoptosis and pericytes attachment to endothelial cells is loosed (5). New vessels could be recruited to tumour site by vessel cooption (6). Finally mechanisms involved in tumor anti-angiogenesis resistances lead to select more invasive and metastatic tumour cells (3).

controlled by HIF-1, and viable circulating endothelial cells [13, 34, 36].

Hepatocyte Growth Factor (HGF) is a potent mitogenic, motogenic, morphogenic factor, and also an important actor in angiogenesis and tumour growth [37–39]. HGF induces and activates its membrane tyrosine kinase receptor c-MET. Whereas HGF is mostly produced by mesenchymal cells [40], c-MET is expressed by different cell types such as vascular and lymphatic endothelial cells and pericytes [41]. Thereby, HGF effects are not limited to endothelial cells. HGF binds c-MET and induces its homodimerization and autophosphorylation; then c-MET activates signal transduction pathways such as Src, Akt, MEK, STAT3 [42] and leads to increase expression of VEGF and VEGF/R in endothelial cells [43]. C-MET and HGF are deregulated and correlated with poor prognosis in a lot of human cancers. The receptor can be constitutively phosphorylated, its gene mutated or amplified in tumours. HGF/c-MET promotes cell invasiveness and triggers metastases through angiogenic pathway [44]. It has been described, in lung cancer patient specimens, that HGF is colocalized with fibroblasts. When lung cancer cells and HGF-producing fibroblasts are injected into mice, the tumour becomes resistant to EGFR-TKIs treatment. The stromal fibroblasts seem to an actor in TKI treatment resistance by producing angiogenic factors such as HGF [45]. In a pancreatic cancer model it has been shown that HIF-1 increases c-MET expression in cancer cells and HGF secretion by fibroblast cells [46]. Hypoxia induces HGF/c-MET signaling pathway that leads to matrice membrane degradation and increase of cell migration towards blood or lymphatic vessels (Figure 1(5)).

The plasma-membrane bound Notch receptor ligand, Delta-like ligand 4 (DLL4), can be an alternative angiogenic pathway which participates to anti-angiogenic treatment failure. DLL4 is highly expressed by vascular endothelial cells and induced by VEGF. It interacts with Notch cell-surface receptors to act as a negative feedback inhibitor downstream of VEGF signaling to restrain the sprouting and branching of new blood vessels [47, 48]. Inhibition of DLL4-Notch signaling induces an increase in vessel density

but these blood vessels are abnormal and not perfused. Therefore intratumour hypoxia is increased and leads to induction of transcription of proangiogenic genes regulated by HIF-1 [48–50]. Moreover, tumours that have an intrinsic resistance to anti-VEGF therapy are responsive to inhibition of DLL4/Notch signaling [51].

2.3. Selection of More Invasive Tumour Cells. The angiogenic inhibitors normalize tumour vasculature, reduce tumour size, but increase local hypoxia (Figure 1(3)). It has been shown that tumour cells cultured under hypoxia conditions can become more invasive and metastatic [52]. More recently, two studies support the hypothesis that under anti-angiogenic treatment, cancer cells become more invasive and metastatic to migrate to normoxic location. Thereby, in mouse models of pancreatic neuron-endocrine carcinoma and glioblastoma, the primary tumour size decreases after one week of Sunitinib. This anti-angiogenic treatment seems to select more aggressive cancer cells and local tumour cell invasion and distant metastasis are increased. Furthermore, tumours and disseminated liver metastases of animals present more regions of hypoxia compared to untreated control tumours. The proportion of invasive tumours during long-term continuous treatment and the invasive phenotype are not reverse even when the anti-angiogenic treatment is lifted [53]. Another study suggests, by metastasis assays, that a short-term of TKIs treatments reduces tumour growth but increases the incidence of metastasis, facilitates metastatic dissemination of tumour cells, and decreases overall survival of animals. This observation is not reverted when mice are treated one week before tumour cells implantation [54].

2.4. Recruitment of Bone Marrow Derived Proangiogenic Cells and Inflammatory Cells Invasion. Antiangiogenic therapies normalize vessels but increase intratumoral hypoxia which lead to recruitment of bone marrow derived cells (BMDCs, endothelial and pericytes progenitors, tumour associated macrophages, immature monocytic cell and myeloid cells) (Figure 1(4)). These cells produce a lot of different proangiogenic factors and can constitute an adaptive mechanism of resistance in low oxygen context. Preclinical and clinical studies have revealed that the number of myeloid cell-derived suppressor cells (MDSCs), such as CD11b+Gr1+ cells, is increased in tumours and peripheral blood of tumour-bearing animals and in blood and spleen of cancer patients [55]. Furthermore the invasion of tumour by these myeloid cells is associated with tumour growth and progression and also contributes to refractoriness to anti-VEGF antibody treatment [56, 57]. Tumours and stromal cells secrete interleukin 6 (IL-6), SDF-1α, and granulocyte colony-stimulating factor (G-CSF), three factors implicated in CD11b+Gr1+ cells mobilization and activation [58, 59]. G-CSF expression by tumour or stromal cells is crucial for refractoriness [56]. Moreover, patients treated by tyrosine kinase inhibitors, such as Sorafenib or Sunitinib, or by Bevacizumab plus chemotherapy could be neutropenic [60, 61]. Studies have shown that with a recombinant G-CSF treatment, the use of hematopoietic growth factors for treating patients with neutropenia can mobilize endothelial-cell progenitors and CD11b+Gr1+ [62–64].

Furthermore, SDF-1α expression by endothelial cells is increased by HIF-1 following hypoxic conditions. BMDCs expressed the SDF-1α receptor CXCR4 and are recruited to the ischemic tissue by cell tropism to SDF-1α. SDF-1α upregulation is associated with anti-angiogenic treatment resistance in patients with glioblastoma [13]. Following Sunitinib treatments, levels of SDF-1α and G-CSF are increased dose-dependently in healthy mice and cancer patients [26]. Thereby, in hepatocellular carcinoma, the plasmatic level increase of SDF-1α and IL-6, in patients treated with Sunitinib, is associated with a poor outcome [65]. During Bevacizumab therapy, plasmatic levels of SDF-1α are increased in patients with rectal cancer and seem to be associated with distant metastasis after three years [66]. Could SDF-1α be considered as a biomarker of response or resistance to anti-angiogenic treatment [67]? In response to SDF-1α and Lysyl Oxidase gradient, CD11b+Gr1+ cells will be recruited at premetastatic sites and promote tumour metastasis through matrix metalloproteinase 2 (MMP2) production [68, 69]. In the tumour microenvironment and proangiogenic culture conditions, these cells acquire endothelial cell properties [64]. CD11b+Gr1+ cells promote tumour angiogenesis by production of angiogenic factors such as Bv8, a protein related to endocrine-gland derived-VEGF [63] and matrix degrading enzymes like the matrix metalloproteinase 9. Some tumours recruit CD11b+Gr1+ cells from the bone marrow to the tumour site and others do it in response to anti-VEGF treatment [70].

2.5. Tumour-Associated Endothelial Cells and Pericytes. Tumour-associated endothelial cells and pericytes were generally assumed to be genetically stable and to have a low mutational rate [71]. Antiangiogenic therapies target endothelial cells which develop less drug resistance than tumour cells. Finally, recent studies have shown that normal and tumour-associated endothelial cells are different and can be tumour type-dependent, aneuploid, and acquire genotypic alterations [72, 73]. Therefore, these endothelial cell alterations in tumour environment can induce a complex crosstalk between angiogenic pathways and increase anti-angiogenic therapies resistances. Endothelial cells secrete PDGF-bb. It mediates proliferation and migration of pericytes which express PDGF-R β [74]. Endothelial cells can induce pericyte recruitment in order to be protected from anti-VEGF therapy. This leads to an increase of mature vessels covered with pericytes [75, 76]. Pericytes are vascular smooth muscle lineage cells closely associated with the endothelial cells; this contact enhances endothelial cell survival [77]. These cells are important for vascular development, endothelial cells permeability, vessel stabilization by matrix deposition and/or release, vessel maturation, and remodeling [78]. Pericytes secrete paracrine factors that stimulate signaling pathways implicated in endothelial cell differentiation and survival and then modulating vessel stabilization and maturation [74, 79, 80]. Moreover, in context of tumours or in response to treatments, pericytes express different proteins compared to normal conditions

[81]. Anti-VEGF therapies lead to a 80% destruction of the tumour vasculature [82]. Furthermore, vascular sprouting is suppressed, blood flow arrested in some vessels, and finally some tumour vessels regress and others are normalized [32, 83–85]. Inhibition of VEGF pathway may induce endothelial cell apoptosis and/or selection of endothelial cells which express less VEGFR-2 [86] (Figure 1(5)). This reduction of expression is reversible and high reexpression of this receptor corresponds with the return of tumour vessels' dependence for VEGF [20]. VEGFR-2 blockade can lead to the upregulation of angiopoietin 1 that increases pericyte coverage of vessels [87]. Despite the endothelial cells' regression and pericyte changes, the vascular basal membrane of tumours persists and provides a potential scaffold for tumour revascularization and a storage site for angiogenic growth factors [82, 88]. In preclinical study, after 7 days of treatment, endothelial sprouts grew into empty sleeves of basal membrane just one day after anti-VEGF withdrawal. After 7 days, the tumour's regrowth is complete and pericyte phenotypes reverse to return to baseline. Furthermore, tumour vessels become functional, reacquire the dependence to VEGF, and remain sensible to anti-VEGF therapy [20]. Targeting endothelial cells and pericytes by inhibition of VEGF pathway and PDGF receptor with tyrosine kinase inhibitors can increase the efficacy of treatments [89]. Moreover, tumour pericytes have abnormal shapes, lose the attachment to endothelial cells, and contribute to the aberrant tumour vasculature [90]. Contrariwise, the loss of pericyte attachment may disrupt the vascular integrity, increases the risk of hemorrhage, and facilitates the transit and dissemination of tumour cells in circulatory system.

2.6. Vessel Cooption. Patients treated by anti-angiogenic therapy are mainly patients with metastases for which other treatments are no longer available. In vasculature-rich organs such as brain, liver, and lung, primary tumours cells and metastases could coopt with the neighboring quiescent normal blood vessels [91] (Figure 1(6)). In rat brain, one or two weeks after C6 glioma cell implantation, small tumours are vascularized without angiogenic response [92]. In early stage of cooption, the tumour growth is angiogenesis independent. Tumours vessels display characteristics of normal vessels. Hence, they will be less sensitive to anti-angiogenic molecules [93, 94].

3. Conclusion

The establishment of a new vascular network by angiogenic process is one part of the basis of solid tumour development and dissemination of tumour cells in the organism as metastasis. The core of the tumour rapidly undergoes hypoxic and tumour cells counteract this process by producing angiogenic factors responsible for growth and migration of quiescent endothelial cells of proximal blood vessels. Among the angiogenic factors, the VEGF is one of the most important. Targeting the VEGF/VEGFR pathway represents a major advance in cancer treatments and an important therapeutic option. Despite the transient effect of these expansive anti-VEGF treatments, the enduring clinical responses are rare. Pre-clinical and clinical trials suggest a host response to VEGF inhibition implicated in treatment failure and can participate to progression of secondary disease. Furthermore, the mechanisms of action of anti-angiogenic drugs and resistances against these molecules vary according to the tumour. Resistance or evasion to anti-angiogenic therapies in preclinical models is more and more reported. For example, anti-angiogenic treatments not only normalize tumour vasculature but also reduce pericyte coverage and increase tumour hypoxia and tumour cells can acquire a more invasive; however the molecular mechanisms are not fully understood. Hence, it is crucial to highlight molecular mechanisms or actors implicated in this phenomenon of resistance in order to anticipate the best responders to the treatment and to improve anti-angiogenic drugs or to develop new agents. Tumours produce multiple factors and animal models suggest that different pathways are activated under or following anti-angiogenic therapies. Hence, targeting complementary pathways implicated in tumour angiogenesis would be more efficient. At last, it is also important to find predictive biological markers of objective response or involved in resistance to anti-angiogenic drugs in order to improve therapy efficacy or to propose alternative anti-angiogenic therapy in case of treatment failure.

Acknowledgment

This work was supported by grants from the National Institute of Cancer (INCA), the French Association for Cancer Research (ARC, contract N° 4932), and Roche France.

References

[1] J. Folkman, "Tumor angiogenesis: therapeutic implications," *New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[2] J. Folkman, "What is the evidence that tumors are angiogenesis dependent?" *Journal of the National Cancer Institute*, vol. 82, no. 1, pp. 4–6, 1990.

[3] T. R. Geiger and D. S. Peeper, "Metastasis mechanisms," *Biochimica et Biophysica Acta*, vol. 1796, no. 2, pp. 293–308, 2009.

[4] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[5] J. M. Brown and W. R. Wilson, "Exploiting tumour hypoxia in cancer treatment," *Nature Reviews Cancer*, vol. 4, no. 6, pp. 437–447, 2004.

[6] G. Bergers and L. E. Benjamin, "Tumorigenesis and the angiogenic switch," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 401–410, 2003.

[7] J.-L. Su, P.-C. Yang, J.-Y. Shih, et al., "The VEGF-C/Flt-4 axis promotes invasion and metastasis of cancer cells," *Cancer Cell*, vol. 9, no. 3, pp. 209–223, 2006.

[8] G. Xia, S. R. Kumar, D. Hawes, et al., "Expression and significance of vascular endothelial growth factor receptor 2 in bladder cancer," *Journal of Urology*, vol. 175, no. 4, pp. 1245–1252, 2006.

[9] A. D. Yang, E. R. Camp, F. Fan, et al., "Vascular endothelial growth factor receptor-1 activation mediates epithelial to mesenchymal transition in human pancreatic carcinoma cells," *Cancer Research*, vol. 66, no. 1, pp. 46–51, 2006.

[10] S. E. Duff, M. Jeziorska, D. D. Rosa, et al., "Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy," *European Journal of Cancer*, vol. 42, no. 1, pp. 112–117, 2006.

[11] P. M. Lacal, F. Ruffini, E. Pagani, and S. D'Atri, "An autocrine loop directed by the vascular endothelial growth factor promotes invasiveness of human melanoma cells," *International Journal of Oncology*, vol. 27, no. 6, pp. 1625–1632, 2005.

[12] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[13] T. T. Batchelor, A. G. Sorensen, E. di Tomaso, et al., "AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients," *Cancer Cell*, vol. 11, no. 1, pp. 83–95, 2007.

[14] J. Ma and D. J. Waxman, "Combination of antiangiogenesis with chemotherapy for more effective cancer treatment," *Molecular Cancer Therapeutics*, vol. 7, no. 12, pp. 3670–3684, 2008.

[15] L. B. Saltz, S. Clarke, E. Diaz-Rubio, et al., "Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study," *Journal of Clinical Oncology*, vol. 26, no. 12, pp. 2013–2019, 2008.

[16] A. Sandler, R. Gray, M. C. Perry, et al., "Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer," *New England Journal of Medicine*, vol. 355, no. 24, pp. 2542–2550, 2006.

[17] C. J. Allegra, G. Yothers, M. J. O'Connell, et al., "Initial safety report of NSABP C-08: a randomized phase III study of modified FOLFOX6 with or without bevacizumab for the adjuvant treatment of patients with stage II or III colon cancer," *Journal of Clinical Oncology*, vol. 27, no. 20, pp. 3385–3390, 2009.

[18] H. J. Burstein, A. D. Elias, H. S. Rugo, et al., "Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane," *Journal of Clinical Oncology*, vol. 26, no. 11, pp. 1810–1816, 2008.

[19] M. Johannsen, A. Florcken, A. Bex, et al., "Can tyrosine kinase inhibitors be discontinued in patients with metastatic renal cell carcinoma and a complete response to treatment? A multicentre, retrospective analysis," *European Urology*, vol. 55, no. 6, pp. 1430–1439, 2009.

[20] M. R. Mancuso, R. Davis, S. M. Norberg, et al., "Rapid vascular regrowth in tumors after reversal of VEGF inhibition," *Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2610–2621, 2006.

[21] H. Burris III and C. Rocha-Lima, "New therapeutic directions for advanced pancreatic cancer: targeting the epidermal growth factor and vascular endothelial growth factor pathways," *Oncologist*, vol. 13, no. 3, pp. 289–298, 2008.

[22] O. Casanovas, D. J. Hicklin, G. Bergers, and D. Hanahan, "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors," *Cancer Cell*, vol. 8, no. 4, pp. 299–309, 2005.

[23] M. Relf, S. LeJeune, P. A. E. Scott, et al., "Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor β-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis," *Cancer Research*, vol. 57, no. 5, pp. 963–969, 1997.

[24] C. Fischer, B. Jonckx, M. Mazzone, et al., "Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels," *Cell*, vol. 131, no. 3, pp. 463–475, 2007.

[25] A. Eggert, N. Ikegaki, J. Kwiatkowski, H. Zhao, G. M. Brodeur, and B. P. Himelstein, "High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas," *Clinical Cancer Research*, vol. 6, no. 5, pp. 1900–1908, 2000.

[26] J. M. L. Ebos, C. R. Lee, J. G. Christensen, A. J. Mutsaers, and R. S. Kerbel, "Multiple circulating proangiogenic factors induced by sunitinib malate are tumor-independent and correlate with antitumor efficacy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 43, pp. 17069–17074, 2007.

[27] L. J. Nissen, R. Cao, E.-M. Hedlund, et al., "Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis," *Journal of Clinical Investigation*, vol. 117, no. 10, pp. 2766–2777, 2007.

[28] R. Cao, E. Brakenhielm, R. Pawliuk, et al., "Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2," *Nature Medicine*, vol. 9, no. 5, pp. 604–613, 2003.

[29] C. G. Willett, Y. Boucher, D. G. Duda, et al., "Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients," *Journal of Clinical Oncology*, vol. 23, no. 31, pp. 8136–8139, 2005.

[30] V. Levina, Y. Su, B. Nolen, et al., "Chemotherapeutic drugs and human tumor cells cytokine network," *International Journal of Cancer*, vol. 123, no. 9, pp. 2031–2040, 2008.

[31] R. Kerbel and J. Folkman, "Clinical translation of angiogenesis inhibitors," *Nature Reviews Cancer*, vol. 2, no. 10, pp. 727–739, 2002.

[32] R. K. Jain, "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy," *Science*, vol. 307, no. 5706, pp. 58–62, 2005.

[33] A. L. Harris, "Hypoxia—a key regulatory factor in tumour growth," *Nature Reviews Cancer*, vol. 2, no. 1, pp. 38–47, 2002.

[34] K. Hirota and G. L. Semenza, "Regulation of angiogenesis by hypoxia-inducible factor 1," *Critical Reviews in Oncology/Hematology*, vol. 59, no. 1, pp. 15–26, 2006.

[35] A. Rapisarda and G. Melillo, "Role of the hypoxic tumor microenvironment in the resistance to anti-angiogenic therapies," *Drug Resistance Updates*, vol. 12, no. 3, pp. 74–80, 2009.

[36] M. Calvani, A. Rapisarda, B. Uranchimeg, R. H. Shoemaker, and G. Melillo, "Hypoxic induction of an HIF-1α-dependent bFGF autocrine loop drives angiogenesis in human endothelial cells," *Blood*, vol. 107, no. 7, pp. 2705–2712, 2006.

[37] E. Gherard and M. Stoker, "Hepatocytes and scatter factor," *Nature*, vol. 346, no. 6281, p. 228, 1990.

[38] D. S. Grant, H. K. Kleinman, I. D. Goldberg, et al., "Scatter factor induces blood vessel formation in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 5, pp. 1937–1941, 1993.

[39] T. I. Merkulova, K. Y. Kropachev, O. A. Timofeeva, et al., "Species specific hepatocarcinogen inhibition of the glucocorticoid induction of tyrosine aminotransferase gene in mouse and rat liver," *Biochemistry*, vol. 68, no. 5, pp. 520–528, 2003.

[40] R. Zarnegar, "Regulation of HGF and HGFR gene expression," *EXS*, vol. 74, pp. 33–49, 1995.

[41] K. Kajiya, S. Hirakawa, B. Ma, I. Drinnenberg, and M. Detmar, "Hepatocyte growth factor promotes lymphatic vessel

formation and function," *EMBO Journal*, vol. 24, no. 16, pp. 2885–2895, 2005.

[42] W. W. Ma and A. A. Adjei, "Novel agents on the horizon for cancer therapy," *CA: A Cancer Journal for Clinicians*, vol. 59, no. 2, pp. 111–137, 2009.

[43] J. Wojta, C. Kaun, J. M. Breuss, et al., "Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells," *Laboratory Investigation*, vol. 79, no. 4, pp. 427–438, 1999.

[44] Y.-W. Zhang, Y. Su, O. V. Volpert, and G. F. Vande Woude, "Hepatocyte growth factor/scatter factor mediates angiogenesis through positive VEGF and negative thrombospondin 1 regulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 22, pp. 12718–12723, 2003.

[45] W. Wang, Q. Li, T. Yamada, et al., "Crosstalk to stromal fibroblasts induces resistance of lung cancer to epidermal growth factor receptor tyrosine kinase inhibitors," *Clinical Cancer Research*, vol. 15, no. 21, pp. 6630–6638, 2009.

[46] T. Ide, Y. Kitajima, A. Miyoshi, et al., "Tumor-stromal cell interaction under hypoxia increases the invasiveness of pancreatic cancer cells through the hepatocyte growth factor/c-Met pathway," *International Journal of Cancer*, vol. 119, no. 12, pp. 2750–2759, 2006.

[47] H. Diez, A. Fischer, A. Winkler, et al., "Hypoxia-mediated activation of Dll4-Notch-Hey2 signaling in endothelial progenitor cells and adoption of arterial cell fate," *Experimental Cell Research*, vol. 313, no. 1, pp. 1–9, 2007.

[48] G. Thurston, I. Noguera-Troise, and G. D. Yancopoulos, "The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth," *Nature Reviews Cancer*, vol. 7, no. 5, pp. 327–331, 2007.

[49] R. C. A. Sainson and A. L. Harris, "Regulation of angiogenesis by homotypic and heterotypic notch signalling in endothelial cells and pericytes: from basic research to potential therapies," *Angiogenesis*, vol. 11, no. 1, pp. 41–51, 2008.

[50] J. Ridgway, G. Zhang, Y. Wu, et al., "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis," *Nature*, vol. 444, no. 7122, pp. 1083–1087, 2006.

[51] M. Yan and G. D. Plowman, "Delta-like 4/notch signaling and its therapeutic implications," *Clinical Cancer Research*, vol. 13, no. 24, pp. 7243–7246, 2007.

[52] S. Pennacchietti, P. Michieli, M. Galluzzo, M. Mazzone, S. Giordano, and P. M. Comoglio, "Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene," *Cancer Cell*, vol. 3, no. 4, pp. 347–361, 2003.

[53] M. Paez-Ribes, E. Allen, J. Hudock, et al., "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[54] J. M. L. Ebos, C. R. Lee, W. Cruz-Munoz, G. A. Bjarnason, J. G. Christensen, and R. S. Kerbel, "Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis," *Cancer Cell*, vol. 15, no. 3, pp. 232–239, 2009.

[55] J. A. Joyce and J. W. Pollard, "Microenvironmental regulation of metastasis," *Nature Reviews Cancer*, vol. 9, no. 4, pp. 239–252, 2009.

[56] F. Shojaei, X. Wu, X. Qu, et al., "G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 16, pp. 6742–6747, 2009.

[57] S. Ostrand-Rosenberg, "Immune surveillance: a balance between protumor and antitumor immunity," *Current Opinion in Genetics and Development*, vol. 18, no. 1, pp. 11–18, 2008.

[58] F. Shojaei, X. Wu, A. K. Malik, et al., "Tumor refractoriness to anti-VEGF treatment is mediated by $CD11b^+Gr1^+$ myeloid cells," *Nature Biotechnology*, vol. 25, no. 8, pp. 911–920, 2007.

[59] I. Marigo, L. Dolcetti, P. Serafini, P. Zanovello, and V. Bronte, "Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells," *Immunological Reviews*, vol. 222, no. 1, pp. 162–179, 2008.

[60] G. Di Lorenzo, G. Carteni, R. Autorino, et al., "Phase II study of sorafenib in patients with sunitinib-refractory metastatic renal cell cancer," *Journal of Clinical Oncology*, vol. 27, no. 27, pp. 4469–4474, 2009.

[61] B. I. Rini, S. Halabi, J. E. Rosenberg, et al., "Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206," *Journal of Clinical Oncology*, vol. 26, no. 33, pp. 5422–5428, 2008.

[62] T. Natori, M. Sata, M. Washida, Y. Hirata, R. Nagai, and M. Makuuchi, "G-CSF stimulates angiogenesis and promotes tumor growth: potential contribution of bone marrow-derived endothelial progenitor cells," *Biochemical and Biophysical Research Communications*, vol. 297, no. 4, pp. 1058–1061, 2002.

[63] F. Shojaei, X. Wu, C. Zhong, et al., "Bv8 regulates myeloid-cell-dependent tumour angiogenesis," *Nature*, vol. 450, no. 7171, pp. 825–831, 2007.

[64] L. Yang, L. M. DeBusk, K. Fukuda, et al., "Expansion of myeloid immune suppressor Gr^+CD11b^+ cells in tumor-bearing host directly promotes tumor angiogenesis," *Cancer Cell*, vol. 6, no. 4, pp. 409–421, 2004.

[65] A. X. Zhu, D. V. Sahani, D. G. Duda, et al., "Efficacy, safety, and potential biomarkers of sunitinib monotherapy in advanced hepatocellular carcinoma: a phase II study," *Journal of Clinical Oncology*, vol. 27, no. 18, pp. 3027–3035, 2009.

[66] L. Xu, D. G. Duda, E. di Tomaso, et al., "Direct evidence that bevacizumab, an anti-VEGF antibody, up-regulates SDF1alpha, CXCR4, CXCL6, and neuropilin 1 in tumors from patients with rectal cancer," *Cancer Research*, vol. 69, no. 20, pp. 7905–7910, 2009.

[67] R. K. Jain, D. G. Duda, C. G. Willett, et al., "Biomarkers of response and resistance to antiangiogenic therapy," *Nature Reviews Clinical Oncology*, vol. 6, no. 6, pp. 327–338, 2009.

[68] J. T. Erler, K. L. Bennewith, T. R. Cox, et al., "Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche," *Cancer Cell*, vol. 15, no. 1, pp. 35–44, 2009.

[69] L. Yang, J. Huang, X. Ren, et al., "Abrogation of TGFβ signaling in mammary carcinomas recruits $Gr\text{-}1^+CD11b^+$ myeloid cells that promote metastasis," *Cancer Cell*, vol. 13, no. 1, pp. 23–35, 2008.

[70] F. Shojaei, M. Singh, J. D. Thompson, and N. Ferrara, "Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 7, pp. 2640–2645, 2008.

[71] T. Boehm, J. Folkman, T. Browder, and M. S. O'Reilly, "Antiangiogenic therapy of experimental cancer does not

induce acquired drug resistance," *Nature*, vol. 390, no. 6658, pp. 404–407, 1997.

[72] K. E. Fathers, C. M. Stone, K. Minhas, et al., "Heterogeneity of Tie2 expression in tumor microcirculation: influence of cancer type, implantation site, and response to therapy," *American Journal of Pathology*, vol. 167, no. 6, pp. 1753–1762, 2005.

[73] S. Seaman, J. Stevens, M. Y. Yang, D. Logsdon, C. Graff-Cherry, and B. St. Croix, "Genes that distinguish physiological and pathological angiogenesis," *Cancer Cell*, vol. 11, no. 6, pp. 539–554, 2007.

[74] N. Reinmuth, W. Liu, Y. D. Jung, et al., "Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival," *The FASEB Journal*, vol. 15, no. 7, pp. 1239–1241, 2001.

[75] G. Bergers, S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan, "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1287–1295, 2003.

[76] L. E. Benjamin, I. Hemo, and E. Keshet, "A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF," *Development*, vol. 125, no. 9, pp. 1591–1598, 1998.

[77] P. A. D'Amore, "Vascular endothelial cell growth factor-a: not just for endothelial cells anymore," *American Journal of Pathology*, vol. 171, no. 1, pp. 14–18, 2007.

[78] P. Baluk, H. Hashizume, and D. M. McDonald, "Cellular abnormalities of blood vessels as targets in cancer," *Current Opinion in Genetics and Development*, vol. 15, no. 1, pp. 102–111, 2005.

[79] D. C. Darland, L. J. Massingham, S. R. Smith, E. Piek, M. Saint-Geniez, and P. A. D'Amore, "Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival," *Developmental Biology*, vol. 264, no. 1, pp. 275–288, 2003.

[80] A. Armulik, A. Abramsson, and C. Betsholtz, "Endothelial/pericyte interactions," *Circulation Research*, vol. 97, no. 6, pp. 512–523, 2005.

[81] G. Allt and J. G. Lawrenson, "Pericytes: cell biology and pathology," *Cells Tissues Organs*, vol. 169, no. 1, pp. 1–11, 2001.

[82] T. Inai, M. Mancuso, H. Hashizume, et al., "Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts," *American Journal of Pathology*, vol. 165, no. 1, pp. 35–52, 2004.

[83] K. Hori, S. Saito, Y. Sato, et al., "Differential relationship between changes in tumour size and microcirculatory functions induced by therapy with an antivascular drug and with cytotoxic drugs: implications for the evaluation of therapeutic efficacy of AC7700 (AVE8062)," *European Journal of Cancer*, vol. 39, no. 13, pp. 1957–1966, 2003.

[84] R. T. Tong, Y. Boucher, S. V. Kozin, F. Winkler, D. J. Hicklin, and R. K. Jain, "Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors," *Cancer Research*, vol. 64, no. 11, pp. 3731–3736, 2004.

[85] M. Czabanka, M. Vinci, F. Heppner, A. Ullrich, and P. Vajkoczy, "Effects of sunitinib on tumor hemodynamics and delivery of chemotherapy," *International Journal of Cancer*, vol. 124, no. 6, pp. 1293–1300, 2009.

[86] J. L. Wilkinson-Berka, S. Babic, T. De Gooyer, et al., "Inhibition of platelet-derived growth factor promotes pericyte loss and angiogenesis in ischemic retinopathy," *American Journal of Pathology*, vol. 164, no. 4, pp. 1263–1273, 2004.

[87] F. Winkler, S. V. Kozin, R. T. Tong, et al., "Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases," *Cancer Cell*, vol. 6, no. 6, pp. 553–563, 2004.

[88] R. Kalluri, "Basement membranes: structure, assembly and role in tumour angiogenesis," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 422–433, 2003.

[89] R. M. Shaheen, W. W. Tseng, D. W. Davis, et al., "Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms," *Cancer Research*, vol. 61, no. 4, pp. 1464–1468, 2001.

[90] S. Morikawa, P. Baluk, T. Kaidoh, A. Haskell, R. K. Jain, and D. M. McDonald, "Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors," *American Journal of Pathology*, vol. 160, no. 3, pp. 985–1000, 2002.

[91] W. P. J. Leenders, B. Kusters, K. Verrijp, et al., "Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option," *Clinical Cancer Research*, vol. 10, no. 18, part 1, pp. 6222–6230, 2004.

[92] J. Holash, P. C. Maisonpierre, D. Compton, et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF," *Science*, vol. 284, no. 5422, pp. 1994–1998, 1999.

[93] P. Sardari Nia, J. Hendriks, G. Friedel, P. Van Schil, and E. Van Marck, "Distinct angiogenic and non-angiogenic growth patterns of lung metastases from renal cell carcinoma," *Histopathology*, vol. 51, no. 3, pp. 354–361, 2007.

[94] E. R. Gerstner, A. G. Sorensen, R. K. Jain, and T. T. Batchelor, "Anti-vascular endothelial growth factor therapy for malignant glioma," *Current Neurology and Neuroscience Reports*, vol. 9, no. 3, pp. 254–262, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 652320, 12 pages
doi:10.1155/2010/652320

Review Article

Molecular and Clinical Aspects of Targeting the VEGF Pathway in Tumors

Grzegorz Korpanty,[1] Laura A. Sullivan,[2] Elizabeth Smyth,[1] Desmond N. Carney,[1] and Rolf A. Brekken[2,3]

[1] *Department of Medical Oncology, Mater Misericordiae University Hospital, Eccles St, Dublin 7, Ireland*
[2] *Division of Surgical Oncology, Department of Surgery, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA*
[3] *Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA*

Correspondence should be addressed to Grzegorz Korpanty, greg.korpanty@gmail.com

Received 25 November 2009; Accepted 21 April 2010

Academic Editor: Arkadiusz Dudek

Tumor angiogenesis is a complex process resulting from many signals from the tumor microenvironment. From preclinical animal models to clinical trials and practice, targeting tumors with antiangiogenic therapy remains an exciting area of study. Although many scientific advances have been achieved, leading to the development and clinical use of antiangiogenic drugs such as bevacizumab, sorafenib, and sunitinib, these therapies fall short of their anticipated benefits and leave many questions unanswered. Continued research into the complex signaling cascades that promote tumor angiogenesis may yield new targets or improve upon current therapies. In addition, the development of reliable tools to track tumor responses to antiangiogenic therapy will enable a better understanding of current therapeutic efficacy and may elucidate mechanisms to predict patient response to therapy.

1. Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vascular network, is a crucial process during tumor development. New vessels are needed to supply the tumor with nutrients for sustained local growth and to enable distant metastases [1]. The idea of tumors promoting their own angiogenesis through the secretion of then unknown factor(s) was first proposed in the 1930s by Gordon Ide [2]. In 1945, Glenn Algire [3] noticed that tumors grew significantly faster than normal tissues in part because of the ability to stimulate the growth of new vessels to provide oxygen and nutrients. In late 1960s, work by Bruce Warren, Melvin Greenblatt and Philippe Shubik [4, 5] supported the crucial role of tumor angiogenesis in malignant tumorigenesis. Their studies confirmed the hypothesis that tumors secrete soluble substances that promote vessel formation. Folkman reported the isolation of such a substance from a Walker 256 carcinoma grown in rats and called it a "tumor angiogenic factor" (TAF). In 1971, Folkman proposed that tumors cannot grow beyond a certain size without inducing angiogenesis and proposed that inhibiting tumor angiogenesis could prevent local tumor growth and formation of distant metastases [6, 7]. Since then extensive research has focused on the identification of proangiogenic factors produced by tumor cells and strategies to block their action.

In 2004, bevacizumab (Avastin, Genentech, Inc.) became the first antiangiogenic drug approved by the Food and Drug Administration (FDA) for use in patients with metastatic colorectal cancer [8]. Since then bevacizumab has been approved for several other tumor types including breast, renal cell carcinoma, nonsmall cell lung cancer (NSCLC), and glioblastoma. Additionally, other antiangiogenic drugs were developed, such as sunitinib malate (Sutent, Pfizer, Inc.) and sorafenib tosylate (Nexavar, Bayer Pharmaceuticals Corp.), which are FDA approved for renal cell carcinoma and for gastrointestinal stromal tumors and hepatocellular carcinoma, respectively. The results of the antiangiogenic therapy in clinic have been disappointing as compared to the promising data from preclinical animal studies. Therefore,

there is much to be learned about tumor angiogenesis and how best to utilize antiangiogenic therapy. In this review we will discuss the mechanisms of tumor angiogenesis and clinical application of antiangiogenic therapy.

2. Molecular Mechanisms of Tumor Angiogenesis

Growing tumors secrete a number of growth factors that can induce angiogenesis. One predominant factor that stimulates tumor angiogenesis is vascular endothelial growth factor A (VEGF). VEGF was initially identified as Vascular Permeability Factor (VPF) in 1983 by Harold Dvorak and Donald Senger in the conditioned medium of a guinea pig cancer cell line [9]. In 1989, Napoleone Ferrara's group reported the isolation and sequencing of an endothelial cell specific mitogen from pituitary cells and called it VEGF [10]. In the same issue of Science, Daniel T. Connolly reported cloning a gene encoding VPF that turned out to be identical with VEGF [11].

VEGF stimulates proliferation and migration of vascular endothelial cells (ECs). It also promotes survival, inhibits apoptosis, and regulates permeability of ECs. VEGF belongs to a family of growth factors that includes VEGF-B, -C, -D, -E, and placental growth factor (PlGF) [12]. Alternative splicing of the VEGF gene results in formation of four major isoforms of VEGF of varying molecular weights ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$). The main difference between these isoforms is bioavailability of VEGF for receptor binding. $VEGF_{121}$ exists as a highly soluble circulating form while $VEGF_{206}$ remains bound exclusively to the extracellular matrix (ECM) and is released upon proteolytic cleavage by metalloproteinases (MMPs) or plasmin [13]. $VEGF_{165}$ is the predominantly active isoform that can be found both circulating in plasma and bound to ECM [12].

The biological functions of VEGF are mediated upon binding to receptor tyrosine kinases Vascular Endothelial Growth Factor Receptor-1, -2 (VEGFR1, 2). VEGFR1 binds VEGF, VEGF-B, and PlGF [14, 15]. VEGFR1 participates in embryonic vessel development, and is proposed to facilitate hematopoiesis and recruitment of endothelial cell progenitors to tumor blood vessels from bone marrow [12]. VEGFR1 binds VEGF with high affinity ($K_D \sim$ 10–20 pM), which is significantly stronger than the affinity of VEGF-VEGFR2 interaction. The mechanisms through which VEGFR1 functions have not been elucidated completely. Genetic data suggest that during embryonic development VEGFR1 functions as a negative regulator of VEGF activity. Mice deficient in VEGFR1 (e.g., *VEGFR1*$^{-/-}$) die *in utero* from an over abundance of endothelial cells that are disorganized [16]. Furthermore, mice engineered to express VEGFR1 lacking the tyrosine kinase domain (*flt-1*$^{TK-/-}$) develop normally with a fully functional vascular network [16]. Additionally, the extent of VEGFR1 involvement in adult angiogenesis is not well-defined [17, 18]. There are numerous reports of selective blockade of VEGFR2 activity in tumors resulting in reduced angiogenesis and tumor growth [19–23] suggesting that VEGFR1 activity is not required for VEGF-induced angiogenesis in pathological conditions. However, the function of VEGFR2 is defined more clearly.

VEGFR2 is the key mediator of VEGF-driven angiogenesis. VEGFR2 is crucial during embryonic vascular development. Heterozygous and homozygous VEGFR-2 knockout mice die *in utero* due to disrupted vasculogenesis and hematopoiesis [24]. Upon VEGF binding, VEGFR2 undergoes auto-transphosphorylation and downstream effectors including phospholipase C gamma, protein kinase C, Raf, the MAP kinase signaling cascades, and the PI3K and FAK pathways are activated, leading to endothelial cell proliferation, migration, and survival (Figure 1) [25, 26]. VEGFR3 binds VEGF-C and -D and is directly involved in formation of the lymphatic vasculature physiologic and tumor development [27, 28]. There is also experimental evidence that VEGFR3 mediated activation of lymphatic endothelial cells is crucial for metastasis [29]. Neuropilin-1 (Nrp-1) and Neuropilin-2 (Nrp-2) are coreceptors originally identified for their involvement in neuronal guidance, and that bind members of collapsin/semaphorin protein family [30]. The Nrps can also bind to certain heparin binding isofoms of VEGF (e.g., $VEGF_{165}$) to enhance the binding of VEGF to VEGFR1, and VEGFR2 (Figure 1) [31, 32]. Nrps lack tyrosine kinase domains but do contain an intracellular PDZ domain, which has been suggested to facilitate VEGF specific signaling.

VEGF expression within tumors is regulated by oxygen levels, growth factors and cytokines, and oncogene activation/tumor suppressor inactivation [26]. Hypoxia in the tumor microenvironment is one of the most important factors influencing expression of VEGF. Hypoxia inducible factor-1 (HIF-1) is a transcription factor that regulates expression of certain genes in response to intracellular oxygen levels [33, 34]. It consists of two subunits: alpha (α) and beta (β). Normoxic conditions favor ubiquitin-dependent proteosome-mediated degradation of HIF-1α subunit, while oxygen deprivation stabilizes and enhances HIF-1α/HIF-1β dimerization. These dimers interact with a hypoxia response element (HRE) in the promoter region of many genes, including VEGF [35–37]. VEGF expression is also regulated via paracrine or autocrine release of growth factors and cytokines such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), keratinocyte growth factor, insulin-like growth factor (IGF), transforming growth factors alpha and beta (TGF-α, -β), interleukin 1α and 6 (IL-1α, -6) and prostaglandins (PGE2) [38–43]. During tumorigenesis, certain genetic mutations in the ras oncogene or Wnt-signaling pathways may also lead to elevated expression of VEGF [44, 45]. Tumor-derived VEGF may also function in an autocrine manner [46]. Receptors for VEGF (e.g., VEGFR1, VEGF2, Nrp1, Nrp2) are expressed on multiple cancer cell lines [47, 48], and there is evidence that VEGF can function as a cell survival factor for tumor cells and vascular endothelial cells within the tumor [49, 50].

The idea of vascular progenitor cells derived from bone marrow that incorporate into the tumor vasculature is exciting and controversial [51]. Circulating VEGF as well as other growth factors produced by tumor can mobilize variety of hematopoietic cell populations that express CD45,

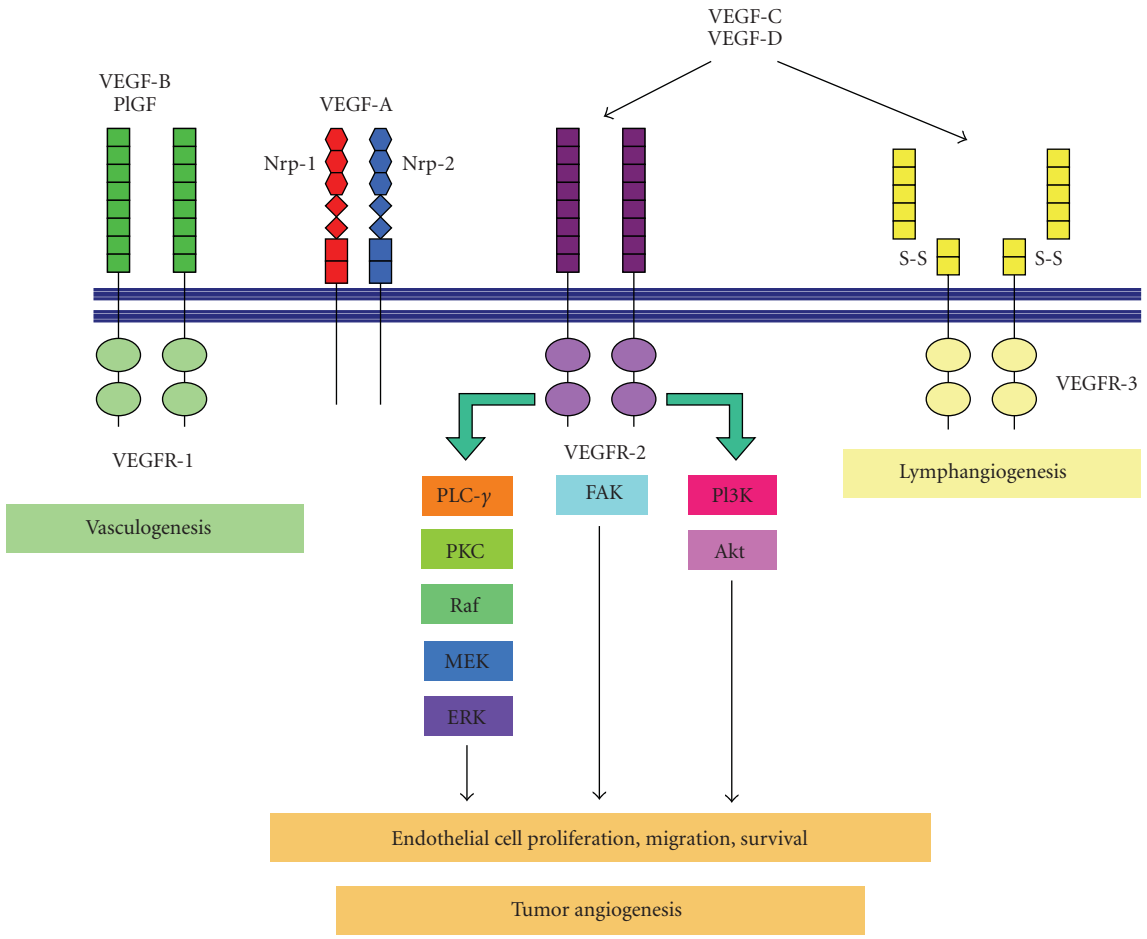


Figure 1: VEGF signaling interactions. The VEGF family can bind to VEGFR1, VEGFR2, and VEGFR3 inducing signaling cascades to promote vasculogenesis, angiogenesis, and lymphangiogenesis, respectively.

VEGFR1, VEGFR2, VE-cadherin, tie-2 or CXC chemokine receptor 4 [52–56]. There is a significant discrepancy among the published studies regarding the percentage contribution of bone marrow-derived cells into the formation of tumor vasculature—numbers vary between as high as 50% to as low as 5% [52, 57–59]. However, Robert Kerbel's group observed that after exposure to chemotherapy or vascular disrupting agents (VDAs), there is a significant efflux of circulating bone marrow-derived cells (BMDC) homing to the sites of tumor vasculature [60, 61]. This phenomenon may have a potent clinical application if confirmed in human studies. Using agents that can block incorporation of BMDCs may contribute to better outcomes of chemotherapy by interfering with tumor angiogenesis.

3. Clinical Applications of Antiangiogenic Therapy

The VEGF pathway can be targeted therapeutically at various molecular levels. Currently two major concepts are studied in the clinical setting: blocking VEGF from binding to its extracellular receptors with VEGF antagonists (antibodies, VEGF-Trap) or inhibiting VEGF signaling with tyrosine kinase inhibitors (TKIs) [62]. As previously mentioned, bevacizumab is a humanized, VEGF-neutralizing antibody that was the first antiangiogenic agent approved by the FDA for use in cancer patients. In 2004, a pivotal phase III clinical trial demonstrated a 4.6 months survival benefit of adding bevacizumab to chemotherapy in patients with metastatic colorectal cancer [8]. After the encouraging data from this trial were published, patients with other solid malignancies were enrolled into a multitude of clinical trials that added bevacizumab into the standard treatment of care. However, the results from many of these clinical trials are disappointing. Most patients fail to achieve long-term benefits with bevacizumab plus chemotherapy [63]. Selected groups of patients respond with tumor shrinkage, disease stabilization, or improvements in survival that are counted in months rather than years [64].

A new approach to anti-VEGF therapy currently being evaluated is genetically engineered fusion proteins that function as molecular "traps" for VEGF. Aflibercept (VEGF-Trap, Regeneron Pharmaceuticals, Inc.) is a recombinant fusion protein that binds both VEGF and PlGF with high affinity. It is composed of the extracellular domains of VEGFR1 and VEGFR2 that are fused to the Fc region of

human IgG [65]. Currently, there are more than 40 ongoing trials (http://clinicaltrials.gov/) that explore this therapy in solid and hematologic malignancies.

Small molecule TKIs with antiangiogenic activity are another important area of active clinical research. Unlike monoclonal antibodies (i.e., bevacizumab) or fusion proteins (i.e., aflibercept), TKIs are small molecules that interfere directly with tyrosine kinase activity (Figure 2). Since the intracellular domain targeted by TKIs is structurally similar in many tyrosine kinase receptors, a single TKI usually interferes with the activity of multiple receptors [66]. Sunitinib and sorafenib are multitargeting TKIs that can block activity angiogenic targets such as of VEGFR1, 2, 3, platelet-derived growth factor (PDGF) receptors and c-Kit or RET. After confirmed clinical benefit for sunitinib and sorafenib in selected patient groups [67, 68], there are now a variety of ongoing clinical trials recruiting patients from a broad spectrum of solid malignancies (http://www.clinicaltrials.gov/).

4. Mechanisms of Action of Antiangiogenic Agents

Various agents that target tumor angiogenesis are currently under investigation in different cancer types in many clinical trials [62]. While some of these agents show more encouraging results than the others, a common clinical problem is the lack of effective tools to monitor tumor response to these novel therapies [69]. The *Response Evaluation Criteria in Solid Tumors* (RECIST) criteria that are commonly used to monitor tumor response may not be an effective or even accurate measure of response to antiangiogenic agents. As an example, antiangiogenic agents will often enhance the central necrosis of tumors without changing the overall tumor size, which is a central parameter in RECIST evaluation [70].

An area of intense debate is how antiangiogenic agents actually work in terms of combating cancer [71]. According to the Folkman hypothesis, interference with tumor angiogenesis results in either inhibition of new vessel formation or progressive loss of existing vessels supporting tumor growth. An inadequate blood supply caused by a reduction of the vascular network in response to antiangiogenic therapy, slows and eventually prevents tumor growth and causes the tumor to regress to a "state of dormancy", which can be clinically undetectable [7]. Evidence for this paradigm can be found in preclinical studies where fast-growing human tumors are treated with anti-VEGF therapy for long periods of time [72, 73].

An alternative explanation for anti-VEGF activity and possibly antiangiogenic agents in general is anchored in the heterogeneity of tumor vasculature. A minority of tumor blood vessels are associated intimately with pericytes and as a result are more functional and stable [74]. These vessels are not as dependent on VEGF stimulation for survival. In contrast, a large proportion of tumor blood vessels are tortuous, leaky, and immature, lacking interactions with pericytes. Furthermore, these vessels are more dependent on survival signals provided by VEGF and other growth factors. When VEGF levels are decreased via therapy these vessels regress, leaving behind a more stable vascular network. There is also compelling evidence that VEGF actively suppresses pericyte recruitment, therefore blocking VEGF activity may also result in the active recruitment of pericytes to remaining blood vessels [75]. As a result, the vasculature that remains in the face of anti-VEGF therapy consists of a higher percentage of pericyte associated blood vessels that are more efficient in function. This process has been termed "normalization" by Jain who hypothesizes that anti-VEGF therapy actually "normalizes" tumor vasculature and transiently improves blood flow within the tumor, thus enhancing the delivery of chemotherapy [76, 77]. Additionally, because stable vessels within the tumor are less leaky, interstitial pressure may decrease and thereby facilitate tissue penetration of chemotherapy. A supportive corollary to this is that antiangiogenic therapy has been shown to increase the efficacy of radiation therapy due to transient improvement in tumor oxygenation as a result of antiangiogenic treatment and vascular normalization [71, 78].

5. Monitoring Clinical Response to Antiangiogenic Therapies

The majority of noninvasive techniques used to assess the effects of antiangiogenic therapy do not directly visualize tumor blood vessels. Rather surrogate markers for vascular function such as blood flow are used commonly. These techniques rely on the fact that during the course of treatment blood flow within the tumor changes, either increasing due to normalization or decreasing due to diminished blood supply and vessel regression [79–81]. Hemodynamic changes within the tumor vasculature remain the major surrogate markers for majority of these techniques. Clinically relevant imaging techniques include magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), and ultrasound (US). Each of these techniques can be used with appropriate contrast media to evaluate hemodynamic function within tissues including solid tumors.

Perfusion dynamic contrast-enhanced (DCE) MRI has been used successfully in both preclinical and clinical models to follow hemodynamic function [82]. DCE-MRI makes use of paramagnetic tracers, mostly consisting of a low-molecular-weight gadolinium (Gd) and is the standard method for measurement of vascular function in clinical trials of antiangiogenic drugs [83]. Signal enhancement obtained by DCE-MRI depends on tissue perfusion and permeability, contrast concentration, and extravascular space volume [84]. DCE-MRI has been especially useful in clinical studies of patients with liver and brain tumors [85–90], and has been investigated as a possible pharmacodynamic biomarker sorafenib therapy in metastatic renal carcinoma [91].

CT-based perfusion imaging techniques are also used to assess the vascular effects of antiangiogenic treatments [92, 93]. Although DCE-MRI gives better spatial resolution and is a superior method for brain imaging studies, CT still remains a preferred method for imaging structures within the thorax, abdomen, and pelvis. Thus some clinical studies

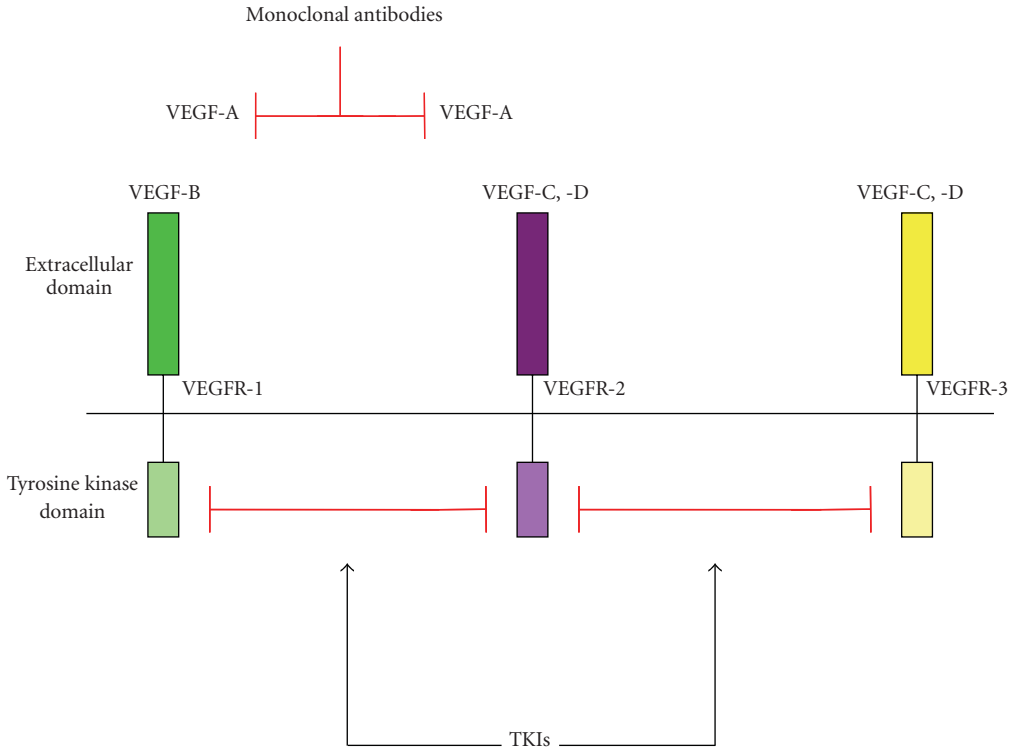


Figure 2: Inhibition of VEGF signaling pathways. Several classes of drugs have been developed to combat VEGF-mediated tumor angiogenesis. Monoclonal anti-VEGF antibodies (e.g., bevacizumab) and soluble receptor constructs (e.g., VEGF-Trap) bind to the VEGF and PlGF preventing their interaction and signaling through VEGFR1 and VEGFR2. Tyrosine kinase inhibitors (TKIs) inhibit the intracellular tyrosine kinase activity of VEGF receptors, blocking downstream signaling.

investigating antiangiogenic agents have used perfusion CT rather than DCE-MRI to evaluate tumor blood flow [94–96].

In addition, PET-based imaging techniques are widely used in clinical oncology [97]. PET uses positron-emitting tracers, of which $H_2^{15}O$ can be used to study tumor blood flow and this method has been used in clinical trials with good results [98]. $H_2^{15}O$ is a positron-emitting tracer that can diffuse freely into the tissues and its tissue uptake correlates with blood perfusion [99]. Both $H_2^{15}O$ PET and DCE-MRI are useful for monitoring tumor microvasculature. $H_2^{15}O$ PET is particularly useful in the assessment of tissue perfusion while DCE-MRI measures also vascular permeability. A major disadvantage of both methods is their limited availability for patients because they require highly skilled and trained staff, that is, typically only available in large radiology or nuclear medicine departments.

Worldwide, ultrasound (US) is one of the most commonly used noninvasive imaging techniques. It provides anatomical information and can also be used to assess physiological function (e.g., blood flow with doppler ultrasound) or to serve as a therapeutic tool (e.g., high frequency ultrasound ablation of the tissue) [100, 101]. Because blood is only slightly less echogenic than surrounding tissue, US is not very effective for imaging small blood vessels. However, the introduction of US contrast agents expanded the clinical and research applications of US especially in the area of vascular imaging. Microbubbles (MB) are small particles (1–10 μm) consisting of a gaseous core and a shell of protein (e.g., albumin) or lipid mixture [102] that can be injected intravenously and are promising US contrast agents. MBs are intravascular tracers that do not extravasate unless there is structural damage to the vessel wall. When injected intravenously, MBs enhance the echogenicity of the blood pool and enable distinction of vascular structures from the surrounding tissue. Within in an ultrasound field MB resonate in response to the ultrasound wave and can enhance both grey scale images and flow mediated doppler signals. Their high echogenic properties are due to the difference of compressibility of the gaseous core within the MB and the surrounding blood components and tissue [103]. MB have proven their usefulness in clinical echocardiography, especially in the evaluation of systolic myocardial function, ejection fraction, delineating endocardial border, and myocardial blood flow [104–106]. Imaging metastatic deposits or primary liver tumors (e.g., hepatocellular carcinoma) with contrast US is an example of the clinical application for MB-enhanced US imaging [107, 108]. The liver is one of the organs, that is, most commonly affected by distant metastases, and early detection of small (subcentimeter) lesions by contrast-enhanced US is of clinical significance [109–111]. Comparative studies of the sensitivity and specificity of PET, CT, DCE-MRI, and MB-enhanced US for detection of tumor perfusion showed

that contrast US is an effective and correlative method with significant clinical potential [112, 113].

MB behave hemodynamically like red blood cells, circulate freely after injection and are small enough to reach the capillary microcirculation [114]. The idea of targeted imaging using contrast US is based on the selective accumulation of MB in specific vascular beds that can be reached by US wave and subsequently imaged. MB with an albumin-containing shell can adhere to endothelial cells that are activated by inflammatory cytokines, or activated leukocytes, which enables MB to be targeted passively to the areas of vascular inflammation [115–117]. MB can be also targeted actively to specific vascular beds by conjugation of targeting moieties (e.g., antibodies or peptides) to the MB shell [118–120]. In preclinical studies, MB have been targeted to various endothelial markers expressed on inflamed or ischemic tissues such as the myocardium or kidney [121–123]. Although tumor endothelial cells are often thought to be genetically normal, work by Hida et al. has demonstrated that mouse endothelial cells harvested from tumor xenografts are aneuploid and have abnormal centromeres [124]. Perhaps related to this cytogenetic abnormality, tumor endothelial cells express specific molecules that are absent or expressed at a much lower levels on endothelium in normal noncancerous tissue. Thus, the tumor vasculature is an attractive subject for imaging with targeted MB and US [125, 126]. The list of potential target molecules selective for tumor vasculature is growing and includes growth factor receptors, integrins, ephrins, endoglin, tumor endothelial markers (TEMs), and markers of cell stress (see Table 1 at supplementary material available at 10.1155/2010/652320).

The development of surrogate markers of pathological angiogenesis to monitor the response of patients to antiangiogenic therapy is of critical importance if antiangiogenic strategies are to be a viable modality for cancer therapy. Contrast US using targeted MB can be an efficient tool to monitor the expression of surface markers by tumor endothelial cells. This strategy can be used to visualize tumor blood vessels and in addition can follow the expression level of markers that are known to be altered by antiangiogenic therapy. VEGFR2 is a commonly used marker of vascular endothelial cells and has been used by multiple groups as a molecular target for MB. Animal models of angiosarcoma, glioma, and breast cancer showed that VEGFR2 targeted MB enhanced US imaging in evaluation of tumor angiogenesis [127, 128]. Recently, our group evaluated vascular response to antiangiogenic and chemotherapy in mouse models of pancreatic cancer using MB targeted against VEGFR2, the VEGF:VEGFR complex, and endoglin [20]. Using three different formulations of tumor vessel specific MB and US, we were able to noninvasively monitor vascular function of subcutaneous and orthotopic pancreatic tumors in mice. We found that targeting to VEGFR2, endoglin, or the VEGF:VEGFR complex was specific for tumor vasculature as there was no signal enhancement in nontumor tumor tissue. Further, we found that anti-VEGF therapy or treatment with gemcitabine reduced the expression of the molecular targets bound by targeted MB. Our contrast US intensity data correlated with immunohistochemical analysis of tumor samples, providing the first indication that targeted MB could be used to follow expression of a cell surface target. Additionally, these studies also validated that gemcitabine can effect endothelial cells in tumors. Other groups have since confirmed our findings using targeted MB to image the response of tumor vessels to the therapy [129]. These data and the work of others [130–133] that conclusively demonstrate the utility of contrast US using targeted MB support the clinical evaluation of such strategies as a method for following response to antiangiogenic therapy in cancer patients.

6. Toxicities of Antiangiogenic Therapies

VEGF signaling is involved in many normal physiologic processes such as hemostasis, vascular homeostasis and integrity, and the maintenance of endothelial function in kidney glomeruli [134]. Following the introduction of bevacizumab into the clinic, toxic side effects became apparent. The most common side effects of bevacizumab and other antiangiogenic agents are hypertension (3–36 % of patients) and proteinuria (21–64 % of patients) [135]. Although the exact pathophysiological mechanism is not yet fully understood, there is evidence coming from both animal and clinical models, that bevacizumab increases the risk of renal thrombotic microangiopathy [136]. It has been shown in animal models that after binding to VEGF, bevacizumab-VEGF immune complexes can be deposited in the glomerular basement membrane contributing to the development of both proteinuria and hypertension [137]. Bevacizumab has also been shown to increase the incidence of hemorrhagic and thrombotic events in cancer patients. One of the most serious side effects observed in lung cancer patients are hemoptysis and pulmonary hemorrhage. In a phase II clinical trial, NSCLC patients with squamous histology were at a higher risk of developing fatal bleeding, that was most likely related to tumor necrosis and proximity of tumor to the large vessels [138]. Based on this observation bevacizumab is not recommended for squamous NSCLC. In addition, bevacizumab is not recommended for patients with pre-existing conditions that may predispose for either thrombotic or hemorrhagic events (e.g., brain metastases). Bevacizumab can also potentiate the incidence of side effects that are specific to chemotherapy treatment like neutropenia, infections, and thrombocytopenia [139]. There have also been reports of potentially serious toxicities such as nasal septum perforation, reversible posterior leukoencephalopathy syndrome (severe hypertension, cortical blindness, and seizures) or osteonecrosis of the jaw, although these events are very rare [140–142]. TKIs have a unique toxicity profile and are more commonly associated with rash due to blocking EGFR activity and gastrointestinal symptoms like nausea, diarrhea due to the administration of the drug. Hypertension is the predominate toxicity associated with sorafenib and sunitinib treatment due to their antiangiogenic specificities [143]. Endothelial cell production of nitric oxide and prostacyclin is required for mediating vasodilatation and controlling blood pressure. These mediators are stimulated by VEGF-induced VEGFR2 signaling, which is blocked

by sorafenib and sunitinib treatment. Further, TKIs have recently been reported to increase patient risk of bleeding events due to interruption of VEGF-mediated vascular homeostasis [144].

7. Future Directions

Despite the modest survival benefits observed in clinical practice, antiangiogenic therapy remains an attractive concept. Only five years have passed since the first antiangiogenic drug was approved by the FDA for a clinical use. Although we have learned important lessons about this new class of cancer drugs, many questions still remain. The multitude of ongoing clinical trials testing both the new agents and different combinations of agents with already established clinical benefits, may shed light on multiple questions regarding antiangiogenic therapy [145]. Better selection of patients, including the therapeutic schedule of antiangiogenic therapy (i.e., adjuvant versus palliative), monitoring of clinical response, and better toxicity profile of antiangiogenic drugs are among the most important clinical aspects that the ongoing clinical trials will address. In addition, intrinsic and acquired resistance of tumors to antiangiogenic therapies is a growing concern in the clinic, as most patients fail to show sustained benefit with continuous therapy [146]. There are many possible mechanisms of resistance to antiangiogenic therapy that are being actively investigated. One possibility is the activation of alternative molecular pathways resulting in ongoing angiogenesis in response to the presence of a selective inhibitor (e.g., fibroblast growth factor (FGF), interleukin-8, ephrins, angiopoietins, SDF-1 pathway activation, and increased VEGF expression following epidermal growth factor blockade) [147–149]. Also, resistance has been linked to an unresponsiveness of pericyte-covered tumor vessels to antiangiogenic therapies [150, 151], and to the hypovascularity and low levels of *de novo* angiogenesis characteristic of some tumors (e.g., pancreatic cancer) [152]. Although antiangiogenic therapies have entered into clinical practice, we still lack a reliable marker(s) of treatment efficacy. Studies on noninvasive marker(s) such as blood levels of circulating growth factors, cytokines and/or endothelial progenitor cells gave mixed results and are not validated at the present time for a clinical use [153]. Future studies that involve complex proteomic-based analysis may help to find a noninvasive way to not only monitor the effects by also to better select patients who may benefit from antiangiogenic therapies.

Acknowledgment

This work was supported in part by the Randall Bridwell Lung Cancer Research Grant and the Effie Marie Cain Scholarship in Angiogenesis Research (to R. Brekken).

References

[1] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.

[2] N. Ferrara, "VEGF and the quest for tumour angiogenesis factors," *Nature Reviews Cancer*, vol. 2, no. 10, pp. 795–803, 2002.

[3] M. Narazaki and G. Tosato, "Targeting coagulation to the tumor microvasculature: perspectives and therapeutic implications from preclinical studies," *Journal of the National Cancer Institute*, vol. 97, no. 10, pp. 705–707, 2005.

[4] B. A. Warren and P. Shubik, "The growth of the blood supply to melanoma transplants in the hamster cheek pouch," *Laboratory Investigation*, vol. 15, no. 2, pp. 464–478, 1966.

[5] M. Greenblatt and P. Shubi, "Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique," *Journal of the National Cancer Institute*, vol. 41, no. 1, pp. 111–124, 1968.

[6] J. Folkman, E. Merler, C. Abernathy, and G. Williams, "Isolation of a tumor factor responsible or angiogenesis," *Journal of Experimental Medicine*, vol. 133, no. 2, pp. 275–288, 1971.

[7] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[8] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *The New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[9] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. Susan Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, 1983.

[10] D. W. Leung, G. Cachianes, W.-J. Kuang, D. V. Goeddel, and N. Ferrara, "Vascular endothelial growth factor is a secreted angiogenic mitogen," *Science*, vol. 246, no. 4935, pp. 1306–1309, 1989.

[11] P. J. Keck, S. D. Hauser, G. Krivi, et al., "Vascular permeability factor, an endothelial cell mitogen related to PDGF," *Science*, vol. 246, no. 4935, pp. 1309–1312, 1989.

[12] N. Ferrara, H.-P. Gerber, and J. LeCouter, "The biology of VEGF and its receptors," *Nature Medicine*, vol. 9, no. 6, pp. 669–676, 2003.

[13] H. Takahashi and M. Shibuya, "The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions," *Clinical Science*, vol. 109, no. 3, pp. 227–241, 2005.

[14] J. E. Park, H. H. Chen, J. Winer, K. A. Houck, and N. Ferrara, "Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR," *Journal of Biological Chemistry*, vol. 269, no. 41, pp. 25646–25654, 1994.

[15] B. Olofsson, E. Korpelainen, M. S. Pepper, et al., "Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11709–11714, 1998.

[16] S. Hiratsuka, O. Minowa, J. Kuno, T. Noda, and M. Shibuya, "Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 16, pp. 9349–9354, 1998.

[17] D. Lyden, K. Hattori, S. Dias, et al., "Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth," *Nature Medicine*, vol. 7, no. 11, pp. 1194–1201, 2001.

[18] G.-H. Fong, J. Rossant, M. Gertsenstein, and M. L. Breitman, "Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium," *Nature*, vol. 376, no. 6535, pp. 66–70, 1995.

[19] R. A. Brekken, J. P. Overholser, V. A. Stastny, J. Waltenberger, J. D. Minna, and P. E. Thorpe, "Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice," *Cancer Research*, vol. 60, no. 18, pp. 5117–5124, 2000.

[20] G. Korpanty, J. G. Carbon, P. A. Grayburn, J. B. Fleming, and R. A. Brekken, "Monitoring response to anticancer therapy by targeting microbubbles to tumor vasculature," *Clinical Cancer Research*, vol. 13, no. 1, pp. 323–330, 2007.

[21] S. P. Dineen, K. D. Lynn, S. E. Holloway, et al., "Vascular endothelial growth factor receptor 2 mediates macrophage infiltration into orthotopic pancreatic tumors in mice," *Cancer Research*, vol. 68, no. 11, pp. 4340–4346, 2008.

[22] L. Witte, D. J. Hicklin, Z. Zhu, et al., "Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy," *Cancer and Metastasis Reviews*, vol. 17, no. 2, pp. 155–161, 1998.

[23] Y. Izumi, E. Di Tomaso, A. Hooper, et al., "Responses to antiangiogenesis treatment of spontaneous autochthonous tumors and their isografts," *Cancer Research*, vol. 63, no. 4, pp. 747–751, 2003.

[24] M. Shibuya, "Vascular endothelial growth factor (VEGF)-receptor2: its biological functions, major signaling pathway, and specific ligand VEGF-E," *Endothelium*, vol. 13, no. 2, pp. 63–69, 2006.

[25] K. Holmes, O. L. Roberts, A. M. Thomas, and M. J. Cross, "Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition," *Cellular Signalling*, vol. 19, no. 10, pp. 2003–2012, 2007.

[26] N. Ferrara, "Vascular endothelial growth factor: basic science and clinical progress," *Endocrine Reviews*, vol. 25, no. 4, pp. 581–611, 2004.

[27] K. Paavonen, P. Puolakkainen, L. Jussila, T. Jahkola, and K. Alitalo, "Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing," *American Journal of Pathology*, vol. 156, no. 5, pp. 1499–1504, 2000.

[28] Y. He, I. Rajantie, K. Pajusola, et al., "Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels," *Cancer Research*, vol. 65, no. 11, pp. 4739–4746, 2005.

[29] Y. He, K.-I. Kozaki, T. Karpanen, et al., "Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling," *Journal of the National Cancer Institute*, vol. 94, no. 11, pp. 819–825, 2002.

[30] G. Neufeld, T. Cohen, N. Shraga, T. Lange, O. Kessler, and Y. Herzog, "The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis," *Trends in Cardiovascular Medicine*, vol. 12, no. 1, pp. 13–19, 2002.

[31] S. Soker, H. Fidder, G. Neufeld, and M. Klagsbrun, "Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain," *Journal of Biological Chemistry*, vol. 271, no. 10, pp. 5761–5767, 1996.

[32] S. Soker, S. Takashima, H. Q. Miao, G. Neufeld, and M. Klagsbrun, "Neuropilin-1 is expressed by endothelial and tumor cells as an isoform- specific receptor for vascular endothelial growth factor," *Cell*, vol. 92, no. 6, pp. 735–745, 1998.

[33] M. Safran and W. G. Kaelin Jr., "HIF hydroxylation and the mammalian oxygen-sensing pathway," *Journal of Clinical Investigation*, vol. 111, no. 6, pp. 779–783, 2003.

[34] G. L. Wang and G. L. Semenza, "Purification and characterization of hypoxia-inducible factor 1," *Journal of Biological Chemistry*, vol. 270, no. 3, pp. 1230–1237, 1995.

[35] G. L. Wang, B.-H. Jiang, E. A. Rue, and G. L. Semenza, "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 12, pp. 5510–5514, 1995.

[36] G. L. Semenza, "HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus," *Cell*, vol. 107, no. 1, pp. 1–3, 2001.

[37] A. P. Levy, N. S. Levy, S. Wegner, and M. A. Goldberg, "Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia," *Journal of Biological Chemistry*, vol. 270, no. 22, pp. 13333–13340, 1995.

[38] T. Cohen, D. Nahari, L. W. Cerem, G. Neufeld, and B.-Z. Levin, "Interleukin 6 induces the expression of vascular endothelial growth factor," *Journal of Biological Chemistry*, vol. 271, no. 2, pp. 736–741, 1996.

[39] S. Frank, G. Hubner, G. Breier, M. T. Longaker, D. G. Greenhalgh, and S. Werner, "Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing," *Journal of Biological Chemistry*, vol. 270, no. 21, pp. 12607–12613, 1995.

[40] L. Pertovaara, A. Kaipainen, T. Mustonen, et al., "Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells," *Journal of Biological Chemistry*, vol. 269, no. 9, pp. 6271–6274, 1994.

[41] R. S. Warren, H. Yuan, M. R. Matli, N. Ferrara, and D. B. Donner, "Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma," *Journal of Biological Chemistry*, vol. 271, no. 46, pp. 29483–29488, 1996.

[42] P. Ben-Av, L. J. Crofford, R. L. Wilder, and T. Hla, "Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis," *FEBS Letters*, vol. 372, no. 1, pp. 83–87, 1995.

[43] Y.-Q. Li, K.-S. Tao, N. Ren, and Y.-H. Wang, "Effect of c-fos antisense probe on prostaglandin E2-induced upregulation of vascular endothelial growth factor mRNA in human liver cancer cells," *World Journal of Gastroenterology*, vol. 11, no. 28, pp. 4427–4430, 2005.

[44] S. Grugel, G. Finkenzeller, K. Weindel, B. Barleon, and D. Marme, "Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells," *Journal of Biological Chemistry*, vol. 270, no. 43, pp. 25915–25919, 1995.

[45] F. Okada, J. W. Rak, B. St. Croix, et al., "Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3609–3614, 1998.

[46] J. S. Wey, O. Stoeltzing, and L. M. Ellis, "Vascular endothelial growth factor receptors: expression and function in solid

tumors," *Clinical Advances in Hematology & Oncology*, vol. 2, no. 1, pp. 37–45, 2004.

[47] M. Decaussin, H. Sartelet, C. Robert, et al., "Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell lung carcinomas (NSCLCs): correlation with angiogenesis and survival," *Journal of Pathology*, vol. 188, no. 4, pp. 369–377, 1999.

[48] S. Lantuéjoul, B. Constantin, H. Drabkin, C. Brambilla, J. Roche, and E. Brambilla, "Expression of VEGF, semaphorin SEMA3F, and their common receptors neuropilins NP1 and NP2 in preinvasive bronchial lesions, lung tumours, and cell lines," *Journal of Pathology*, vol. 200, no. 3, pp. 336–347, 2003.

[49] E. Castro-Rivera, S. Ran, P. Thorpe, and J. D. Minna, "Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 31, pp. 11432–11437, 2004.

[50] P. Nasarre, B. Constantin, L. Rouhaud, et al., "Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading," *Neoplasia*, vol. 5, no. 1, pp. 83–92, 2003.

[51] F. Bertolini, Y. Shaked, P. Mancuso, and R. S. Kerbel, "The multifaceted circulating endothelial cell in cancer: towards marker and target identification," *Nature Reviews Cancer*, vol. 6, no. 11, pp. 835–845, 2006.

[52] T. Okazaki, S. Ebihara, M. Asada, A. Kanda, H. Sasaki, and M. Yamaya, "Granulocyte colony-stimulating factor promotes tumor angiogenesis via increasing circulating endothelial progenitor cells and Gr1+CD11b+ cells in cancer animal models," *International Immunology*, vol. 18, no. 1, pp. 1–9, 2006.

[53] M. De Palma, M. A. Venneri, R. Galli, et al., "Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors," *Cancer Cell*, vol. 8, no. 3, pp. 211–226, 2005.

[54] M. A. Venneri, M. De Palma, M. Ponzoni, et al., "Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer," *Blood*, vol. 109, no. 12, pp. 5276–5285, 2007.

[55] J. R. Conejo-Garcia, R. J. Buckanovich, F. Benencia, et al., "Vascular leukocytes contribute to tumor vascularization," *Blood*, vol. 105, no. 2, pp. 679–681, 2005.

[56] M. Grunewald, I. Avraham, Y. Dor, et al., "VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells," *Cell*, vol. 124, no. 1, pp. 175–189, 2006.

[57] B. A. Peters, L. A. Diaz Jr., K. Polyak, et al., "Contribution of bone marrow-derived endothelial cells to human tumor vasculature," *Nature Medicine*, vol. 11, no. 3, pp. 261–262, 2005.

[58] M. Garcia-Barros, F. Paris, C. Cordon-Cardo, et al., "Tumor response to radiotherapy regulated by endothelial cell apoptosis," *Science*, vol. 300, no. 5622, pp. 1155–1159, 2003.

[59] H. Spring, T. Schüler, B. Arnold, G. J. Hämmerling, and R. Ganss, "Chemokines direct endothelial progenitors into tumor neovessels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18111–18116, 2005.

[60] Y. Shaked, A. Ciarrocchi, M. Franco, et al., "Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors," *Science*, vol. 313, no. 5794, pp. 1785–1787, 2006.

[61] D. W. Siemann, D. J. Chaplin, and M. R. Horsman, "Vascular-targeting therapies for treatment of malignant disease," *Cancer*, vol. 100, no. 12, pp. 2491–2499, 2004.

[62] N. Ferrara and R. S. Kerbel, "Angiogenesis as a therapeutic target," *Nature*, vol. 438, no. 7070, pp. 967–974, 2005.

[63] R. K. Jain, D. G. Duda, J. W. Clark, and J. S. Loeffler, "Lessons from phase III clinical trials on anti-VEGF therapy for cancer," *Nature Clinical Practice Oncology*, vol. 3, no. 1, pp. 24–40, 2006.

[64] M. A. Medina, R. Munoz-Chapuli, and A. R. Quesada, "Challenges of antiangiogenic cancer therapy: trials and errors, and renewed hope," *Journal of Cellular and Molecular Medicine*, vol. 11, no. 3, pp. 374–382, 2007.

[65] "Aflibercept: AVE 0005, AVE 005, AVE0005, VEGF Trap—regeneron, VEGF Trap (R1R2), VEGF Trap-eye," *Drugs in R and D*, vol. 9, no. 4, pp. 261–269, 2008.

[66] D. S. Krause and R. A. Van Etten, "Tyrosine kinases as targets for cancer therapy," *The New England Journal of Medicine*, vol. 353, no. 2, pp. 172–187, 2005.

[67] R. J. Motzer, T. E. Hutson, P. Tomczak, et al., "Sunitinib versus interferon alfa in metastatic renal-cell carcinoma," *The New England Journal of Medicine*, vol. 356, no. 2, pp. 115–124, 2007.

[68] J. M. Llovet, S. Ricci, V. Mazzaferro, et al., "Sorafenib in advanced hepatocellular carcinoma," *The New England Journal of Medicine*, vol. 359, no. 4, pp. 378–390, 2008.

[69] A. M. Jubb, A. J. Oates, S. Holden, and H. Koeppen, "Predicting benefit from anti-angiogenic agents in malignancy," *Nature Reviews Cancer*, vol. 6, no. 8, pp. 626–635, 2006.

[70] C. C. Jaffe, "Measures of response: RECIST, WHO, and new alternatives," *Journal of Clinical Oncology*, vol. 24, no. 20, pp. 3245–3251, 2006.

[71] D. G. Duda, R. K. Jain, and C. G. Willett, "Antiangiogenics: the potential role of integrating this novel treatment modality with chemoradiation for solid cancers," *Journal of Clinical Oncology*, vol. 25, no. 26, pp. 4033–4042, 2007.

[72] L. A. Akslen and G. N. Naumov, "Tumor dormancy—from basic mechanisms to clinical practice," *APMIS*, vol. 116, no. 7-8, pp. 545–547, 2008.

[73] G. N. Naumov, J. Folkman, O. Straume, and L. A. Akslen, "Tumor-vascular interactions and tumor dormancy," *APMIS*, vol. 116, no. 7-8, pp. 569–585, 2008.

[74] P. Carmeliet, "Angiogenesis in life, disease and medicine," *Nature*, vol. 438, no. 7070, pp. 932–936, 2005.

[75] J. I. Greenberg, D. J. Shields, S. G. Barillas, et al., "A role for VEGF as a negative regulator of pericyte function and vessel maturation," *Nature*, vol. 456, no. 7223, pp. 809–814, 2008.

[76] R. K. Jain, "Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy," *Nature Medicine*, vol. 7, no. 9, pp. 987–989, 2001.

[77] R. K. Jain, "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy," *Science*, vol. 307, no. 5706, pp. 58–62, 2005.

[78] R. P. M. Dings, M. Loren, H. Heun, et al., "Scheduling of radiation with angiogenesis inhibitors anginex and avastin improves therapeutic outcome via vessel normalization," *Clinical Cancer Research*, vol. 13, no. 11, pp. 3395–3402, 2007.

[79] D. M. McDonald and P. L. Choyke, "Imaging of angiogenesis: from microscope to clinic," *Nature Medicine*, vol. 9, no. 6, pp. 713–725, 2003.

[80] J. M. Provenzale, "Imaging of angiogenesis: clinical techniques and novel imaging methods," *American Journal of Roentgenology*, vol. 188, no. 1, pp. 11–23, 2007.

[81] Y. Shaked, F. Bertolini, S. Man, et al., "Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis," *Cancer Cell*, vol. 7, no. 1, pp. 101–111, 2005.

[82] A. R. Padhani and M. O. Leach, "Antivascular cancer treatments: functional assessments by dynamic contrast-enhanced magnetic resonance imaging," *Abdominal Imaging*, vol. 30, no. 3, pp. 324–341, 2005.

[83] T. Barrett, M. Brechbiel, M. Bernardo, and P. L. Choyke, "MRI of tumor angiogenesis," *Journal of Magnetic Resonance Imaging*, vol. 26, no. 2, pp. 235–249, 2007.

[84] P. S. Tofts, G. Brix, D. L. Buckley, et al., "Estimating kinetic parameters from dynamic contrast-enhanced T1- weighted MRI of a diffusable tracer: standardized quantities and symbols," *Journal of Magnetic Resonance Imaging*, vol. 10, no. 3, pp. 223–232, 1999.

[85] B. Morgan, A. L. Thomas, J. Drevs, et al., "Dynamic contrast-enhanced magnetic resonance imaging as a biomarker for the pharmacological response of PTK787/ZK 222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases, in patients with advanced colorectal cancer and liver metastases: results from two phase I studies," *Journal of Clinical Oncology*, vol. 21, no. 21, pp. 3955–3964, 2003.

[86] A. Gossmann, T. H. Helbich, N. Kuriyama, et al., "Dynamic contrast-enhanced magnetic resonance imaging as a surrogate marker of tumor response to anti-angiogenic therapy in a xenograft model of glioblastoma multiforme," *Journal of Magnetic Resonance Imaging*, vol. 15, no. 3, pp. 233–240, 2002.

[87] M. O. Leach, K. M. Brindle, J. L. Evelhoch, et al., "Assessment of antiangiogenic and antivascular therapeutics using MRI: recommendations for appropriate methodology for clinical trials," *British Journal of Radiology*, vol. 76, no. 1, pp. S87–S91, 2003.

[88] J. C. Miller, H. H. Pien, D. Sahani, A. G. Sorensen, and J. H. Thrall, "Imaging angiogenesis: application and potential for drug development," *Journal of the National Cancer Institute*, vol. 97, no. 3, pp. 172–187, 2005.

[89] M. A. Rosen and M. D. Schnall, "Dynamic contrast-enhanced magnetic resonance imaging for assessing tumor vascularity and vascular effects of targeted therapies in renal cell carcinoma," *Clinical Cancer Research*, vol. 13, no. 2, part 2, pp. 770s–776s, 2007.

[90] C. G. Willett, Y. Boucher, E. Di Tomaso, et al., "Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer," *Nature Medicine*, vol. 10, no. 2, pp. 145–147, 2004.

[91] O. M. Hahn, C. Yang, M. Medved, et al., "Dynamic contrast-enhanced magnetic resonance imaging pharmacodynamic biomarker study of sorafenib in metastatic renal carcinoma," *Journal of Clinical Oncology*, vol. 26, no. 28, pp. 4572–4578, 2008.

[92] A. X. Zhu, N. S. Holalkere, A. Muzikansky, K. Horgan, and D. V. Sahani, "Early antiangiogenic activity of bevacizumab evaluated by computed tomography perfusion scan in patients with advanced hepatocellular carcinoma," *Oncologist*, vol. 13, no. 2, pp. 120–125, 2008.

[93] A. R. Broumas, R. E. Pollard, S. H. Bloch, E. R. Wisner, S. Griffey, and K. W. Ferrara, "Contrast-enhanced computed tomography and ultrasound for the evaluation of tumor blood flow," *Investigative Radiology*, vol. 40, no. 3, pp. 134–147, 2005.

[94] P. E. Dugdale, K. A. Miles, I. Bunce, B. B. Kelley, and D. A. C. Leggett, "CT measurement of perfusion and permeability within lymphoma masses and its ability to assess grade, activity, and chemotherapeutic response," *Journal of Computer Assisted Tomography*, vol. 23, no. 4, pp. 540–547, 1999.

[95] R. E. Pollard, T. C. Garcia, S. M. Stieger, K. W. Ferrara, A. R. Sadlowski, and E. R. Wisner, "Quantitative evaluation of perfusion and permeability of peripheral tumors using contrast-enhanced computed tomography," *Investigative Radiology*, vol. 39, no. 6, pp. 340–349, 2004.

[96] Y. Tsushima, S. Funabasama, J. Aoki, S. Sanada, and K. Endo, "Quantitative perfusion map of malignant liver tumors, created from dynamic computed tomography data," *Academic Radiology*, vol. 11, no. 2, pp. 215–223, 2004.

[97] F. Castell and G. J. R. Cook, "Quantitative techniques in 18FDG PET scanning in oncology," *British Journal of Cancer*, vol. 98, no. 10, pp. 1597–1601, 2008.

[98] M. A. Lodge, R. E. Carson, J. A. Carrasquillo, M. Whatley, S. K. Libutti, and S. L. Bacharach, "Parametric images of blood flow in oncology PET studies using [15O]water," *Journal of Nuclear Medicine*, vol. 41, no. 11, pp. 1784–1792, 2000.

[99] A. J. De Langen, V. E. M. Van Den Boogaart, J. T. Marcus, and M. Lubberink, "Use of H215O-PET and DCE-MRI to measure tumor blood flow," *Oncologist*, vol. 13, no. 6, pp. 631–644, 2008.

[100] D. L. Franklin, W. Schlegel, and R. F. Rushmer, "Blood flow measured by Doppler frequency shift of back-scattered ultrasound," *Science*, vol. 134, no. 3478, pp. 564–565, 1961.

[101] W. J. Fry, et al., "Production of focal destructive lesions in the central nervous system with ultrasound," *Journal of Neurosurgery*, vol. 11, no. 5, pp. 417–418, 1954.

[102] J.-M. Correas, L. Bridal, A. Lesavre, A. Méjean, M. Claudon, and O. Hélénon, "Ultrasound contrast agents: properties, principles of action, tolerance, and artifacts," *European Radiology*, vol. 11, no. 8, pp. 1316–1328, 2001.

[103] K. E. Morgan, "Experimental and theoretical evaluation of microbubble behavior: effect of transmitted phase and bubble size," *IEEE Transactions on Ultrasonics, Ferroelectrics, and Frequency Control*, vol. 47, no. 6, pp. 1494–1509, 2000.

[104] J. Cwajg, et al., "Detection of angiographically significant coronary artery disease with accelerated intermittent imaging after intravenous administration of ultrasound contrast material," *American Heart Journal*, vol. 139, no. 4, pp. 675–683, 2000.

[105] S.-C. Cheng, T. C. Dy, and S. B. Feinstein, "Contrast echocardiography: review and future directions," *American Journal of Cardiology*, vol. 81, no. 12 A, pp. 41G–48G, 1998.

[106] J. L. Cohen, J. Cheirif, D. S. Segar, et al., "Improved left ventricular endocardial border delineation and opacification with OPTISON (FS069), a new echocardiographic contrast agent: results of a phase III multicenter trial," *Journal of the American College of Cardiology*, vol. 32, no. 3, pp. 746–752, 1998.

[107] M. Krix, "Quantification of enhancement in contrast ultrasound: a tool for monitoring of therapies in liver metastases," *European Radiology, Supplement*, vol. 15, no. 5, pp. E104–E108, 2005.

[108] M. Krix, et al., "Monitoring of liver metastases after stereotactic radiotherapy using low-MI contrast-enhanced ultrasound–initial results," *European Radiology*, vol. 15, no. 4, pp. 677–684, 2005.

[109] T. K. Kim, B. I. Choi, J. K. Han, H.-S. Hong, S. H. Park, and S. G. Moon, "Hepatic tumors: contrast agent-enhancement patterns with pulse-inversion harmonic US," *Radiology*, vol. 216, no. 2, pp. 411–417, 2000.

[110] F. Forsberg, B. B. Goldberg, J.-B. Liu, D. A. Merton, N. M. Rawool, and W. T. Shi, "Tissue-specific US contrast agent for evaluation of hepatic and splenic parenchyma," *Radiology*, vol. 210, no. 1, pp. 125–132, 1999.

[111] C. J. Harvey, M. J. K. Blomley, R. J. Eckersley, R. A. Heckemann, J. Butler-Barnes, and D. O. Cosgrove, "Pulse-inversion mode imaging of liver specific microbubbles: improved detection of subcentimetre metastases," *Lancet*, vol. 355, no. 9206, pp. 807–808, 2000.

[112] K. J. Niermann, A. C. Fleischer, J. Huamani, et al., "Measuring tumor perfusion in control and treated murine tumors: correlation of microbubble contrast-enhanced sonography to dynamic contrast-enhanced magnetic resonance imaging and fluorodeoxyglucose positron emission tomography," *Journal of Ultrasound in Medicine*, vol. 26, no. 6, pp. 749–756, 2007.

[113] T. E. Yankeelov, et al., "Correlation between estimates of tumor perfusion from microbubble contrast-enhanced sonography and dynamic contrast-enhanced magnetic resonance imaging," *Journal of Ultrasound in Medicine*, vol. 25, no. 4, pp. 487–497, 2006.

[114] S. Ismail, et al., "Relation between air-filled albumin microbubble and red blood cell rheology in the human myocardium. Influence of echocardiographic systems and chest wall attenuation," *Circulation*, vol. 94, no. 3, pp. 445–451, 1996.

[115] F. S. Villanueva, R. J. Jankowski, C. Manaugh, and W. R. Wagner, "Albumin microbubble adherence to human coronary endotheliumml: implications for assessment of endothelial function using myocardial contrast echocardiography," *Journal of the American College of Cardiology*, vol. 30, no. 3, pp. 689–693, 1997.

[116] J. R. Lindner, M. P. Coggins, S. Kaul, A. L. Klibanov, G. H. Brandenburger, and K. Ley, "Microbubble persistence in the microcirculation during ischemia/reperfusion and inflammation is caused by integrin- and complement- mediated adherence to activated leukocytes," *Circulation*, vol. 101, no. 6, pp. 668–675, 2000.

[117] J. R. Lindner, P. A. Dayton, M. P. Coggins, et al., "Noninvasive imaging of inflammation by ultrasound detection of Phagocytosed microbubbles," *Circulation*, vol. 102, no. 5, pp. 531–538, 2000.

[118] P. A. Dayton and K. W. Ferrara, "Targeted imaging using ultrasound," *Journal of Magnetic Resonance Imaging*, vol. 16, no. 4, pp. 362–377, 2002.

[119] G. Korpanty, P. A. Grayburn, R. V. Shohet, and R. A. Brekken, "Targeting vascular endothelium with avidin microbubbles," *Ultrasound in Medicine and Biology*, vol. 31, no. 9, pp. 1279–1283, 2005.

[120] G. M. Lanza and S. A. Wickline, "Targeted ultrasonic contrast agents for molecular imaging and therapy," *Progress in Cardiovascular Diseases*, vol. 44, no. 1, pp. 13–31, 2001.

[121] J. R. Lindner, J. Song, J. Christiansen, A. L. Klibanov, F. Xu, and K. Ley, "Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-selectin," *Circulation*, vol. 104, no. 17, pp. 2107–2112, 2001.

[122] F. S. Villanueva, R. J. Jankowski, S. Klibanov, et al., "Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells," *Circulation*, vol. 98, no. 1, pp. 1–5, 1998.

[123] F. S. Villanueva and W. R. Wagner, "Ultrasound molecular imaging of cardiovascular disease," *Nature Clinical Practice Cardiovascular Medicine*, vol. 5, no. 2, pp. S26–S32, 2008.

[124] K. Hida, Y. Hida, D. N. Amin, et al., "Tumor-associated endothelial cells with cytogenetic abnormalities," *Cancer Research*, vol. 64, no. 22, pp. 8249–8255, 2004.

[125] B. St. Croix, C. Rago, V. Velculescu, et al., "Genes expressed in human tumor endothelium," *Science*, vol. 289, no. 5482, pp. 1197–1202, 2000.

[126] A. Eberhard, S. Kahlert, V. Goede, B. Hemmerlein, K. H. Plate, and H. G. Augustin, "Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies," *Cancer Research*, vol. 60, no. 5, pp. 1388–1393, 2000.

[127] D. J. Lee, et al., "Relationship between retention of a vascular endothelial growth factor receptor 2 (VEGFR2)-targeted ultrasonographic contrast agent and the level of VEGFR2 expression in an in vivo breast cancer model," *Journal of Ultrasound in Medicine*, vol. 27, no. 6, pp. 855–866, 2008.

[128] A. Lyshchik, A. C. Fleischer, J. Huamani, D. E. Hallahan, M. Brissova, and J. C. Gore, "Molecular imaging of vascular endothelial growth factor receptor 2 expression using targeted contrast-enhanced high-frequency ultrasonography," *Journal of Ultrasound in Medicine*, vol. 26, no. 11, pp. 1575–1586, 2007.

[129] M. Palmowski, J. Huppert, G. Ladewig, et al., "Molecular profiling of angiogenesis with targeted ultrasound imaging: early assessment of antiangiogenic therapy effects," *Molecular Cancer Therapeutics*, vol. 7, no. 1, pp. 101–109, 2008.

[130] H. Leong-Poi, "Molecular imaging using contrast-enhanced ultrasound: evaluation of angiogenesis and cell therapy," *Cardiovascular Research*, vol. 84, no. 2, pp. 190–200, 2009.

[131] M. A. Kuliszewski, H. Fuj II, C. Liao, et al., "Molecular imaging of endothelial progenitor cell engraftment using contrast-enhanced ultrasound and targeted microbubbles," *Cardiovascular Research*, vol. 83, no. 4, pp. 653–662, 2009.

[132] J. K. Willmann, Z. Cheng, C. Davis, et al., "Targeted microbubbles for imaging tumor angiogenesis: assessment of whole-body biodistribution with dynamic micro-pet in Mice," *Radiology*, vol. 249, no. 1, pp. 212–219, 2008.

[133] J. K. Willmann, A. M. Lutz, R. Paulmurugan, et al., "Dual-targeted contrast agent for US assessment of tumor angiogenesis in vivo," *Radiology*, vol. 248, no. 3, pp. 936–944, 2008.

[134] H. M. W. Verheul and H. M. Pinedo, "Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition," *Nature Reviews Cancer*, vol. 7, no. 6, pp. 475–485, 2007.

[135] X. Zhu, S. Wu, W. L. Dahut, and C. R. Parikh, "Risks of proteinuria and hypertension with bevacizumab, an antibody against vascular endothelial growth factor: systematic review and meta-analysis," *American Journal of Kidney Diseases*, vol. 49, no. 2, pp. 186–193, 2007.

[136] V. Eremina, J. A. Jefferson, J. Kowalewska, et al., "VEGF inhibition and renal thrombotic microangiopathy," *The New England Journal of Medicine*, vol. 358, no. 11, pp. 1129–1136, 2008.

[137] J. S. Rudge, et al., "Inaugural article: VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade," *Proceedings of the National Academy of Sciences of USA*, vol. 104, no. 47, pp. 18363–18370, 2007.

[138] R. S. Herbst and A. B. Sandler, "Non-small cell lung cancer and antiangiogenic therapy: what can be expected of bevacizumab?" *Oncologist*, vol. 9, no. 1, pp. 19–26, 2004.

[139] A. Sandler, R. Gray, M. C. Perry, et al., "Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer," *The New England Journal of Medicine*, vol. 355, no. 24, pp. 2542–2550, 2006.

[140] T. A. Traina, L. Norton, K. Drucker, and B. Singh, "Nasal septum perforation in a bevacizumab-treated patient with

metastatic breast cancer," *Oncologist*, vol. 11, no. 10, pp. 1070–1071, 2006.

[141] C. Ozcan, S. J. Wong, and P. Hari, "Reversible posterior leukoencephalopathy syndrome and bevacizumab," *The New England Journal of Medicine*, vol. 354, no. 9, pp. 981–982, 2006.

[142] C. L. Estilo, M. Fornier, A. Farooki, D. Carlson, G. Bohle I II, and J. M. Huryn, "Osteonecrosis of the jaw related to bevacizumab," *Journal of Clinical Oncology*, vol. 26, no. 24, pp. 4037–4038, 2008.

[143] J. M. Roodhart, M. H. Langenberg, E. Witteveen, and E. E. Voest, "The molecular basis of class side effects due to treatment with inhibitors of the VEGF/VEGFR pathway," *Current Clinical Pharmacology*, vol. 3, no. 2, pp. 132–143, 2008.

[144] Y. Je, F. A. Schutz, and T. K. Choueiri, "Risk of bleeding with vascular endothelial growth factor receptor tyrosine-kinase inhibitors sunitinib and sorafenib: a systematic review and meta-analysis of clinical trials," *The Lancet Oncology*, vol. 10, no. 10, pp. 967–974, 2009.

[145] V. Brower, "Antiangiogenesis research is booming, as questions and studies proliferate," *Journal of the National Cancer Institute*, vol. 101, no. 11, pp. 780–781, 2009.

[146] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[147] T. T. Batchelor, A. G. Sorensen, E. di Tomaso, et al., "AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients," *Cancer Cell*, vol. 11, no. 1, pp. 83–95, 2007.

[148] O. Casanovas, D. J. Hicklin, G. Bergers, and D. Hanahan, "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors," *Cancer Cell*, vol. 8, no. 4, pp. 299–309, 2005.

[149] Y. Mizukami, W.-S. Jo, E.-M. Duerr, et al., "Induction of interleukin-8 preserves the angiogenic response in HIF-1α-deficient colon cancer cells," *Nature Medicine*, vol. 11, no. 9, pp. 992–997, 2005.

[150] M. R. Mancuso, R. Davis, S. M. Norberg, et al., "Rapid vascular regrowth in tumors after reversal of VEGF inhibition," *Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2610–2621, 2006.

[151] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[152] J. L. Yu, J. W. Rak, P. Carmeliet, A. Nagy, R. S. Kerbel, and B. L. Coomber, "Heterogeneous vascular dependence of tumor cell populations," *American Journal of Pathology*, vol. 158, no. 4, pp. 1325–1334, 2001.

[153] R. S. Bhatt, P. Seth, and V. P. Sukhatme, "Biomarkers for monitoring antiangiogenic therapy," *Clinical Cancer Research*, vol. 13, no. 2, part 2, pp. 777s–780s, 2007.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 317068, 10 pages
doi:10.1155/2010/317068

Review Article

TGF-β Superfamily Receptors—Targets for Antiangiogenic Therapy?

Jasmin Otten, Carsten Bokemeyer, and Walter Fiedler

Sections of Pneumonology and Bone Marrow Transplantation, Department of Oncology and Hematology, Hubertus Wald University Cancer Center, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany

Correspondence should be addressed to Walter Fiedler, fiedler@uke.uni-hamburg.de

Received 23 November 2009; Accepted 23 February 2010

Academic Editor: Arkadiusz Dudek

The TGF-β pathway controls a broad range of cellular behavior including cell proliferation, differentiation, and apoptosis of various cell types including tumor cells, endothelial cells, immune cells, and fibroblasts. Besides TGF-β's direct effects on tumor growth and its involvement in neoangiogenesis have received recent attention. Germline mutations in TGF-β receptors or coreceptors causing Hereditary Hemorrhagic Teleangiectasia and the Loeys-Dietz syndrome underline the involvement of TGF-β in vessel formation and maturation. Several therapeutic approaches are evaluated at present targeting the TGF-β pathway including utilization of antisense oligonucleotides against TGF-β itself or antibodies or small molecule inhibitors of TGF-β receptors. Some of these therapeutic agents have already entered the clinical arena including an antibody against the endothelium specific TGF-β class I receptor ALK-1 targeting tumor vasculature. In conclusion, therapeutic manipulation of the TGF-β pathway opens great opportunities in future cancer therapy.

1. TGF-β Pathway

The TGF-β superfamily consists of over 30 structurally related multifunctional proteins, including three TGF-β isoforms (TGF-β1, 2, and 3), three forms of activin, and over 20 bone morphogenic proteins (BMPs), which control a broad range of cellular behavior such as cell growth, differentiation and apoptosis in various cell types including tumor, immune, and endothelial cells as well as fibroblasts [1–5].

Ligand signaling is mediated through two related single transmembrane type I and type II receptors, which together comprise the only known family of serine/threonine kinases [6–8]. In mammals, there are five different type II (TGFBR2, ActR-IIa, ActR-IIb, BMPR2, AMHRII) and seven type I receptors, also named activin receptor-like kinases (ALK-1-7) [7, 9]. In most cases, the receptor combination is important for the binding of a specific ligand, but the TGF-β family members often bind to more than one type II and type I receptor combination [10]. Upon ligand binding, the type I and type II receptors form a heteromeric complex, presumably consisting of two type I and two type II receptors. The type II receptor exhibits a constitutively active kinase which transphosphorylates and activates the type I receptor in a glycine- and serine-rich region known as GS-box [11]. The activated type I receptor propagates the downstream signaling by phosphorylating specific receptor-regulated SMAD proteins (R-SMAD) [12, 13]. R-SMADs interact with SMAD-4, the only known common mediator SMAD (CoSMAD) in mammals, and form heteromeric complexes which translocate to the nucleus where they influence gene expression (by binding to the DNA and acting as transcription factors, coactivators, and corepressors) [14–17].

The TGF-β pathway has several feedback mechanisms, which regulate the duration of the signaling. One of the feedback mechanisms is mediated by inhibitory SMADs (I-SMAD), in humans SMAD-6 and SMAD-7, which compete with the R-SMADs for binding to the type I receptor, but without the ability to transduce the downstream signal. I-SMADs also recruit the E3 ubiquitin ligases SMAD ubiquitin related factor-1 and -2 (Smurf-1 and -2), which ubiquitinate

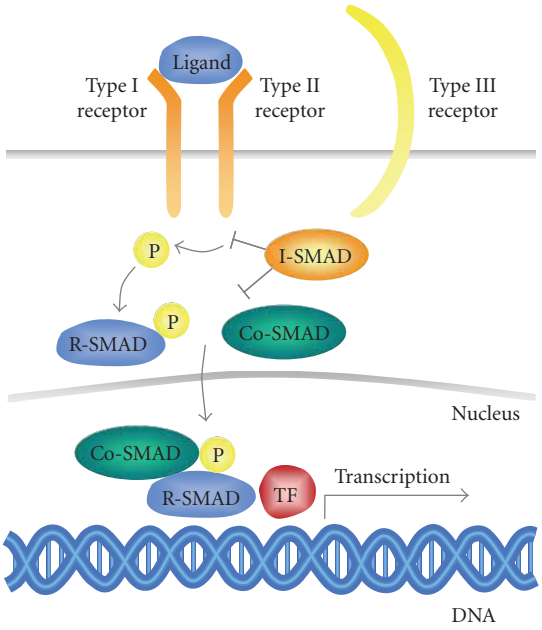


Figure 1: TGF-β signaling cascade. Upon ligand binding the constitutively active kinase of the type II receptor transphosphorylates and activates the type I receptor. Type III receptors lack any kinase activity but they act as accessory receptors and facilitate ligand binding to the type I and II receptors. Downstream signaling is mediated via R-SMADs which are phosphorylated by the activated type I receptor and form a complex with CoSMADs. This complex translocates to the nucleus where it induces transcription of downstream signaling. I-SMAD proteins represent important negative feedback structures, since they can block the signaling via competitive binding to the type I receptors or R-SMADs. R-SMAD: receptor-regulated SMAD; CoSMAD: common mediator SMAD; I-SMAD: inhibitory SMAD; TF: transcription factor.

the SMADs and type I receptors, resulting in protein degradation [18–23].

In humans, two accessory TGF-β superfamily receptors have been described which have a more indirect role in TGF-β signaling: betaglycan and endoglin. The later is mainly expressed in endothelial cells [24–26]. These type III receptors are structurally related transmembrane receptors with short intracellular domains that lack any enzymatic motif but contain many serine and threonine residues. They facilitate the binding of ligand to the type I and type II receptors [27]. A soluble form of endoglin has been described, most likely generated by proteolytic shedding, that antagonizes the membrane bound form [28]. The components of the TGF-β pathway are shown schematically in Figure 1.

2. TGF-β Signaling in Cancer

2.1. Hereditary Cancer Syndromes. Several hereditary cancer syndromes with mutations in TGF-β superfamily members are known. The autosomal dominant familial juvenile polyposis syndrome (JPS) is the most common of the hamartomatous syndromes which occurs with an incidence of about one per 100.000 births [29]. Patients develop numerous polyps not only in the colon or rectum but also in the proximal gastrointestinal tract. Although most juvenile polyps are benign, malignant transformation occurs with a lifetime risk of colorectal carcinoma of approximately 70%. In addition, the risk of pancreatic, gastric, and duodenal carcinoma is increased [29]. Germline mutations in different members of the TGF-β superfamily have been described in JPS. In every fourth patient a mutation in the type I receptor ALK-3 (BMPR1A) is found [30]. In 15% of cases SMAD-4 is mutated [30]. Furthermore, mutations in the endoglin gene have been described, but the incidence is unknown [31].

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common hereditary predisposition for the development of colorectal cancer. HNPCC results from germline mutations within genes involved in the DNA mismatch repair system, leading to microsatellite instability. Since the TGFBR2 gene contains a 10-base pair polyadenine repeat microsatellite sequence, it is an apparent target for inactivation caused by errors of the DNA mismatch repair. Indeed, a mutated form of TGFBR2 can be observed in up to 80% of colon cancer patients with HNPCC [32, 33].

The autosomal cancer syndrome Cowden Syndrome (CS) and Bannayan-Riley-Ruvalcaba (BRR) disease are normally associated with a phosphatase and tensin homolog (PTEN) gene mutation. However, in one patient with CS and BRR symptoms but without PTEN mutation an ALK-3 mutation was found [34].

2.2. Dysregulated Expression in Cancer Patients. For several pathologies, especially cancer, a correlation between the expression level of a TGF-β superfamily member and the severity of the related disease has been identified, which makes the concerning TGF-β family member a diagnostic, prognostic, or predictive marker.

2.2.1. Transforming Growth Factors. In 1986, Nishimura et al. detected elevated TGF-β levels in the urine of patients suffering from advanced cancer stages compared to healthy donors [35]. Since then, increased serum levels of TGF-β1 have been implicated as a prognostic marker of advanced disease and poor prognosis in multiple cancer types such as gastric carcinoma, colorectal cancer, bladder carcinoma, prostate cancer, breast cancer, lung cancer, esophageal adenocarcinoma, and melanoma [36–44]. But nevertheless TGF-β levels are not yet used as tumor markers in clinical routine.

2.2.2. Bone Morphogenic Proteins. Bone morphogenic proteins can also serve as prognostic markers, since the BMP-7 expression is increased in malignant melanomas and their metastases, which correlates with a shorter time to tumor recurrence [45]. Furthermore, high BMP-6 levels predicted development of distant metastasis in primary prostate cancer [46]. On the other hand, the mRNA level of BMP-2 was significantly decreased in breast cancer tumors compared to normal breast tissue [47].

2.2.3. TGF-β Receptors. The expression of TGF-β superfamily receptors within tumor cells can be a prognostic marker. Reduced ALK-5 and TGFBR2 expression correlates with a shorter survival rate of colon cancer patients, as does reduced expression of the coreceptor betaglycan in breast and prostate cancer patients [48–50]. Low expression levels of TGFBR2 have been observed in patients with chronic myeloid leukemia [51]. In addition, mutations in ALK-5 and TGFBR2 have been described for other haematological malignancies, but it seems to be a rare event [52, 53]. A significant association between loss of BMPR2 expression and tumor grade was found in bladder transitional cell carcinoma [54]. In contrast, high expression of type III coreceptor endoglin was mainly detected on immature blood vessels in prostate tumors and had a negative impact on patient's survival as well as with response rates in breast cancer or cervical cancer [55–58]. Calabro et al. detected elevated levels of soluble endoglin that correlated with low TGF-β1 levels in patients with acute myeloid leukemia or chronic myeloproliferative disorders [59]. However none of these markers is used in routine clinical practice.

3. TGF-β Signaling in Endothelial Cells

Several members of the TGF-β superfamily are expressed in endothelial cells and play an important role in angiogenesis and vasculogenesis. The targeted inactivation of TGF-β signaling components in mice revealed the pathway's crucial role in vascular morphogenesis. For example, animals lacking TGF-β1, ALK-5, ALK-1, endoglin, or various SMAD proteins die at midgestation during embryogenesis due to defects in vascular development of the yolk sac [10, 60–62].

In humans, the Hereditary Hemorrhagic Teleangiectasia (HHT, also named Rendu-Osler-Weber syndrome) is an autosomal dominant disease in which vascular dysplasia results in teleangiectasia and arteriovenous malformations. Two forms with different clinical characteristics have been described: HHT type 1 patients have a mutation in the endoglin gene whereas HHT type 2 is characterized by a mutation in the ALK-1 gene. Together these mutations account for about 80% of all HHT patients [6, 63–65]. In 2005, another autosomal dominant syndrome with mutations in TGF-β receptors was described: the Loeys-Dietz syndrome. Patients have a very high risk for aortic dissection or rupture. Analysis of 52 families with a history of Loeys-Dietz syndrome revealed somatic mutations either in the type I receptor ALK-5 or in the type II receptor TGFBR2 [66, 67].

3.1. Functional Aspects of TGF-β Signaling in Endothelial Cells. In endothelial cells the type I TGF-β receptors, which have been investigated most thoroughly, are ubiquitously expressed ALK-5 and endothel-specific ALK-1. Previously, it was believed that ALK-5 and ALK-1 had opposite roles in angiogenesis and might balance the activation state of endothelium. Several investigators observed increased proliferation and migration when the TGF-β/ALK-1 pathway had been stimulated whereas stimulation of the TGF-β/ALK-5 pathway led to inhibition of endothelial cell proliferation and migration [68, 69]. This opposing effect was thought to be mediated by activation of SMAD-1/5/8 by ALK-1 and SMAD-2/3 by ALK-5 [68]. Due to activation of different intracellular pathways, specific changes in gene transcription can be observed. Goumans et al. revealed that the inhibitor of DNA binding 1 (ID-1), a helix-loop-helix (HLH) protein that can form heterodimers with members of the *basic HLH* family of *transcription factors*, is a specific downstream signal of ALK-1, whereas the proteinase inhibitor plasminogen activator inhibitor-1 (PAI-1) is induced by ALK-5 activation [68].

More recently published data might alter the presumed relationship between ALK-1 and ALK-5. David et al. showed that not TGF-β1 but the bone morphogenic proteins 9 and 10 are likely to be the physiological ligands for ALK-1. Binding of BMP-9 to the ALK-1 and BMPR2 complex potently inhibited endothelial cell proliferation and migration [70]. The increase of angiogenesis in ECs upon ALK-1 activation in former studies was due to TGF-β1 binding to the ALK-1/TGFBR2 complex. Thus, the role of ALK-1 is dependant of type II receptor expression and ligand availability. Interestingly, both pathways signal via activation of SMAD-1, -5, and -8 although these SMADs have been described as characteristic BMP downstream signals [71]. Hence, additional elements must be involved in regulation of the ALK-1 pathway driving it either to the pro or antiangiogenic direction. Indeed, cross-talk between the TGF-β pathway with other pathways such as the mitogen-activated protein kinase (MAPK), the phosphatidylinositol-3 kinase (PI3K) or the Hedgehog pathways have been described [72].

Very recently, a possible explanation for the requisite role of ALK-1 and ALK-5 in angiogenesis has been described. Shao et al. demonstrated that ALK-1 and ALK-5 are both essential for the regulation of vascular endothelial growth factor (VEGF), which is believed to be the central growth factor in angiogenesis. TGF-β1/ALK-5 stimulation elevated the m-RNA levels of VEGF in bovine aortic ECs, whereas BMP-9/ALK-1 stimulation led to decreased VEGF m-RNA levels. Proliferation and migration assays were in line with these observations [73].

A remaining question is the interdependence between ALK-1 and ALK-5. Whereas Goumans et al. proposed that ALK-5 mediates a TGF-β-dependent recruitment of ALK-1 into the receptor complex and that ALK-5 kinase activity is essential for optimal ALK-1 activity [68]. Shao et al. observed opposite effects. They found some hints that ALK-1 acts independently of ALK-5 but that ALK-5 might actually be dependent of ALK-1 [73]. Hence, interdependence between ALK-1 and ALK-5 seems to be apparent, yet it has to be clarified which of the receptors is the leading force.

4. TGF-β Receptor Expression in Leukemia

Since endothelial and hematopoietic cells have a common stem cell, the so-called hemangioblast, many immature

hematopoietic cells share cell surface receptors with endothelial cells, such as receptors for hematopoieitc growth factors, for example, GM-CSF or erythropoietin [74, 75]. Our group investigated expression of ALK-1 and ALK-5 in various leukemic cells lines and samples from patients with acute myeloid leukemia (AML). We found that both receptors are expressed in most cases implying that both ALK-1 and ALK-5 are involved in autocrine or paracrine growth stimulation in AML (manuscript in preparation).

In a recent study, an association between high ID-1 expression and poor prognosis in patients with AML has been described. ID-1 is the typical downstream mediator of ALK-1 signaling although enhancement of ID-1 expression by other tryrosine kinase receptors such as FLT3 cannot be excluded in a subgroup of patients [76]. However, data about dysregulated TGF-β signaling in hematologic malignancies are rare, since only few reports in lymphoid neoplasms or myeloid leukemia have been published [52, 53, 77, 78].

5. TGF-β Signaling Pathway as a Therapeutic Target

Because of the enormous number of observed alterations in the TGF-β pathway in cancer patients, the development of therapeutic substances seems to be evident.

In fact, there are different reasons why the inhibition of the TGF-β pathway might be a promising target for anticancer therapies. First, the direct effect on tumor cells has to be stressed. Secondly, as described above, the TGF-β pathway plays an important role in endothelial cell behavior and therefore in angiogenesis. Antiangiogenic therapies belong to the most promising therapeutic concepts which are currently under development. Thirdly, TGF-β is one of the most potent naturally immunosuppressors [79, 80]. Mice deficient for TGF-β1 develop a harmful syndrome with multifocal, mixed inflammatory cell response, and tissue necrosis, leading to organ failure and death [4]. Furthermore, suppression of TGF-β signaling in T cells by transduction with a truncated TGFBR2 resulted in severe autoimmune reactions [81]. The immune response of cancer patients is often suppressed, since many advanced tumors overexpress TGF-β resulting in inhibition of IL-2-dependant proliferation and differentiation of NK and T cells [82, 83]. In addition, TGF-β recruits different immune cells to the tumor microenvironment: monocytes and macrophages promote tumor invasion, angiogenesis and metastasis whereas mast cells secrete numerous tumor promoting factors [83]. Figure 2 summarizes the main tumor promoting effects of dysregulated TGF-β signaling.

Targeting the TGF-β pathway should therefore not only affect the tumor cells by itself; moreover a decreased tumor vascularization and strengthening the patient's immune responses should be achieved. Numerous in vitro and in vivo studies have been performed, accounting for these different strategies to inhibit tumor growth and to target various components within the TGF-β pathway including ligands, receptors and even downstream signals. Some of these studies passed the preclinical phase with success and

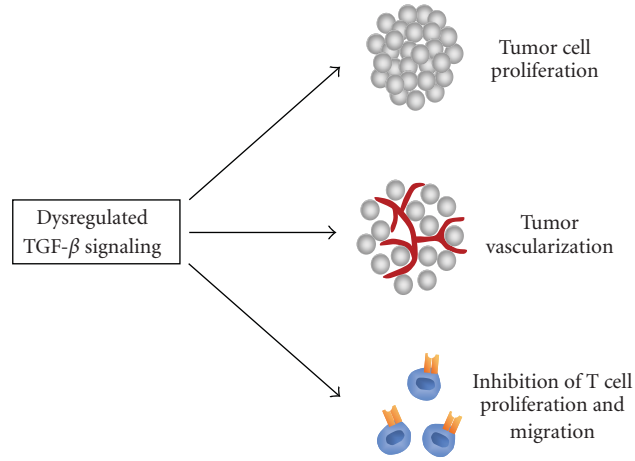


Figure 2: Dysregulation of the TGF-β pathway promotes tumor growth. An unbalanced TGF-β pathway can cause advanced tumorigenesis due to several cellular changes. On the one hand, the dysregulation has a direct effect on tumor cells leading to elevated tumor cell proliferation. Secondly, endothelial cells are affected which results in increased angiogenesis and therefore in tumor vascularization. Finally immune responses are attenuated due to inhibition of T cell proliferation and migration caused by dysregulated TGF-β signaling.

phase I and II clinical studies have been started. Table 1 gives a short overview of preclinical and clinical studies using agents targeted at TGF-β family members.

Representing the central factor of the pathway, TGF-β is the preferred target structure in most cases. For example, Yang et al. developed transgenic mice expressing a TGF-β antagonist consisting of a soluble TGF-β type II receptor fused with the Fc domain of a human IgG1. The number of metastases was reduced both in a tail vein metastasis assay with melanoma cells and in crosses with a transgenic mouse model of metastatic breast cancer [101]. A neutralizing pan-TGF-β antibody prevented radiation-induced acceleration of metastatic cancer progression in a transgenic mouse model of metastatic breast cancer [102]. The pan-TGF-β antibody GC-1008 was tested in a phase I clinical study with 22 patients with renal cell carcinoma or malignant melanoma (NCT00356460). Treatment was well tolerated with mainly grade 1-2 toxicity including skin rash, fatigue, headache and gastrointestinal symptoms. 5 patients achieved stable disease or better and one patient with skin disease achieved a partial response with >75% reduction of target lesions [84].

Several studies concentrated on the restoration of immune responses. In a prostate cancer xenograft model a reduction in tumor weight was observed after implantation of tumor-reactive $CD8^+$ T cells which were TGF-β insensitive due to introduction of a dominant-negative TGF-β type II receptor vector [103]. Yamamoto et al. utilized direct hemoperfusion treatment with specific immunosuppressive substance adsorption columns for TGF-β ablation in rats bearing a TGF-β-producing hepatocellular carcinoma. TGF-β serum levels were decreased after hemoperfusion treatment leading to restored T lymphocyte response,

Table 1: Overview of preclinical and clinical studies using agents targeted at TGF-β family members.

Class of substance	Target	Drug	Study
Human anti-TGF-β mAb	TGF-β	GC1008	Phase I study on renal cell carcinoma and malignant melanoma (NCT00356460 and NCT00899444) [84]
TGF-β2 antisense compound	TGF-β2	AP12009	Phase I study on pancreatic and colorectal neoplasms and melanoma (NCT00844064)
			Phase II study on glioblastoma and anaplastic astrocytoma (NCT00431561) [85, 86]
			Phase III study on anaplastic astrocytoma (NCT00761280)
		Belagenpumatucel-L	Phase II study on advanced nonsmall lung cancer (NCT01058785) [87]
TGF-β type I and type II receptor small molecule inhibitor	TGF-β type I and type II receptors	LY2109761	Preclinical studies [88–94]
Human anti-ALK-1 mAb	ALK-1	PF-03446962	Phase I on advanced solid tumors (NCT00557856)
ALK-5 small molecule inhibitor	ALK-5	SB431542	Preclinical studies [95, 96]
		SD208	Preclinical studies [97, 98]
		SM16	Preclinical studies [78, 99]
Chimeric antiEndoglin antibody	Endoglin	TRC105	Phase I on advanced or metastatic solid cancer (NCT00582985) [100]

decelerated tumor growth and longer survival times [104]. Fujita et al. observed similar results using plasmid DNA encoding the extracellular domain of the TGF-β type II receptor fused to the human IgG heavy chain; after plasmid injection in the proximity of established murine lymphomas an increased number of tumor antigen-specific $CD4^+$ and $CD8^+$ cells could be detected in tumor-draining lymph nodes [105].

Another promising approach which has entered clinical phase I and II trials is to inhibit TGF-β function by means of antisense oligonucleotides (AS-ODNs). In a preclinical trial, local intracranial administration of TGF-β2 AS-ODNs was combined with systemic tumor vaccine in a rat glioma model. Only the combination of both substances led to a significantly prolonged survival [106]. Increased survival of glioma patients who had received whole-cell vaccines comprising autologous tumor cells genetically modified by a TGF-β2 antisense vector was observed in a phase I study [107]. A phase II trial with belagenpumatucel-L, a TGF-β2 antisense gene-modified allogeneic tumor vaccine, is ongoing in patients with advanced nonsmall cell lung cancer (NCT01058785) [87]. The antitumorigenic effect of antisense oligonucleotides was supported by phase II trials with the TGF-β2 inhibitor AP12009. In comparison to standard chemotherapy, treatment with AP12009 resulted in prolonged survival of patients with anaplastic astrocytoma [85]. Consistently, patients with high-grade glioma achieved a higher survival rate at 24 months and showed significantly more responders after 14 months when AP12009 treatment was compared to standard chemotherapy protocols [86].

Therapeutic concepts against TGF-β receptors were almost exclusively targeted at ALK-5, in most cases using small molecule inhibitors such as SB431542 which showed similar results in several in vitro and in vivo studies. SB431542 caused inhibition of proliferation, TGF-β-mediated morphognic changes, and cellular motility of glioma cells in vitro. This effect was due to blocked phosphorylation of SMADs leading to reduced transcription of PAI-1 and VEGF which are key mediators in cell invasion and neoangiogenesis [95]. Javelaud and collegues analyzed the role of TGF-β in murine melanoma metastasis to bone. Both the therapy with SB431542 as well as tumors transduced with the inhibitory protein SMAD-7, showed significantly less osteolyses, longer survival and lower expression levels of osteolytic factors such as parathyroid hormone-related protein and interleukin-11 [96].

Another ALK-5 small molecule inhibitor, SD208, led to decreased tumor growth and metastasis in a murine mamma carcinoma and pancreatic adenocarcinoma model. Furthermore, antiangiogenic effects could be observed in both studies with a reduced microvessel density and altered expression levels of angiogenesis-related factors like FLT-1, Neuropillin-2 and VEGF-C, respectively [97, 98]. In addition, treatment of $CD34^+$ cells isolated from patients with myelodysplastic syndrome with SD208 led to in vitro enhancement of hematopoiesis [78]. Furthermore in a malignant mesothelioma mouse model, the ALK-5 inhibitor SM16 significantly decreased tumor growth which could be ascribed to a $CD8^+$ antitumor response [99].

The substance LY2109761 inhibits both TGF-β type I and type II receptors [88]. An orthotopic murine model of metastatic pancreatic cancer and a liver metastasis model proved the efficacy of LY2109761, since tumor growth and spontaneous metastases were reduced whereas the animals' survival was prolonged [89]. Similar effects were observed in an experimental colorectal cancer mouse model [90]. Gianelli's group performed several studies with LY2109761

in hepatocellular carcinoma. Tumor progression was delayed due to inhibition of vascular invasion as well as disturbance of cross-talk between hepatocellular carcinoma cells and stroma or endothelial cells. In a xenograft chick embryo model, LY210976 treatment caused even enhanced inhibition of tumor growth and reduced microvessel density compared to bevacizumab-treated animals. However, the strongest antitumoral effect was observed when combining both substances [91–93]. Myelo-monocytic leukemic cells cocultured with bone marrow derived mesenchymal stem cells were stimulated with TGF-β1 which led to inhibition of spontaneous and cytarabine-induced apoptosis. This prosurvival signaling was neutralized with LY2109761 [94].

Although no in vitro data about specific ALK-1 inhibitors have been published so far, a clinical phase I study testing a human antiALK-1 antibody in patients with advanced solid tumors is ongoing (NCT00557856).

Since its expression is restricted to endothelial cells with higher expression levels in tumor-associated endothelium compared to normal tissue, the accessory receptor endoglin may represent a promising target for anticancer therapy [108]. Antitumorigenic and antiangiogenic effects could be observed in several in vivo tumor models using antiendoglin antibodies [109–112]. For example, Uneda et al. used multiple metastasis models with murine mamma carcinoma and colon adenocarcinoma cells to test the effect of several antiendoglin antibodies targeted at different endoglin epitopes. Under treatment, metastases were suppressed and microvessel density was effectively reduced as measured by Matrigel plug assay [109, 113]: a phase I clinical trial with the human/murine chimeric antiendoglin monoclonal antibody TRC105 in 19 patients with solid cancer. Treatment was well tolerated with mainly grade 1-2 toxicity including fatigue, anemia, proteinuria and diarrhea. One patient with hormone refractory prostate cancer obtained a complete PSA response and 3 patients had prolonged stable disease (NCT00582985) [100].

6. Outlook

The results of numerous in vitro studies with cell lines, in vivo mouse models and clinical trials show that the TGF-β pathway plays an important role in cancer progression and represents a promising target for anticancer therapy. Targeting TGF-β isoforms, TGF-β receptors as well as downstream signaling proteins yielded satisfactory results, since a reduction in tumor load was observed in most cases.

Manipulating TGF-β signaling implies the great advantage of affecting at least three important structures in tumor progression: in addition to the direct antitumor effect, endothelial and immune cells will be targeted. Although restoration of the immune system is a desirable achievement in cancer therapy, the complete inhibition of TGF-β1 might have fatal consequences. For example, TGF-β1 deficient mice develop a lethal syndrome accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure [4]. Furthermore, abrogation of TGF-β signaling in T cells by introduction of a truncated TGFBR2 results in severe autoimmune reactions [81].

In addition, TGF-β1 plays an important role in fibroblast biology, since it is a relevant growth factor for extracellular matrix formation in fibroblasts due to its stimulation of collagen, fibronectin and proteoglycan synthesis [114]. Due to TGF-β signaling, fibroblasts suppress the activation of tumor-promoting paracrine signaling which would target epithelial cells and could lead to epithelial to mesenchymal transition [115]. Bhowmick et al. inactivated the TGFBR2 gene in mouse fibroblasts which resulted in intraepithelial neoplasia in prostate and invasive squamous cell carcinoma of the forestomach [115]. Coimplantation of TGFBR2-deficient mammary fibroblasts with mammary carcinoma cells promoted tumor growth and invasion as compared to wild-type fibroblasts [116].

These examples reveal the great difficulty in targeting the TGF-β pathway. Perhaps targeting the type I receptor ALK-1 or the accessory receptor endoglin might represent a solution to this discrepancy since expression of both receptors seems to be restricted to endothelial cells which could limit side effects. Therefore, results of phase I studies are awaited where patients with advanced solid tumors will be treated with an ALK-1 or an endoglin antibody (NCT00557856 and NCT00582985, resp.). These studies might resolve this question.

Integrating all results underlines the complexity in TGF-β signaling in endothelial cells. In some extent, this may be due to different experimental settings since TGF-β superfamily members have often been overexpressed or downregulated in in vitro models using plasmid vectors. Furthermore, although discussed as important ALK-1 ligand in the regulation of angiogenesis, no physiological data about BMP-9 or BMP-10 expression in endothelial or tumor cells exist.

7. Conclusion

We conclude that the TGF-β pathway might be a promising therapeutic target in anticancer therapy due to its involvement in several mechanisms including endothelial and immune cell biology that are most important for tumor progression. On the other hand, since the TGF-β pathway affects a broad range of cellular behavior, it is an ambitious approach to restore the delicate balance of physiological signaling. Therefore manipulation of the pathway bears the risk of adverse effects and of therapeutic success. Comprehensive investigations that comprise the interactions between tumor cells, fibroblasts, endothelial and immune cells are indispensable.

References

[1] M. de Caestecker, "The transforming growth factor-β superfamily of receptors," *Cytokine & Growth Factor Reviews*, vol. 15, no. 1, pp. 1–11, 2004.

[2] D. M. Kingsley, "The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different

organisms," *Genes and Development*, vol. 8, no. 2, pp. 133–146, 1994.

[3] J. Massagué, "How cells read TGF-β signals," *Nature Reviews Molecular Cell Biology*, vol. 1, no. 3, pp. 169–178, 2000.

[4] M. M. Shull, I. Ormsby, A. B. Kier, et al., "Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease," *Nature*, vol. 359, no. 6397, pp. 693–699, 1992.

[5] P. M. Siegel and J. Massagué, "Cytostatic and apoptotic actions of TGF-β in homeostasis and cancer," *Nature Reviews Cancer*, vol. 3, no. 11, pp. 807–820, 2003.

[6] K. J. Gordon and G. C. Blobe, "Role of transforming growth factor-β superfamily signaling pathways in human disease," *Biochimica et Biophysica Acta*, vol. 1782, no. 4, pp. 197–228, 2008.

[7] C. H. Heldin, K. Miyazono, and P. ten Dijke, "TGF-β signalling from cell membrane to nucleus through SMAD proteins," *Nature*, vol. 390, no. 6659, pp. 465–471, 1997.

[8] B. Schmierer and C. S. Hill, "TGFβ-SMAD signal transduction: molecular specificity and functional flexibility," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 12, pp. 970–982, 2007.

[9] J. A. Grootegoed, W. M. Baarends, and A. P. N. Themmen, "Welcome to the family: the anti-mullerian hormone receptor," *Molecular and Cellular Endocrinology*, vol. 100, no. 1-2, pp. 29–34, 1994.

[10] M.-J. Goumans and C. Mummery, "Functional analysis of the TGFβ receptor/Smad pathway through gene ablation in mice," *International Journal of Developmental Biology*, vol. 44, no. 3, pp. 253–265, 2000.

[11] J. L. Wrana, L. Attisano, R. Wieser, et al., "Mechanism of activation of the TGF-β receptor," *Nature*, vol. 370, no. 6488, pp. 341–347, 1994.

[12] S. Abdollah, M. Macias-Silva, T. Tsukazaki, et al., "TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling," *The Journal of Biological Chemistry*, vol. 272, no. 44, pp. 27678–27685, 1997.

[13] S. Souchelnytskyi, K. Tamaki, U. Engstrom, et al., "Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling," *The Journal of Biological Chemistry*, vol. 272, no. 44, pp. 28107–28115, 1997.

[14] R. Derynck, Y. Zhang, and X.-H. Feng, "Smads: transcriptional activators of TGF-β responses," *Cell*, vol. 95, no. 6, pp. 737–740, 1998.

[15] X. H. Feng, Y. Zhang, R. Y. Wu, et al., "The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-β-induced transcriptional activation," *Genes & Development*, vol. 12, no. 14, pp. 2153–2163, 1998.

[16] G. Lagna, A. Hata, A. Hemmati-Brivanlou, et al., "Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways," *Nature*, vol. 383, no. 6603, pp. 832–836, 1996.

[17] F. Liu, C. Pouponnot, and J. Massagué, "Dual role of the Smad4/DPC4 tumor suppressor in TGFβ-inducible transcriptional complexes," *Genes and Development*, vol. 11, no. 23, pp. 3157–3167, 1997.

[18] A. Hata, G. Lagna, J. Massagué, et al., "Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor," *Genes & Development*, vol. 12, no. 2, pp. 186–197, 1998.

[19] H. Hayashi, S. Abdollah, Y. Qiu, et al., "The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFbeta signaling," *Cell*, vol. 89, no. 7, pp. 1165–1173, 1997.

[20] T. Imamura, M. Takase, A. Nishihara, et al., "Smad6 inhibits signalling by the TGF-β superfamily," *Nature*, vol. 389, no. 6651, pp. 622–626, 1997.

[21] P. Kavsak, R. K. Rasmussen, C. G. Causing, et al., "Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation," *Molecular Cell*, vol. 6, no. 6, pp. 1365–1375, 2000.

[22] A. Nakao, M. Afrakhte, A. Moren, et al., "Identification of Smad7, a TGFβ-inducible antagonist of TGF-β signalling," *Nature*, vol. 389, no. 6651, pp. 631–635, 1997.

[23] C. Suzuki, G. Murakami, M. Fukuchi, et al., "Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane," *The Journal of Biological Chemistry*, vol. 277, no. 42, pp. 39919–39925, 2002.

[24] J. L. Andres, K. Stanley, S. Cheifetz, and J. Massagué, "Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β," *Journal of Cell Biology*, vol. 109, no. 6, pp. 3137–3145, 1989.

[25] S. Cheifetz, T. Bellon, C. Cales, et al., "Endoglin is a component of the transforming growth factor-β receptor system in human endothelial cells," *The Journal of Biological Chemistry*, vol. 267, no. 27, pp. 19027–19030, 1992.

[26] A. Gougos and M. Letarte, "Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line," *Journal of Immunology*, vol. 141, no. 6, pp. 1925–1933, 1988.

[27] L. Attisano, J. L. Wrana, F. Lopez-Casillas, et al., "TGF-β receptors and actions," *Biochimica et Biophysica Acta*, vol. 1222, no. 1, pp. 71–80, 1994.

[28] S. Venkatesha, M. Toporsian, C. Lam, et al., "Soluble endoglin contributes to the pathogenesis of preeclampsia," *Nature Medicine*, vol. 12, no. 6, pp. 642–649, 2006.

[29] Z. Gatalica and E. Torlakovic, "Pathology of the hereditary colorectal carcinoma," *Familial Cancer*, vol. 7, no. 1, pp. 15–26, 2008.

[30] J. R. Howe, M. G. Sayed, A. F. Ahmed, et al., "The prevalence of MADH4 and BMPR1A mutations in juvenile polyposis and absence of BMPR2, BMPR1B, and ACVR1 mutations," *Journal of Medical Genetics*, vol. 41, no. 7, pp. 484–491, 2004.

[31] K. Sweet, J. Willis, X. P. Zhou, et al., "Molecular classification of patients with unexplained hamartomatous and hyperplastic polyposis," *The Journal of the American Medical Association*, vol. 294, no. 19, pp. 2465–2473, 2005.

[32] S. Y. Jeong, K. H. Shin, J. H. Shin, et al., "Microsatellite instability and mutations in DNA mismatch repair genes in sporadic colorectal cancers," *Diseases of the Colon & Rectum*, vol. 46, no. 8, pp. 1069–1077, 2003.

[33] K. H. Shin, Y. J. Park, and J.-G. Park, "Mutational analysis of the transforming growth factor β receptor type II gene in hereditary nonpolyposis colorectal cancer and early-onset colorectal cancer patients," *Clinical Cancer Research*, vol. 6, no. 2, pp. 536–540, 2000.

[34] X. Zhou, H. Hampel, H. Thiele, et al., "Association of germline mutation in the PTEN tumour suppressor gene and Proteus and Proteus-like syndromes," *Lancet*, vol. 358, no. 9277, pp. 210–211, 2001.

[35] R. Nishimura, H. Okumura, K. Noda, et al., "High level of β-type transforming growth factor activity in human urine obtained from cancer patients," *Japanese Journal of Cancer Research*, vol. 77, no. 6, pp. 560–567, 1986.

[36] F. M. Kong, M. S. Anscher, T. Murase, et al., "Elevated plasma transforming growth factor-β 1 levels in breast cancer patients decrease after surgical removal of the tumor," *Annals of Surgery*, vol. 222, no. 2, pp. 155–162, 1995.

[37] F. M. Kong, M. K. Washington, R. L. Jirtle, et al., "Plasma transforming growth factor-β 1 reflects disease status in patients with lung cancer after radiotherapy: a possible tumor marker," *Lung Cancer*, vol. 16, no. 1, pp. 47–59, 1996.

[38] H. Saito, S. Tsujitani, S. Oka, et al., "An elevated serum level of transforming growth factor-β 1 (TGF-β 1) significantly correlated with lymph node metastasis and poor prognosis in patients with gastric carcinoma," *Anticancer Research*, vol. 20, no. 6B, pp. 4489–4493, 2000.

[39] S. F. Shariat, M. W. Kattan, E. Traxel, et al., "Association of pre- and postoperative plasma levels of transforming growth factor $\beta(1)$ and interleukin 6 and its soluble receptor with prostate cancer progression," *Clinical Cancer Research*, vol. 10, no. 6, pp. 1992–1999, 2004.

[40] S. F. Shariat, J. H. Kim, B. Andrews, et al., "Preoperative plasma levels of transforming growth factor $\beta(1)$ strongly predict clinical outcome in patients with bladder carcinoma," *Cancer*, vol. 92, no. 12, pp. 2985–2992, 2001.

[41] S. M. Sheen-Chen, H. S. Chen, C. W. Sheen, et al., "Serum levels of transforming growth factor β1 in patients with breast cancer," *Archives of Surgery*, vol. 136, no. 8, pp. 937–940, 2001.

[42] K. S. Shim, K. H. Kim, W. S. Han, et al., "Elevated serum levels of transforming growth factor-β1 in patients with colorectal carcinoma: its association with tumor progression and its significant decrease after curative surgical resection," *Cancer*, vol. 85, no. 3, pp. 554–561, 1999.

[43] F. Tas, D. Duranyildiz, H. Oguz, et al., "Circulating serum levels of angiogenic factors and vascular endothelial growth factor receptors 1 and 2 in melanoma patients," *Melanoma Research*, vol. 16, no. 5, pp. 405–411, 2006.

[44] B. H. von Rahden, H. J. Stein, M. Feith, et al., "Overexpression of TGF-β1 in esophageal (Barrett's) adenocarcinoma is associated with advanced stage of disease and poor prognosis," *Molecular Carcinogenesis*, vol. 45, no. 10, pp. 786–794, 2006.

[45] T. Rothhammer, P. J. Wild, S. Meyer, et al., "Bone morphogenetic protein 7 (BMP7) expression is a potential novel prognostic marker for recurrence in patients with primary melanoma," *Cancer Biomark*, vol. 3, no. 2, pp. 111–117, 2007.

[46] H. F. Yuen, Y. P. Chan, W. L. Cheung, et al., "The prognostic significance of BMP-6 signaling in prostate cancer," *Modern Pathology*, vol. 21, no. 12, pp. 1436–1443, 2008.

[47] M. M. Reinholz, S. J. Iturria, J. N. Ingle, et al., "Differential gene expression of TGF-β family members and osteopontin in breast tumor tissue: analysis by real-time quantitative PCR," *Breast Cancer Research and Treatment*, vol. 74, no. 3, pp. 255–269, 2002.

[48] D. Bacman, S. Merkel, R. Croner, et al., "TGF-β receptor 2 downregulation in tumour-associated stroma worsens prognosis and high-grade tumours show more tumour-associated macrophages and lower TGF-β1 expression in colon carcinoma: a retrospective study," *BMC Cancer*, vol. 7, article no. 156, 2007.

[49] M. Dong, T. How, K. C. Kirkbride, et al., "The type III TGF-β receptor suppresses breast cancer progression," *Journal of Clinical Investigation*, vol. 117, no. 1, pp. 206–217, 2007.

[50] R. S. Turley, E. C. Finger, N. Hempel, et al., "The type III transforming growth factor-β receptor as a novel tumor suppressor gene in prostate cancer," *Cancer Research*, vol. 67, no. 3, pp. 1090–1098, 2007.

[51] H. M. Rooke, M. R. Vitas, P. S. Crosier, et al., "The TGF-β type II receptor in chronic myeloid leukemia: analysis of microsatellite regions and gene expression," *Leukemia*, vol. 13, no. 4, pp. 535–541, 1999.

[52] P. I. Knaus, D. Lindemann, J. F. DeCoteau, et al., "A dominant inhibitory mutant of the type II transforming growth factor β receptor in the malignant progression of a cutaneous T-cell lymphoma," *Molecular and Cellular Biology*, vol. 16, no. 7, pp. 3480–3489, 1996.

[53] W. P. Schiemann, W. M. Pfeifer, E. Levi, et al., "A deletion in the gene for transforming growth factor β type I receptor abolishes growth regulation by transforming growth factor β in a cutaneous T-cell lymphoma," *Blood*, vol. 94, no. 8, pp. 2854–2861, 1999.

[54] I. Y. Kim, D. H. Lee, D. K. Lee, et al., "Restoration of bone morphogenetic protein receptor type II expression leads to a decreased rate of tumor growth in bladder transitional cell carcinoma cell line TSU-Pr1," *Cancer Research*, vol. 64, no. 20, pp. 7355–7360, 2004.

[55] M. J. Beresford, A. L. Harris, M. Ah-See, F. Daley, A. R. Padhani, and A. Makris, "The relationship of the neo-angiogenic marker, endoglin, with response to neoadjuvant chemotherapy in breast cancer," *British Journal of Cancer*, vol. 95, no. 12, pp. 1683–1688, 2006.

[56] C. A. Brewer, J. J. Setterdahl, M. J. Li, J. M. Johnston, J. L. Mann, and M. E. McAsey, "Endoglin expression as a measure of microvessel density in cervical cancer," *Obstetrics and Gynecology*, vol. 96, no. 2, pp. 224–228, 2000.

[57] Y. M. El-Gohary, J. F. Silverman, P. R. Olson, et al., "Endoglin (CD105) and vascular endothelial growth factor as prognostic markers in prostatic adenocarcinoma," *American Journal of Clinical Pathology*, vol. 127, no. 4, pp. 572–579, 2007.

[58] P. Wikstrom, I. F. Lissbrant, P. Stattin, et al., "Endoglin (CD105) is expressed on immature blood vessels and is a marker for survival in prostate cancer," *Prostate*, vol. 51, no. 4, pp. 268–275, 2002.

[59] L. Calabro, E. Fonsatti, G. Bellomo, et al., "Differential levels of soluble endoglin (CD105) in myeloid malignancies," *Journal of Cellular Physiology*, vol. 194, no. 2, pp. 171–175, 2003.

[60] J. Larsson, M. J. Goumans, L. J. Sjostrand, et al., "Abnormal angiogenesis but intact hematopoietic potential in TGF-β type I receptor-deficient mice," *EMBO Journal*, vol. 20, no. 7, pp. 1663–1673, 2001.

[61] Y. Tang, K. S. Lee, H. Yang, et al., "Epistatic interactions between modifier genes confer strain-specific redundancy for Tgfb1 in developmental angiogenesis," *Genomics*, vol. 85, no. 1, pp. 60–70, 2005.

[62] L. D. Urness, L. K. Sorensen, and D. Y. Li, "Arteriovenous malformations in mice lacking activin receptor-like kinase-1," *Nature Genetics*, vol. 26, no. 3, pp. 328–331, 2000.

[63] H. M. Arthur, J. Ure, A. J. Smith, et al., "Endoglin, an ancillary TGFβ receptor, is required for extraembryonic angiogenesis and plays a key role in heart development," *Developmental Biology*, vol. 217, no. 1, pp. 42–53, 2000.

[64] L. A. Fernández, F. Sanz-Rodriguez, F. J. Blanco, et al., "Hereditary hemorrhagic telangiectasia, a vascular dysplasia affecting the TGF-β signaling pathway," *Clinical Medicine and Research*, vol. 4, no. 1, pp. 66–78, 2006.

[65] D. W. Johnson, J. N. Berg, M. A. Baldwin, et al., "Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2," *Nature Genetics*, vol. 13, no. 2, pp. 189–195, 1996.

[66] B. L. Loeys, J. Chen, E. R. Neptune, et al., "A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2," *Nature Genetics*, vol. 37, no. 3, pp. 275–281, 2005.

[67] B. L. Loeys, U. Schwarze, T. Holm, et al., "Aneurysm syndromes caused by mutations in the TGF-β receptor," *The New England Journal of Medicine*, vol. 355, no. 8, pp. 788–798, 2006.

[68] M.-J. Goumans, G. Valdimarsdottir, S. Itoh, et al., "Balancing the activation state of the endothelium via two distinct TGF-β type I receptors," *EMBO Journal*, vol. 21, no. 7, pp. 1743–1753, 2002.

[69] T. Ota, M. Fujii, T. Sugizaki, et al., "Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells," *Journal of Cellular Physiology*, vol. 193, no. 3, pp. 299–318, 2002.

[70] M. Scharpfenecker, M. van Dinther, Z. Liu, et al., "BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis," *Journal of Cell Science*, vol. 120, no. 6, pp. 964–972, 2007.

[71] J. Massagué, J. Seoane, and D. Wotton, "Smad transcription factors," *Genes and Development*, vol. 19, no. 23, pp. 2783–2810, 2005.

[72] X. Guo and X.-F. Wang, "Signaling cross-talk between TGF-β/BMP and other pathways," *Cell Research*, vol. 19, no. 1, pp. 71–88, 2009.

[73] E. S. Shao, L. Lin, Y. Yao, and K. I. Boström, "Expression of vascular endothelial growth factor is coordinately regulated by the activin-like kinase receptors 1 and 5 in endothelial cells," *Blood*, vol. 114, no. 10, pp. 2197–2206, 2009.

[74] A. Anagnostou, E. S. Lee, N. Kessimian, R. Levinson, and M. Steiner, "Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 15, pp. 5978–5982, 1990.

[75] F. Bussolino, J. M. Wang, P. Defilippi, et al., "Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate," *Nature*, vol. 337, no. 6206, pp. 471–473, 1989.

[76] R. Tang, P. Hirsch, F. Fava, et al., "High Id1 expression is associated with poor prognosis in 237 patients with acute myeloid leukemia," *Blood*, vol. 114, no. 14, pp. 2993–3000, 2009.

[77] Y. Imai, M. Kurokawa, K. Izutsu, et al., "Mutations of the Smad4 gene in acute myelogeneous leukemia and their functional implications in leukemogenesis," *Oncogene*, vol. 20, no. 1, pp. 88–96, 2001.

[78] L. Zhou, A. N. Nguyen, D. Sohal, et al., "Inhibition of the TGF-β receptor I kinase promotes hematopoiesis in MDS," *Blood*, vol. 112, no. 8, pp. 3434–3443, 2008.

[79] M. R. Shalaby and A. J. Ammann, "Suppression of immune cell function in vitro by recombinant human transforming growth factor-β," *Cellular Immunology*, vol. 112, no. 2, pp. 343–350, 1988.

[80] S. M. Wahl, D. A. Hunt, H. L. Wong, et al., "Transforming growth factor-β is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation," *The Journal of Immunology*, vol. 140, no. 9, pp. 3026–3032, 1988.

[81] L. Gorelik and R. A. Flavell, "Abrogation of TGFβ signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease," *Immunity*, vol. 12, no. 2, pp. 171–181, 2000.

[82] J. H. Kehrl, L. M. Wakefield, A. B. Roberts, et al., "Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth," *The Journal of Experimental Medicine*, vol. 163, no. 5, pp. 1037–1050, 1986.

[83] S. H. Wrzesinski, Y. Y. Wan, and R. A. Flavell, "Transforming growth factor-β and the immune response: implications for anticancer therapy," *Clinical Cancer Research*, vol. 13, no. 18, part 1, pp. 5262–5270, 2007.

[84] J. C. Morris, G. I. Shapiro, A. R. Tan, et al., "Phase I/II study of GC1008: a human anti-transforming growth factor-β (TGFβ) monoclonal antibody (MAb) in patients with advanced malignant melanoma (MM) or renal cell carcinoma (RCC)," *Journal of Clinical Oncology*, vol. 26, abstract 9028, 2008.

[85] U. Bogdahn, A. K. Mahapatra, V. Olyushin, et al., "Results of a phase Iib active-controlled study with AP 12009 for patients with recurrent or refractory anaplastic astrocytoma," *Journal of Clinical Oncology*, vol. 26, abstract 2076, 2008.

[86] U. Bogdahn, T. Schneider, V. Oliushine, et al., "Randomized, active-controlled phase IIb study with trabedersen (AP 12009) in recurrent or refractory high-grade glioma patients: basis for phase III endpoints," *Journal of Clinical Oncology*, vol. 27, no. 15s, supplement, 2009, abstract 2037.

[87] J. Nemunaitis, R. O. Dillman, P. O. Schwarzenberger, et al., "Phase II study of belagenpumatucel-L, a transforming growth factor β-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 24, no. 29, pp. 4721–4730, 2006.

[88] E. Fransvea, U. Angelotti, S. Antonaci, and G. Giannelli, "Blocking transforming growth factor-β up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells," *Hepatology*, vol. 47, no. 5, pp. 1557–1566, 2008.

[89] D. Melisi, S. Ishiyama, G. M. Sclabas, et al., "LY2109761, a novel transforming growth factor β receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis," *Molecular Cancer Therapeutics*, vol. 7, no. 4, pp. 829–840, 2008.

[90] B. Zhang, S. K. Halder, S. Zhang, and P. K. Datta, "Targeting transforming growth factor-β signaling in liver metastasis of colon cancer," *Cancer Letters*, vol. 277, no. 1, pp. 114–120, 2009.

[91] E. Fransvea, A. Mazzocca, S. Antonaci, et al., "Targeting transforming growth factor (TGF)-βRI inhibits activation of β1 integrin and blocks vascular invasion in hepatocellular carcinoma," *Hepatology*, vol. 49, no. 3, pp. 839–850, 2009.

[92] A. Mazzocca, E. Fransvea, F. Dituri, L. Lupo, S. Antonaci, and G. Giannelli, "Down-regulation of connective tissue growth factor by inhibition of transforming growth factor β blocks the tumor-stroma cross-talk and tumor progression in hepatocellular carcinoma," *Hepatology*, vol. 51, no. 2, pp. 523–534, 2010.

[93] A. Mazzocca, E. Fransvea, G. Lavezzari, S. Antonaci, and G. Giannelli, "Inhibition of transforming growth factor β receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation," *Hepatology*, vol. 50, no. 4, pp. 1140–1151, 2009.

[94] Y. Xu, Y. Tabe, L. Jin, et al., "TGF-β receptor kinase inhibitor LY2109761 reverses the anti-apoptotic effects of TGF-β1 in

myelo-monocytic leukaemic cells co-cultured with stromal cells," *British Journal of Haematology*, vol. 142, no. 2, pp. 192–201, 2008.

[95] M. D. Hjelmeland, A. B. Hjelmeland, S. Sathornsumetee, et al., "SB-431542, a small molecule transforming growth factor-β-receptor antagonist, inhibits human glioma cell line proliferation and motility," *Molecular Cancer Therapeutics*, vol. 3, no. 6, pp. 737–745, 2004.

[96] D. Javelaud, K. S. Mohammad, C. R. McKenna, et al., "Stable overexpression of Smad7 in human melanoma cells impairs bone metastasis," *Cancer Research*, vol. 67, no. 5, pp. 2317–2324, 2007.

[97] N. J. Gaspar, L. Li, A. M. Kapoun, et al., "Inhibition of transforming growth factor β signaling reduces pancreatic adenocarcinoma growth and invasiveness," *Molecular Pharmacology*, vol. 72, no. 1, pp. 152–161, 2007.

[98] R. Ge, V. Rajeev, P. Ray, et al., "Inhibition of growth and metastasis of mouse mammary carcinoma by selective inhibitor of transforming growth factor-β type I receptor kinase in vivo," *Clinical Cancer Research*, vol. 12, no. 14, part 1, pp. 4315–4330, 2006.

[99] E. Suzuki, S. Kim, H. K. Cheung, et al., "A novel small-molecule inhibitor of transforming growth factor β type I receptor kinase (SM16) inhibits murine mesothelioma tumor growth in vivo and prevents tumor recurrence after surgical resection," *Cancer Research*, vol. 67, no. 5, pp. 2351–2359, 2007.

[100] L. Rosen, M. S. Gordon, H. I. Hurwitz, et al., "Early evidence of tolerability and clinical activity from a phase I study of TRC105 (anti-CD105 antibody) in patients with advanced refractory cancer," *Journal of Clinical Oncology*, vol. 27, no. 15s, supplement, 2009, abstract 3518.

[101] Y. A. Yang, O. Dukhanina, B. Tang, et al., "Lifetime exposure to a soluble TGF-β antagonist protects mice against metastasis without adverse side effects," *Journal of Clinical Investigation*, vol. 109, no. 12, pp. 1607–1615, 2002.

[102] S. Biswas, M. Guix, C. Rinehart, et al., "Inhibition of TGF-β with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression," *Journal of Clinical Investigation*, vol. 117, no. 5, pp. 1305–1313, 2007.

[103] Q. Zhang, X. J. Yang, S. D. Kundu, et al., "Blockade of transforming growth factor-β signaling in tumor-reactive $CD8^{(+)}$ T cells activates the antitumor immune response cycle," *Molecular Cancer Therapeutics*, vol. 5, no. 7, pp. 1733–1743, 2006.

[104] Y. Yamamoto, Y. Ueda, T. Itoh, et al., "A novel immunotherapeutic modality with direct hemoperfusion targeting transforming growth factor-β prolongs the survival of tumor-bearing rats," *Oncology Reports*, vol. 16, no. 6, pp. 1277–1284, 2006.

[105] T. Fujita, K. Teramoto, Y. Ozaki, et al., "Inhibition of transforming growth factor-β-mediated immunosuppression in tumor-draining lymph nodes augments antitumor responses by various immunologic cell types," *Cancer Research*, vol. 69, no. 12, pp. 5142–5150, 2009.

[106] Y. Liu, Q. Wang, B. K. Kleinschmidt-DeMasters, et al., "TGF-β2 inhibition augments the effect of tumor vaccine and improves the survival of animals with pre-established brain tumors," *Journal of Neuro-Oncology*, vol. 81, no. 2, pp. 149–162, 2007.

[107] H. Fakhrai, J. C. Mantil, L. Liu, et al., "Phase I clinical trial of a TGF-β antisense-modified tumor cell vaccine in patients with advanced glioma," *Cancer Gene Therapy*, vol. 13, no. 12, pp. 1052–1060, 2006.

[108] F. J. Burrows, E. J. Derbyshire, P. L. Tazzari, et al., "Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy," *Clinical Cancer Research*, vol. 1, no. 12, pp. 1623–1634, 1995.

[109] F. Matsuno, Y. Haruta, M. Kondo, H. Tsai, M. Barcos, and B. K. Seon, "Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies," *Clinical Cancer Research*, vol. 5, no. 2, pp. 371–382, 1999.

[110] B. K. Seon, F. Matsuno, Y. Haruta, M. Kondo, and M. Barcos, "Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin," *Clinical Cancer Research*, vol. 3, no. 7, pp. 1031–1044, 1997.

[111] K. Shiozaki, N. Harada, W. R. Greco, et al., "Antiangiogenic chimeric anti-endoglin (CD105) antibody: pharmacokinetics and immunogenicity in nonhuman primates and effects of doxorubicin," *Cancer Immunology, Immunotherapy*, vol. 55, no. 2, pp. 140–150, 2006.

[112] N. Takahashi, A. Haba, F. Matsuno, and B. K. Seon, "Antiangiogenic therapy of established tumors in human skin/severe combined immunodeficiency mouse chimeras by anti-endoglin (CD105) monoclonal antibodies, and synergy between anti-endoglin antibody and cyclophosphamide," *Cancer Research*, vol. 61, no. 21, pp. 7846–7854, 2001.

[113] S. Uneda, H. Toi, T. Tsujie, et al., "Anti-endoglin monoclonal antibodies are effective for suppressing metastasis and the primary tumors by targeting tumor vasculature," *International Journal of Cancer*, vol. 125, no. 6, pp. 1446–1453, 2009.

[114] J. Taipale, K. Miyazono, C.-H. Heldin, and J. Keski-Oja, "Latent transforming growth factor-β1 associates to fibroblast extracellular matrix via latent TGF-β binding protein," *Journal of Cell Biology*, vol. 124, no. 1-2, pp. 171–181, 1994.

[115] N. A. Bhowmick, A. Chytil, D. Plieth, et al., "TGF-β signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia," *Science*, vol. 303, no. 5659, pp. 848–851, 2004.

[116] N. Cheng, N. A. Bhowmick, A. Chytil, et al., "Loss of TGF-β type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-α-, MSP- and HGF-mediated signaling networks," *Oncogene*, vol. 24, no. 32, pp. 5053–5068, 2005.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 396286, 13 pages
doi:10.1155/2010/396286

Research Article

Architectural Heterogeneity in Tumors Caused by Differentiation Alters Intratumoral Drug Distribution and Affects Therapeutic Synergy of Antiangiogenic Organoselenium Compound

Youcef M. Rustum,[1] Károly Tóth,[1] Mukund Seshadri,[1] Arindam Sen,[2] Farukh A. Durrani,[1] Emily Stott,[1] Carl D. Morrison,[3] Shousong Cao,[1] and Arup Bhattacharya[4]

[1] *Department of Cancer Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*
[2] *Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*
[3] *Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*
[4] *Department of Cancer Prevention & Control, Roswell Park Cancer Institute, Buffalo, NY 14263, USA*

Correspondence should be addressed to Arup Bhattacharya, arup.bhattacharya@roswellpark.org

Received 25 December 2009; Accepted 22 January 2010

Academic Editor: Arkadiusz Dudek

Tumor differentiation enhances morphologic and microvascular heterogeneity fostering hypoxia that retards intratumoral drug delivery, distribution, and compromise therapeutic efficacy. In this study, the influence of tumor biologic heterogeneity on the interaction between cytotoxic chemotherapy and selenium was examined using a panel of human tumor xenografts representing cancers of the head and neck and lung along with tissue microarray analysis of human surgical samples. Tumor differentiation status, microvessel density, interstitial fluid pressure, vascular phenotype, and drug delivery were correlated with the degree of enhancement of chemotherapeutic efficacy by selenium. Marked potentiation of antitumor activity was observed in H69 tumors that exhibited a well-vascularized, poorly differentiated phenotype. In comparison, modulation of chemotherapeutic efficacy by antiangiogenic selenium was generally lower or absent in well-differentiated tumors with multiple avascular hypoxic, differentiated regions. Tumor histomorphologic heterogeneity was found prevalent in the clinical samples studied and represents a primary and critical physiological barrier to chemotherapy.

1. Introduction

Despite concerted efforts for more than five decades, curative response to chemotherapy remains elusive in majority of solid malignancies [1]. Efforts in discovering novel anticancer agents without similar efforts in understanding and overcoming physical barriers within tumors are unlikely to radically change chemotherapeutic efficacy in the clinic. Tumor being a heterogeneous three-dimensional composite of various components, factors at coarser physiological scales can and does influence tumor response [2]. For an optimum chemotherapeutic efficacy, the drug has to extravasate, diffuse to distant tumor cells, be transported inside the cells, bind to the specific target, and induce cell death. Only when an anticancer agent is able to affect each and every individual proliferating cancer cells in effectively inducing tumor cell death, can it result in a complete remission (CR) or a cure. Factors within the tumor microenvironment that impede drug delivery and distribution are likely to result in tumor regrowth and resistance irrespective of the drug's efficacy in vitro.

Tumor drug delivery system constituted predominantly of abnormal vasculature, lack pericyte coverage, have abnormal branching patterns and shunt perfusion, prestasis, stasis and reversal of flow [3]. This dilated, chaotic and leaky vasculature with an intermittent or unstable blood flow contributes to an adverse and high intratumoral interstitial fluid pressure (IFP) that retards delivery and distribution of drugs from the vessels into the tumor [4]. We have previously demonstrated that differentiated regions in squamous cell

carcinoma and adenocarcinoma do not contain blood vessel and contribute to heterogeneity in microvessel distribution that physiologically further retards an optimal intratumoral drug delivery and distribution [5–7]. Various strategies for improving drug penetration include improving tumor blood flow, enhancing vascular permeability, reducing IFP, and modifying the extracellular matrix [2]. For example, use of antiangiogenic agents has been shown to result in a favorable improvement in extent and quality of tumor perfusion while reducing vascular permeability and tumor IFP as a result of tumor vascular normalization [5, 7]. While there has been an ongoing effort in improving tumor drug penetration in order to improve therapeutic efficacy, newer approaches are required to overcome tumor architectural and morphologic barriers that foster therapeutic resistance by adversely affecting tumor drug delivery.

In our earlier studies, we have observed that high but nontoxic doses of antiangiogenic organoselenium compounds such as 5-methylselenocysteine (MSC) and selenomethionine (SLM) act as selective modulators of chemotherapeutic efficacy of a broad range of anticancer drugs (irinotecan, taxanes, platinum complexes, doxorubicin and cyclophosphamide) that are currently used in the clinic [8]. Administration of MSC (0.2 mg/mice/day per oral starting 7 days before irinotecan) in combination with irinotecan (100 mg/kg i.v. weekly $\times$ 4) was found to enhance therapeutic response from 20% and 30% CR with the drug alone to 100% CR in uniformly well-vascularized poorly differentiated colorectal carcinoma HCT-8 and head and neck squamous cell carcinoma (HNSCC) FaDu, respectively [8]. In contrast, this therapeutic synergy was less dramatic in well differentiated xenografts such as the colorectal adenocarcinoma HT-29 and HNSCC A253 (0% and 10% CR with the drug alone to 20% and 60% CR with the combination, resp.). In subsequent studies, we demonstrated that this therapeutic synergy was the result of enhanced tumor drug delivery and distribution as a consequence of an improved tumor vascular normalization and tumor IFP [5, 7].

In this study, we examined the influence of tumor histologic heterogeneity on the interaction between cytotoxic chemotherapy and selenium (Se) using a panel of surgical human tumor xenografts representing cancers of the head and neck (A253, well differentiated and poorly differentiated patient tumor derived HNSCC xenografts) and lung (H69, A549). Tumor differentiation status, microvessel density, interstitial fluid pressure, tumor blood, volume and permeability were correlated with the degree of enhancement of chemotherapeutic efficacy by selenium. Additionally, tissue microarray (TMA) analysis of human surgical samples was also performed to examine the occurrence and relevance of the observed biologic heterogeneity.

2. Materials and Methods

2.1. Tumor Model. The human cancer cell lines H69 (small cell lung cancer, SCLC), A549 (nonsmall cell epithelial lung carcinoma, NSCLC), and A253 (well differentiated head and neck squamous cell carcinoma) were originally obtained from American Type Culture Collection (Manassas, VA) and xenografts were established in ~8 week old female athymic nude mice (Foxn1^{nu}, Harlan Sprague Dawley, Inc. Indianapolis, IN) as previously described [6]. Human poorly differentiated squamous cell carcinoma (SCC) surgical sample #17073 (maxillary sinus, PDSCC) and well differentiated SCC #16653 (larynx, WDSCC) were obtained in house at Roswell Park Cancer Institute and were maintained in SCID (C.B-Igh-1^{b}IcrTac-Prkdc^{scid}/Ros) mice. Mice were assessed for tumor growth using digital vernier calipers for measuring tumor burden (mm^3) using the formulae: 1/2 ($L \times W^2$), where L and W are the longest and shortest axis in mm as per established method [8]. All studies were performed in accordance with Institute Animal Care and Use Committee-approved protocols and each treatment group had a minimum of 4 animals per group.

2.2. Patient Samples of Cancer. Formalin/paraffin sections of human cancer surgical TMA containing 0.6 mm cores from head and neck, colorectal, and lung cancer were studied for presence or absence of differentiated structure, microvessel distribution (MVD), and tumor hypoxia. Tumor vessels were detected using CD34 marker for endothelial cells and hypoxia was determined using carbonic anhydrase IX (CAIX) staining as per methods described earlier [9].

2.3. Drugs and Chemicals. MSC or SLM (1 mg/mL, Sigma, St. Louis, MO) was administered orally as a sterile saline solution at the maximum tolerated dose of 0.2 mg/mouse/day [8] or 8 mg/kg/day for 14 days, starting at least 3 days after tumor implantation. Taxotere (Sanofi-Aventis, Bridgewater, NJ) was administered as a single intravenous dose of 60 mg/kg while irinotecan (Pharmacia & Upjohn, New York, NY) was administered at a weekly schedule of 100 or 200 mg/kg $\times$4. Doxorubicin (Bedford Laboratories, Bedford, OH) was administered as a single intravenous dose of 30 mg/kg, 24 hours after the MSC dose on day 14. Albumin-GdDTPA was obtained from the Contrast Media Laboratory, Department of Radiology (Dr. Robert C. Brasch), University of California at San Francisco (San Francisco, CA). For the combination chemotherapy with MSC/SLM, MSC or SLM was given 7 days before therapy and continued daily for 7 more days after the last dose of the chemotherapeutic drug.

2.4. Immunohistochemistry. MVD determination was done using CD31 in mouse and CD34 in human tumor TMAs. CD31 and alpha-smooth muscle actin (α-SMA) double staining was used to detect endothelial cells and pericytes, respectively, as described previously [5]. Briefly, 5–8 μm cryosections were fixed in cold acetone (-20°C) for 15 minutes and following quenching with endogenous peroxidase were incubated with rabbit polyclonal SMA antibody (1 μg/mL or 1/500) (Abcam, Cambridge, MA), biotinylated goat antirabbit secondary antibody (1/250) (Vector Labs) for 30 minutes, and CD31 antibody (B.D. Biosciences Pharmingen, Franklin Lakes, NJ) at a concentration of 10 μg/mL for 60 minutes. An isotype-matched negative control

was used in all cases. Immunostaining of tumor vasculature in the TMAs was done using antihuman CD34 (DAKO, Carpinteria, CA) used at 1/50 dilution for 90 minutes at room temperature followed by 30 minutes incubation with Streptavadin complex (Zymed Lab, Inc., San Francisco, CA) as per methods described earlier [5–7]; CAIX immunostaining, the primary antibody M75 (gift from Dr Pastorek, Institute of Virology, Slovak Republic) was used at 20 μg/mL for 90 minutes followed by 30-minutes incubation with Streptavadin complex (Zymed Lab, Inc., San Francisco, CA) as described earlier [5–7]. Hypoxia inducible factor 1-α (HIF-1α) was detected with a multilayer, amplified method after antigen retrieval with Target Retrieval Solution (TRS, Dako Carpenteria, CA) in a pressure cooker. The method was developed and optimized at our Core Facility [10]. All immunohistochemical interpretation and analysis was carried out under the supervision of a board-certified and experienced pathologist (KT).

2.5. Magnetic Resonance Imaging. Tumor-bearing mice were imaged in a 4.7 T horizontal bore MR scanner (GE NMR Instruments, Fremont, CA). The imaging protocol and data analysis methods for estimating the vascular volume and permeability of tumors using the intravascular contrast agent, albumin-GdDTPA, have been previously described [11, 12]. The change in tumor T1-relaxation rate of tumors (ΔR1) was calculated over approximately 30–50 minutes post contrast. ΔR1 values were measured in the kidneys as a measure of vascular relaxation enhancement and used to compute vascular volume (y-intercept of the linear regression line) and permeability (slope of the linear regression line) tumor/blood fit. Image processing and analysis were carried out using commercially available medical imaging software Analyze PC, Version 7.0 (AnalyzeDirect, Lenexa, KS).

2.6. In Vivo Tumor IFP Measurements. MSC-induced changes in tumor IFP were monitored in A253, H69, and A549 xenografts in vivo real-time using microcatheter pressure transducers in externally accessible tumors and in normal tissue as per method described earlier [5, 7]. The interstitial fluid pressure within the tumor microenvironment was measured-using a modified "wick-in-needle" technique using custom-designed instrumentation broadly based upon an earlier published design [13]. Briefly, measurements were made with a MikroTip Catheter Transducer (Model SPR-524, Millar Instruments) via a 23.5 gauge wing-tipped infusion needle catheter. The transducer was interfaced to a PC using a pressure control unit (PCU-2000, Millar Instruments) via an USB analog-to-digital converter (Model DT9816 Data Translation, Marlboro MA). The software used to acquire the data was developed in the laboratory using DT Measure Foundry Ver. 4.0.7 (Data Translation, Marlboro, MA). The needle was inserted into the tumor, with measurements made every few millimeters; thus three to six measurements were made within each tumor, and the average value was used. Prior to each measurement, the apparatus was aspirated to ensure that no tissue was clogging the needle, and the instrument was zeroed. The instrument was calibrated before and after each experiment to ensure proper function, using a custom built water-column manometer. Mice with subcutaneous tumors (N = 4–8 per group) were used to assess the IFP measurements under anesthesia at time points equivalent to 24 hours post 14 days of MSC.

2.7. Determination of Intratumoral Doxorubicin Distribution Gradient. The influence of MSC on drug distribution gradient ($N = 47$ or more linear paths 90 pixels long using multiple sections from tumors) was assessed using fluorescence microscopy as described earlier [5]. Two hours post doxorubicin administration, animals were euthanized and the harvested tumor frozen and ~5–10 μm thick frozen sections were used. An average of 4 maximum intensity projection images with a resolution of 0.23 μm was acquired using identical acquisition parameters under 63× objective of Leica confocal microscope [5] and the digitized images were used further for image analysis.

2.8. Image Analysis. CD31 positive endothelial cell clusters in multiple high-power fields (400×) covering non-necrotic areas of the whole tumor sections were used to determine MVD. Tumor vascular maturation index (VMI) was derived by calculating the total number of CD31+ α-SMA+ areas and areas positive for CD31 alone in double-stained (CD31/α-SMA) tissue sections using Analyze (AnalyzeDirect, OverlandPark, KS) [5]. The mean intensity of doxorubicin autofluorescence at various distances away from the blood vessels was calculated using Analyze.

2.9. Statistical Analysis. Results are expressed as mean ± standard error of the mean and the differences between the mean of the groups were analyzed using unpaired two-tailed Student t-test (GraphPad Version 5.00, GraphPad Software, San Diego, CA). Linear regression analysis was carried out to determine statistical significance of DCE-MRI-measured parameters and intratumoral drug distribution gradient. A P value <.05 considered statistically significant.

3. Results

3.1. Histological Characteristics of Tumor Xenografts. While H69 (Figure 1(d)) is a poorly differentiated small cell lung carcinoma, HNSCC A253 (Figure 1(a)) and the non-small cell lung adenocarcinoma A549 (Figure 1(g)) are well and moderately differentiated tumors, respectively. CD31 immunostaining shows H69 (Figure 1(e)) to be uniformly well-vascularized while A253 (Figure 1(b)) and A549 (Figure 1(h)) xenografts have differentiated regions that are devoid of blood vessels and are thus consequently hypoxic as seen by presence of CAIX immunostaining (*arrows,* Figures 1(c) and 1(i), resp.). While very few cells were found to be CAIX positive in smaller H69 tumors (<1000 mg, Figure 1(f(a))), the larger H69 tumors (>2000 mg, Figure 1(f(b))) had the characteristic perinecrotic hypoxia. As shown in Figure 1(j), #17073 is a PDSCC while #16653 is a WDSCC (Figure 1(m)). The hallmark of surgical PDSCC

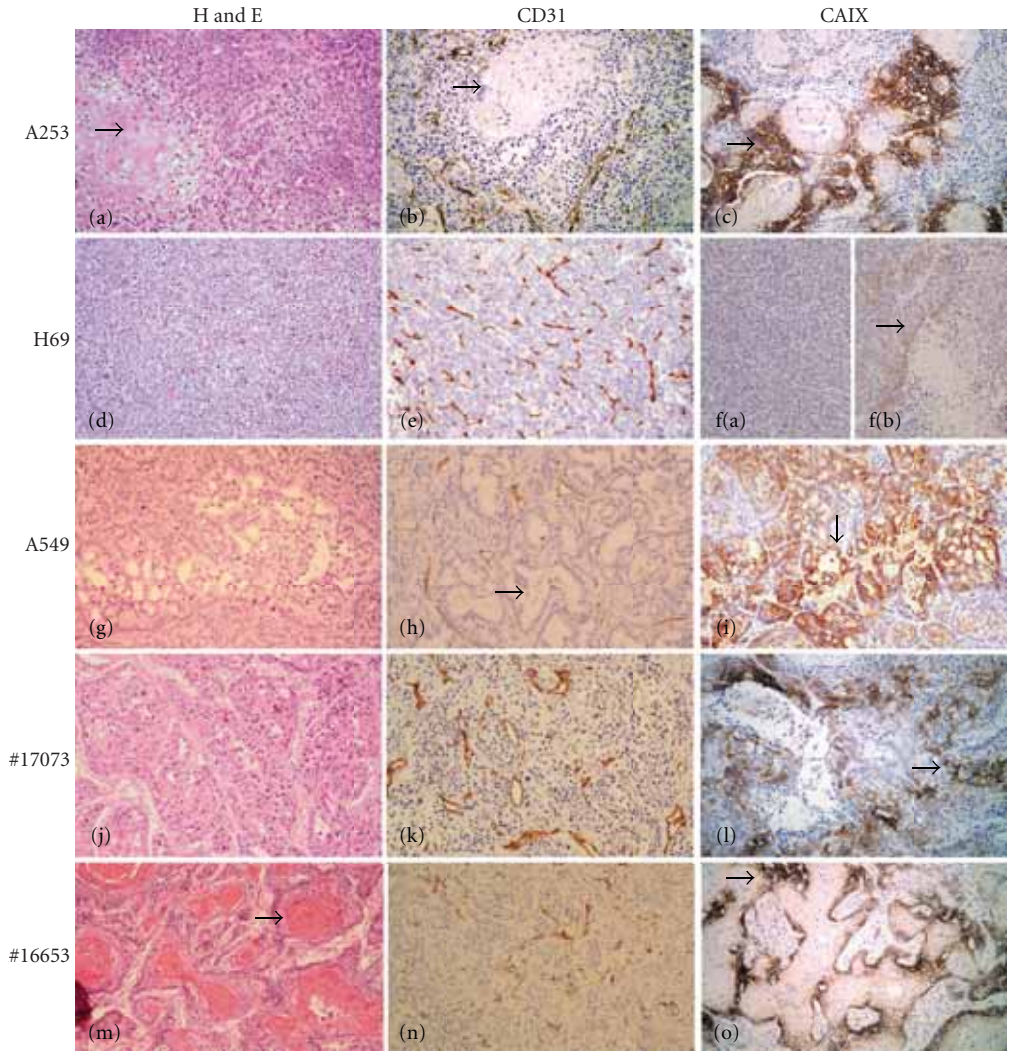


Figure 1: Photomicrographs of human cancer cell line xenografts A253, H69, A549 along with human HNSCC surgical samples-derived xenografts #17073 and #16653. (Left panels, H&E; middle panels, CD31 immunostaining to visualize microvessels; right panels, CAIX immunostaining to visualize tumor hypoxic regions; original magnifications, ×100). Poorly differentiated H69 (d) is uniformly well vascularised (e) and has no regions of hypoxia (f(a)) in the ~250 mg tumor but has perinecrotic hypoxic regions in larger (>2000 mg) tumor (f(b), *arrow*). Similar vascular arrangement (k) is seen in viable regions of the poorly differentiated HNSCC #17073 (PDSCC) which though has hypoxic regions ((l), *arrow*) around the many necrotic regions seen in the growing tumor. In contrast, A253 and A549 have differentiated regions ((a), *arrow* & (g)) that being avascular ((b), (h), *arrows)* are hypoxic ((c), (i), *arrows*). Surgical sample derived well differentiated HNSCC #16653(WDSCC) has a highly differentiated morphology due to presence of many well differentiated ((m), *arrow*) regions that are avascular (n) and hypoxic ((o), *arrow*).

xenografts was the presence of many necrotic regions throughout the tumor. These necrotic regions did not contain viable tumor cells or any vasculature and were surrounded by CAIX positive hypoxic cells (*arrow,* Figure 1(l)). Though the other regions in the PDSCC exhibited a uniform microvessel distribution (Figure 1(k)), these necrotic regions contribute to a histomorphological heterogeneity that is not conducive to an optimal intratumoral drug delivery and distribution. The surgical WDSCC xenograft contained several well differentiated regions (*arrow,* Figure 1(m)) that are without microvessels (Figure 1(n)) and are surrounded by rims of CAIX positive hypoxic proliferating cells (*arrow,* Figure 1(o)).

3.2. Effect of MSC on Tumor Growth. As shown in Figure 2(a), the HNSCC A253 grew at a faster pace than the lung xenografts H69 and A549. Treatment with MSC resulted in a reduction of tumor burden when compared to the untreated controls (Figure 2(b)) by 30%, 62%, and 36% in A253 (1952 ± 32.07 versus 1360 ± 59.75, $N = 6$), H69 (279.5 ± 106.60 versus 105 ± 31.84, $N = 4$) and A549 (552.50 ± 60.62 versus 353.40 ± 68.16, $N = 4$), respectively, albeit significantly only in A253 ($P < .0001$). Untreated PDSCC and WDSCC xenografts grew slower than control A253 tumors (Figure 2(c)). Treatment with MSC for 2 weeks did not lead to a significant reduction in tumor burden when compared to untreated controls in both

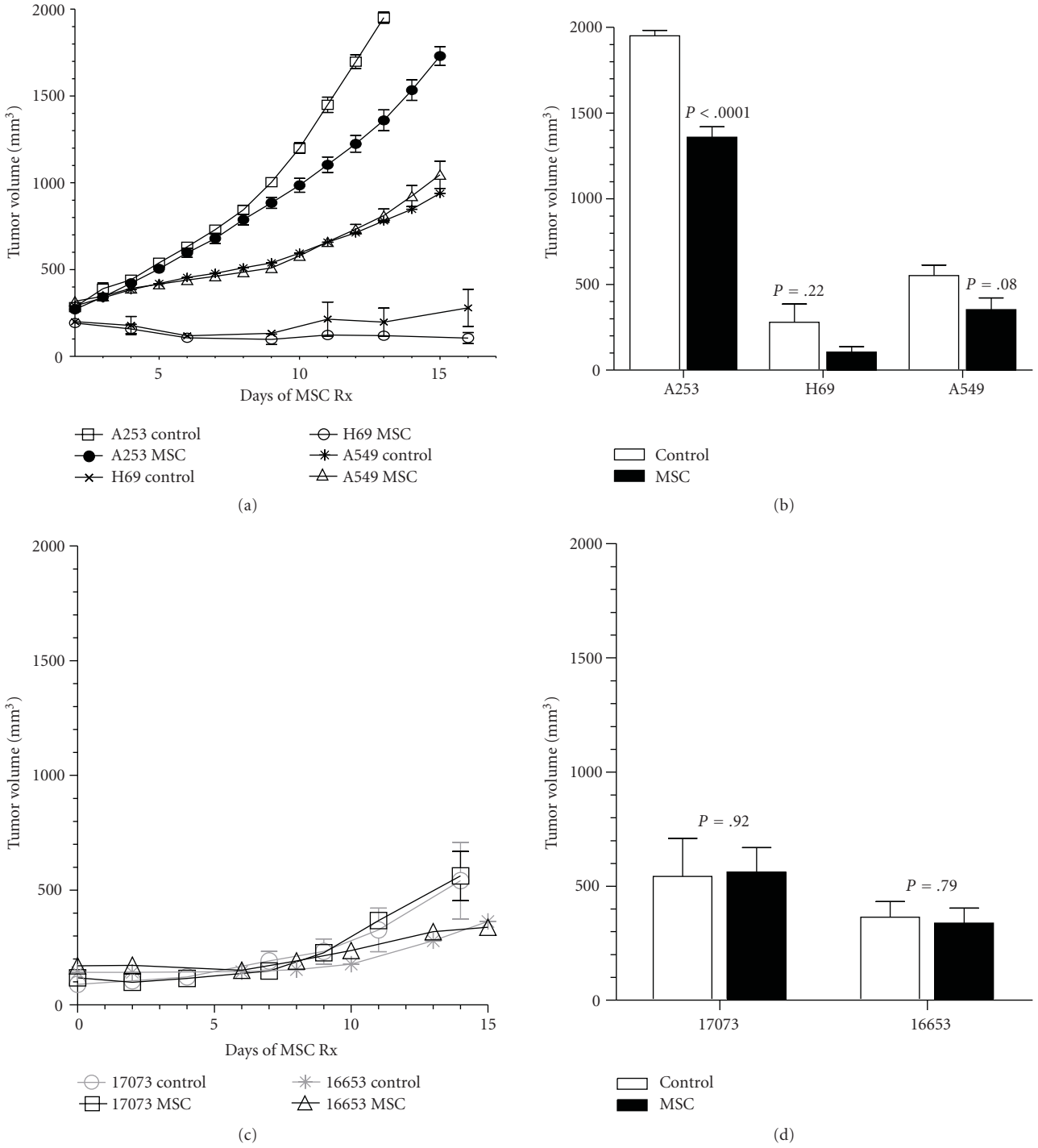


Figure 2: Effect of MSC on tumor growth ((a), (c)) and tumor volume ((b), (d)) at the end of 14 days treatment.

PDSCC (541.9 ± 167.2 versus 561.80 ± 107.30, $N = 6$) and WDSCC (363.80 ± 68.53 versus 338.50 ± 65.63, $N = 6$–8) xenografts (Figure 2(d)).

3.3. Effect of MSC on MVD, IFP, and Drug Delivery. Poorly differentiated untreated H69 xenografts were highly vascularized compared to the untreated well differentiated HNSCC A253 ($P = .002$) and NSCLC A549 ($P = .008$) tumors. As shown in Figure 3(a), the antiangiogenic effect of MSC (0.2 mg/mouse/day × 14) led to a 59%, 62%, and 6% reduction in MVD compared to untreated control tumors in A253 (4.63 ± 0.87 versus 11.23 ± 1.87, $P = .03$), H69 (9.28 ± 0.82 versus 24.29 ± 1.45, $P = .008$) and A549 (6.60 ± 1.50 versus 7.05 ± 2.23, $P = .88$) respectively. No differences in MVD of normal mouse liver tissue were observed (data not shown). Tumor vascular maturation index (VMI), that is,

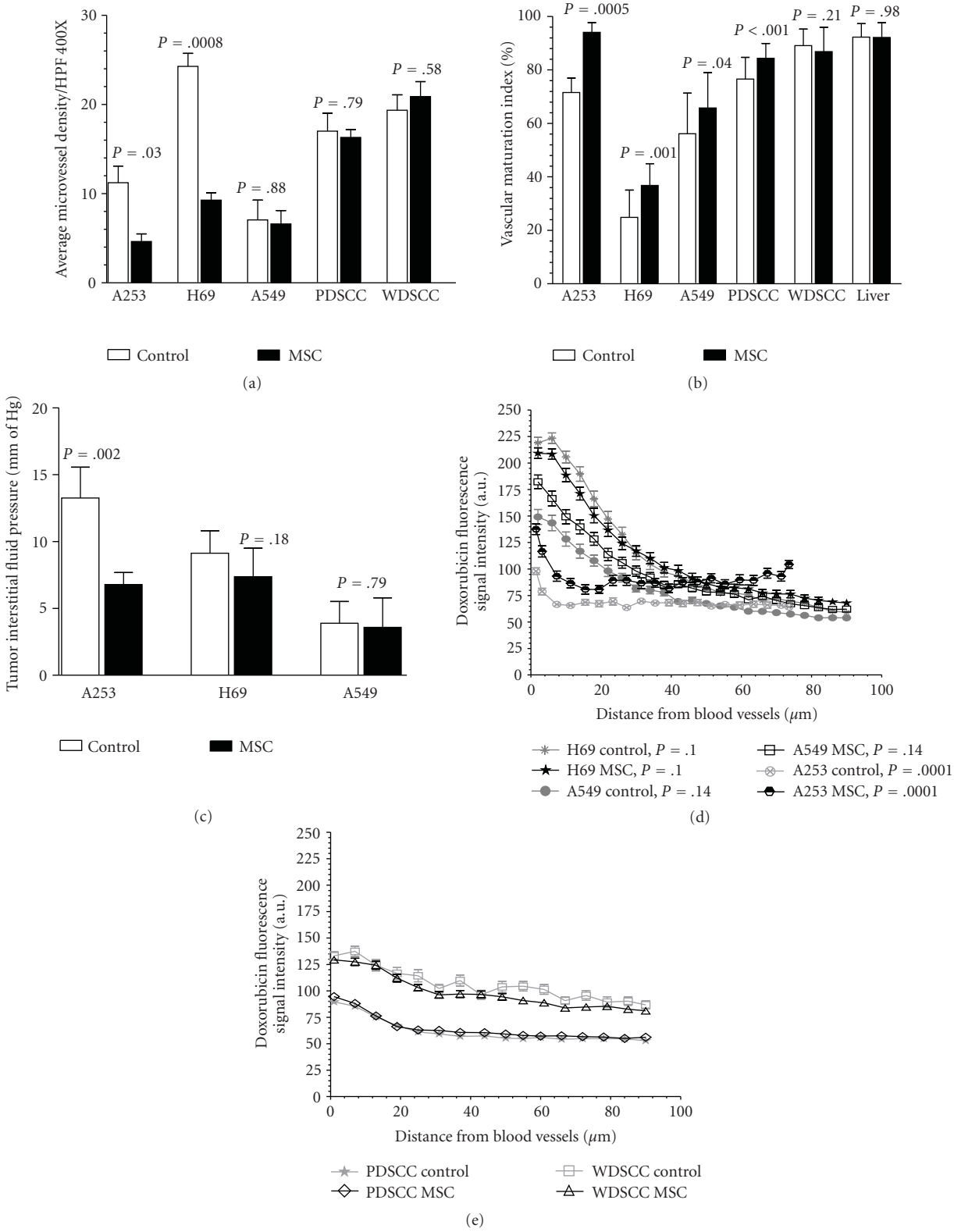


Figure 3: (a) Bar graphs show MVD counts per high-power field (HPF) (original magnification, 400×) in the untreated versus MSC-treated xenografts and surgical samples following 14 days of MSC treatment (0.2 mg/mouse/day). Significant reduction in MVD was seen in A253 and H69 xenografts after 14 days of MSC treatment with no changes seen in A549 xenografts or the surgical samples. (b) MSC led to an improved vascular maturation in A253, H69, A549 xenografts and PDSCC but did not induce any change in WDSCC and normal liver. (c) Treatment with MSC (0.2 mg/mouse/day × 14) led to a lowering of interstitial fluid pressure in A253 but not in H69 and A549 xenografts. (d) As a consequence, there was a significant improvement in tumor doxorubicin fluorescence intensity gradient in A253 xenografts but not in H69 or A549 xenografts. (e) MSC did not result in an improvement in tumor doxorubicin fluorescence intensity gradient in the surgical samples PDSCC and WDSCC.

the percentage of endothelial cells associated with pericytes, showed an increase of 31.38%, 49.93% and 6.38% in A253, H69 and A549 xenografts, respectively (Figure 3(b)). Tumor IFP was higher in the untreated HNSCC xenograft A253 compared to the untreated lung tumor xenografts that were lower by 31% and 71% in H69 and A549, respectively. As shown in Figure 3(c), treatment with MSC led to a significant reduction in tumor IFP in A253 compared to the untreated controls (6.78 ± 0.45 versus 13.26 ± 1.15, $P = .002$) but this reduction was less dramatic in the lung xenografts—H69 (7.38 ± 0.75 versus 9.13 ± 0.84, $P = .18$) and A549 (3.58 ± 0.91 versus 3.89 ± 0.66, $P = .79$). The net effect of MSC on tumor vasculature and IFP was a significantly ($P = .0001$) increased intratumoral doxorubicin gradient in A253 xenografts while there was no significant increase in the overall drug gradient in the lung xenografts (Figure 3(d)). Interestingly, at a distance of ~90 μm from tumor blood vessels, all the MSC-treated tumors showed a significantly higher doxorubicin concentration as measured by the fluorescence intensity compared to the non-MSC-treated A253 (91.38 ± 4.81 versus 66.90 ± 3.21, $P = .001$), H69 (68.06 ± 2.60 versus 59.17 ± 1.41, $P = .003$), and A549 (62.21 ± 1.84 versus 53.94 ± 1.58, $P = .0008$) xenografts.

In contrast, the untreated human surgical samples of HNSCC had a higher MVD (17 ± 0.3 in PDSCC, and 19 ± 0.41 in WDSCC) compared to HNSCC A253 xenograft (11.23 ± 1.87) (Figure 3(a)). Though treatment with MSC did not lead to a significant reduction in MVD in PDSCC compared to untreated controls (16 ± 0.13 versus 17 ± 0.3), there was a significant increase in tumor vascular maturation (84.39 ± 1.06 versus 76.57 ± 1.48, $P < .001$) (Figure 3(b)). In WDSCC, MSC did not lead to any reduction in MVD (20.90 ± 0.35 versus 19.36 ± 0.4) or improvement in VMI (86.82 ± 1.45 versus 89.10 ± 1.03). The intratumoral doxorubicin gradient was not significantly different in both the surgical samples (Figure 3(e)). At a distance of ~90 μm from tumor blood vessels, MSC-treated tumors showed a significantly higher doxorubicin concentration as measured by the fluorescence intensity compared to the non-MSC treated PDSCC (56.13 ± 0.94 versus 52.96 ± 0.94, $P = .047$). A similar increase was not seen in the well differentiated WDSCC at ~90 μm from tumor blood vessels. Instead, a significant increase in doxorubicin fluorescence intensity was seen at a distance of ~50 μm within the avascular well differentiated hypoxic foci in the MSC-treated tumors compared to the non-MSC-treated tumors (63.62 ± 2.11 versus 56.32 ± 1.96, $P = .02$).

3.4. Influence of Histologic and Vascular Heterogeneity on Tumor Vascular Response to Selenium. In order to examine changes in vascular function following selenium treatment, noninvasive MRI was utilized. Changes in vascular volume and permeability were estimated in tumor-bearing mice following treatment with MSC (0.2 mg/day × 14) and compared to untreated controls. As shown in Figure 4, only A549 tumors showed a significant reduction ($P < .001$) in vascular volume compared to untreated control tumors ($n = 4$ per group). No significant differences in tumor vascular volume and permeability were observed between control and selenium-treated mice for H69 ($P > .05$; $N = 3$ controls, $N = 4$ MSC), A253 ($P > .05$; $N = 9$ controls, $N = 7$ MSC), poorly differentiated ($P > .05$; $N = 8$ controls, $N = 12$ MSC), and the well differentiated patient tumor-derived HNSCC xenografts ($P > .05$; $N = 6$ per group).

3.5. Modulation of Antitumor Activity. We had earlier reported that MSC (0.2 mg/mouse/day p.o. starting 7 days prior to and continuing daily till end of chemotherapy) in combination with the maximum tolerated dose of irinotecan (100 mg/kg i.v. weekly × 4) resulted in an increase in cure rates (CR) in A253 xenografts from 10% with irinotecan (100 mg/kg weekly × 4 i.v.) alone to 60% with the combination [8]. As can be seen in Figure 5(a), the small cell lung cancer xenograft H69 gives a 100% CR either with irinotecan (100 mg/kg weekly × 4 i.v.) alone or in combination with SLM. The CR with taxotere (60 mg/kg × 1 i.v) alone was 80% that increased to 100% when used in combination with SLM. These high cure rates were obtained when treatments were initiated at ~100 mm^3 size of tumor in H69 tumor xenografts. H69, a uniformly well vascularised tumor with relatively small pockets of necrosis, is an attractive preclinical model for determining the influence of tumor morphological heterogeneity on synergistic chemotherapeutic efficacy in combination with antiangiogenic agents. To determine if this activity of combination treatment was persistent against larger tumors, studies were carried out using larger H69 tumors (~1000–1500 mm^3). These large H69 tumors contain multiple necrotic regions surrounded by hypoxic CAIX positive tumor cells (Figure 1(f(b)) and, thus, are less homogeneous compared to the smaller (<1000 mg) H69 tumors. As seen in Figure 5(b), a CR of 0% and 20% with taxotere alone and in combination with SLM and a CR of 20% and 60% with irinotecan alone and in combination with SLM, respectively were achieved. In contrast, the moderately differentiated non-small cell lung carcinoma A549 had 0% cures either with the drug alone or in combination with MSC.

In order to further investigate this therapeutic efficacy of Se in combination chemotherapy, 2-patient tumor-derived HNSCC xenografts with varying differentiation status were used. Both xenografts were strongly positive for hypoxia as determined by CAIX immunostaining. As can be seen in Figure 5(c) and Figure 5(d), treatment with irinotecan (100 mg/kg weekly × 4 i.v.) alone gave a cure rate of 0% and 37.5% in WDSCC and PDSCC, respectively. In combination with MSC there was no significant improvement in CR except in WDSCC where the CR increased to 14%. Since our earlier studies [8] have indicated the possibility of dose escalation to double the normal MTD of irinotecan, due to the additional chemoprotective properties of MSC, we used this higher dose of irinotecan (200 mg/kg weekly × 4, i.v.) in another group of mice bearing these human cancer surgical samples. As can be seen in Figure 5(d), MSC in combination with this higher irinotecan dose resulted in a higher response of 75% CR and 25% partial response in PDSCC. No such improvement in response was seen in the surgical WDSCC. Thus, enhancement in therapeutic efficacy by antiangiogenic MSC in combination of chemotherapy is higher in tumors

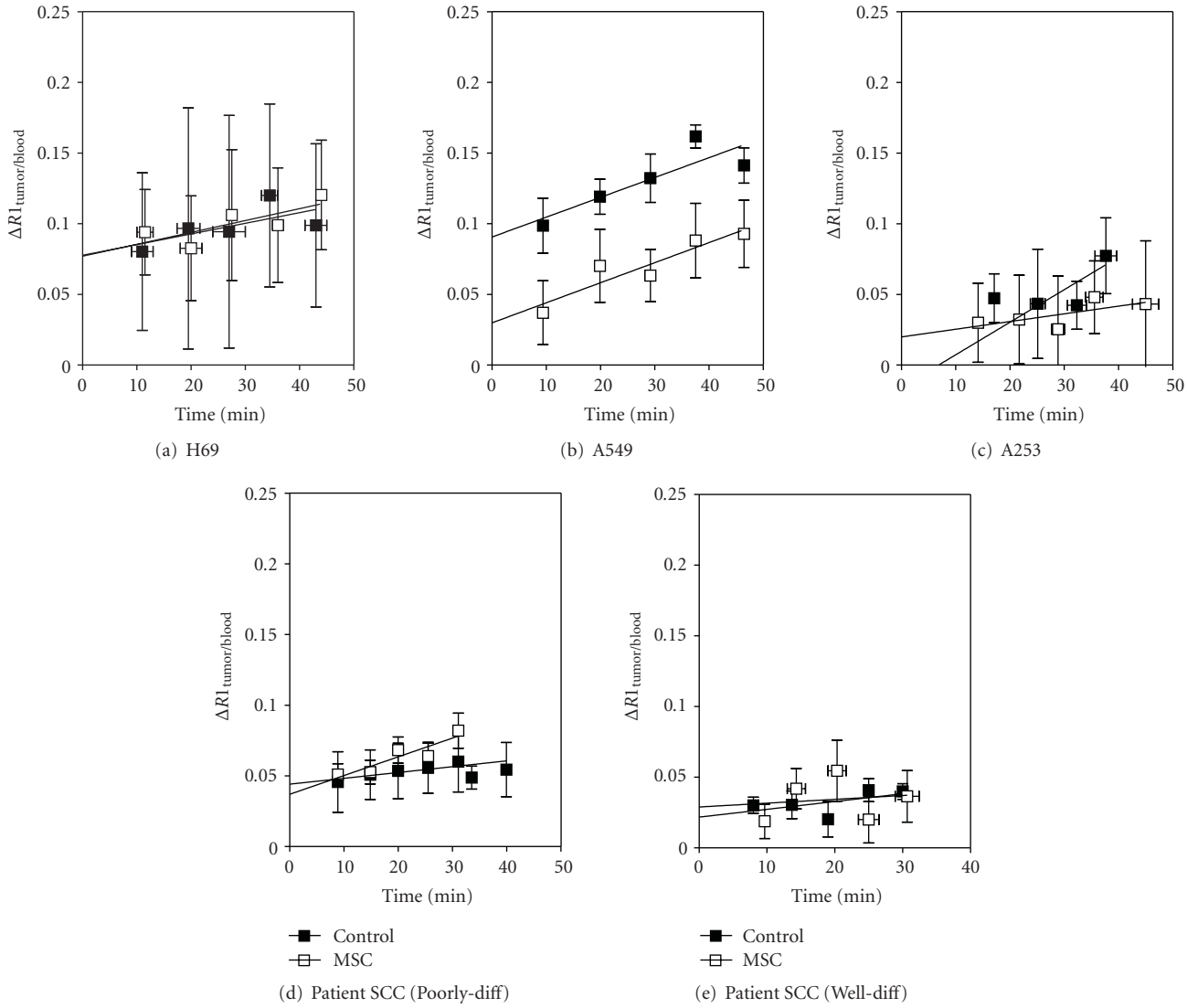


Figure 4: Change in relative vascular permeability and vascular volume as a result of treatment with MSC in H69 (a), A549 (b), A253 (c), PDSCC (d), and WDSCC (e) tumors as assessed by DCE-MRI.

that are relatively more homogeneous versus in tumors that are highly heterogeneous such as in the WDSCC.

3.6. Patient Samples of HNSCC, Colorectal, and Lung Cancer. In order to determine the relevance of tumor morphologic heterogeneity in influencing efficacy of antiangiogenic therapy such as MSC/SLM, we studied the prevalence of architectural heterogeneity in solid tumor malignancies in terms of tumor cell differentiation, vascularization, and hypoxia using TMAs derived from patient surgical cancer specimens from three different disease sites—head and neck, colorectal, and lung. As summarized in Table 1, 51% of HNSCCs are poorly differentiated while 41% of HNSCCs are well differentiated (41%). Most (47%) HNSCC show presence of hypoxia marker CAIX. Figures 6(a) and 6(d) shows the representative tumor vascular distribution (*arrow, brown*) in poorly and well differentiated HNSCC with regions of hypoxia stained for CAIX immunostaining (Figures 6(b) and 6(e)) and HIF-1α (Figures 6(c) and 6(f)). In colorectal cancers, the majority were found to be moderately differentiated cancers (79%) and hypoxic (CAIX, 63%) (Table 1). Figures 6(g) and 6(j) show typical distribution of tumor MVD (*arrow, brown*) with regions of hypoxia stained for CAIX (Figure 6(k), *arrow, brown*) and HIF-1α (Figure 6(l), *brown*) immunostaining in the well differentiated colorectal cancers. NSCLC also presented itself predominantly (86%) as a moderately differentiated cancer with 41% of tumors being hypoxic (CAIX positive). Figure 6(m) shows the distribution of vessels (*brown*) in lung cancer (*arrow, brown*) while Figure 6(n) and 6(o) depict the distribution of hypoxia as assessed using CAIX (*brown, arrow*) and HIF-1α, respectively in lung cancers. The majority of solid malignancies studied was found to have the hallmark of a heterogeneous tumor architecture that is not conducive for an optimal

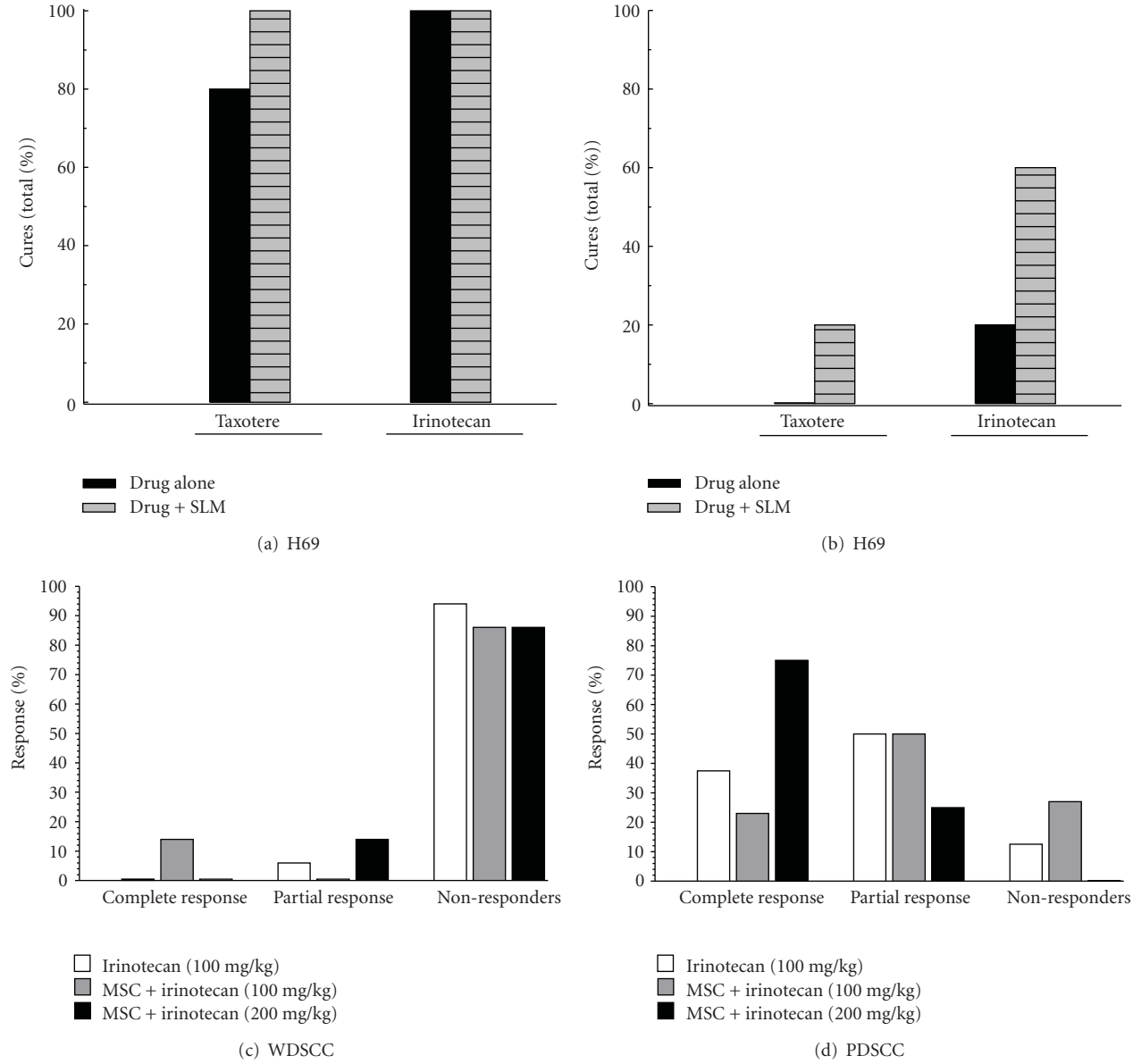


Figure 5: Response to cytotoxic drugs alone and in combination with MSC in H69 lung xenografts when treatment was started at ~100 mm^3 tumor volume (a), at ~1000 mm^3 tumor volume (b), and in human surgical samples of HNSCC WDSCC and PDSCC.

intratumoral drug delivery and distribution and, thus, will not respond optimally to monotherapy or to the synergistic modulation by an antiangiogenic agents such as MSC/SLM used in combination setting.

4. Discussion

The effectiveness of traditional and novel anticancer agents is limited by physical barriers that compromise tumor drug delivery at therapeutically meaningful concentrations. Our knowledge and understanding of tumor vasculature and hypoxia has increased over the past few decades, but if this understanding is not tied to the morphologic or cellular heterogeneity arising out of tumor tissue differentiation, progress in treating solid malignancies will remain inadequate. Drug penetration in the tumor tissues is by convection and/or diffusion. Convection depends on both hydrostatic and osmotic pressure gradients between vascular space and interstitial space, vascular permeability, surface area; and the volume and structure of extracellular matrix [2]. Diffusion is determined by concentration gradients. Since tumor IFP is often elevated to the level of microvascular pressure, it retards extravasation of macromolecules that must penetrate distances of up to 200 μm within the tumors in order to reach all viable cells [2]. While many studies have reported an association between low IFP and therapeutic response [14, 15], some others have not found such association [2, 16]. Obviously, a lowered IFP by itself may not be the panacea for improving drug delivery and therapeutic response.

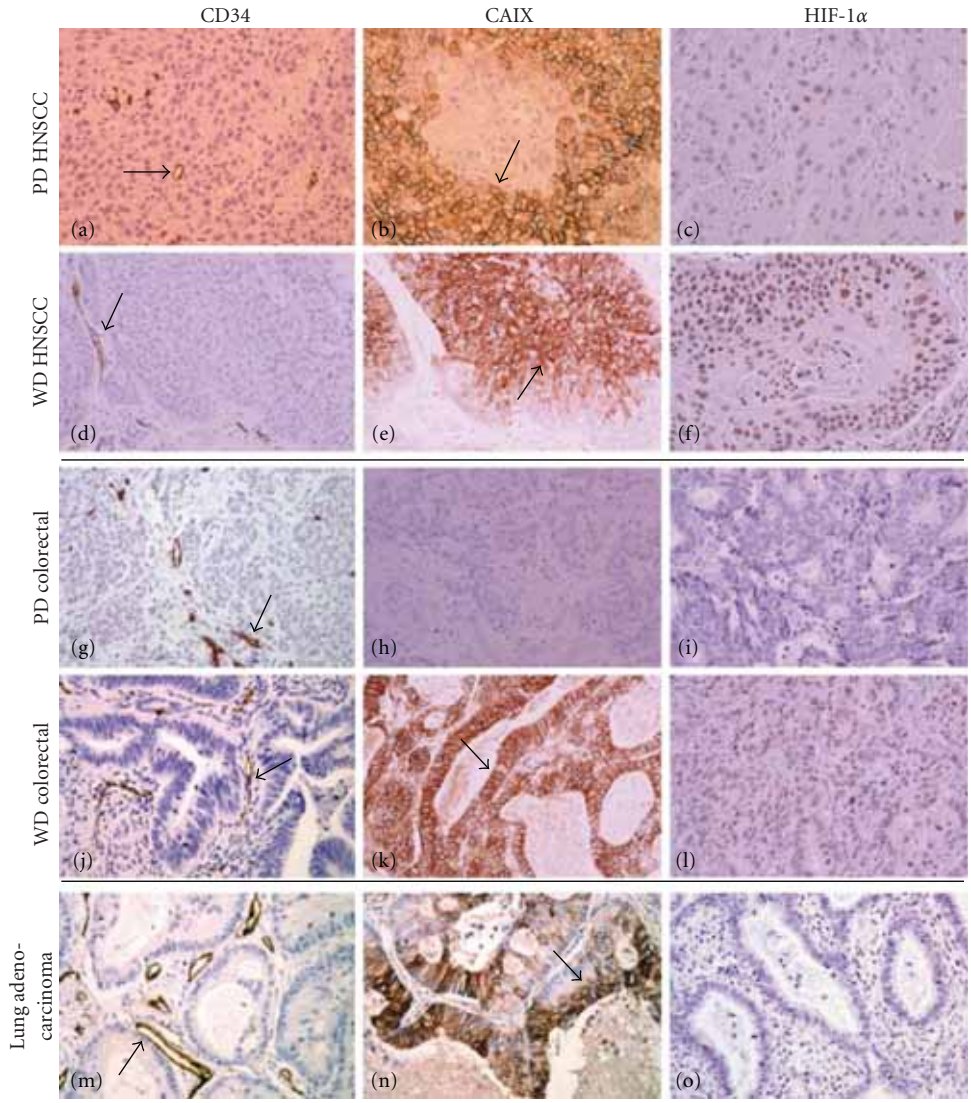


Figure 6: Photomicrographs of tumor tissue microarrays (TMAs) from surgical samples of head and neck squamous cell, colorectal, and lung carcinoma. (Left panels, CD34 immunostaining to visualize microvessels; middle panels, CAIX immunostaining and right panels, HIF-1α immunostaining (brown, nuclear) to visualize tumor hypoxic regions; original magnifications, $\times$200). Poorly differentiated TMAs are relatively uniformly well vascularised ((a), (g), *arrows*) with some regions of hypoxia as measured by CAIX ((b), *arrow*, (h)) and HIF-1α ((c), (i)). In contrast, the well differentiated tumors have large differentiated regions that are avascular ((d), (j), (m), *arrows*) and more strongly hypoxic as seen with CAIX ((e), (k), (n), *arrows*) and HIF-1α ((f), (l)) immunostaining. Most lung adenocarcinomas were negative for HIF-1α staining (o).

Differentiated cells do not lead to tumor propagation and metastasis but can result in a morphologic heterogeneity that hinders optimal intratumoral drug delivery [6]. While many studies have focused on the individual cancer cells, stromal cells and the vascular network, there are few studies on the effect of tumor histomorphologic heterogeneity in drug resistance. Tumor tissue architecture is an important factor that influences drug delivery. For instance, the higher cell density in differentiated regions of tumors prevents the sprouting and growth of blood vessels [6]. This combined with a reduced interstitial space and volume of extracellular matrix results in lower intratumoral drug penetration [2].

SCC and adenocarcinoma are the most common histological types of solid malignancies. In well differentiated forms of SCC, tumor cells nests are often observed with a closely packed arrangement of individual tumor cells without stroma or microvessels. Tumor cells in these differentiated regions (Figure 1(a), *arrow*) or the lumen structure (Figure 1(h), *arrows*) seen in adenocarcinoma do not receive adequate blood supply causing hypoxia, limited drug delivery and resulting in sanctuaries for proliferating cells that escape therapy. In contrast, the poorly differentiated parts of these tumors or in tumors that are wholly poorly differentiated, microvessels are uniformly distributed providing blood supply to most such parts of the tumors. In our study,

Table 1: Prevalence of histomorphologic heterogeneity in human cancers.

Cancer disease site	Total evaluable tumors	Tumor differentiation status (% of Total)			CAlX immunostaining (% of Total)			HIF-1α immunostaining (% of Total)		
		Well Diff	Mod. Diff	Poorly Diff	<33%	33–67%	>67%	<33%	33–67%	>67%
Head and neck	198	41% (72% CAIX+)	8% (50% CAIX+)	51% (38% CAIX+)	25%	8%	14%	9%	4%	8%
					Total—47% of all tumors			Total—21% of all tumors		
Colorectal	57	12% (57% CAIX+)	79% (64% CAIX+)	9% (60% CAIX+)	28%	5%	30%	7%	2%	12%
					Total—63% of all tumors			Total—21% of all tumors		
Lung	102	3% (0% CAIX+)	86% (42% CAIX+)	11% (36% CAIX+)	23%	10%	8%	3%	1%	0%
					Total—41% of all tumors			Total—4% of all tumors		

MSC treatment led to a lower tumor burden in all three human tumor xenografts albeit only significantly in A253 xenografts. This effect was not seen in the human surgical samples of head and neck cancers-PDSCC and WDSCC. Greater optimal therapeutic synergy of antiangiogenic Se compounds in combination to anticancer drug was observed in the morphologically less heterogeneous large human SCLC cell line xenograft H69 compared to more heterogeneous NSLC A549 that had a lower, relatively normalized MVD, IFP, or an improved drug delivery. In contrast, A253 despite being well differentiated shows a better response to the combination therapy as reported earlier [8]. This is as a consequence of MSC-induced vascular modulation that led to a significant lowering of MVD, an improved VMI and IFP, thereby resulting in an increased intratumoral drug gradient. The heterogeneous differentiated human NSCLC A549 with a lower but relatively matured MVD did not show a marked modulation to antiangiogenic Se in terms of reduction in MVD, IFP or an improved VMI. Hence, it did not respond with a higher efficacy to the combination chemotherapy with Se. Interestingly, in SCLC H69, the synergy persisted even when the tumors were allowed to grow to ~1–1.5 g before the start of therapy. H69, a representative SCLC with uniformly poorly differentiated, well-vascularised tumor, contains few small necrotic foci and had the least morphologic heterogeneity in terms of its cellular architecture and vascular distribution amongst the xenografts studied. As shown in Figure 1(d), the more homogeneous morphologic feature of this tumor facilitates a better drug penetration and distribution within the tumor. This translates into a better therapeutic response. We had earlier reported similar results in the poorly differentiated human tumor xenografts HNSCC FaDu and colorectal HCT-8 that has a similar uniform vasculalarized homogeneous morphology and in which treatment with MSC resulted in a significant modulation of the vascular parameters and IFP [5, 7]. The antiangiogenic effect in terms of reduction in MVD was the highest in H69 (62%) and A253 (59%) and was minimal in A549. Despite a significant reduction in MVD and a dramatic improvement in VMI (49.93%), there was no significant difference in IFP or in the vascular parameters assessed with DCE-MRI in H69 xenografts. In all three tumor xenografts, treatment with MSC led to increased doxorubicin fluorescent intensity at a distance of ~90 μm from vessel wall. These MSC-induced changes in vascular parameters and IFP resulted in a significant increase in response rates in H69 and A253 xenografts. Despite the minimal reduction in MVD, MRI revealed a statistically significant reduction in vascular volume of A459 tumors following selenium treatment (Figure 4). It is plausible that this dramatic reduction in perfusion contributes to decreased chemotherapeutic delivery leading to a minimal therapeutic response with combination therapy in this tumor model.

SCLC, being poorly differentiated, homogeneous, and well vascularized, is sensitive to chemotherapy. Regrowth and resistance in the clinic has been associated with cellular differentiation in these cancers induced in part by the agents themselves [17]. Thus, an effective first line chemotherapeutic regime that can kill most of the cancer cells is likely to be more efficacious in such tumors and the use of antiangiogenic agents such as Se in the combinatorial setting is likely to be the most promising especially in chemo-naive patients. In contrast, the heterogeneous surgical samples of HNSCC did not show any significant MVD reduction by Se. The improvement in VMI was significant but modest (~8%) in the poorly differentiated surgical sample #17073 but was not significant in well differentiated #16653. Both these surgical samples did not show any MSC-induced increase in doxorubicin concentration gradient immediately adjacent to tumor vasculature and MSC did not result in an enhanced synergistic therapeutic response of irinotecan at its MTD in both these tumors. Unlike the poorly differentiated and uniformly vascularized FaDu, HCT8, and H69 xenografts, PDSCC is relatively less homogeneous due to the presence of irregular and large regions of hypoxia and necrosis that does not facilitate therapeutic exposure of drugs in meaningful concentration to all proliferating cancer cells (Figure 1(l)). Since Se has chemoprotective effect [8] and allows for dose escalation to higher than MTD, we treated a subset of the mice bearing these surgical specimens with MSC in combination with double the MTD of irinotecan. At this dose, MSC did result in a significant increase in CR (75%) in the surgical sample of PDSCC but showed no change in the surgical sample WDSCC. The histological structure of WDSCC is extremely heterogeneous due to the presence of many keratinized well differentiated avascular (Figure 1(m), *arrow*), hypoxic regions (Figure 1(o), *arrow*).

Such regions do not allow penetration of an effective drug in a therapeutically meaningful concentration in the rim region surrounding these differentiated regions. This causes proliferating cells within these rims to escape therapy and allow tumor repopulation and regrowth [6]. Further, the remnant tumor after therapy generally contained mainly differentiated regions remaining as survivor—a pointer to their contribution to drug resistance [6].

As previously reported [18], poorly differentiated tumor xenografts such as FaDu and HCT-8 have a slightly faster doubling time (~2.5 to 3 days) compared to their well differentiated counterparts such as A253 and HT-29 (~3.5 days), respectively. In our studies, the PDSCC had a similar doubling time of 3.5–4 days while the WDSCC had a doubling time of more than 5 days (Figure 2(c)). The lung cancer xenografts had a slower proliferation rate with the poorly differentiated SCLC xenografts H69 showing a doubling time of 6.1 days [19] while the differentiated NSCLC was found to have a doubling time of ~8.3 days in our studies (Figure 2(a)). It is likely that due to the presence of more uniformly distributed and often higher number of blood vessel, the poorly differentiated tumors tend to proliferate and grow faster. Proliferating cells are more sensitive to chemotherapy while the uniform distribution of vessels in poorly differentiated tumors makes the tumor easily accessible to anticancer drugs especially after tumor vascular normalization by antiangiogenic agents including MSC/SLM. From the results, one can surmise that chemomodulation of an antiangiogenic agent such as MSC will be most optimal in tumors where it can significantly reduce MVD, increase VMI, and as a consequence lead to a reduced IFP. In tumors such as the A549 and WDSCC where the untreated controls showed a presence of either high (WDSCC) or a low (A549) MVD, and the addition of the antiangiogenic agent did not change the VMI, the chemomodulation effect of antiangiogenic agents in combination chemotherapy is unlikely to lead to a significantly high response. The higher the degree of tumor morphologic heterogeneity, the less likely is the synergistic effect of antiangiogenic agents in combination chemotherapy. There remains an urgent need in developing novel agents that can abrogate tumor morphological heterogeneity in order to increase chemotherapeutic response in the clinic.

Similar to observations by others, our results indicate that CAIX and HIF-1α do not always co-localize in the same tumor regions [20, 21]. CAIX is a more robust indicator of physiologic hypoxia while HIF-1α can often be transiently upregulated by acute hypoxia or by other non-hypoxia related factors [22].

The results of our TMA analysis, even with limited tissue, indicate presence of histological architectural heterogeneity in majority of HNSCC, colorectal and lung cancers. This heterogeneity is not conducive for optimal intratumoral drug delivery and distribution leading to the suboptimal chemotherapeutic response seen in the clinic. It is also likely to compromise synergistic efficacy of antiangiogenic agents in combination with anticancer agents. Morphologically homogeneous tumors such as SCLC, FaDu, and HCT-8 are more likely to respond to antiangiogenic agents in combinatorial chemotherapy setting especially in chemo-naive cases.

Currently available antiangiogenic agents are limited by host tissue toxicity, are cost prohibitive [23], and suffer from the lacunae that most tumors can easily overcome the blockade of a single specific proangiogenic molecule through bypassing onto other proangiogenic markers/pathways. In fact, recent reports suggest an alarming trend towards increased metastatic and aggressive disease in the tumors surviving antiangiogenic therapy in both the clinical and preclinical model [24, 25]. Since Se is part of the mammalian physiology, it is relatively well tolerated and affects multiple upstream targets such as HIF-1α, Cox-2, and iNos [26] important for cancer survival and progression and is likely to have a better success as an antiangiogenic agent in combination chemotherapy [27]. Inhibition of HIF-1α, a critical master gene that regulates tumor angiogenesis, growth, survival, and resistance, has been shown recently to be through downregulation of reactive oxygen species and stabilization of prolylhydoxylase 2 and 3 by MSC [28]. This inhibition results in the observed antiangiogenic effects of MSC besides sensitizing the small but therapy resistant population of HIF-1α positive, hypoxic cell population to the cytotoxic effects of the chemotherapeutic agents used in the combination therapy with MSC [28]. Unlike other antiangiogenic agents, Se also asserts its anticancer efficacy independent of antiangiogenic effects through various mechanisms such as redox cycling, altering protein-thiol redox status and methionine mimicry [29]. Since the dose levels of Se used in the preclinical studies have been attained in the clinic [30], it is a clinically viable antiangiogenic agent that can enhance therapeutic efficacy of chemotherapy in various solid malignancies especially those with a relatively well-vascularised and homogenous tumor architecture. Further, the protective effect of Se on healthy tissues from the adverse effects of cytotoxic drugs [8] allows for drug dose escalation to higher than their MTDs. Our current data and our earlier published data [5, 7] demonstrate that a nontoxic dose of Se is a promising antiangiogenic agent for use in combination chemotherapy especially against solid tumors with little or no morphological heterogeneity such as what is seen in SCLC H69, HNSCC FaDu and the colorectal cancer HCT-8.

Acknowledgments

Drs. Haikuo Tang and Elizabeth Repasky, Department of Immunology, Roswell Park Cancer Institute, are acknowledged for generously making the human surgical xenografts of SCC available. This research is Supported by the National Cancer Institute Grant 1R21 CA133682-01A2 (A. Bhattacharya) and a Comprehensive Cancer Center Support Grant CA016056 from the National Cancer Institute, Bethesda, Md, USA.

References

[1] P. Savage, J. Stebbing, M. Bower, and T. Crook, "Why does cytotoxic chemotherapy cure only some cancers?" *Nature Clinical Practice Oncology*, vol. 6, no. 1, pp. 43–52, 2009.

[2] O. Tredan, C. M. Galmarini, K. Patel, and I. F. Tannock, "Drug resistance and the solid tumor microenvironment," *Journal of the National Cancer Institute*, vol. 99, no. 19, pp. 1441–1454, 2007.

[3] P. Carmeliet and R. K. Jain, "Angiogenesis in cancer and other diseases," *Nature*, vol. 407, no. 6801, pp. 249–257, 2000.

[4] R. K. Jain, "Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy," *Nature Medicine*, vol. 7, no. 9, pp. 987–989, 2001.

[5] A. Bhattacharya, M. Seshadri, S. D. Oven, K. Tóth, M. M. Vaughan, and Y. M. Rustum, "Tumor vascular maturation and improved drug delivery induced by methylselenocysteine leads to therapeutic synergy with anticancer drugs," *Clinical Cancer Research*, vol. 14, no. 12, pp. 3926–3932, 2008.

[6] A. Bhattacharya, K. Tóth, R. Mazurchuk, et al., "Lack of microvessels in well-differentiated regions of human head and neck squamous cell carcinoma A253 associated with functional magnetic resonance imaging detectable hypoxia, limited drug delivery, and resistance to irinotecan therapy," *Clinical Cancer Research*, vol. 10, no. 23, pp. 8005–8017, 2004.

[7] A. Bhattacharya, K. Tóth, A. Sen, et al., "Inhibition of colon cancer growth by methylselenocysteine-induced angiogenic chemomodulation is influenced by histologic characteristics of the tumor," *Clinical Colorectal Cancer*, vol. 8, no. 3, pp. 155–162, 2009.

[8] S. Cao, F. A. Durrani, and Y. M. Rustum, "Selective modulation of the therapeutic efficacy of anticancer drugs by selenium containing compounds against human tumor xenografts," *Clinical Cancer Research*, vol. 10, no. 7, pp. 2561–2569, 2004.

[9] A. Bhattacharya, K. Tóth, F. A. Durrani, et al., "Hypoxia-specific drug tirapazamine does not abrogate hypoxic tumor cells in combination therapy with irinotecan and methylselenocysteine in well-differentiated human head and neck squamous cell carcinoma A253 xenografts," *Neoplasia*, vol. 10, no. 8, pp. 857–865, 2008.

[10] M. M. Vaughan, K. Tóth, S. Chintala, and Y. M. Rustum, "Double immunohistochemical staining method for HIF-1a and its regulators PHD2 and PHD3 in formalin fixed paraffin embedded tissues," *Applied Immunohistochemistry and Molecular Morphology*, 2010.

[11] F. Demsar, T. P. L. Roberts, H. C. Schwickert, et al., "A MRI spatial mapping technique for microvascular permeability and tissue blood volume based on macromolecular contrast agent distribution," *Magnetic Resonance in Medicine*, vol. 37, no. 2, pp. 236–242, 1997.

[12] M. Seshadri, R. Mazurchuk, J. A. Spernyak, A. Bhattacharya, Y. M. Rustum, and D. A. Bellnier, "Activity of the vascular-disrupting agent 5,6-dimethylxanthenone-4-acetic acid against human head and neck carcinoma xenografts," *Neoplasia*, vol. 8, no. 7, pp. 534–542, 2006.

[13] U. Ozerdem and A. R. Hargens, "A simple method for measuring interstitial fluid pressure in cancer tissues," *Microvascular Research*, vol. 70, no. 1-2, pp. 116–120, 2005.

[14] C.-H. Heldin, K. Rubin, K. Pietras, and A. Ostman, "High interstitial fluid pressure—an obstacle in cancer therapy," *Nature Reviews Cancer*, vol. 4, no. 10, pp. 806–813, 2004.

[15] B. D. Curti, W. J. Urba, W. G. Alvord, et al., "Interstitial pressure of subcutaneous nodules in melanoma and lymphoma patients: changes during treatment," *Cancer Research*, vol. 53, no. 10, pp. 2204–2207, 1993.

[16] F. G. Herrera, P. Chan, C. Doll, et al., "A prospective phase I-II trial of the cyclooxygenase-2 inhibitor celecoxib in patients with carcinoma of the cervix with biomarker assessment of the tumor microenvironment," *International Journal of Radiation Oncology Biology Physics*, vol. 67, no. 1, pp. 97–103, 2007.

[17] E. Brambilla, D. Moro, S. Gazzeri, et al., "Cytotoxic chemotherapy induces cell differentiation in small-cell lung carcinoma," *Journal of Clinical Oncology*, vol. 9, no. 1, pp. 50–61, 1991.

[18] R. G. Azrak, S. Cao, H. K. Slocum, et al., "Therapeutic synergy between irinotecan and 5-fluorouracil against human tumor xenografts," *Clinical Cancer Research*, vol. 10, no. 3, pp. 1121–1129, 2004.

[19] C. A. Kanashiro, A. V. Schally, M. Zarandi, B. D. Hammann, and J. L. Varga, "Suppression of growth of H-69 small cell lung carcinoma by antagonists of growth hormone releasing hormone and bombesin is associated with an inhibition of protein kinase C signaling," *International Journal of Cancer*, vol. 112, no. 4, pp. 570–576, 2004.

[20] A. Mayer, M. Höckel, and P. Vaupel, "Carbonic anhydrase IX expression end tumor oxygenation status do not correlate at the microregional level in locally advanced cancers of the uterine cervix," *Clinical Cancer Research*, vol. 11, no. 20, pp. 7220–7225, 2005.

[21] D. Hedley, M. Pintilie, J. Woo, et al., "Carbonic anhydrase IX expression, hypoxia, and prognosis in patients with uterine cervical carcinomas," *Clinical Cancer Research*, vol. 9, no. 15, pp. 5666–5674, 2003.

[22] S. Kaluz, M. Kaluzová, S.-Y. Liao, M. Lerman, and E. J. Stanbridge, "Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: a one transcription factor (HIF-1) show?" *Biochimica et Biophysica Acta*, vol. 1795, no. 2, pp. 162–172, 2009.

[23] H. M. W. Verheul and H. M. Pinedo, "Possible molecular mechanisms involved in the toxicity of angiogenesis inhibition," *Nature Reviews Cancer*, vol. 7, no. 6, pp. 475–485, 2007.

[24] L. M. Ellis and D. A. Reardon, "Cancer: the nuances of therapy," *Nature*, vol. 458, no. 7236, pp. 290–292, 2009.

[25] M. Paez-Ribes, E. Allen, J. Hudock, et al., "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[26] M.-B. Yin, Z.-R. Li, K. Tóth, et al., "Potentiation of irinotecan sensitivity by Se-methylselenocysteine in an in vivo tumor model is associated with downregulation of cyclooxygenase-2, inducible nitric oxide synthase, and hypoxia-inducible factor 1α expression, resulting in reduced angiogenesis," *Oncogene*, vol. 25, no. 17, pp. 2509–2519, 2006.

[27] P. D. Whanger, "Selenium and its relationship to cancer: an update," *The British Journal of Nutrition*, vol. 91, no. 1, pp. 11–28, 2004.

[28] S. Chintala, K. Tth, S. Cao, et al., "Se-methylselenocysteine sensitizes hypoxic tumor cells to irinotecan by targeting hypoxia-inducible factor 1α," *Cancer Chemotherapy and Pharmacology*, 2010.

[29] M. I. Jackson and G. F. Combs Jr., "Selenium and anticarcinogenesis: underlying mechanisms," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 11, no. 6, pp. 718–726, 2008.

[30] M. G. Fakih, L. Pendyala, W. Brady, et al., "A phase I and pharmacokinetic study of selenomethionine in combination with a fixed dose of irinotecan in solid tumors," *Cancer Chemotherapy and Pharmacology*, vol. 62, no. 3, pp. 499–508, 2008.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 723798, 8 pages
doi:10.1155/2010/723798

Review Article

Peptide-Mediated Liposomal Drug Delivery System Targeting Tumor Blood Vessels in Anticancer Therapy

Han-Chung Wu and De-Kuan Chang

Institute of Cellular and Organismic Biology, Academia Sinica, Taipei 11529, Taiwan

Correspondence should be addressed to Han-Chung Wu, hcw0928@gate.sinica.edu.tw

Received 3 November 2009; Revised 13 January 2010; Accepted 3 March 2010

Academic Editor: Arkadiusz Dudek

Solid tumors are known to recruit new blood vessels to support their growth. Therefore, unique molecules expressed on tumor endothelial cells can function as targets for the antiangiogenic therapy of cancer. Current efforts are focusing on developing therapeutic agents capable of specifically targeting cancer cells and tumor-associated microenvironments including tumor blood vessels. These therapies hold the promise of high efficacy and low toxicity. One recognized strategy for improving the therapeutic effectiveness of conventional chemotherapeutics is to encapsulate anticancer drugs into targeting liposomes that bind to the cell surface receptors expressed on tumor-associated endothelial cells. These anti-angiogenic drug delivery systems could be used to target both tumor blood vessels as well as the tumor cells, themselves. This article reviews the mechanisms and advantages of various present and potential methods using peptide-conjugated liposomes to specifically destroy tumor blood vessels in anticancer therapy.

1. Introduction

One of the primary goals of a successful cancer treatment regimen is to deliver sufficient amounts of drug to tumors while minimizing damage to normal tissues. Most chemotherapeutic agents enter normal tissues in the body with indiscriminate cytotoxicity and do not preferentially accumulate at tumor sites. At times the dose reaching the tumor may be as little as 5% to 10% of the doses accumulating in normal organs [1, 2]. One reason for the inability for drugs to accumulate at target sites is that the interstitial fluid pressure (IFP) in solid tumors is higher than in normal tissues, that blocking transcapillary transport of chemotherapeutic drugs or antibodies [3–5]. In this way, the anticancer effect is decreased and toxic effect to normal cells is increased. Fear of severely harming the patients often limits the dose of anticancer drugs that can be given to a patient. These lower than optimal doses elicit incomplete tumor responses which leads to disease relapse and drug resistance. Therefore, most cancer drugs fail in clinical studies not because they are ineffective in killing cancer cells but because they cannot be administered in doses high enough to eradicate the tumor without severely harming the patient.

Several approaches have been developed to improve the ability of anticancer drug to more specifically target tumors and avoid normal organs. One of the most effective strategies is to encapsulate drugs in particles that deliver them preferentially to tumor sites. For example, liposome particles have been found able to deliver radionuclides, genes, and chemotherapeutic agents to tumor sites. [6–10]. Another promising strategy is to encapsulate anticancer drugs in liposomes conjugated with moieties, such as antibodies and peptides, that target particular types of target tumor cells or tumor vasculatures [11–13]. Use of internalizing ligands for targeting liposomes conjugated with such moieties makes it possible to deliver the chemotherapeutic drugs encapsulated within them to the cytosol through the receptor-mediated endocytosis [14–17]. This article reviews the current research in developing liposomal drug delivery systems that use peptide ligands to target blood vessels in solid tumors. We discuss the identification of peptides that can target tumor blood vessels and the use of targeting and nontargeting

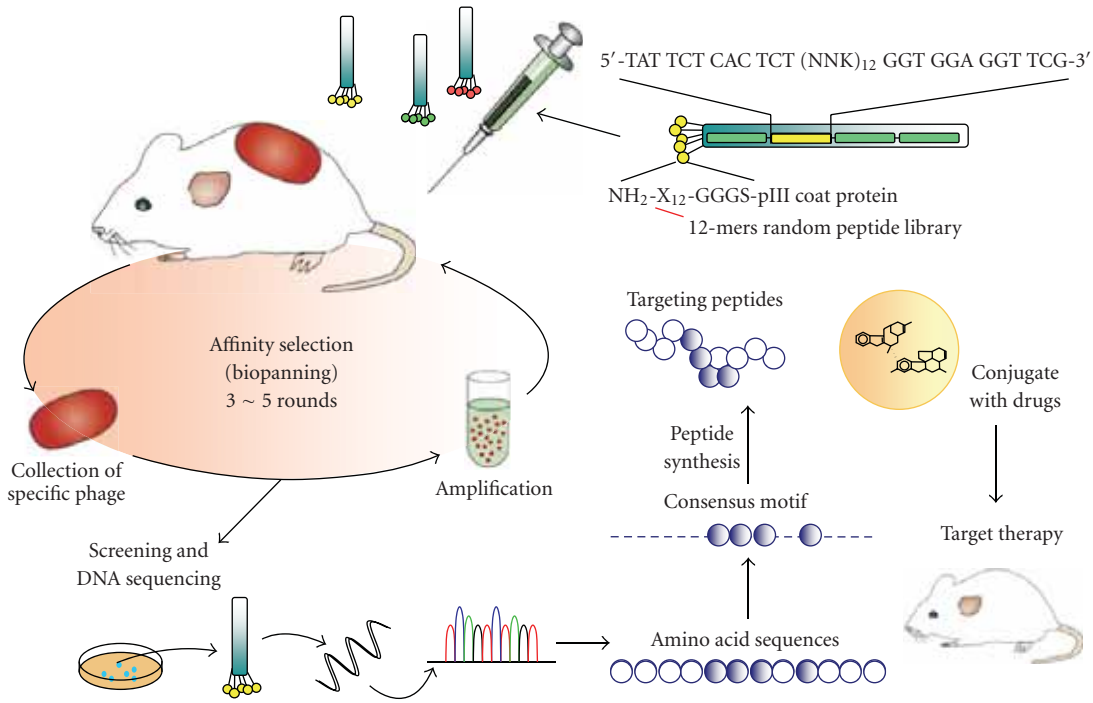


Figure 1: Selection of peptides that target tumor blood vessels using in vivo phage display. Peptide or antibody libraries are expressed as fusion proteins with a coat protein (pIII) of a bacteriophage, and the fused proteins are displayed on the surface of the virion. A phage-displayed peptide library was injected through the tail vein of tumor-bearing mice. Eight minutes after injection, the mice were perfused through the heart. Phage recovered from the tumor was amplified and reinjected in mice for another four rounds. Tumor-targeting phages were further identified by in vivo tumor-homing assay, synthetic peptide binding and competition assay, and immunohistochemical staining. The identified peptides can be used as ligands to recognize cell surface markers or tumor antigens to develop targeted therapy. SCID mice bearing human cancer xenografts were successfully treated with ligand-conjugated antiangiogenic targeting liposomes.

liposomes to encapsulate and deliver chemotherapeutic drugs to tumor sites.

2. Inhibiting Angiogenesis

Virtually every conventional cytotoxic drug has been found to be antiangiogenic in in vitro and in vivo models [18]. One treatment approach known as metronomic therapy uses frequent administrations of low-dose antiangiogenic agents to destroy vessels in tumors while decreasing the toxicity to normal tissues [19–21]. For example, it has been found in mice that frequent administration of relatively low, noncytotoxic doses of liposome-encapsulated doxorubicin can shrink various solid tumor xenografts [13, 16]. The antiangiogenic agent bevacizumab (Avastin), a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), has been used with some success to treat advanced colon cancer. One study compared the effect using three chemotherapeutic agents alone to treat advanced colon cancer with using the three agents combined with bevacizumab [22]. They found that the combined use of chemotherapeutic agents and bevacizumab extended overall survival by approximately 4.7 months compared to the use of chemotherapeutic agents alone [22]. Other angiogenesis inhibitors, including sunitinib and sorafenib, have also been found to improve clinical outcomes when used to treat various cancer types [23, 24].

The targeting of proliferating endothelial cells in the blood vessels of tumors has several advantages. First, endothelial cells in malignant tumors are genetically stable, nonmalignant, and rarely drug resistant, compared to the cancer cells [19, 21]. However, some recent studies show that tumor-associated endothelial cells can acquire cytogenetic abnormalities while they are in the tumor microenvironment [25, 26]. Second, the destruction of endothelial cells using this method amplifies the drugs antitumor effect. It has been reported that the elimination of one endothelial cell can inhibit the growth of as many as a hundred tumor cells [27, 28]. Third, antiangiogenic therapy decreases IFP within the tumor allowing better penetration by chemotherapeutic agents [29–32]. For example, Jain found that bevacizumab could decrease IFP by normalizing tumor vasculature and decreasing vascular leakage [29, 33]. Fourth, antiangiogenic therapy is known to inhibit the growth of both primary and metastatic solid tumors. Finally, intravenously injected angiogenesis inhibitors can directly reach endothelial cells.

In addition, we can take advantage of the differences between endothelial cell plasma membrane proteins (i.e., vascular zip codes) to develop drug delivery systems capable of guiding therapeutic or imaging agents to a particular

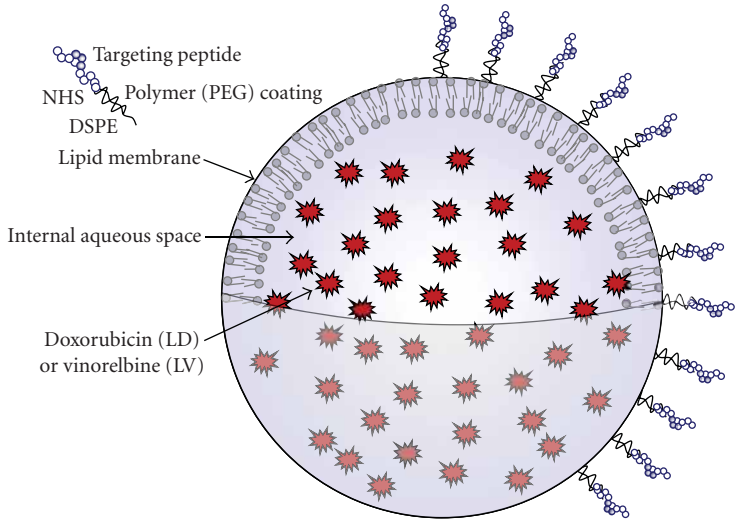


Figure 2: Generation of peptide-conjugated liposomes targeting tumor blood vessels. A single lipid bilayer membrane separates an internal aqueous compartment from the external medium. Doxorubicin was encapsulated in the internal compartment. Drug molecules are tightly packed (10,000 to 15,000 molecules per liposome) in a gel phase. Tumor-homing peptide ligands were coupled to NHS-PEG-DSPE [N-hydroxysuccinimido-carboxyl-polyethylene glycol-derived distearoylphosphatidylethanolamine] in a 1 : 1.5 molar ratio [13, 14, 50]. The reaction was completed and confirmed by quantifying the remaining amino groups using TNBS (Trinitrobenzenesulfonate) reagent [51]. Peptidyl-PEG-DSPE was transferred to preformed liposomes after coincubation at a temperature above the transition temperature of the lipid bilayer [52]. There were 500 peptide molecules per liposome [53]. The mean diameter of the targeting liposome is approximately 75 nm [2, 13].

organ or tumor [34, 35]. Endothelial cells of blood vessels within solid tumors express certain molecular structures that are absent or minimally detectable in normal blood vessels [13, 36, 37]. These structures can be used as molecular targets for antitumor treatment.

3. Identifying Peptides That Target Tumor Blood Vessels

The key to delivering drugs specifically to these targets is to identify and use ligands that specifically bind to and that can be internalized by endothelial cells in tumors. Combinatorial peptide libraries displayed on microorganisms have become a research tool for identifying cell surface-binding peptides that can become targets for antitumor treatment. Of the many molecular display techniques, phage display has been the most popular approach. Phage display is a selection technique in which a peptide or protein is fused with a coat protein of bacteriophage and displayed on the surface of the virion. Phage-displayed random peptide libraries have helped researchers map B-cell epitopes [38–40], discover protein-protein contacts [41, 42], and identify bioactive peptides bound to receptors [43, 44] or proteins [45, 46]. Peptide libraries can be used to find disease-specific antigens [47, 48] and cell- [2, 49] and organ-specific peptides [16, 35, 36].

Recently, using affinity selection (biopanning) of phage-displayed peptide libraries, researchers have discovered molecules that are expressed on tumor blood vessels exclusively [16, 34–36]. The strategy for identifying tumor-targeting ligands and developing ligand-mediated targeted therapy is shown in Figure 1. Researchers have used in vivo affinity selection of phage libraries to identify peptides that interact with the molecules found on endothelia in tumors [34, 36]. The NGR peptide motif targets angiogenic blood vessels [36] and the tumor-homing property of NGR motif relies on recognition of a CD13 isoform selectively expressed within tumor blood vessels [54]. Compared with the nontargeting liposomal doxorubicin (Caelyx), NGR peptide-conjugated Caelyx significant improvements in survival was seen in clinically relevant animal models of neuroblastoma, ovarian, and lung cancers [17]. Another peptide, SP5-52, has been found to recognize blood vessels created in tumors but not normal blood vessels in severe combined immunodeficiency (SCID) mice bearing solid tumors. Several selected phage clones display Pro-Ser-Pro, a motif crucial to peptide binding to tumor neovasculature [13]. Several tumor homing peptides have been found to bind to blood vessels in surgical specimens of human cancer and they have also been found to home to tumor tissues of different human tumor xenografts as confirmed by in vivo homing assays [16]. These studies found a greater correlation between increased tumoral accumulation of the targeting liposomes and antitumor efficacy than the accumulation of free drugs or drugs formulated in the nontargeting liposomes [2, 13, 16].

4. Drug-Encapsulated Liposomes

Most of the drug delivery systems approved for marketing are liposomal- or lipid-based formulations or therapeutic molecules linked to polyethylene glycol (PEG) [6, 10, 55, 56]. One such product is PEGylated liposomal doxorubicin,

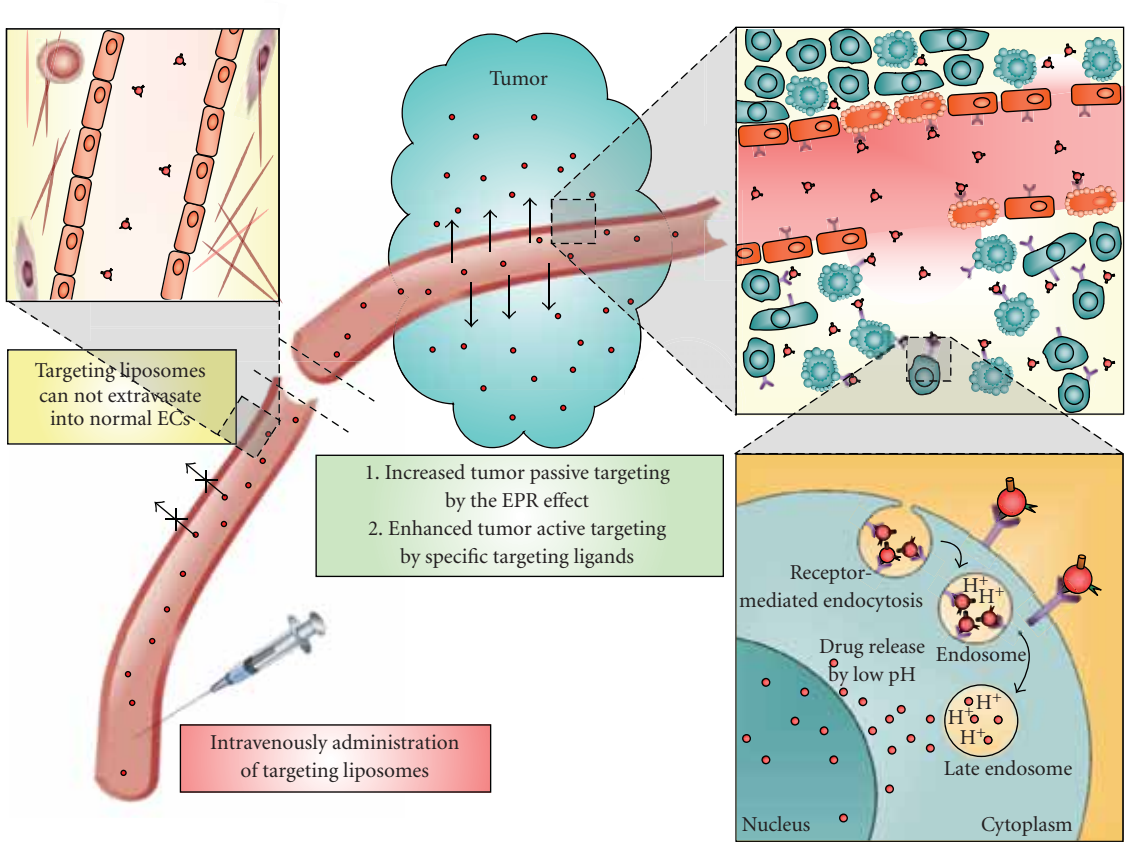


Figure 3: Diagram of the molecular mechanism of peptide-conjugated liposomes on cancer therapy. These liposomes prolong circulation time in blood and improve pharmacokinetic and biodistribution of their encapsulated drugs. After intravenous administration, liposomes are large enough to be excluded from normal endothelium. In solid tumors, the angiogenic tumor vasculature becomes leakiness that particulate liposomes can extravasate and localize in the tissue interstitial space making it possible for more drug delivering liposomes to accumulate within the tumor by EPR effect. Coupling liposomes with peptides targeted to tumor cells or tumor vasculature further enhances the specificity and accumulation of liposomes in the tumor. On arrival in the tumor tissues, the liposomes are bound and internalized by tumor cells or tumor-associated endothelial cells through receptor-mediated endocytosis, fused with the low pH compartments of the endosomes, and subsequently broken down the liposomes and to release encapsulated drugs into the intracellular space of the cells.

which is known as Doxil in the US and Caelyx in Europe [57]. It is currently approved for the treatment of AIDS-related Kaposi's sarcoma and recurrent ovarian cancer in North America, Europe, and other countries, and for metastatic breast cancer in Europe. Liposome-encapsulated doxorubicin has been found to significantly improve the therapeutic index of doxorubicin both in preclinical [58–60] and in clinical studies [61–64]. An important advantage of PEGylated liposomal doxorubicin is that the heart muscle uptakes much less of it than free doxorubicin [58, 65]. One study found no cardiotoxicity in 40 patients receiving cumulative doses of 500–1500 mg/m^2 of doxorubicin [62]. Free doxorubicin, on the other hand, is limited to a maximum recommended cumulative dose of 450–550 mg/m^2. Colbern et al. found that the activity of PEGylated liposomal doxorubicin 1-2 mg/kg was almost equivalent to that of free doxorubicin 9 mg/kg in mouse Lewis lung carcinoma [59]. One clinical study reported that most (>98%) of the drug circulating in the blood stream remains in encapsulated in liposomes [61], suggesting that little of the liposomal drugs will be leaked to the circulation system during its journey to the tumor tissues.

The hyperpermeability of tumor vasculature is a key factor for the success of liposome-delivered chemotherapy agents. The "leakiness" of the angiogenic tumor vasculature is estimated to have an average pore size of 100–600 nm [66]. These pores are significantly larger than the gap junction found in normal endothelium, which are typically <6 nm wide [67]. Liposomes with diameters of approximately 65–75 nm [13, 14, 50] are small enough to passively infiltrate tumor endothelium but large enough to be excluded from normal endothelium. Hence, they selectively extravasate into the tumor interstitial space. In the tissue of solid tumors, vasculature becomes so permeable that particulate liposomes can extravasate and localize in the tissue interstitial space [6, 10]. In addition, tumor tissues frequently lack effective lymphatic drainage [3], which means that the liposomes can be retained longer. Together, these factors increase the

accumulation of the drug within the tumor, which has been referred to as the "enhanced permeability and retention (EPR) effect" by Maeda et al. [68, 69]. EPR-mediated passive tumor targeting by liposomes can increase the concentration of drugs in solid tumors by as much as ten times, compared to free drugs [70].

Passively targeted liposomal drug delivery systems have some disadvantages. Normal organ uptake of liposomes leads to accumulation of the encapsulated drug in mononuclear phagocytic system cells in the liver, spleen, and bone marrow [63], which may present hazards to these tissues. For example, with increased circulation time of these drugs may come increased toxicity inducing such problems as hand-foot syndrome, mucositis, and hematological toxicities such as neutropenia, thrombocytopenia, and leucopenia [71–74]. Therefore, ongoing research aims at enhancing the tumor site-specific action of the liposomes by attaching ligands to surface molecules of tumor cells and tumor vasculature, a process called active or ligand-mediated targeting liposomes [5, 6, 13, 75].

5. Peptide-Mediated Targeting Liposomes

The disadvantage of the passive PEGylated liposomes can be overcome by creating ligand-mediated targeting liposomes with more selective anticancer activity. The activity of anticancer drugs can be enhanced by coupling targeting moieties to the surface of liposomes to promote selective binding to tumor-associated antigens and facilitate the delivery of drug-containing liposomes to the intended cellular sites. This drug delivery system has a higher drug-to-carrier ratio than immunoconjugates and multivalent presentation of ligands, which increases their binding avidity [11].

Antibodies that bind to tumor-specific antigens have so far yielded little success as a drug delivery system for solid tumors, which make up more than 90% of all cancers in humans. Although monoclonal antibodies have shown clinical potential as tumor targeting agents, they are limited by their large molecular size and poor tumor penetration [76], by the immunogenicity associated with immunoliposomes, and by their toxicity to liver and bone marrow from nonspecific antibody uptake. These limitations can be overcome by using peptide ligands, which are smaller, less immunogenic molecules, and easier to produce and manipulate. Furthermore, peptide ligands have moderate affinity to antigens, which is beneficial because extremely high affinity of antibody-binding can impair tumor penetration [77]. Compared with antibody ligands, peptide ligands can improve tumor penetration and decrease MPS clearance of conjugated liposomes [50, 78]. The increasing use of peptides as targeting ligands has been aided by the use of phage display to identify novel ligands (Figure 1). Researchers have already produced liposomes conjugated with ligands that specifically target tumor cells or tumor vasculature [5, 16, 17].

Peptide-conjugated liposomes have three main components: anticancer drug, a liposome carrier, and targeting peptide (Figure 2). Remote loading methods such as the ammonium sulfate method [13, 79] and the pH gradient method [80] can encapsulate weak bases such as doxorubicin or vinorelbine into the liposomes with more than 95% efficiency. Schedule-dependent drugs such as vinca alkaloids, topotecan, and 5-fluorouracil are also potential candidates for liposomal delivery because they can extend the time when cancer cells are exposed to therapeutic levels of the drug.

The bioavailability and pharmacodynamics of liposome-encapsulated chemotherapeutic drugs must be considered in developing these delivery systems. To take advantage of the EPR effect, liposomes need to have long half-lives so that the drug stays within the carrier as long as possible in blood circulation until it accumulates in diseased tissues [81]. Once liposomes are localized to a solid tumor, the drug they contain must be released and become bioavailable at a rate remains therapeutically effective for a period of time. The rate of active drug's release into tumor cells, not the total drug concentration in the tumor tissues, is critical for measuring the actual bioavailability of the liposomal drug [16]. Some targeting liposomes have not been found to have greater therapeutic efficacy than passive liposomal drugs, possibly because the lack of internalizing ligands does not give the drug greater access inside tumor cells [82, 83]. Drug delivery can be further enhanced if the liposome-attached ligands bind selectively to internalizing antigens which would help increase the concentration of drugs inside tumor or tumor-associated endothelial cells resulting in higher drug concentration inside the cells [13, 15, 84, 85]. This binding to internalizing antigens by ligands can induce receptor-mediated endocytosis of liposomes into endosome compartments with low pH, where the liposomes break down and release the encapsulated drug into the intracellular space (Figure 3). These steps lead to higher intracellular drug concentration and greater destruction or inhibition of tumor cells. Studies have confirmed greater cytotoxic effects produced by liposomes with peptides that target internalizing antigens through enhanced specificity and improved drug bioavailability [2, 16].

The use of drug-encapsulated liposomes with ligands to target tumor blood vessels allows us to destroy both tumor blood vessels and tumor cells. In mice bearing human cancer xenograft, low dose of peptide-conjugated liposomal doxorubicin has been found to markedly inhibit vascularization and reduce total volume and weight of tumors [13, 16, 17]. The immunofluorescent analysis of the tumors in several studies has revealed an association between significant decreases in microvessel density and increases in the apoptosis of tumor cells and tumor-associated endothelial cells. The severe damage to tumor vasculature caused by peptide-conjugated liposomal doxorubicin throughout the tumors suggested an improvement in chemotherapeutic efficacy over nontargeting liposomes and conventional drugs [13, 16, 17]. This dual action may produce a greater, more durable anticancer effect than is found with the use of simple antiangiogenic therapy.

One peptide-conjugated liposome can deliver over ten thousand anticancer drug molecules directly into target tumor cells efficiently and effectively. The targeted and sustained release of the drug molecules can increase the maximum tolerated dose (MTD) of the cytotoxic drugs

and dramatically lower dose-limiting toxicities, and in turn prevent treatment delay or discontinuation. The affinity of targeting ligands may allow the liposomes to move past the high IFP barrier within tumors [4, 5, 13, 16].

Advances in nanotechnology and molecular biology are moving us closer to developing an ideal "multifunctional smart nanodrug delivery system" using various types of ligands and drugs based on the kinds of diagnosis, imaging, or therapy needed. Such smart nanodrug delivery systems will allow accurate, specific, and noninvasive disease treatment, early diagnosis, and monitoring. In the future, combining ligands that specifically bind to cancer cells (including cancer stem cells) and tumor blood vessels with multifunctional liposomal drug delivery systems may help improve the effectiveness of cancer treatment and minimize the side effects traditionally associated with chemotherapy.

6. Conclusions

The development of highly selective anticancer drugs that can discriminate between tumor cells and normal cells is the most important goal of current oncology research. The potential use of ligand-conjugated liposome-encapsulated drugs to target tumor cells and vasculature is very promising. Peptides that specifically bind to tumor targets can be coupled to the PEG terminus of sterically stabilized liposomes and subsequently precisely deliver chemotherapeutic agents to tumor cells or blood vessels. Peptide-mediated liposomes that target vasculature are a new generation of chemotherapy delivery systems with superior pharmacokinetics, controlled biodistribution, efficacy, and safety profiles.

Acknowledgment

H-C. Wu is supported by the funding from Academia Sinica and National Science Council, Taiwan.

References

[1] K. Bosslet, R. Straub, M. Blumrich, et al., "Elucidation of the mechanism enabling tumor selective prodrug monotherapy," *Cancer Research*, vol. 58, no. 6, pp. 1195–1201, 1998.

[2] D. E. K. Chang, C. T. Lin, C. H. Wu, and H. A. N. C. Wu, "A novel peptide enhances therapeutic efficacy of liposomal anti-cancer drugs in mice models of human lung cancer," *PLoS ONE*, vol. 4, no. 1, article e4171, 2009.

[3] R. K. Jain, "Transport of molecules in the tumor interstitium: a review," *Cancer Research*, vol. 47, no. 12, pp. 3039–3051, 1987.

[4] C. H. Heldin, K. Rubin, K. Pietras, and A. Ostman, "High interstitial fluid pressure—an obstacle in cancer therapy," *Nature Reviews Cancer*, vol. 4, no. 10, pp. 806–813, 2004.

[5] H. C. Wu, D. K. Chang, and C. T. Huang, "Targeted-therapy for cancer," *Journal of Cancer Molecules*, vol. 2, pp. 57–66, 2006.

[6] T. M. Allen and P. R. Cullis, "Drug delivery systems: entering the mainstream," *Science*, vol. 303, no. 5665, pp. 1818–1822, 2004.

[7] D. A. Sipkins, D. A. Cheresh, M. R. Kazemi, L. M. Nevin, M. D. Bednarski, and K. C. P. Li, "Detection of tumor angiogenesis in vivo by alphaVbeta-targeted magnetic resonance imaging," *Nature Medicine*, vol. 4, no. 5, pp. 623–626, 1998.

[8] A. G. Niethammer, R. Xiang, J. C. Becker, et al., "A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth," *Nature Medicine*, vol. 8, no. 12, pp. 1369–1375, 2002.

[9] J. D. Hood, M. Bednarski, R. Frausto, et al., "Tumor regression by targeted gene delivery to the neovasculature," *Science*, vol. 296, no. 5577, pp. 2404–2407, 2002.

[10] K. Cho, X. U. Wang, S. Nie, Z. Chen, and D. M. Shin, "Therapeutic nanoparticles for drug delivery in cancer," *Clinical Cancer Research*, vol. 14, no. 5, pp. 1310–1316, 2008.

[11] T. M. Allen, "Ligand-targeted therapeutics in anticancer therapy," *Nature Reviews Cancer*, vol. 2, no. 10, pp. 750–763, 2002.

[12] F. Pastorino, C. Brignole, D. Marimpietri, et al., "Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy," *Cancer Research*, vol. 63, no. 21, pp. 7400–7409, 2003.

[13] T. Y. Lee, C. T. Lin, S. Z. U. Y. Kuo, D. E. K. Chang, and H. A. N. C. Wu, "Peptide-mediated targeting to tumor blood vessels of lung cancer for drug delivery," *Cancer Research*, vol. 67, no. 22, pp. 10959–10965, 2007.

[14] T. Y. Lee, H. A. N. C. Wu, Y. U. N. L. Tseng, and C. T. Lin, "A novel peptide specifically binding to nasopharyngeal carcinoma for targeted drug delivery," *Cancer Research*, vol. 64, no. 21, pp. 8002–8008, 2004.

[15] P. Sapra and T. M. Allen, "Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs," *Cancer Research*, vol. 62, no. 24, pp. 7190–7194, 2002.

[16] D.-K. Chang, C.-Y. Chiu, S.-Y. Kuo, et al., "Antiangiogenic targeting liposomes increase therapeutic efficacy for solid tumors," *Journal of Biological Chemistry*, vol. 284, no. 19, pp. 12905–12916, 2009.

[17] F. Pastorino, D. D. Paolo, F. Piccardi, et al., "Enhanced antitumor efficacy of clinical-grade vasculature-targeted liposomal doxorubicin," *Clinical Cancer Research*, vol. 14, no. 22, pp. 7320–7329, 2008.

[18] G. Bocci, K. C. Nicolaou, and R. S. Kerbel, "Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs," *Cancer Research*, vol. 62, no. 23, pp. 6938–6943, 2002.

[19] T. Browder, C. E. Butterfield, B. M. Kräling, et al., "Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer," *Cancer Research*, vol. 60, no. 7, pp. 1878–1886, 2000.

[20] D. Hanahan, G. Bergers, and E. Bergsland, "Less is, more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice," *The Journal of Clinical Investigation*, vol. 105, no. 8, pp. 1045–1047, 2000.

[21] G. Klement, S. Baruchel, J. Rak, et al., "Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity," *The Journal of Clinical Investigation*, vol. 105, no. 8, pp. 15–24, 2000.

[22] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *The New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[23] R. J. Motzer, T. E. Hutson, P. Tomczak, et al., "Sunitinib versus interferon alfa in metastatic renal-cell carcinoma," *The New England Journal of Medicine*, vol. 356, no. 2, pp. 115–124, 2007.

[24] M. J. Ratain, T. I. M. Eisen, W. M. Stadler, et al., "Phase II placebo-controlled randomized discontinuation trial of

sorafenib in patients with metastatic renal cell carcinoma," *Journal of Clinical Oncology*, vol. 24, no. 16, pp. 2505–2512, 2006.

[25] T. Akino, K. Hida, Y. Hida, et al., "Cytogenetic abnormalities of tumor-associated endothelial cells in human malignant tumors," *The American Journal of Pathology*, vol. 175, no. 6, pp. 2657–2667, 2009.

[26] K. Hida, Y. Hida, D. N. Amin, et al., "Tumor-associated endothelial cells with cytogenetic abnormalities," *Cancer Research*, vol. 64, no. 22, pp. 8249–8255, 2004.

[27] J. Denekamp, "Angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy," *The British Journal of Radiology*, vol. 66, no. 783, pp. 181–196, 1993.

[28] H. A. N. C. Wu and P. I. C. Li, "Proteins expressed on tumor endothelial cells as potential targets for anti-angiogenic therapy," *Journal of Cancer Molecules*, vol. 4, no. 1, pp. 17–22, 2008.

[29] R. K. Jain, "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy," *Science*, vol. 307, no. 5706, pp. 58–62, 2005.

[30] K. Pietras, K. Rubin, T. Sjoblom, et al., "Inhibition of PDGF receptor signaling in tumor stroma enhances antitumor effect of chemotherapy," *Cancer Research*, vol. 62, no. 19, pp. 5476–5484, 2002.

[31] H. Wildiers, G. Guetens, G. De Boeck, et al., "Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11," *British Journal of Cancer*, vol. 88, no. 12, pp. 1979–1986, 2003.

[32] C. G. Willett, Y. Boucher, E. di Tomaso, et al., "Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer," *Nature Medicine*, vol. 10, no. 2, pp. 145–147, 2004.

[33] R. K. Jain, "Antiangiogenic therapy for cancer: current and emerging concepts," *Oncology*, vol. 19, no. 4, supplement 3, pp. 7–16, 2005.

[34] W. Arap, M. G. Kolonin, M. Trepel, et al., "Steps toward mapping the human vasculature by phage display," *Nature Medicine*, vol. 8, no. 2, pp. 121–127, 2002.

[35] M. L. Balestrieri and C. Napoli, "Novel challenges in exploring peptide ligands and corresponding tissue-specific endothelial receptors," *European Journal of Cancer*, vol. 43, no. 8, pp. 1242–1250, 2007.

[36] W. Arap, R. Pasqualini, and E. Ruoslahti, "Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model," *Science*, vol. 279, no. 5349, pp. 377–380, 1998.

[37] R. Pasqualini, E. Koivunen, R. Kain, et al., "Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis," *Cancer Research*, vol. 60, no. 3, pp. 722–727, 2000.

[38] H. C. Wu, Y. L. Huang, T. T. Chao, et al., "Identification of B-cell epitope of dengue virus type 1 and its application in diagnosis of patients," *Journal of Clinical Microbiology*, vol. 39, no. 3, pp. 977–982, 2001.

[39] H. A. N. C. Wu, M. E. I. Y. Jung, C. Y. Chiu, et al., "Identification of a dengue virus type 2 (DEN-2) serotype-specific B-cell epitope and detection of DEN-2-immunized animal serum samples using an epitope-based peptide antigen," *The Journal of General Virology*, vol. 84, no. 10, pp. 2771–2779, 2003.

[40] Y. U. N. C. Chen, H. N. Huang, C. T. Lin, Y. I. F. Chen, C. C. King, and H. A. N. C. Wu, "Generation and characterization of monoclonal antibodies against dengue virus type 1 for epitope mapping and serological detection by epitope-based peptide antigens," *Clinical and Vaccine Immunology*, vol. 14, no. 4, pp. 404–411, 2007.

[41] S. Atwell, M. Ultsch, A. M. De Vos, and J. A. Wells, "Structural plasticity in a remodeled protein-protein interface," *Science*, vol. 278, no. 5340, pp. 1125–1128, 1997.

[42] K. Nord, E. Gunneriusson, J. Ringdahl, S. Stahl, M. Uhlén, and P. E. R. A. Nygren, "Binding proteins selected from combinatorial libraries of an α-helical bacterial receptor domain," *Nature Biotechnology*, vol. 15, no. 8, pp. 772–777, 1997.

[43] B. Li, J. Y. K. Tom, D. Oare, et al., "Minimization of a polypeptide hormone," *Science*, vol. 270, no. 5242, pp. 1657–1660, 1995.

[44] K. N. Sugahara, T. Teesalu, P. P. Karmali, et al., "Tissue-penetrating delivery of compounds and nanoparticles into tumors," *Cancer Cell*, vol. 16, no. 6, pp. 510–520, 2009.

[45] A. R. Castano, S. Tangri, J. E. W. Miller, et al., "Peptide binding and presentation by mouse CD1," *Science*, vol. 269, no. 5221, pp. 223–226, 1995.

[46] J. Mai, S. Song, M. Rui, et al., "A synthetic peptide mediated active targeting of cisplatin liposomes to Tie2 expressing cells," *Journal of Controlled Release*, vol. 139, no. 3, pp. 174–181, 2009.

[47] A. Folgori, R. Tafi, A. Meola, et al., "A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera," *EMBO Journal*, vol. 13, no. 9, pp. 2236–2243, 1994.

[48] I. J. Liu, P. O. R. Hsueh, C. T. Lin, et al., "Disease-specific B cell epitopes for serum antibodies from patients with severe acute respiratory syndrome (SARS) and serologic detection of SARS antibodies by epitope-based peptide antigens," *The Journal of Infectious Diseases*, vol. 190, no. 4, pp. 797–809, 2004.

[49] L. Mazzucchelli, J. B. Burritt, A. J. Jesaitis, et al., "Cell-specific peptide binding by human neutrophils," *Blood*, vol. 93, no. 5, pp. 1738–1748, 1999.

[50] A. Lo, C. T. Lin, and H. A. N. C. Wu, "Hepatocellular carcinoma cell-specific peptide ligand for targeted drug delivery," *Molecular Cancer Therapeutics*, vol. 7, no. 3, pp. 579–589, 2008.

[51] A. F. S. A. Habeeb, "Determination of free amino groups in proteins by trinitrobenzenesulfonic acid," *Analytical Biochemistry*, vol. 14, no. 3, pp. 328–336, 1966.

[52] S. Zalipsky, N. Mullah, J. A. Harding, J. Gittelman, L. Guo, and S. A. DeFrees, "Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains," *Bioconjugate Chemistry*, vol. 8, no. 2, pp. 111–118, 1997.

[53] D. Kirpotin, J. W. Park, K. Hong, et al., "Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro," *Biochemistry*, vol. 36, no. 1, pp. 66–75, 1997.

[54] F. Curnis, G. Arrigoni, A. Sacchi, et al., "Differential binding of drugs containing the NGR motif to CD13 isoforms in tumor vessels, epithelia, and myeloid cells," *Cancer Research*, vol. 62, no. 3, pp. 867–874, 2002.

[55] L. Brannon-Peppas and J. O. Blanchette, "Nanoparticle and targeted systems for cancer therapy," *Advanced Drug Delivery Reviews*, vol. 56, no. 11, pp. 1649–1659, 2004.

[56] R. Duncan, "Polymer conjugates as anticancer nanomedicines," *Nature Reviews Cancer*, vol. 6, no. 9, pp. 688–701, 2006.

[57] F. Muggia and A. Hamilton, "Phase III data on Caelyx in ovarian cancer," *European Journal of Cancer*, vol. 37, supplement 9, pp. 15–18, 2001.

[58] D. Papahadjopoulos, T. M. Allen, A. Gabizon, et al., "Sterically stabilized liposomes: improvements in pharmacokinetics and

antitumor therapeutic efficacy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 24, pp. 11460–11464, 1991.

[59] G. T. Colbern, A. J. Hiller, R. S. Musterer, E. Pegg, I. C. Henderson, and P. K. Working, "Significant increase in antitumor potency of doxorubicin HCl by its encapsulation in pegylated liposomes," *Journal of Liposome Research*, vol. 9, no. 4, pp. 523–538, 1999.

[60] A. S. Abu Lila, Y. Doi, K. Nakamura, T. Ishida, and H. Kiwada, "Sequential administration with oxaliplatin-containing PEG-coated cationic liposomes promotes a significant delivery of subsequent dose into murine solid tumor," *Journal of Controlled Release*, vol. 142, no. 2, pp. 167–173, 2010.

[61] R. Z. Orlowski, A. Nagler, P. Sonneveld, et al., "Randomized phase III study of pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone in relapsed or refractory multiple myeloma: combination therapy improves time to progression," *Journal of Clinical Oncology*, vol. 25, no. 25, pp. 3892–3901, 2007.

[62] T. Safra, F. Muggia, S. Jeffers, et al., "Pegylated liposomal doxorubicin (doxil): reduced clinical cardiotoxicity in patients reaching or exceeding cumulative doses of 500 mg/m^2," *Annals of Oncology*, vol. 11, no. 8, pp. 1029–1033, 2000.

[63] K. J. Harrington, S. Mohammadtaghi, P. S. Uster, et al., "Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes," *Clinical Cancer Research*, vol. 7, no. 2, pp. 243–254, 2001.

[64] N. M. Marina, D. Cochrane, E. Harney, et al., "Dose escalation and pharmacokinetics of pegylated liposomal doxorubicin (Doxil) in children with solid tumors: a pediatric oncology group study," *Clinical Cancer Research*, vol. 8, no. 2, pp. 413–418, 2002.

[65] G. Batist, "Cardiac safety of liposomal anthracyclines," *Cardiovascular Toxicology*, vol. 7, no. 2, pp. 72–74, 2007.

[66] H. Hashizume, P. Baluk, S. Morikawa, et al., "Openings between defective endothelial cells explain tumor vessel leakiness," *American Journal of Pathology*, vol. 156, no. 4, pp. 1363–1380, 2000.

[67] D. C. Drummond, O. Meyer, K. Hong, D. B. Kirpotin, and D. Papahadjopoulos, "Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors," *Pharmacological Reviews*, vol. 51, no. 4, pp. 691–743, 1999.

[68] Y. Matsumura and H. Maeda, "A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs," *Cancer Research*, vol. 46, no. 12, pp. 6387–6392, 1986.

[69] H. Maeda, J. Wu, T. Sawa, Y. Matsumura, and K. Hori, "Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review," *Journal of Controlled Release*, vol. 65, no. 1-2, pp. 271–284, 2000.

[70] D. W. Northfelt, F. J. Martin, P. Working, et al., "Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: pharmacokinetics, tumor localization, and safety in patients with AIDS-related Kaposi's sarcoma," *Journal of Clinical Pharmacology*, vol. 36, no. 1, pp. 55–63, 1996.

[71] B. Uziely, S. Jeffers, R. Isacson, et al., "Liposomal doxorubicin: antitumor activity and unique toxicities during two complementary phase I studies," *Journal of Clinical Oncology*, vol. 13, no. 7, pp. 1777–1785, 1995.

[72] K. B. Gordon, A. Tajuddin, J. Guitart, T. M. Kuzel, L. R. Eramo, and J. VonRoenn, "Hand-foot syndrome associated with liposome-encapsulated doxorubicin therapy," *Cancer*, vol. 75, no. 8, pp. 2169–2173, 1995.

[73] Y. Matsumura, M. Gotoh, K. Muro, et al., "Phase I and pharmacokinetic study of MCC-465, a doxorubicin (DXR) encapsulated in PEG immunoliposome, in patients with metastatic stomach cancer," *Annals of Oncology*, vol. 15, no. 3, pp. 517–525, 2004.

[74] S. E. Al-Batran, J. Bischoff, G. Von Minckwitz, et al., "The clinical benefit of pegylated liposomal doxorubicin in patients with metastatic breast cancer previously treated with conventional anthracyclines: a multicentre phase II trial," *British Journal of Cancer*, vol. 94, no. 11, pp. 1615–1620, 2006.

[75] F. Pastorino, C. Brignole, D. Paolo, et al., "Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy," *Cancer Research*, vol. 66, no. 20, pp. 10073–10082, 2006.

[76] T. R. Shockley, K. Lin, J. A. Nagy, R. G. Tompkins, H. F. Dvorak, and M. L. Yarmush, "Penetration of tumor tissue by antibodies and other immunoproteins," *Annals of the New York Academy of Sciences*, vol. 618, pp. 367–382, 1991.

[77] G. P. Adams, R. Schier, A. M. McCall, et al., "High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules," *Cancer Research*, vol. 61, no. 12, pp. 4750–4755, 2001.

[78] T. Mori, "Cancer-specific ligands identified from screening of peptide-display libraries," *Current Pharmaceutical Design*, vol. 10, no. 19, pp. 2335–2343, 2004.

[79] E. M. Bolotin, R. Cohen, L. K. Bar, et al., "Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandoliposomes," *Journal of Liposome Research*, vol. 4, no. 1, pp. 455–479, 1994.

[80] N. L. Boman, D. Masin, L. D. Mayer, P. R. Cullis, and M. B. Bally, "Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors," *Cancer Research*, vol. 54, no. 11, pp. 2830–2833, 1994.

[81] A. Gabizon, H. Shmeeda, and Y. Barenholz, "Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies," *Clinical Pharmacokinetics*, vol. 42, no. 5, pp. 419–436, 2003.

[82] M. H. Vingerhoeds, P. A. Steerenberg, J. J. G. W. Hendriks, et al., "Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma in vitro and in vivo," *British Journal of Cancer*, vol. 74, no. 7, pp. 1023–1029, 1996.

[83] D. Goren, A. T. Horowitz, D. Tzemach, M. Tarshish, S. Zalipsky, and A. Gabizon, "Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump," *Clinical Cancer Research*, vol. 6, no. 5, pp. 1949–1957, 2000.

[84] M. Sugano, N. K. Egilmez, S. J. Yokota, et al., "Antibody targeting of doxorubicin-loaded liposomes suppresses the growth and metastatic spread of established human lung tumor xenografts in severe combined immunodeficient mice," *Cancer Research*, vol. 60, no. 24, pp. 6942–6949, 2000.

[85] J. W. Park, K. Hong, D. B. Kirpotin, et al., "Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery," *Clinical Cancer Research*, vol. 8, no. 4, pp. 1172–1181, 2002.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 361836, 7 pages
doi:10.1155/2010/361836

Review Article

Angiogenesis Inhibition in Prostate Cancer: Current Uses and Future Promises

Jeanny B. Aragon-Ching,[1] Ravi A. Madan,[2] and William L. Dahut[3]

[1] *Division of Hematology and Oncology, Department of Medicine, The George Washington University Medical Center, 2150 Pennsylvania Avenue Northwest, Washington, DC 20037, USA*
[2] *Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*
[3] *Medical Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA*

Correspondence should be addressed to Jeanny B. Aragon-Ching, jaragonching@mfa.gwu.edu

Received 31 October 2009; Accepted 30 December 2009

Academic Editor: Arkadiusz Dudek

Angiogenesis has been well recognized as a fundamental part of a multistep process in the evolution of cancer progression, invasion, and metastasis. Strategies for inhibiting angiogenesis have been one of the most robust fields of cancer investigation, focusing on the vascular endothelial growth factor (VEGF) family and its receptors. There are numerous regulatory drug approvals to date for the use of these agents in treating a variety of solid tumors. While therapeutic efficacy has been established, challenges remain with regards to overcoming resistance and assessing response to antiangiogenic therapies. Prostate cancer is the most common noncutaneous malignancy among American men and angiogenesis plays a role in disease progression. The use of antiangiogenesis agents in prostate cancer has been promising and is hereby explored.

1. Introduction

Angiogenesis is a complex, dynamic process that involves multiple pathways that converge to affect carcinogenesis, proliferation, and tumor growth. Since the inception of the concept of angiogenesis by Dr. Judah Folkman decades ago, a substantial body of research has emerged and currently forms the groundwork for establishing antiangiogenesis as an important part of the armamentarium in cancer therapy. Furthermore, investigation of signaling pathways, molecules, drugs, as well as mechanisms of resistance may lead to a better understanding of angiogenesis and the development of strategies incorporating antiangiogenic drugs with chemotherapy in various tumor types, such as prostate cancer.

Prostate cancer is the leading noncutaneous malignancy among American men in North America. In 2009 alone, it is estimated that 192,280 new cases will be diagnosed and more than 27,000 deaths will occur from this disease [1]. Hormonal therapy remains the cornerstone of treatment for men who have androgen-responsive metastatic disease. While most men will respond to sequential hormonal manipulations, castration resistant prostate cancer (CRPC) eventually ensues. The demonstration of survival benefit using docetaxel-based therapy [2, 3] led to the approval by the Food and Drug Administration (FDA) in 2004 of docetaxel and prednisone for the treatment of metastatic CRPC. Since then, no other drug has been approved for metastatic CRPC, thus creating an area of increased unmet medical need.

Extensive studies on angiogenesis in prostate cancer to date have revealed that angiogenesis plays a role in the progression of prostate cancer. Microvessel density, a measurement of prostate cancer angiogenesis, has been shown to be a predictor of metastasis and survival [4]. Thus, targeting angiogenesis has been the subject of several clinical investigations.

2. Pathways Involved in the Angiogenic Process

Since the introduction of the concept that tumors would not grow beyond a pinhead size in the absence of blood vessel

growth [5], our understanding of the role that angiogenesis plays in cancer has robustly expanded. Angiogenesis is a complex process that involves an interplay between various regulatory proteins, proangiogenic stimuli, endothelial cell activation, as well as proliferation and migration, governed by molecular and cellular mechanisms, resulting in reorganization into new blood vessels [6]. The theory of the "angiogenic switch" describes the dynamic transition to a malignant tumor phenotype that promotes neovascularization, the absence of which is a rate-limiting step in carcinogenesis [7–10]. Several factors may trigger proangiogenic factor expression. Hypoxia, for instance, regulates the production of several angiogenic cytokines such as fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and interleukin-8 (IL-8), among others. The overall tumor microenvironment is also instrumental in the recruitment of proangiogenic factors [11], although the resulting tumor neovasculatures are characterized by inefficient, permeable, and leaky vessels [12].

Vascular Endothelial Growth Factor Pathway (VEGF). Perhaps the most widely studied pathway in the angiogenic signaling process involves triggering VEGF and its receptors. The VEGF family has more than 7 members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, PlGF, and *Trimeresurus flavoviridis* (*T.f.*) svVEGFs [13]. VEGF-A is the prototype VEGF ligand, playing a key role in tumor angiogenesis. VEGF-A binds and activates two receptors, namely, VEGFR1 (Flt-1 or fms-like tyrosine kinase receptor 1) and VEGFR2 (KDR/Flk-1), and it has varying roles in the promotion of endothelial cell differentiation, cell growth, tubular formation, and migration [14]. Neutralization of the VEGF-A ligand has, therefore, been the subject of investigation in the last decade, leading to the first FDA drug approval of its class, Bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA). Other strategies for targeting the VEGF pathway involve the inhibition of key enzymes such as tyrosine kinase inhibitors or use of decoy receptor fusion proteins.

3. Prostate Cancer: Experience with Use of Antiangiogenic Agents

Early studies yielded promising results of VEGF inhibition in various murine tumor models [15, 16]. In prostate cancer, elevated circulating VEGF and other soluble growth factors have been demonstrated to be predictive of biochemical progression in men undergoing radical prostatectomy [17]. Measurements of angiogenesis using microvessel density have also been shown to be a prognosticator for survival and metastasis [4, 18, 19]. The utility of microvessel density has also been shown to improve prediction of cancer staging from prostate biopsies, beyond the well-known contributory factors afforded by existing risk features such as Gleason scores and prostate-specific antigen (PSA) levels [20]. In a prospective study among 572 men diagnosed with prostate cancer in the Health Professionals Follow-Up Study, those with the most irregular and primitive microvessel density as measured by staining CD34 were more likely to have lethal and aggressive prostate cancer [21]. This section will discuss varying agents used in the treatment of prostate cancer including thalidomide, bevacizumab, and the tyrosine kinase inhibitors.

3.1. Thalidomide. Thalidomide (alpha-N-phthalimidoglutarimide; Thalomid, Celgene, Summit, NJ) has emerged as a potent treatment for several disease entities and has been currently approved by the FDA in the United States, including for the treatment of Multiple Myeloma [22]. Although its antiangiogenic properties are not clearly understood, several in vitro assays have suggested that the antiangiogenic properties could be secondary to the inhibition of secretion of two angiogenic cytokines, namely, VEGF and FGF from both tumor and stromal cells [23, 24]. Evidence to date suggests that this occurs independently of thalidomide's immunomodulatory properties and that the downregulation of integrins perhaps results in the inhibition of endothelial cell migration and adhesion [25]. Although thalidomide has predominantly been studied in hematologic malignancies, activity in solid tumors has also been demonstrated. Thalidomide in prostate cancer has been used alone or in combination with chemotherapy in CRPC. An earlier study using thalidomide in an open-label phase II randomized trial compared low-dose (200 mg/day) and high-dose (up to 1200 mg/day) thalidomide in 63 patients [26]. Results showed a modest response with 27% of the patients having a reduction in the serum PSA levels of $\geq$40%. Four patients in the low-dose arm showed sustainable response of >150 days with a >50% decrease in PSA. The demonstration of the potential activity of thalidomide and preclinical evidence demonstrating that chemotherapy could enhance the activity of antiangiogenic agents [27] led to the combination of thalidomide with docetaxel [28]. This randomized phase II study of docetaxel, with or without 200 mg of thalidomide, enrolled 75 patients with chemotherapy-naive metastatic CRPC. Docetaxel, at a dose of 30 mg/m^2 given intravenously, was administered weekly for 3 out of 4 weeks, with a 1-week rest period during the 4th week. The trial was launched prior to the TAX 327 trial which demonstrated the superiority of docetaxel every 3 weeks; thus, the weekly dose was administered. PSA declines of at least 50% were greater in the combined docetaxel and thalidomide arm compared to the docetaxel alone arm, with better progression-free survival (PFS) which was not statistically significant (median PFS of 3.7 months in docetaxel group and 5.9 months in the combined group ($P = .32$)) [29]. The side effects were manageable although thrombotic events were seen in the combination arm which was later alleviated by institution of thromboprophylaxis. Further overall survival analysis of this trial showed an improvement in the 18-month survival in the combination arm versus docetaxel alone arm (69.3% versus 47.2%, $P < .05$) [30].

To this end, a search for a more efficacious and less toxic thalidomide analog such as lenalidomide was studied in prostate cancer. A phase I study to determine the maximum tolerated dose (MTD) and to characterize the side-effect

profile and pharmacokinetics (PKs) of lenalidomide in patients with advanced refractory solid tumors was launched [31]. Forty-five patients were enrolled, of whom 36 patients had prostate cancer. Dose levels used were 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, and 40 mg. The dosing schedule was modified from continuous daily to 21 out of 28 days of dosing due to the observed side effects. Interestingly, stable disease was seen in 12 out of 44 evaluable patients, 9 of whom had prostate cancer. A phase I dose-escalation trial using docetaxel at 60 mg/m^2 and 75 mg/m^2 every 21 days, combined with lenalidomide at varying doses from 10 mg to 30 mg on days 1–21, showed promising responses with manageable toxicity [32]. There were 31 patients evaluable for PSA response. PSA declines of >50% were seen in 47% (8 out of 17) previously untreated patients and 50% (7 out of 14) previously treated patients. The promising activity seen in prostate cancer, as well as the demonstrated activity using thalidomide, has led to an ongoing phase II trial using the combination of lenalidomide, docetaxel, prednisone, and bevacizumab, in chemotherapy-naive patients with metastatic CRPC at the National Cancer Institute [33].

3.2. Bevacizumab. Bevacizumab is the most widely studied antiangiogenic inhibitor and the first of its class to be approved by the United States FDA. It has gained approval predominantly in combination with chemotherapy for metastatic colorectal cancer, lung, breast, renal cell cancer, and Glioblastoma multiforme [34]. Bevacizumab is a humanized IgG1 monoclonal antibody developed from a murine monoclonal antibody targeting the human VEGF ligand, specifically the major isoforms of VEGF-A. The initial studies using a murine monoclonal antibody targeting VEGF on several human cancer cell lines in vivo showed promising results [15, 35]. However, initial single agent studies in prostate cancer were disappointing[36]. Fifteen patients with metastatic CRPC were treated at a dose of 10 mg/kg of bevacizumab every 2 weeks for 6 total infusions. No objective or partial responses were observed by day 70 in the 8 patients who had measurable disease. Only 4 patients had PSA declines, none more than 50%. Although single-agent bevacizumab lacked significant activity in prostate cancer, as in most other solid tumors, the encouraging results from other clinical trials using combined bevacizumab and chemotherapy led to a Cancer and Leukemia Group B (CALGB) trial 90006 that combined bevacizumab with docetaxel and estramustine [37]. The CALGB 90006 trial enrolled 79 patients, and a 77% PSA decline rate of 50% was observed (in the 58 of 75 patients with sufficient PSA data) [37, 38]. The estimated time to progression was 9.7 months and the overall survival was 21 months.

Similar promising results were shown in a phase II, open-label trial at the National Cancer Institute (NCI) utilizing a combination of bevacizumab at 15 mg/kg every 3 weeks, in combination with docetaxel 75 mg/m^2 every 3 weeks, thalidomide 200 mg daily, and prednisone 10 mg daily, with thromboprophylaxis using a low-molecular weight heparin. This trial enrolled 60 patients with metastatic CRPC, with a median age of 66 years (range 44–79), and predominantly high-risk features, including a median Gleason score of 8, on-study PSA of 99 ng/mL (range: 6.0–4,399), and prestudy PSA doubling time of 1.6 months (0.3–18.2, 81% <3 months). As of the last followup [39], 51 patients (88%) had PSA declines of >50%, with median >50% PSA decline duration of 11 cycles (0–45). Furthermore, the overall response rate was 63% of the 32 patients with measurable disease, with a complete response (CR) seen in 2 patients, partial response (PR) in 18 patients, and stable disease (SD) in 11 patients. This combination was tolerable with expected adverse-effects that included febrile neutropenia in 5 patients, syncope in 5 patients, gastrointestinal perforation or fistula in 3 patients, and thrombosis or bleeding in 5 patients. The estimated median PFS was 18.2 months.

Another CALGB study that evaluated bevacizumab in prostate cancer was CALGB 90401 which had the primary objective of comparing overall survival between men with chemotherapy-naive metastatic CRPC treated with standard of care docetaxel 75 mg/m^2 every 21 days and prednisone 5 mg twice daily versus docetaxel 75 mg/m^2, prednisone 5 mg twice daily, and bevacizumab 15 mg/kg every 21 days. The study completed accrual in December 2007 and results are currently awaited. The study was powered to detect a 25% improvement in overall survival in the bevacizumab arm [40].

3.3. VEGF Trap. Another strategy for targeting VEGF is through blocking the VEGF receptors. One of the most potent VEGF-R blockers is a novel decoy receptor fusion protein comprised of the extracellular domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused to the constant region (Fc fragment) of human IgG1 [41]. Earlier studies using truncated soluble VEGF-R1 inhibitors exhibited effective inhibition of VEGF but had poor pharmacokinetic profile and had to be administered more frequently and at high concentrations [42, 43]. VEGF Trap (Aflibercept; Sanofi Aventis, Paris, France and Regeneron, Tarrytown, New York) is a human fusion protein that binds and neutralizes the major VEGF isoforms including VEGF-A, VEGF-B, as well as platelet-derived growth factor (PlGF) [44]. A phase I dose escalation study using aflibercept in combination with docetaxel 75 mg/m^2 every 3 weeks has been reported [45], with recommended dosing of aflibercept at 6 mg/kg. A phase III trial has been launched in metastatic CRPC patients with a primary objective of improvement in overall survival for metastatic CRPC and a planned accrual of 1,200 patients who will be randomized to either VEGF Trap in combination with standard docetaxel and prednisone or standard docetaxel and prednisone alone [46].

3.4. Tyrosine Kinase Inhibitors. Tyrosine kinases are key enzymes that modulate various cellular processes that affect signaling for tumor growth, proliferation, and survival [47]. Several tyrosine kinase inhibitors have been used in the treatment of prostate cancer. Sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals Inc., Wayne, NJ) and sunitinib (Sutent; Pfizer, New York, NY) lead the agents that have been used. Sorafenib not only inhibits VEGF but functions as a multi-tyrosine kinase inhibitor that has been shown

in preclinical models to inhibit wild-type and mutant b-Raf and c-Raf kinase isoforms in vitro. In addition, this agent also inhibits various pathways, including p38, c-kit, VEGFR-2, and PDGFR-β in varying concentrations, affecting tumor growth as well as possibly promoting apoptosis by events downstream of c-Raf [48, 49]. Clinical studies using sorafenib have been done in various phase II studies but have shown only modest activity and no robust PSA declines. In a phase II study using sorafenib in 22 patients, no PSA declines of 50% were noted [50]. However, there was discordance between PSA rise and improvement in bone lesions by bone scintigraphy scan in two patients. This led to further accrual of the trial to the accrual goal of 46 patients [51]. Other phase II studies using sorafenib showed similar minimal PSA activity in spite of radiographic improvements [52–56].

Sunitinib malate is another small molecule inhibitor targeting VEGFR-1 and VEGFR-2, along with PDGF-R, c-kit, and *RET* kinases [57]. This agent has been shown to exhibit some activity in prostate cancer. However, similar to sorafenib, sunitinib exhibited few PSA declines and several patients had clinical and radiographic improvements despite PSA rises [58]. The use of these agents, therefore, raises the question of whether adequate assessments are being used in analyzing the treatment effects using these multikinase inhibitors or perhaps they are best combined with other agents, such as chemotherapy.

Cediranib (Recentin; AstraZeneca, London, UK) is another agent that inhibits tyrosine kinases of VEGF receptors. This is an oral, potent, indole-ether quinazoline ATP-competitive compound that inhibits proliferation via inhibition of all 3 VEGF receptors [59]. A phase I study using cediranib in prostate cancer showed dose-limiting toxicity occurring at 30 mg dose, with a dose of 20 mg/day being the MTD [60]. A phase II study in docetaxel-resistant metastatic CRPC showed encouraging results, with 13 of 23 evaluable patients having decreases in soft tissue lesions, 4 of whom met criteria for partial response [61]. Shrinkage of metastatic visceral disease to the lymph nodes, lung, liver, and bone was observed although the PSA levels have not corresponded with imaging responses. Similar to sorafenib, posttreatment PSA declines were noted in patients following drug discontinuation, in the absence of administration of a new drug treatment.

4. Mechanisms of Resistance

It was initially believed that angiogenesis inhibition carries little potential risk for resistance [62]. However, recent studies suggest that this may not be the case. For instance, experiments using mouse endothelial cells isolated from human tumor xenografts when compared to normal endothelial cell counterparts showed acquired cytogenetic abnormalities [63]. Furthermore, regrowth of tumors during treatment with antibodies to VEGFR1 and R2 after an initial period of growth suppression was seen in a pancreatic islet cell tumor murine model, suggesting development of phenotypic resistance to VEGFR2 blockade [64]. This resistance to VEGF blockade involves various possible mechanisms, including an adaptive evasion or intrinsic nonresponsiveness of tumors [10, 65]. Adaptive evasion suggests upregulation of alternative signaling pathways to circumvent the blocked angiogenic pathway, recruitment of bone-marrow derived proangiogenic cells, or increased surrounding pericyte coverage. Intrinsic nonresponsiveness, on the other hand, suggests innate indifference of the tumor to antiangiogenic therapy, which supports the clinical observation that not all patients respond to antiangiogenic therapy. These observations certainly have clinical implications since strategies to obviate these acquired or inherent resistances must be sought. Strategies that include combination or sequential approach of antiangiogenic therapies may be one approach to address this. In addition, genetic variability in the VEGF promoter can be used as a potential predictive biomarker for bevacizumab activity.

5. Future Directions

Angiogenesis inhibitors have the potential to enhance therapeutic options for patients with prostate cancer. Various combinations, not limited to chemotherapy, have been used with promising results. For instance, interesting results have been seen using a combination of bevacizumab with the autologous dendritic cell-based vaccine sipuleucel-T (Provenge; Dendreon, Seattle, WA) in patients with biochemical recurrence [66]. This vaccine has demonstrated improved overall survival relative to placebo in a phase III trial in metastatic CRPC [67] and may warrant further investigation with bevacizumab. With the success seen in various other tumor types using antiangiogenesis, similar results are likely seen in prostate cancer. However, several challenges remain, including the use of appropriate assessment tools for measuring response in metastatic prostate cancer and the use of candidate surrogate biomarkers for correlating response. Perhaps the use of pharmacogenetics may help in identifying patients who may benefit or develop undue toxicity from the use of antiangiogenic agents. For instance, in a trial using bevacizumab in breast cancer patients, varying single nucleotide polymorphisms (SNPs) in the regulatory regions of the VEGF gene may predict for improved median overall survival or protection from hypertension, which is one of the most commonly encountered toxicity with the use of bevacizumab [68]. The benefit of angiogenesis inhibitors has become a reality in several tumor types, with significant potential in prostate cancer.

Acknowledgments

This project has been supported by the Intramural Research Program of the National Cancer Institute, Center for Cancer Research, National Institutes of Health. The content of this publication does not reflect the views or policies of the Department of Health and Human Services nor does it mention trade names, commercial products, or organizations implying endorsement by the U.S. Government.

References

[1] A. Jemal, R. Siegel, E. Ward, Y. Hao, J. Xu, and M. J. Thun, "Cancer statistics, 2009," *CA: A Cancer Journal for Clinicians*, vol. 59, no. 4, pp. 225–249, 2009.

[2] I. F. Tannock, R. de Wit, W. R. Berry, et al., "Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer," *The New England Journal of Medicine*, vol. 351, no. 15, pp. 1502–1512, 2004.

[3] D. P. Petrylak, C. M. Tangen, M. H. A. Hussain, et al., "Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer," *The New England Journal of Medicine*, vol. 351, no. 15, pp. 1513–1520, 2004.

[4] N. Weidner, P. R. Carroll, J. Flax, W. Blumenfeld, and J. Folkman, "Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma," *American Journal of Pathology*, vol. 143, no. 2, pp. 401–409, 1993.

[5] J. Folkman, "Tumor angiogenesis: therapeutic implications.," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[6] J. Folkman, K. Watson, D. Ingber, and D. Hanahan, "Induction of angiogenesis during the transition from hyperplasia to neoplasia," *Nature*, vol. 339, no. 6219, pp. 58–61, 1989.

[7] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[8] G. Bergers, K. Javaherian, K.-M. Lo, J. Folkman, and D. Hanahan, "Effects of angiogenesis inhibitors on multistage carcinogenesis in mice," *Science*, vol. 284, no. 5415, pp. 808–812, 1999.

[9] G. Bergers and L. E. Benjamin, "Tumorigenesis and the angiogenic switch," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 401–410, 2003.

[10] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[11] K. Hattori, B. Heissig, Y. Wu, et al., "Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1$^+$ stem cells from bone-marrow microenvironment," *Nature Medicine*, vol. 8, no. 8, pp. 841–849, 2002.

[12] A. Pettersson, J. A. Nagy, L. F. Brown, et al., "Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor," *Laboratory Investigation*, vol. 80, no. 1, pp. 99–115, 2000.

[13] M. Shibuya, "Vascular permeability/vascular endothelial growth factor," in *Angiogenesis: An Integrative Approach from Science to Medicine*, J. Folkman and W. D. Figg, Eds., pp. 89–98, Springer, New York, NY, USA, 2008.

[14] N. Ferrara, R. D. Mass, C. Campa, and R. Kim, "Targeting VEGF-A to treat cancer and age-related macular degeneration," *Annual Review of Medicine*, vol. 58, pp. 491–504, 2007.

[15] K. J. Kim, B. Li, J. Winer, et al., "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo," *Nature*, vol. 362, no. 6423, pp. 841–844, 1993.

[16] H.-P. Gerber and N. Ferrara, "Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies," *Cancer Research*, vol. 65, no. 3, pp. 671–680, 2005.

[17] S. F. Shariat, V. A. Anwuri, D. J. Lamb, N. V. Shah, T. M. Wheeler, and K. M. Slawin, "Association of preoperative plasma levels of vascular endothelial growth factor and soluble vascular cell adhesion molecule-1 with lymph node status and biochemical progression after radical prostatectomy," *Journal of Clinical Oncology*, vol. 22, no. 9, pp. 1655–1663, 2004.

[18] M. Borre, B. V. Offersen, B. Nerstrom, and J. Overgaard, "Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting," *British Journal of Cancer*, vol. 78, no. 7, pp. 940–944, 1998.

[19] M. Borre, S. M. Bentzen, B. Nerstrom, and J. Overgaard, "Tumor cell proliferation and survival in patients with prostate cancer followed expectantly," *Journal of Urology*, vol. 159, no. 5, pp. 1609–1614, 1998.

[20] D. G. Bostwick, T. M. Wheeler, M. Blute, et al., "Optimized microvessel density analysis improves prediction of cancer stage from prostate needle biopsies," *Urology*, vol. 48, no. 1, pp. 47–57, 1996.

[21] L. A. Mucci, A. Powolny, E. Giovannucci, et al., "Prospective study of prostate tumor angiogenesis and cancer-specific mortality in the health professionals follow-up study," *Journal of Clinical Oncology*, vol. 27, pp. 5627–5633, 2009.

[22] Thalidomide FDA Approval—National Cancer Institute, October 2009, http://www.cancer.gov/cancertopics/druginfo/thalidomide.

[23] R. J. D'Amato, M. S. Loughnan, E. Flynn, and J. Folkman, "Thalidomide is an inhibitor of angiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 9, pp. 4082–4085, 1994.

[24] B. M. Kenyon, F. Browne, and R. J. D'Amato, "Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization," *Experimental Eye Research*, vol. 64, no. 6, pp. 971–978, 1997.

[25] K. Dredge, J. B. Marriott, C. D. Macdonald, et al., "Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects," *British Journal of Cancer*, vol. 87, no. 10, pp. 1166–1172, 2002.

[26] W. D. Figg, W. Dahut, P. Duray, et al., "A randomized phase II trial of thalidomide, an angiogenesis inhibitor, in patients with androgen-independent prostate cancer," *Clinical Cancer Research*, vol. 7, no. 7, pp. 1888–1893, 2001.

[27] B. A. Teicher, E. A. Sotomayor, and Z. D. Huang, "Antiangiogenic agents potentiate cytotoxic cancer therapies against primary and metastatic disease," *Cancer Research*, vol. 52, no. 23, pp. 6702–6704, 1992.

[28] W. D. Figg, P. Arlen, J. Gulley, et al., "A randomized phase II trial of docetaxel (taxotere) plus thalidomide in androgen-independent prostate cancer," *Seminars in Oncology*, vol. 28, no. 4, supplement 15, pp. 62–66, 2001.

[29] W. L. Dahut, J. L. Gulley, P. M. Arlen, et al., "Randomized phase II trial of docetaxel plus thalidomide in androgen-independent prostate cancer," *Journal of Clinical Oncology*, vol. 22, no. 13, pp. 2532–2539, 2004.

[30] W. D. Figg, A. Retter, S. M. Steinberg, and W. L. Dahut, "In reply," *Journal of Clinical Oncology*, vol. 23, no. 9, pp. 2113–2114, 2005.

[31] W. L. Dahut, J. B. Aragon-Ching, S. Woo, et al., "Phase I study of oral lenalidomide in patients with refractory metastatic cancer," *Journal of Clinical Pharmacology*, vol. 49, no. 6, pp. 650–660, 2009.

[32] D. P. Petrylak, K. Resto-Garces, M. Tibyan, et al., "A phase I open-label study using lenalidomide and docetaxel in castration-resistant prostate cancer," *Journal of Clinical Oncology*, vol. 27, supplement 15, 2009, abstract 5156.

[33] "A Phase 2 Trial of Bevacizumab, Lenalidomide, Docetaxel, and Prednisone (ART-P) for Treatment of Metastatic Castrate-Resistant Prostate Cancer," October 2009, http://clinicaltrials.gov/ct2/show/NCT00942578?term=lenalidomide+and+docetaxel+and+bevacizumab&rank=1.

[34] Bevacizumab approval: National Cancer Institute, October 2009, http://www.cancer.gov/cancertopics/druginfo/bevacizumab.

[35] K. J. Kim, B. Li, K. Houck, J. Winer, and N. Ferrara, "The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies," *Growth Factors*, vol. 7, no. 1, pp. 53–64, 1992.

[36] D. M. Reese, P. Fratesi, M. Corry, W. Novotny, E. Holmgren, and E. J. Small, "A phase II trial of humanized anti-vascular endothelial growth factor antibody for the treatment of androgen-independent prostate cancer," *Prostate Journal*, vol. 3, no. 2, pp. 65–70, 2001.

[37] C. J. Ryan, A. M. Lin, and E. J. Small, "Angiogenesis inhibition plus chemotherapy for metastatic hormone refractory prostate cancer: history and rationale," *Urologic Oncology*, vol. 24, no. 3, pp. 250–253, 2006.

[38] J. Picus, S. Halabi, B. Rini, et al., "The use of bevacizumab with docetaxel and estramustine in hormone refractory prostate cancer: initial results of CALGB 90006," *Proceedings of the American Society of Clinical Oncology*, vol. 22, p. 392, 2003.

[39] Y. M. Ning, P. M. Arlen, J. L. Gulley, et al., "Phase II trial of thalidomide (T), bevacizumab (Bv), and docetaxel (Doc) in patients (pts) with metastatic castration-refractory prostate cancer (mCRPC)," *Journal of Clinical Oncology*, vol. 26, supplement 20, 2008, abstract 5000.

[40] "Docetaxel and Prednisone With or Without Bevacizumab in Treating Patients With Prostate Cancer That Did Not Respond to Hormone Therapy," October 2009, http://clinicaltrials.gov/ct2/show/NCT00110214?term=prostate+avastin+docetaxel&rank=3.

[41] J. Holash, S. Davis, N. Papadopoulos, et al., "VEGF-Trap: a VEGF blocker with potent antitumor effects," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11393–11398, 2002.

[42] H.-P. Gerber, T. H. Vu, A. M. Ryan, J. Kowalski, Z. Werb, and N. Ferrara, "VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation," *Nature Medicine*, vol. 5, no. 6, pp. 623–628, 1999.

[43] N. Ferrara, H. Chen, T. Davis-Smyth, et al., "Vascular endothelial growth factor is essential for corpus luteum angiogenesis," *Nature Medicine*, vol. 4, no. 3, pp. 336–340, 1998.

[44] Q. S.-C. Chu, "Aflibercept (AVE0005): an alternative strategy for inhibiting tumour angiogenesis by vascular endothelial growth factors," *Expert Opinion on Biological Therapy*, vol. 9, no. 2, pp. 263–271, 2009.

[45] N. Isambert, G. Freyer, S. Zanetta, et al., "A phase I dose escalation and pharmacokinetic (PK) study of intravenous aflibercept (VEGF trap) plus docetaxel (D) in patients (pts) with advanced solid tumors: preliminary results," *Journal of Clinical Oncology*, vol. 26, supplement 15, 2008, abstract 3599.

[46] "Aflibercept in Combination With Docetaxel in Metastatic Androgen Independent Prostate Cancer (VENICE)," October 2009, http://www.clinicaltrials.gov/ct2/show/NCT00519285?term=Aflibercept+prostate&rank=1.

[47] A. Arora and E. M. Scholar, "Role of tyrosine kinase inhibitors in cancer therapy," *Journal of Pharmacology and Experimental Therapeutics*, vol. 315, no. 3, pp. 971–979, 2005.

[48] S. Wilhelm and D.-S. Chien, "BAY 43-9006: preclinical data," *Current Pharmaceutical Design*, vol. 8, no. 25, pp. 2255–2257, 2002.

[49] S. M. Wilhelm, C. Carter, L. Tang, et al., "BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis," *Cancer Research*, vol. 64, no. 19, pp. 7099–7109, 2004.

[50] W. L. Dahut, C. Scripture, E. Posadas, et al., "A phase II clinical trial of sorafenib in androgen-independent prostate cancer," *Clinical Cancer Research*, vol. 14, no. 1, pp. 209–214, 2008.

[51] J. B. Aragon-Ching, L. Jain, J. L. Gulley, et al., "Final analysis of a phase II trial using sorafenib for metastaticcastration-resistant prostate cancer," *British Journal of Urology International*, vol. 103, no. 12, pp. 1636–1640, 2009.

[52] K. N. Chi, S. L. Ellard, S. J. Hotte, et al., "A phase II study of sorafenib in patients with chemo-naive castration-resistant prostate cancer," *Annals of Oncology*, vol. 19, no. 4, pp. 746–751, 2008.

[53] G. Colloca, F. Checcaglini, and A. Venturino, "About sorafenib in castration-resistant prostate cancer," *Annals of Oncology*, vol. 19, no. 10, pp. 1812–1813, 2008.

[54] K. N. Chi and L. Seymour, "Reply to the letter "About sorafenib in castration-resistant prostate cancer" by G. Colloca, F. Checcaglini and A. Venturino," *Annals of Oncology*, vol. 19, no. 10, pp. 1813–1814, 2008.

[55] S. Steinbild, K. Mross, A. Frost, et al., "A clinical phase II study with sorafenib in patients with progressive hormone-refractory prostate cancer: a study of the CESAR Central European Society for Anticancer Drug Research-EWIV," *British Journal of Cancer*, vol. 97, no. 11, pp. 1480–1485, 2007.

[56] M. R. Safarinejad, "Safety and efficacy of sorafenib in patients with castrate resistant prostate cancer: a Phase II study," *Urologic Oncology*, vol. 28, no. 1, pp. 21–27, 2010.

[57] L. Q. M. Chow and S. G. Eckhardt, "Sunitinib: from rational design to clinical efficacy," *Journal of Clinical Oncology*, vol. 25, no. 7, pp. 884–896, 2007.

[58] M. Dror Michaelson, M. M. Regan, W. K. Oh, et al., "Phase II study of sunitinib in men with advanced prostate cancer," *Annals of Oncology*, vol. 20, no. 5, pp. 913–920, 2009.

[59] S. R. Wedge, J. Kendrew, L. F. Hennequin, et al., "AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer," *Cancer Research*, vol. 65, no. 10, pp. 4389–4400, 2005.

[60] C. J. Ryan, W. M. Stadler, B. Roth, et al., "Phase I dose escalation and pharmacokinetic study of AZD2171, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinase, in patients with hormone refractory prostate cancer (HRPC)," *Investigational New Drugs*, vol. 25, no. 5, pp. 445–451, 2007.

[61] J. J. Karakunnel, J. L. Gulley, P. M. Arlen, et al., "Phase II trial of cediranib (AZD2171) in docetaxel-resistant, castrate-resistant prostate cancer (CRPC)," *Journal of Clinical Oncology*, vol. 26, supplement 15, 2008, abstract 5136.

[62] R. S. Kerbel, "Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents," *BioEssays*, vol. 13, no. 1, pp. 31–36, 1991.

[63] K. Hida, Y. Hida, D. N. Amin, et al., "Tumor-associated endothelial cells with cytogenetic abnormalities," *Cancer Research*, vol. 64, no. 22, pp. 8249–8255, 2004.

[64] O. Casanovas, D. J. Hicklin, G. Bergers, and D. Hanahan, "Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors," *Cancer Cell*, vol. 8, no. 4, pp. 299–309, 2005.

[65] K. D. Miller, C. J. Sweeney, and G. W. Sledge Jr., "The Snark is a Boojum: the continuing problem of drug resistance in the antiangiogenic era," *Annals of Oncology*, vol. 14, no. 1, pp. 20–28, 2003.

[66] B. I. Rini, V. Weinberg, L. Fong, S. Conry, R. M. Hershberg, and E. J. Small, "Combination immunotherapy with prostatic acid phosphatase pulsed antigen-presenting cells (provenge) plus bevacizumab in patients with serologic progression of

prostate cancer after definitive local therapy," *Cancer*, vol. 107, no. 1, pp. 67–74, 2006.

[67] "Late-Breaking Science Forum: Review of the Provenge Trial," October 2009, http://www.aua2009.org/program/lbsci-forum.asp.

[68] B. P. Schneider, M. Radovich, and K. D. Miller, "The role of vascular endothelial growth factor genetic variability in cancer," *Clinical Cancer Research*, vol. 15, no. 17, pp. 5297–5302, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 939407, 10 pages
doi:10.1155/2010/939407

Research Article

CXCL7-Mediated Stimulation of Lymphangiogenic Factors VEGF-C, VEGF-D in Human Breast Cancer Cells

Minghuan Yu,[1] Richard Berk,[2] and Mary Ann Kosir[1,3,4]

[1] *Department of Surgery, Wayne State University, Detroit, MI 48201, USA*
[2] *Department of Immunology and Microbiology, Wayne State University, Detroit, MI 48201, USA*
[3] *Surgical Service, John D. Dingell VA Medical Center, Detroit, MI 48201, USA*
[4] *Breast Biology Program, Karmanos Cancer Institute, Detroit, MI 48201, USA*

Correspondence should be addressed to Mary Ann Kosir, kosirm@karmanos.org

Received 1 November 2009; Revised 27 March 2010; Accepted 3 May 2010

Academic Editor: Arkadiusz Dudek

Increased expression of lymphangiogenesis factors VEGF-C/D and heparanase has been correlated with the invasion of cancer. Furthermore, chemokines may modify matrix to facilitate metastasis, and they are associated with VEGF-C and heparanase. The chemokine CXCL7 binds heparin and the G-protein-linked receptor CXCR2. We investigatedthe effect of CXCR2 blockade on the expression of VEGF-C/D, heparanase, and on invasion. CXCL7 siRNA and a specific antagonist of CXCR2 (SB225002) were used to treat CXCL7 stably transfected MCF10AT cells. Matrigel invasion assays were performed. VEGF-C/D expression and secretion were determined by real-time PCR and ELISA assay, and heparanase activity was quantified by ELISA. SB225002 blocked VEGF-C/D expression and secretion ($P < .01$). CXCL7 siRNA knockdown decreased heparanase ($P < .01$). Both SB225002 and CXCL7 siRNA reduced the Matrigel invasion ($P < .01$). The MAP kinase signaling pathway was not involved. The CXCL7/CXCR2 axis is important for cell invasion and the expression of VEGF-C/D and heparanase, all linked to invasion.

1. Introduction

Breast cancer is the most common malignancy in women, and metastasis is the number one cause of mortality in breast cancer [1]. Breast cancer treatments that target the steps of metastasis are needed. An important part of metastasis is invasion [2, 3]. Invasion depends in part on degradation of extracellular matrix (ECM) and interaction with molecules formed in the process. Recently, increasing attention has been paid to chemokines that may modify breast cancer cells and the surrounding matrix to facilitate metastasis. Furthermore, there is evidence that chemokines, VEGF-C, and heparanase are interrelated in the process of invasion [4–6]. Increased expression of the lymphangiogenesis factors and heparanase has been correlated with progressive disease in certain cancers [6]. Peritumoral lymphangiogenesis involves the secretion of specific glycoproteins designated vascular endothelial growth factor C (VEGF-C) and (VEGF-D) that act on lymphatic endothelium, and are components of an established signaling system for tumor lymphangiogenesis [7]. Increased lymph node metastases are correlated with increased expression of VEGF-D and VEGFR3 by immunohistochemistry in invasive breast cancer [8].

Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate (HS) side chains of heparin sulfate proteoglycan (HSPG). Heparanase activity has been correlated with cell invasion associated with breast cancer metastasis, a consequence of structural modification of HS that alters the extracellular matrix [9, 10]. However, classical mammalian heparanase is an intracellular enzyme and is not specific to metastatic sites [11–13]. Therefore, another source of heparanase may come from chemokines which are secreted.

Chemokines are a family of small molecular weight proteins. CXCL7 is a member of the CXC subfamily of chemokines which can be further subdivided on the basis of the presence of the tripeptide motif glutamate-leucine-arginine (ELR). All ELR^+-CXC chemokines act through CXC chemokine receptor type1 or type2 (CXCR1 and CXCR2) [14]. CXCL7, has dual functions of heparin binding and is a ligand to the G-protein linked receptor CXCR2 [15].

Until recently, CXCL7 gene expression was thought to be restricted to cells within the megakaryocytic lineage [16, 17], as well as to neutrophils and lymphocytes. Recent reports have suggested that other cell types may produce this chemokine as well [18–23]. Despite studies on increased expression of CXCR2 in breast cancer, reports on CXCL7 in breast cancer are limited.

Earlier studies from our laboratory have shown that the malignant breast cancer cells express more CXCL7 than premalignant MCF10AT cells. CXCL7-transfected MCF10AT breast cells have much higher heparanase activity than premalignant MCF10AT cells. CXCL7-transfected MCF10AT cells are as invasive as malignant breast cancer cells [24]. In addition, the expression of VEGF C and D is increased in these transfectants [5].

Heparanase and the lymphangiogenesis factors VEGF-C and VEGF-D are two important markers closely related to the metastasic capabilty of breast cancer. In this paper, we hypothesize that CXCL7 increases the expression of VEGF-C, VEGF-D, and heparanase, and increases cell invasion via CXCR2 signaling, all linked to tumor lymphangiogenesis and metastasis.

2. Methods

2.1. Cell Culture and Plasmid Stable Transfection. The MCF10AT cells [25] were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F 12 (1 : 1) containing 5% horse serum, supplemented with 10 μg/ml bovine insulin, EGF (20 ng/ml), hydrocortisone (0.5 μg/ml), and cholera toxin (100 ng/ml). Cells were cultured at 37°C in a 5% CO_2 atmosphere. All cell culture reagents were obtained from Gibco (Grand Island, NY). Approximately, 1×10^6 cells/well were plated in 6-well plates in medium containing 5% horse serum to grow overnight to 60%–70% confluency. Transfection of the plasmid was performed by using Lipofectamine 2000 (Invitrogen, USA). The cells were divided into blank control group, negative control group, and the test group. Only Lipofectamine 2000 was used for transfection in the blank control group. Plasmid $_P$EGFP-N1 was used for transfection in the negative control group. Plasmid $_P$EGFP-N1-CXCL7 was used for transfection in the test group. The cells were transfected with the mixture of plasmid and Lipofectamine 2000 (1:3) in 2 ml serum-free medium. At 24 hours after transfection, the medium was replaced by normal medium containing 5% FBS and antibiotics up to 72 hours post-transfection. Since the MCF10AT cells were transfected cells, G418 could not be used for the selection of the stably transfected cell line. Therefore, we submitted the cells for the flow cytometry sorting by using the EGFP antibody. The transfected cells were picked out for subculture. For sh-RNA plasmid transfection we used the same method the described above. The sh-PPBP plasmids were purchased from ORIGENE (Rockville, MD).

2.2. In Vitro Invasion Assays. Briefly, BioCoat Matrigel invasion chambers (Becton-Dickinson, Bedford, MA) were rehydrated according to the manufacturer's instructions. Cell suspensions (2.5×10^5 cells per 2 ml serum-free medium) were added to the top chamber, and complete medium in the lower chamber. For control, inserts without Matrigel were used. The cells were allowed to invade the Matrigel at 37°C in 5% CO_2 for 48 hours. The noninvading cells on the upper surface of membrane were removed from the chamber by gentle scrubbing with a cotton swab, and the invading cells on the lower surface of the membrane were stained with the Quick-Diff stain kit (Becton-Dickinson). After two washes with water, the chambers were allowed to air dry. Membranes were mounted on glass slides and counted manually under a light microscope. Cells were counted in five high power fields (40x magnification). The number of invading cells was expressed as a percentage by the following: the mean number of the cells invading through the Matrigel insert membrane divided by the mean number of cells migrating under control insert membrane conditions multiplied by 100. All assays were performed in triplicate.

2.3. Quantitative Real-Time PCR. RNA was extracted using the RNeasy Mini Kit (catalog no. 74,804; Qiagen, Valencia, CA), including treatment with DNase I to prevent genomic DNA contamination using RURBO DNA-free Kit (catalog no. AM1907; Ambion, Foster City, CA) according to the manufacturer's instructions. Total RNA (two micrograms) was reverse-transcribed to cDNA by using the SuperScript III First-Strand Synthesis System (catalog no. 18080-051; Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Three replicate samples were used for the three cell lines. The primer sequences used for the reactions are in Table 1 along with expected products and GenBank accession numbers. The thermocycler parameters were as follows: an initial step at 95°C for 10 min., 40 cycles of 95°C for 20 sec., 58°C for 30 sec., and 72°C for 20 seconds. The cycle threshold values were used to calculate the normalized expression of VEGFC/D against β-actin. qRT-PCR was performed in ABI 7500 Sequence Detection System using a SYBR Green detection system (catalog no. 170- 8880; Bio-Rad Laboratories). By identifying the threshold cycle (C_T) for expression of mRNA, the ΔC_T for VEGF-C/D was calculated and compared to vector transfected control cells; CXCL7 transfected cells and SB225002-treated CXCL7 transfected cells. The ΔC_T was converted into a ratio (target A/target B = $2^{-\Delta CT}$) describing the comparison of the relative expression of VEGF-C/D (target A) to β-actin (target B) for each of the lines. The Delta-delta model was used for comparison of relative expression RT-PCR results for VEGF-C/D.

2.4. Protein Quantification. VEGF-C and VEGF-D protein concentrations in conditioned media were measured by enzyme-linked immunosorbent assay (ELISA) using human VEGF-C or VEGF-D ELISA Development System (catalog no. DVEC 00 and DVED00, R&D Systems, Minneapolis, MN). Measurements were done at least in duplicate for 2 dilutions. The optical density at 570 nm and 450 nm was determined for each well using the plate reader. Then the reading at 570 nm was subtracted from the reading at 450 nm for each well.

Table 1: Summary of primers for Real-Time PCR.

Gene: VEGF-C
GenBank Accession No.: NM_005429
Product size: 128 bp
Primers:
Forward: 5′-GCCACGGCTTATGCAAGCAAAGAT-3′
Reverse: 5′-AGTTGAGGTTGGCCTGTTCTCTGT -3′
Gene: VEGF-D
GenBank Accession No.: NM_004469
Product size: 132 bp
Primers:
Forward: 5′-CGATGTGGTGGCTGTTGCAATGAA -3′
Reverse: 5′-GCTGTTGGCAAGCACTTACAACCT -3′
Gene: beta-actin
GenBank Accession No.: X00351
Product size: 125 bp
Primers:
Forward: 5′-GGACTTCGAGCAAGAGATGG-3′
Reverse: 5′- AGCACTGTGTTGGCGTACAG -3′

2.5. Heparanase Activity Measurement. The protein concentration of the serum-free conditioned medium was measured by the Bradford assay (BioRad, Richmond, CA, USA). The heparanase activity of the conditioned medium was assessed as heparan sulfate degrading enzyme activity using the Heparan Degrading Enzyme Assay Kit (catalog no. MK412, Takara, Ootsu, Japan). The duration of a series of assays was 100 min., including 45 min. of enzyme degradation reaction. One unit of heparanase activity defined the activity which degraded 0.063 ng of biotinylated heparin sulfate in 1 min. at 37°C and pH 5.8. The detection limit of this assay was 0.1 U/mL. Each value of heparanase activity was normalized by protein concentration (U/g protein).

2.6. Western Blotting. Cells were processed for protein extraction and western blotting using standard procedures. Briefly, the cells were harvested in PBS, counted, and lysed in the RIPA buffer (catalog no. R0278, SIGMA, St. Louis, MO) with protease inhibitor cocktail (catalog no. P8340, SIGMA, St. Louis, MO), and samples were kept at 4°C. Protein concentration was determined for all samples using the Bio-Rad protein assay (catalog no. 500-0006, Bio-Rad, Richmond, CA). The equal-volume samples (50 μg) were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto nitrocellulose membrane (catalog no. 162-0114, Bio-Rad, Richmond, CA) using transfer tank. Immunodetection was performed using pERK1/2 (catalog no. 9101, Cell Signaling Technology) and ERK1/2 (catalog no.9102, Cell Signaling Technology), then developed by ECL (catalog no. PRN 2106, GE Healthcare, Waukesha, WI) and was photodocumented.

2.7. Statistical Analysis. Results are expressed as mean ± standard error of the mean (SEM), unless indicated otherwise. For statistical analysis, one-way ANOVA was used, and significance was defined as $P < .05$. Graphs were generated using GraphPad Prism (GraphPad Software, San Diego, CA).

3. Results

3.1. CXCR2 Antagonist SB225002 Reduced Invasion of Matrigel by Stably CXCL7-Transfected MCF10AT Cells. To further verify CXCL7 function in cell invasion, a selective nonpeptide CXCR2 antagonist SB225002 [26] was used to check whether it can inhibit the invasive ability of MCF10AT cells transfected with CXCL7. MCF10AT cells were stably transfected with CXCL7 plasmid. After 48 hours of incubation, cells were added into the Matrigel invasion chamber; meanwhile, SB225002 (1.1 μM) was also added into the chamber. Several concentrations of SB225002 were tested (0.5, 0.7, 0.9, 1.1, 1.3, and 1.5 μM) and 1.1 μM had the best inhibitory effect similar to that reported by Levashove et al. [23]. After 48 hours, cells were fixed and stained with Diff-Quick. The decrease in the percent invasion of Matrigel by the cells is shown in Figure 1 . Treatment with SB225002 resulted in 18.06% ± 0.76% invasive cells compared with 55.7% ± 1.4% in nontreated CXCL7 stable transfected MCF10AT cells. Thus, the CXCR2 antagonist blocked the invasive ability of CXCL7 stably transfected MCF10AT cells.

3.2. Expression and Secretion of Lymphangiogenesis Factor VEGF-C and VEGF-D by Stable CXCL7 Transfected MCF10AT Cells Are Blocked by SB225002. To investigate the effect of SB225002 on expression and secretion of VEGF-C and VEGF-D by stable CXCL7-transfected MCF10AT cells, we cultured the cells with and without SB225002 (1.1 μM). After 48-hour treatment, the media and the cells were collected. Expression of mRNA for lymphangiogenic factors VEGF-C and VEGF-D was determined by quantitative real-time polymerase chain reaction (qRT-PCR) assay. The expression levels of mRNAs for VEGF-C, VEGF-D, are shown in Figure 2(a). β-actin was used as an internal control. Compared with vector-transfected MCF10AT cells, CXCL7-transfected MCF10AT cells showed 11-fold higher VEGF-C and 18-fold higher VEGF-D expression. Administration of SB225002 antagonist resulted in a significant ($P < .01$) inhibition of VEGF-C (3.5-fold) and VEGF-D (3-fold) mRNA expression. Next, we examined the secretion of the VEGF-C and VEGF-D by ELISA assay. The secretion levels of VEGF-C, VEGF-D are shown in Figure 2(b). Both VEGF-C and VEGF-D secretion from the CXCL7-transfected cells were significantly ($P < .01$) increased compared with the vector-transfected control group (VEGF-C, 3-fold; VEFG-D, 2.5-fold). Our data also showed a significant ($P < .05$) inhibition in VEGF-C and VEGF-D secretion by SB225002-treated cells compared with control treated cells. Thus the CXCR2 antagonist decreases VEGF-C and VEGF-D mRNA and protein expression.

3.3. CXCL7 siRNA Inhibited Heparanase Activity and Invasion of CXCL7 Stably Transfected Cells. Next, we examined whether CXCL7 siRNA inhibited the elevated heparanase

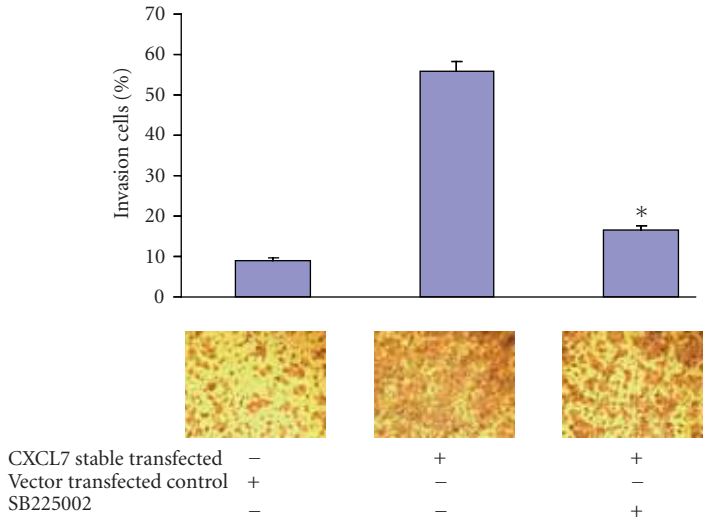


Figure 1: CXCR2 antagonist SB225005 reduced the invasion of MCF10AT stably transfected with CXCL7 using a BD BioCoat Matrigel invasion assay (6-well plates), 1.25 x 10^5 cells/ml were inoculated onto the membrane in serum-free medium. For the blocking experiment, there was serum-free medium containing SB225002 (1.1 μM) in the upper chamber. The lower chamber contained complete medium, and the control membrane did not have Matrigel by which to measure the migration of cells. After 48 hours, membranes were fixed, stained, and photographed, then the percent invasion was determined. Triplicate assays were done. Results are reported per cell line as percent invasion ± (mean number of cells invading Matrigel membrane/mean number of cells migrating through control membrane) ×100 ($P < .01$). (Upper) SB225002 significantly decreased the invasion of CXCL7-stable transfected MCF10AT cells compared with notreated cells. (Lower) Membranes were stained by Diff-Quik kit and were photographed (original magnification x 20).

activity of CXCL7-stably transfected cells. Cells were transfected with CXCL7 siRNA or control siRNA, and after 48 hours, the CM was collected. Compared with control siRNA treatment, CXCL7 siRNA-transfected cells showed significant ($P < .01$) inhibition of heparanase enzymatic activity (Figure 3(a)). The same results were obtained after using a Microcon filter to remove molecules less than 30 kD (which includes CXCL7) from CM, verifying by ELISA that CXCL7 is not present after filtering. And then, retesting for heparanase activity were performed.

To further confirm the increased invasion after transfection by CXCL7, we used the CXCL7 siRNA to block the CXCL7 signal. Matrigel invasion assays were performed with cells transfected with CXCL7 siRNA or control siRNA. The decrease in the percent invasion of Matrigel by the cells is shown in Figure 3(b). Treatment with siRNA resulted in 16% ± 1.2% invasive cells compared with 37% ± 2.5% by MCF10AT cells transfected with CXCL7. Thus, CXCL7 siRNA inhibits the invasion of MCF10AT cells transfected with CXCL7.

3.4. Expression and Secretion of Lymphangiogenesis Factor VEGF-C and VEGF-D by Stable CXCL7 Transfected MCF10AT Cells Are Silenced by sh-PPBP Transfection. To further confirm the increased expression and secretion of lymphangiogenesis factors VEGF-C and VEGF-D after transfection by CXCL7, we used the sh-PPBP to silence the CXCL7 signal. To investigate the effect of sh-PPBP on expression and secretion of VEGF-C, VEGF-D of stable CXCL7-transfected MCF10AT cells, we cultured the cells transfected with sh-PPBP or control (sh-con). After 48 hours of transfection, the media and the cells were collected. Expression of the mRNA of lymphangiogenic factors VEGF-C and VEGF-D was determined by quantitative real-time polymerase chain reaction (qRT-PCR) assay. The expression levels of mRNAs for VEGF-C, VEGF-D, are shown in Figure 4(a). β-actin was used as an internal control. Compared with vector-transfected MCF10AT cells, CXCL7-transfected MCF10AT cells showed 11-fold higher VEGF-C and 18-fold higher VEGF-D expression. The sh-PPBP transfection resulted in a significant ($P < .01$) silencing of VEGF-C (66%) and VEGF-D (68%) mRNA expression. Next, we examined the secretion of the VEGF-C and VEGF-D by ELISA assay. The secretion levels of VEGF-C, VEGF-D are shown in Figure 4(b). Both VEGF-C and VEGF-D secretion from the CXCL7-transfected cells were significantly ($P < .01$) increased compared with the vector-transfected control group (VEGF-C, 3.3-fold; VEFG-D, 4.4-fold). Our data also showed a significant ($P < .05$) silencing in VEGF-C (65%) and VEGF-D (68%) secretion by sh-PPBP transfected cells compared with sh-con transfected cells.

3.5. ERK1/2 Mitogen-Activated Protein Kinase Pathway Is not Involved in CXCL7-CXCR2-Mediated Stimulation of Lymphangiogenic Factors VEGF-C, VEGF-D in Human Breast Cancer Cells. The MAP kinase pathway is important for growth, differentiation, and migration, and is considered a dominant signaling pathway for CXCR2 [27, 28]. To investigate the mechanism by which the CXCL7-CXCR2 axis is involved in the stimulation of lymphangiogenic factors VEGF-C, VEGF-D toward invasion by human breast cancer cells, we investigated the involvement of ERK1/2

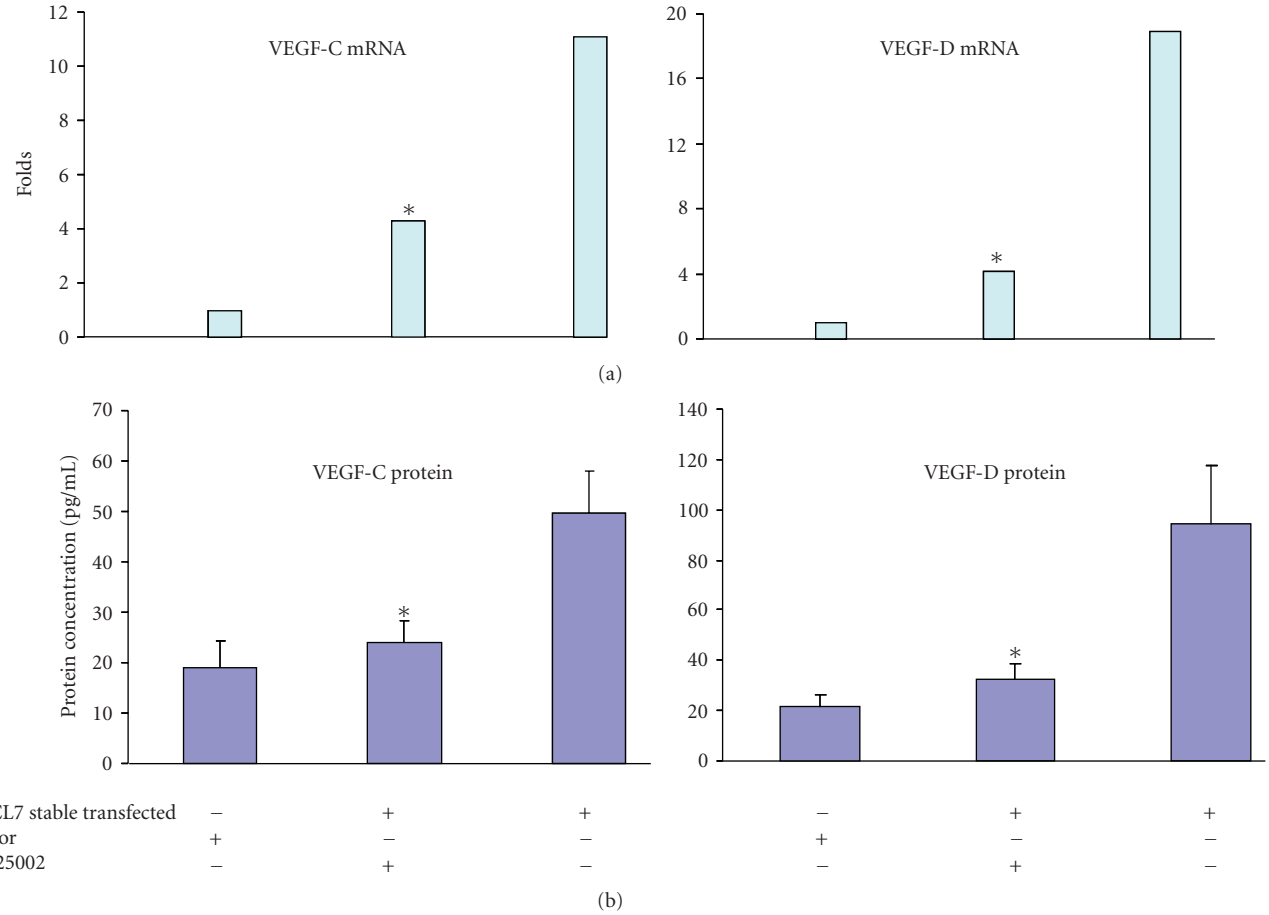


Figure 2: SB225005 blocked VEGF-C and VEG-D mRNA expression by CXCL7-transfected MCF10AT cells. CXCL7-stably transfected MCF10AT cells were cultured with and without SB225002 (1.1 μM). After 48-hour treatment, the media and the cells were collected. (a) Quantitative analysis of VEGF-C and VEGF-D mRNA expression. Total RNA was extracted, and real-time qRT-PCR was performed. β-actin was applied as internal control. Triplicate determinations were performed. The differences between the cell lines were significant ($P < .01$). (b) Quantitative analysis of VEGF-C and VEGF-D protein secretion by the CXCL7-stable transfected MCF10AT cells. Protein concentration in CM was measured by ELISA using human VEGF-C and VEGF-D ELISA Development System. Measurements were done at least in duplicate for 2 dilutions. The optical density of each well was determined using plate reader by subtracting the reading at 570 nm from the reading at 450 nm. VEGF-C/D content in CM of SB225002-treated group was significantly lower than untreated group ($P < .05$).

MAP kinases by western blotting. Stable CXCL7-transfected MCF10AT cells did not induce ERK1/2 phosphorylation in comparison with the vector transfected control cells, and there is no difference in ERK1/ 2 MAP kinase expression in stable CXCL7 transfected MCF10AT cells compared to vector controls (Figure 5).

4. Discussion

Although many molecules have been implicated in cancer metastasis, the detailed mechanism of tumor metastasis is still not completely understood. Recently, the interest in chemokines in cancer research has been increasing as new chemokines are being identified and investigated. Working along with many other molecules, the chemokines and their receptors expressed by both the cancer and its stroma influence growth, dormancy, angiogenesis, and invasion [3, 6, 29]. The CXCR4/CXCL12 (SDF-1) axis was the most common interaction that has been shown to be involved in many different human malignancies, including breast cancer, ovarian cancer, and prostate cancer [30, 31]. However, CXCR4 interactions alone did not completely explain the pattern of metastasis of cancer.

There are 4 families of chemokines (C, CC, CXC, and CX3C) based on the arrangement of cysteine residues near the N terminus [32, 33]. The CXC group can be further subdivided on the basis of presence of the tripeptide motif glutamate-leucine-arginine (ELR) adjacent to the CXC motif. All ELR^+-CXC chemokines act through CXC chemokine receptor type 1 or type 2 (CXCR1 or CXCR2), which are transmembrane G-protein-coupled receptors [32]. ELR^+-CXC chemokines can stimulate angiogenesis. Those without the ELR motif inhibit angiogenesis [34, 35]. Secretion of stromal cell-derived factor-1 (SDF-1)/CXCL12 and expression of CXCR4 have been identified to be associated with breast cancer metastasis [36]. Although CXCR4 was more highly expressed in the breast cancer cells tested by

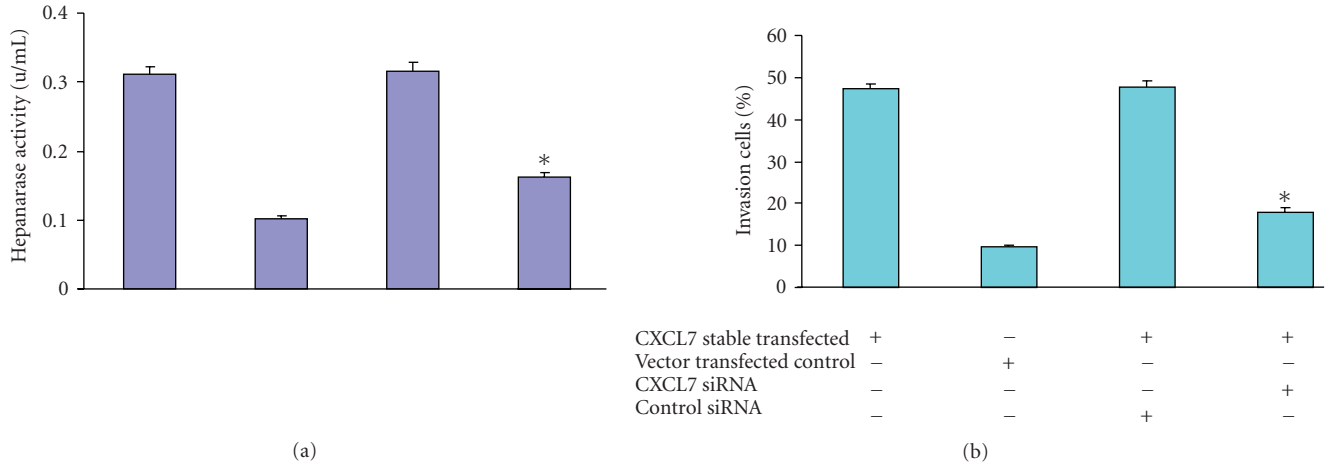


Figure 3: CXCL7 siRNA decreased heparanase activity and invasion of CXCL7-stably transfected cells. The CXCL7 MISSION siRNA was used to effectively knockdown heparanase activity and the invasion of CXCL7-stably transfected cells while the nontargeting siRNA was used as a control. CXCL7-stable transfected MCF10AT cells (2×10^5) were transfected with 100 pmol of each siRNA by using the Lipofectamine 2000 transfection reagent (Invitrogen) after 48 hours in culture. (a) Quantifying heparanase activity. The CM was collected 24 hours later and analyzed by heparanase-degrading enzyme assay kit. Indicated amounts of cell lysate were incubated with biotinylated heparan sulfate at 37°C for 45 minutes, and enzyme activity was determined using an ELISA-type assay. Color was developed using the substrate supplied in the kit, and plates were read at 450 nm using a microplate reader. Decreased heparanase activity in CXCL7 siRNA. (b) Invasion of Matrigel. Using a BD BioCoat Matrigel invasion assay (6-well plates), 1.25×10^5 cells/ml were inoculated onto the membrane in the upper chamber in serum-free medium. The lower chamber contained complete medium, and control membrane did not have Matrigel to measure migration of cells. Triplicate assays per group were completed. Results reported per cell line as % invasion = (mean number of cells invading Matrigel membrane/mean number of cells migrating through control membrane) ×100. CXCL7 siRNA significantly inhibited the invasion of CXCL7 stable transfected MCF10AT cell, while the control siRNA did not. Results compared by one-way ANOVA ($P < .01$).

Müller et al. [37] than the other CXCRs, the expression of CXCR2 was also increased compared with the remaining receptors. CXCL7, which is a member of the ELR-CXC chemokines, binds with CXCR2 receptors, stimulating angiogenesis and association with neutrophils and other immune components [38]. Inhibition of CXCR2 function on endothelial cells has been shown to inhibit tumor growth, for lung cancer and renal cell cancer models [39, 40].

The use of a small selective antagonist for CXCR2 (SB225002) represents an attractive targeted therapeutic approach [26]. Previous work in our laboratory showed that breast cancer cells coexpress CXCL7 and CXCR2, which may act as a potential autocrine mechanism in breast cancer. The malignant cell line MCF10CA1a.cl1 strongly expressed CXCL7 and has much higher invasive ability than MCF10AT. MCF10AT cells gained invasive ability after they were transfected with CXCL7. Thus, CXCL7-transfected MCF10AT cells were as invasive as malignant cells, suggesting that CXCL7 may have a role in the invasion process. Therefore, targeting its receptor, CXCR2, seemed an obvious choice.

By using CXCL7-stably transfected MCF10AT cells treated by SB225002, invasion was decreased significantly compared with CXCL7-transfected cells, which might involve CXCL7 autocrine activity with CXCR2. The present study did not examine whether SB225002 could block the invasive ability of isogenic malignant cells (MCF10CA1a.cl1) or other malignant cell lines, which would be an interesting focus for future study.

Lymphangiogenesis refers to the formation of new lymphatic vessels that may occur in normal developing tissues or in tumors. Overexpression of VEGF-C or VEGF-D can lead to lymphangiogenesis, intralymphatic tumor growth and formation of lymph node metastases [41, 42]. VEGF-C and VEGF-D are ligands for VEGFR-3 (also termed fms-like tyrosine kinase 4, Flt-4), a tyrosine kinase receptor that is expressed predominantly in lymphatic endothelial cells [43]. The breast cancer cells secrete VEGF-C, VEGF-D which directly interact with the receptor. This paracrine relationship may lead to further changes in the breast cancer cells leading to invasion. Chemokine and VEGF-C interactions have been reported in a model of cross-talk for lymphatic endothelial cells and melanoma cells [4]. In our study, both the selective CXCR2 antagonist and sh-PPBP suppresses the elevated expression and secretion of VEGFC/D by the CXCL7-transfected MCF10AT cells. These results support the notion that the CXCL7/CXCR2 axis plays an important role in cancer cell lymphangiogenesis.

The chemokine connective tissue-activating peptide (CTAP-III), which is an N-truncated derivative of CXCL7, has been reported to have heparanase activity [15, 44]. The heparanase activity of chemokines may be important for modification of matrix [15]. Heparanase activity is also responsible for the egress of metastatic tumor cells and other blood-borne cells from the vasculature. Inhibition of heparanase activity results in decreased metastasis. Recently, expression of VEGF-C was shown to be induced

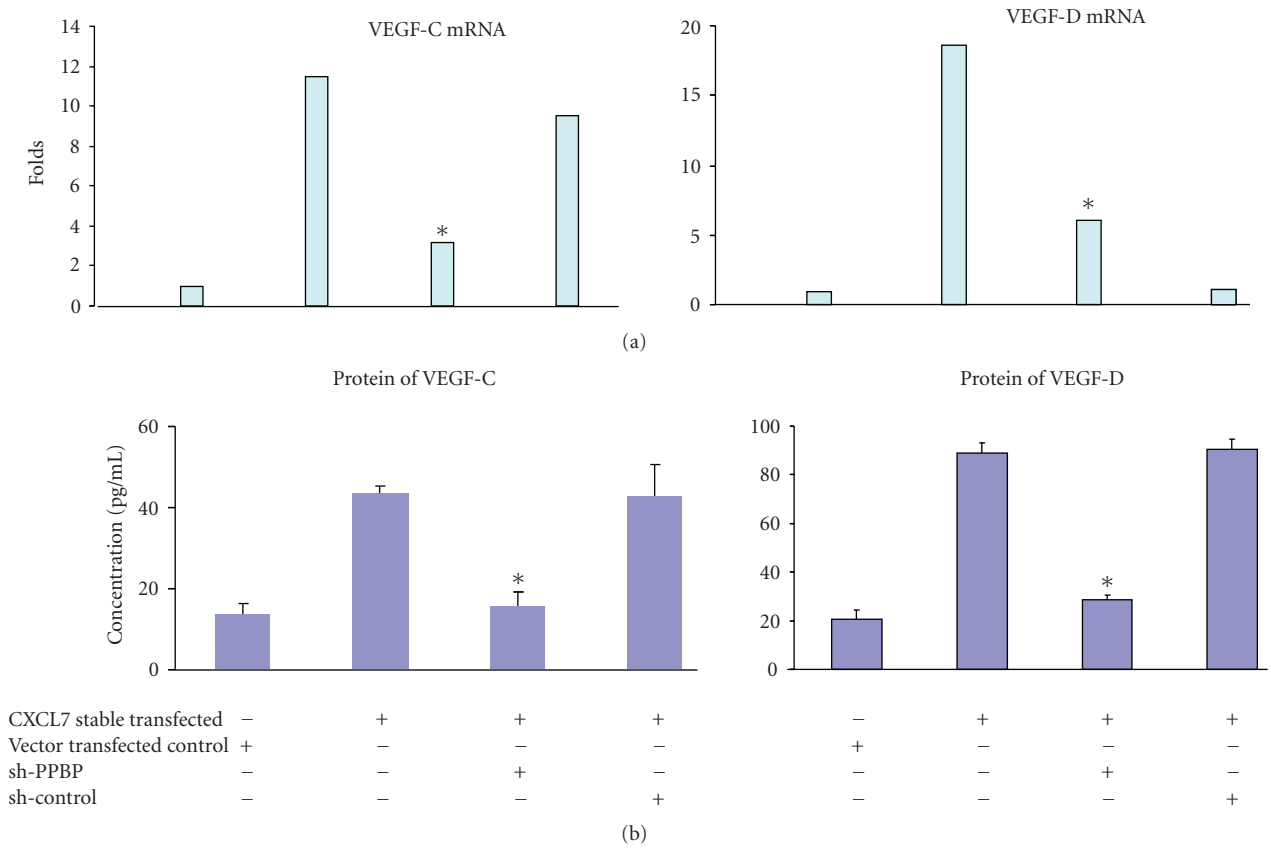


Figure 4: The sh-PPBP suppress VEGF-C and VEGF-D mRNA expression and secretion by CXCL7-transfected MCF10AT cells. We cultured the cells transfected with sh-PPBP or control sh-con. After 48 hours treatment, the media and the cells were collected. (a) Quantitative analysis of VEGF-C and VEGF-D mRNA expression. Total RNA was extracted, and real-time qRT-PCR was performed. β-actin was applied as internal control. Triplicate determinations were performed. The differences between the cell lines were significant ($P < .01$). (b) Quantitative analysis of VEGF-C and VEGF-D protein secretion by the CXCL7 stable transfected MCF10AT cells. Protein concentration in CM was measured by ELISA using human VEGF-C and VEGF-D ELISA Development System. Measurements were done at least in duplicate for 2 dilutions. The optical density of each well was determined using plate reader by subtracting the reading at 570 nm from the reading at 450 nm. VEGF-C and VEGF-D content in CM of sh-PPBP-transfected group was significantly lower than the control group ($P < .05$).

by heparanase in prostate cancer cells, epidermoid cancer cells, breast cancer cells, and melanoma [6]. In this study, heparanase activity in the CM of CXCL7-transfected MCF10AT cells was tested, and it was determined that the CXCL7-transfected cells demonstrated increased secreted heparanase activity. This elevated activity was inhibited by CXCL7 siRNA. In addition, silencing CXCL7 inhibited the invasive ability of CXCL7-transfected MCF10 AT cells which further elucidates its role in invasion. The expression of VEGF-C and VEGF-D mRNA and protein was also decreased in these transfectants by shRNA, thus linking the expression of the chemokine CXCL7 to VEGF-C and VEGF-D, heparanase expression, and invasive ability. The MCF10 model of progressive breast disease provides isogenic cell lines with increasing malignant potential to study the steps of metastasis. Additional breast cancer cell lines and breast cancer tissue can be tested specifically for CXCL7 expression and effects on lymphangiogenesis, heparanase expression and invasion in the future. Lymphangiogenesis in human breast cancer samples can be correlated with clinical parameters and CXCL7/CXCR2 staining.

To explain the mechanism by which the CXCL7-CXCR2 axis is involved in the stimulation of lymphangiogenic factors VEGF-C, VEGF-D, increased invasion and heparanase expression in human breast cancer cells. We investigated the MAP kinase signaling pathway [45, 46]. We demonstrated that CXCL7-stable transfected MCF10AT cells do not activate ERK1/2 MAP kinase signaling. We did not observe a difference of ERK1/2 kinase in CXCL7-stable transfected MCF10AT cells compared with control cells. This result suggests the involvement of different pathways other than MAP kinase signaling for the CXCL7-CXCR2 axis activation. In general, the activation of G-protein coupled receptors (GPCRs) like CXCR2 leads to the dissociation of the α subunit from the β,γ-dimer when GDP is replaced by GTP [47]. Both subunits can activate many signaling pathways including phospholipase C and adenyl cyclase. When specifically considering CXCR2, information from CXCL8 might shed some light on other potential pathways for CXCL7 since these two chemokines have 48% identity in their amino acid sequences and both bind CXCR2 [48]. CXCL8 binding to CXCR2 activates

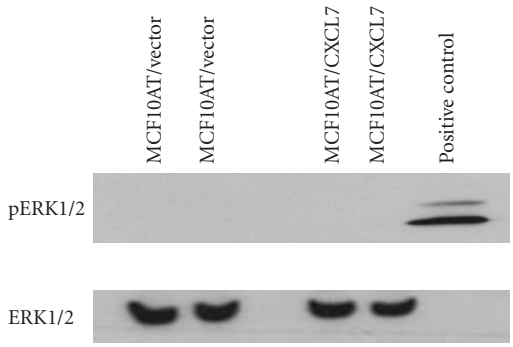


Figure 5: ERK1/2 mitogen-activated protein kinase is not active in CXCL7 stable transfected MCF10AT cells. Briefly, the cells were harvested in PBS, counted and lysed in the RIPA buffer with protease inhibitor cocktail. Protein concentration was determined for all samples using the Bio-Rad protein assay. The equal-volume samples (50 μg) were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto nitrocellulose membrane. Immunodetection was performed using pERK1/2 and ERK1/2, then developed by ECL. Stable CXCL7 transfected MCF10AT cells did not induce ERK1/2 phosphorylation compared to the vector transfected control cells. Furthermore, the expression of ERK1/2 was the same in stable CXCL7 transfected MCF10AT cells compared to vector controls.

the Rac/PI3K, Rho and Ras pathways [49]. Therefore, the CXCL7/CXCR2 axis may activate pathways other than the MAP kinase pathway, including Rac/PI3K, Rho and Ras pathways.

5. Conclusions

In the present paper we showed that both the selective CXCR2 antagonist SB225002 and sh-PPBP suppress VEGF-C, VEGF-D expression and secretion in CXCL7-transfected MCF10AT cells. Furthermore, we also observed that both SB225002 and CXCL7 siRNA reduced the invasion of CXCL7-stably transfected MCF10AT cells, confirming the role of the CXCL7/ CXCR2 axis in cell invasion, possibly through the receptor's signaling mechanism. However, this does not involve the MAP kinase signaling pathway as has been described for other ELR^+ CXC chemokines like IL-8. It has also been shown that CXCL7 siRNA knocked down heparanase activity in transfected MCF10AT cells, suggesting an important role for CXCL7 in heparanase expression. Taken together, these data would support that the CXCL7/CXCR2 axis may be important in breast cancer metastasis. Therapeutics aimed at antagonizing CXC chemokine action, including CXCL7, may be beneficial in preventing invasion and thus the spread of breast cancer.

Acknowledgments

The authors thank Beverly Torok-Storb, M.D., Ph.D., at the Fred Hutchinson Cancer Research Center, for the gift of CXCL7pIRES-enhanced green fluorescence protein plasmid, and Dr. Fred Miller for ongoing discussion about breast cancer models. This paper was supported by funding from a VA Merit Review grant (M. A. Kosir) and research funding from the Office of Vice President of Research (OVPR) at Wayne State University (M.A.Kosir).

References

[1] A. Jemal, R. Siegel, E. Ward et al., "Cancer statistics, 2008," *CA Cancer Journal for Clinicians*, vol. 58, no. 2, pp. 71–96, 2008.

[2] W. Wang, S. Goswami, K. Lapidus et al., "Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors," *Cancer Research*, vol. 64, no. 23, pp. 8585–8594, 2004.

[3] W. Wang, J. B. Wyckoff, S. Goswami et al., "Coordinated regulation of pathways for enhanced cell motility and chemotaxis is conserved in rat and mouse mammary tumors," *Cancer Research*, vol. 67, no. 8, pp. 3505–3511, 2007.

[4] A. Issa, T. X. Le, A. N. Shoushtari, J. D. Shields, and M. A. Swartz, "Vascular endothelial growth factor-C and C-C chemokine receptor 7 in tumor cell-lymphatic cross-talk promote invasive phenotype," *Cancer Research*, vol. 69, no. 1, pp. 349–357, 2009.

[5] M. Yu, Z. Tang, S. Alousi, R. S. Berk, F. Miller, and M. A. Kosir, "Expression patterns of lymphangiogenic and angiogenic factors in a model of breast ductal carcinoma in situ," *American Journal of Surgery*, vol. 194, no. 5, pp. 594–599, 2007.

[6] V. Cohen-Kaplan, I. Naroditsky, A. Zetser, N. Ilan, I. Vlodavsky, and I. Doweck, "Heparanase induces VEGF C and facilitates tumor lymphangiogenesis," *International Journal of Cancer*, vol. 123, no. 11, pp. 2566–2573, 2008.

[7] T. Julian, V. Fourchotte, S. Anderson, et al., "Predictive factors that identify patients not requiring a sentinel node biopsy: continued analysis of the NSABP B-32 sentinel node trial. Poster 2003," San Antonio Breast Cancer Symposium, December 2006.

[8] Y. Nakamura, H. Yasuoka, M. Tsujimoto et al., "Flt-4-positive vessel density correlates with vascular endothelial growth factor-D expression, nodal status, and prognosis in breast cancer," *Clinical Cancer Research*, vol. 9, no. 14, pp. 5313–5317, 2003.

[9] E. Zcharia, S. Metzger, T. Chajek-Shaul et al., "Molecular properties and involvement of heparanase in cancer progression and mammary gland morphogenesis," *Journal of Mammary Gland Biology and Neoplasia*, vol. 6, no. 3, pp. 311–322, 2001.

[10] I. Vlodavsky and Y. Friedmann, "Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis," *Journal of Clinical Investigation*, vol. 108, no. 3, pp. 341–347, 2001.

[11] V. Vreys and G. David, "Mammalian heparanase: what is the message?" *Journal of Cellular and Molecular Medicine*, vol. 11, no. 3, pp. 427–452, 2007.

[12] O. Goldshmidt, E. Zcharia, R. Abramovitch et al., "Cell surface expression and secretion of heparanase markedly promote tumor angiogenesis and metastasis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 15, pp. 10031–10036, 2002.

[13] N. Komatsu, M. Waki, M. Sue et al., "Heparanase expression in B16 melanoma cells and peripheral blood neutrophils before and after extravasation detected by novel anti-mouse heparanase monoclonal antibodies," *Journal of Immunological Methods*, vol. 331, no. 1-2, pp. 82–93, 2008.

[14] A. Ludwig, F. Petersen, S. Zahn et al., "The CXC-chemokine neutrophil-activating peptide-2 induces two distinct optima of neutrophil chemotaxis by differential interaction with interleukin- 8 receptors CXCR-1 and CXCR-2," *Blood*, vol. 90, no. 11, pp. 4588–4597, 1997.

[15] A. J. Hoogewerf, J. W. Leone, I. M. Reardon et al., "CXC chemokines connective tissue activating peptide-III and neutrophil activating peptide-2 are heparin/heparan sulfate-degrading enzymes," *Journal of Biological Chemistry*, vol. 270, no. 7, pp. 3268–3277, 1995.

[16] E. Brandt, A. Ludwig, F. Petersen, and H.-D. Flad, "Platelet-derived CXC chemokines: old players in new games," *Immunological Reviews*, vol. 177, pp. 204–216, 2000.

[17] C. Zhang, P. Gadue, E. Scott, M. Atchison, and M. Poncz, "Activation of the megakaryocyte-specific gene protein (PBP) by the Ets family factor PU.1," *Journal of Biological Chemistry*, vol. 272, no. 42, pp. 26236–26246, 1997.

[18] N. Hda, M. Haisa, A. Igarashi, D. Pencev, and G. R. Grotendorst, "Leukocyte-derived growth factor links the PDGF and CXC chemokine families of peptides," *FASEB Journal*, vol. 10, no. 11, pp. 1336–1345, 1996.

[19] A. Walz and M. Baggiolini, "A novel cleavage product of β-thromboglobulin formed in cultures of stimulated mononuclear cells activates human neutrophils," *Biochemical and Biophysical Research Communications*, vol. 159, no. 3, pp. 969–975, 1989.

[20] A. Schaffner, C. C. King, D. Schaer, and D. G. Guiney, "Induction and antimicrobial activity of platelet basic protein derivatives in human monocytes," *Journal of Leukocyte Biology*, vol. 76, no. 5, pp. 1010–1018, 2004.

[21] A. El-Gedaily, G. Schoedon, M. Schneemann, and A. Schaffner, "Constitutive and regulated expression of platelet basic protein in human monocytes," *Journal of Leukocyte Biology*, vol. 75, no. 3, pp. 495–503, 2004.

[22] L. Kruidenier, T. T. Macdonald, J. E. Collins, S. L. F. Pender, and I. R. Sanderson, "Myofibroblast matrix metalloproteinases activate the neutrophil chemoattractant CXCL7 from intestinal epithelial cells," *Gastroenterology*, vol. 130, no. 1, pp. 127–136, 2006.

[23] Z. B. Levashova, N. Sharma, O. A. Timofeeva, J. S. Dome, and A. O. Perantoni, "ELR$^+$-CXC chemokines and their receptors in early metanephric development," *Journal of the American Society of Nephrology*, vol. 18, no. 8, pp. 2359–2370, 2007.

[24] Z. Tang, M. Yu, F. Miller, R. S. Berk, G. Tromp, and M. A. Kosir, "Increased invasion through basement membrane by CXCL7-transfected breast cells," *American Journal of Surgery*, vol. 196, no. 5, pp. 690–696, 2008.

[25] P. J. Dawson, S. R. Wolman, L. Tait, G. H. Heppner, and F. R. Miller, "MCF10AT: a model for the evolution of cancer from proliferate breast disease," *American Journal of Pathology*, vol. 148, no. 1, pp. 313–319, 1996.

[26] J. R. White, J. M. Lee, P. R. Young et al., "Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration," *Journal of Biological Chemistry*, vol. 273, no. 17, pp. 10095–10098, 1998.

[27] K. L. Dunn, P. S. Espino, B. Drobic, S. He, and J. R. Davie, "The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling," *Biochemistry and Cell Biology*, vol. 83, no. 1, pp. 1–14, 2005.

[28] S. Yoon and R. Seger, "The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions," *Growth Factors*, vol. 24, no. 1, pp. 21–44, 2006.

[29] B. S. Yadav, S. C. Sharma, R. Singh, G. Singh, and V. Kumar, "Postmastectomy radiation and survival in patients with breast cancer," *Journal of Cancer Research and Therapeutics*, vol. 3, no. 4, pp. 218–224, 2007.

[30] A. Muller, B. Homey, H. Soto et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[31] A. Zlotnik, "Involvement of chemokine receptors in organ-specific metastasis," *Contributions to Microbiology*, vol. 13, pp. 191–199, 2006.

[32] T. Kakinuma and S. T. Hwang, "Chemokines, chemokine receptors, and cancer metastasis," *Journal of Leukocyte Biology*, vol. 79, no. 4, pp. 639–651, 2006.

[33] D. Rossi and A. Zlotnik, "The biology of chemokines and their receptors," *Annual Review of Immunology*, vol. 18, pp. 217–243, 2000.

[34] R. M. Strieter, P. J. Polverini, S. L. Kunkel et al., "The functional role of the ELR motif in CXC chemokine-mediated angiogenesis," *Journal of Biological Chemistry*, vol. 270, no. 45, pp. 27348–27357, 1995.

[35] B. J. Rollins, "Chemokines," *Blood*, vol. 90, no. 3, pp. 909–928, 1997.

[36] A. Orimo, P. B. Gupta, D. C. Sgroi et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[37] A. Müller, B. Homey, H. Soto et al., "Involvement of chemokine receptors in breast cancer metastasis," *Nature*, vol. 410, no. 6824, pp. 50–56, 2001.

[38] P. von Hundelshausen, F. Petersen, and E. Brandt, "Platelet-derived chemokines in vascular biology," *Thrombosis and Haemostasis*, vol. 97, no. 5, pp. 704–713, 2007.

[39] M. P. Keane, J. A. Belperio, Y. Y. Xue, M. D. Burdick, and R. M. Strieter, "Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer," *Journal of Immunology*, vol. 172, no. 5, pp. 2853–2860, 2004.

[40] J. Mestas, M. D. Burdick, K. Reckamp, A. Pantuck, R. A. Figlin, and R. M. Strieter, "The role of CXCR2/CXCR2 ligand biological axis in renal cell carcinoma," *Journal of Immunology*, vol. 175, no. 8, pp. 5351–5357, 2005.

[41] M. Jeltsch, A. Kaipainen, V. Joukov et al., "Hyperplasia of lymphatic vessels in VEGF-C transgenic mice," *Science*, vol. 276, no. 5317, pp. 1423–1425, 1997.

[42] S. A. Stacker, C. Caesar, M. E. Baldwin et al., "VEGF-D promotes the metastatic spread of tumor cells via the lymphatics," *Nature Medicine*, vol. 7, no. 2, pp. 186–191, 2001.

[43] A. Kaipainen, J. Korhonen, T. Mustonen et al., "Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 8, pp. 3566–3570, 1995.

[44] M. Rechter, O. Lider, L. Cahalon et al., "A cellulose-binding domain-fused recombinant human T cell connective tissue-activating peptide-III manifests heparanase activity," *Biochemical and Biophysical Research Communications*, vol. 255, no. 3, pp. 657–662, 1999.

[45] S. Singh, K. C. Nannuru, A. Sadanandam, M. L. Varney, and R. K. Singh, "CXCR1 and CXCR2 enhances human melanoma tumourigenesis, growth and invasion," *British Journal of Cancer*, vol. 100, no. 10, pp. 1638–1646, 2009.

[46] J. Heidemann, H. Ogawa, M. B. Dwinell et al., "Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2," *Journal of Biological Chemistry*, vol. 278, no. 10, pp. 8508–8515, 2003.

[47] S. Singh, K. C. Nannuru, A. Sadanandam, et al., "CXCR1 and CXCR2 enhances human melanoma, Premont RT, and Lefkowitz RJ," *Nature Reviews Molecular Cell Biology*, vol. 3, pp. 639–650, 2002.

[48] K. J. Laing and C. J. Secombes, "Chemokines," *Developmental and Comparative Immunology*, vol. 28, no. 5, pp. 443–460, 2004.

[49] J. Gavard, X. Hou, Y. Qu et al., "A role for a CXCR2/phosphatidylinositol 3-kinase γ signaling axis in acute and chronic vascular permeability," *Molecular and Cellular Biology*, vol. 29, no. 9, pp. 2469–2480, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 732680, 8 pages
doi:10.1155/2010/732680

Case Report

Immunomodulatory Agents with Antivascular Activity in the Treatment of Non-Small Cell Lung Cancer: Focus on TLR9 Agonists, IMiDs and NGR-TNF

Angelo Corti,[1] Monica Giovannini,[2] Carmen Belli,[2] and Eugenio Villa[2]

[1] *Division of Molecular Oncology and IIT Network of Molecular Neuroscience, San Raffaele Scientific Institute University Hospital, 60 Olgettina St, 20132 Milan, Italy*
[2] *Oncology Department, San Raffaele Scientific Institute University Hospital, 20132 Milan, Italy*

Correspondence should be addressed to Angelo Corti, corti.angelo@hsr.it and Monica Giovannini, giovannini.monica@hsr.it

Received 23 October 2009; Accepted 18 March 2010

Academic Editor: Arkadiusz Dudek

Standard treatments for nonsmall cell lung cancer (NSCLC), such as surgery, chemotherapy, and radiotherapy, often lead to disappointing results. Unfortunately, also the various immunotherapeutic approaches so far tested have not produced satisfactory results to be widely applied in the clinical practice. However, the recent development of new immunomodulatory agents may open promising therapeutic options. This paper focuses on PF3512676, lenalidomide, and NGR-TNF, that is, drugs belonging to three different classes of immunomodulatory agents, that are also capable to affect tumor blood vessels with different mechanisms, and discusses the potential role of such agents in NSCLC treatment strategy.

1. Introduction

Lung cancer is the leading cause of cancer death in both men and women and one of the leading causes of death worldwide [1]. Non small cell lung cancer (NSCLC) represents ~ 80% of all types of lung cancer. Most patients present with locally advanced (stage III) or metastatic (stage IV) cancer [2, 3]. Despite chemotherapy treatment, sometimes in combination with radiotherapy, most patients die of disease progression, due to acquired or intrinsic resistance to chemotherapeutic drugs. Various immunotherapeutic approaches have been also attempted, ranging from the use of nonspecific immunostimulants like Bacillus Calmette-Guerin (BCG) to more specific strategies, unfortunately often with disappointing results [4, 5]. However, the recent improvement of our understanding of how the immune system works, the identification of new target antigens, and the development of new immunomodulators capable to affect the immune system and the tumor neovasculature could open new therapeutic options that deserve further investigation.

This review discusses the potential role of three immunomodulatory agents that, besides regulating immune cells, can also affect the tumor neovasculature with different mechanisms and to different extent. In particular we discuss: (a) PF3512676, a Toll-like receptor 9 agonist primarily endowed of immunomodulatory activity, (b) lenalidomide, an immunomodulator with antiangiogenic properties and (c) NGR-TNF, an immunomodulatory agent with strong vascular damaging activity.

2. PF3512676 (TLR9 Agonist)

Toll like receptors (TLRs) are a family of highly conserved receptors that regulate innate antigen-specific immunity via the recognition of pathogen-associated molecular pattern [6–9]. TLR9 is expressed in endosomes of B and T lymphocytes, plasmocytoid cells, and dendritic cells [10]. Immunostimulatory oligonucleotides containing certain CpG sequence motifs stimulate the innate and adaptive immune response and have been under investigation for treating infectious diseases, allergies, asthma, and cancer

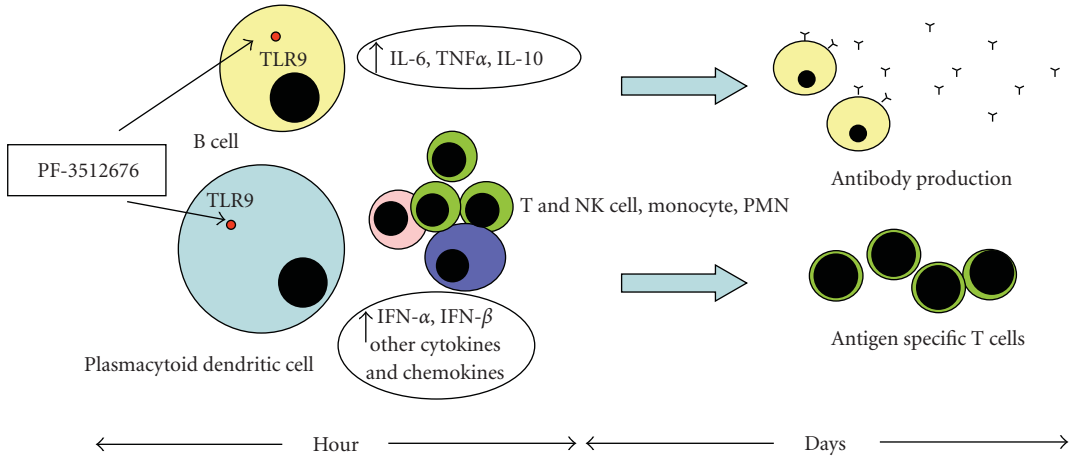


Figure 1: Proposed mechanism of action of PF-3512676. In the first phase, PF-3512676 activates a rapid induction of innate immune response through TLR9 stimulation in B cell and plasmocytoid cells. This may restore defective plasmocytoid dendritic cell function, induces IFN secretion (all IFN types), and activates NK and NKT cell. A later induction of adaptive immune response may then occur through dendritic cell mediated activation of antigen-specific T cells, monocytes and polymorphonuclear leukocytes, promotion of killer T cells and production of antibodies by B cells.

[11–16]. Through TLR9 signalling pathway, immunostimulatory oligonucleotides activate a complex cascade that leads to increased production of proinflammatory cytokines and chemokines and stimulation of an immune response with antitumor effects [17–21]. Bioengineered immunomodulatory oligonucleotides have been developed to stimulate the immune system of various animal species, as well as purified and cultured human immune cells [22–24]. Several new immunomodulatory oligonucleotides have been evaluated in models of human cancer [25–27]. Among these, PF-3512676 (ProMune) is particularly promising. It contains unmethylated cytosine and guanine (CpG) motifs and a nuclease-resistant phosphorothioate backbone [28]. It is an agonist of the TLR9 expressed in plasmocytoid cells and B cells [28]. The anticancer activity of PF-3512676 is related to direct and indirect immunomodulation of both innate and adaptive immune responses [29]. Plasmocytoid dendritic cells stimulated by PF-3512676 express increased levels of MHC I and II and costimulatory molecules (leading to improved antigen presentation) secrete cytokines and chemokines that enhance natural killer (NK) cell activity directed toward tumor cells, present tumor specific antigens and costimulatory molecules to B and T cells, and generate long-living antigen specific cytotoxic T-lymphocytes and antibody responses [30]. A good indicator of activation and maturation of dendritic cells by PF-3512676 is the production of IFNα and the subsequent induction of interferon-inducible protein 10 (IP-10), an antiangiogenic cytokine [28, 31]. Furthermore upregulation of CD86 and CD80 on B cells induced by PF-3512676 and secretion of IL-10 and IL-6 demonstrate its strong stimulatory properties [30]. A schematic representation of the mechanism of action of PF-3512676 is shown in Figure 1. The clinical safety and efficacy of PF-3512676 have been evaluated in 18 clinical studies [30, 32]. Overall, 889 subjects were enrolled in these trials, where PF3512676 was administered via subcutaneous, intravenous, intralesional, or intramuscular routes as monotherapy or in combination with monoclonal antibodies or with chemotherapeutic agents. Immunological responses (such as induction of innate/adaptive immune responses with moderate to abundant cellular infiltrates of lymphocytes proven in tumor biopsies) leading in some cases to tumor regression were observed in patients with melanoma, renal cell carcinoma, non-Hodgkin lymphoma and non small cell lung cancer [28, 30, 33–38]. Focusing on NSCLC, a phase II study enrolling 112 chemonaive patients with NSCLC was conducted. The patients received PF3512676 in combination with platinum and taxane doublet chemotherapy at a dose of 0.2 mg/kg, sc, on the 2nd and 3rd weeks of a 3-week chemotherapy cycle [39, 40]. Twenty-eight (37%) patients had a partial or complete response with the combination of chemotherapy and PF-3512676 and 7 (19%) with chemotherapy alone. The combination of chemotherapy and PF-3512676 was well tolerated even if there was an excess of myelosuppression. The most commonly reported PF-3512676 related events were reversible local injection reactions, such as erythema, pain, induration, warmth and swelling, or systemic flu-like symptoms (fatigue, pyrexia, headache, chills, arthralgia and myalgia). Based on these preliminary data, two phase III trials were conducted to test the efficacy of PF-3512676 in combination with platinum based chemotherapy in advanced NSCLC patients. Unfortunately, both studies failed to prove the superiority of the combination after an interim analysis by an independent data monitoring safety committee [41], showing also a worse toxicity profile for the PF-3512676 arm [42, 43].

3. Lenalidomide (Immunomodulatory Imide Drug)

Another promising drug class among immunomodulatory agents is represented by Immunomodulatory imide Drugs

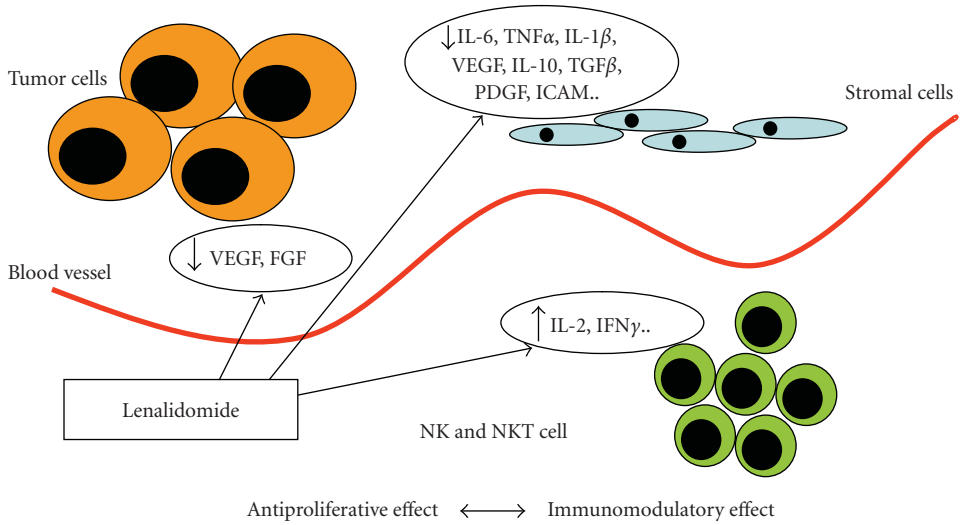


Figure 2: Proposed mechanism of action of lenalidomide. This drug can inhibit endothelial cell migration and adhesion possibly by downregulating endothelial cell integrins and angiogenesis. Lenalidomide can also induce immunomodulatory effect by activating T and NKT cells, which in turn release cytotoxic mediators, and by inhibiting the release of proinflammatory cytokines in the tumor microenvironment. The antiangiogenic effect is a major component of lenalidomide antitumor activity in solid tumors.

(the so-called IMiDs). Thalidomide is the IMiDs progenitor. Second generation IMiDs are lenalidomide, pomalidomide and ENMD-0995. Thalidomide has emerged as a potent treatment for several disease entities. Although originally marketed in Europe as a sedative and antiemetic, reports of teratogenic effects [44] led to its withdrawal in the market in 1961 [45]. Thalidomide-associated congenital malformations were later explained as impaired vasculogenesis suggesting that a similar mechanism may contribute to prevent the growth of tumor blood vessels [46, 47]. In the cancer setting, thalidomide is currently used in multiple myeloma patients [48].

Lenalidomide is the first 2nd generation IMiD to be approved for clinical use. It has been registered as 2nd line treatment in association with dexamethasone in patients with multiple myeloma [49]. However, the therapeutic utility of this drug may not be limited to haematological malignancies. It was synthesized based on the structural backbone of thalidomide, by adding an amino group at position 4 of the phthaloyl ring and removal of the carbonyl group of the 4-amino-substituted phthaloyl ring. Such structural changes were designed to enhance its immunomodulatory and antitumor activity [49, 50]. Despite the proven clinical activity of the IMiDs the exact mechanism of their antitumor activity remains elusive. It is possible that the antitumor activity of lenalidomide is mediated through multiple non-mutually exclusive processes that primarily depend on the type of tumor cells and their microenvironment. Data on lenalidomide's mechanism of action, mostly derived from studies on multiple myeloma and B cell malignancies can likely be applied also to solid tumors. Lenalidomide has been shown to inhibit TNF-α [51, 52], IL-6 and other proapoptotic cytokines and proinflammatory mediators [49, 53], and to activate proapoptotic signals triggered by Fas-mediated cell death, such as caspase-8 (but not caspase-9) [49]. Lenalidomide downregulates antiapoptotic proteins like the cellular inhibitor of apoptosis protein 2 and FLICE inhibitor protein. Nuclear factor-kB is also directly inhibited by lenalidomide [49]. Lenalidomide is a potent stimulator of lipopolysaccharide-induced IL-10, as well as costimulators of T cells that are partially activated through the T-cell receptor, in the CD8+ subset [54, 55]. Furthermore, it induces increase in IL-2 and IFNγ secretion and upregulation of CD40L expression on anti-CD3 stimulated T cells, resulting in activation of natural killer cells, and thus improving host immunity against tumor cells [56]. Compared to thalidomide, lenalidomide is 50 to 2000 times more potent in stimulating T-cell proliferation and activation and 50–100 times more potent in augmenting IL-2 and IFNγ production [49]. In contrast with PF-3512676, which shows a predominant immunomodulatory activity, IMiDs are also proapoptotic agents and strong angiogenesis inhibitors. Indeed, various in vitro assays have demonstrated the antiangiogenic activity of IMiDs [57–60]. This activity is believed to be secondary to the inhibition of secretion of angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), from both tumor and stromal cells. In addition, lenalidomide has been shown to inhibit endothelial cell migration and adhesion, perhaps by downregulating endothelial cell integrins [61, 62]. Lenalidomide is reported to downregulate key cytokines such as TNF-α, IL-6, IL-8 and VEGF, that is, cytokines which favour tumor cell survival, proliferation and, possibly, resistance to therapy, mainly by affecting the tumor vasculature [50]. A schematic representation of lenalidomide's mechanism of action is shown in Figure 2. In solid tumors, lenalidomide proved to have a good safety profile both in monotherapy and in combination with chemotherapy showing results in terms of antitumor

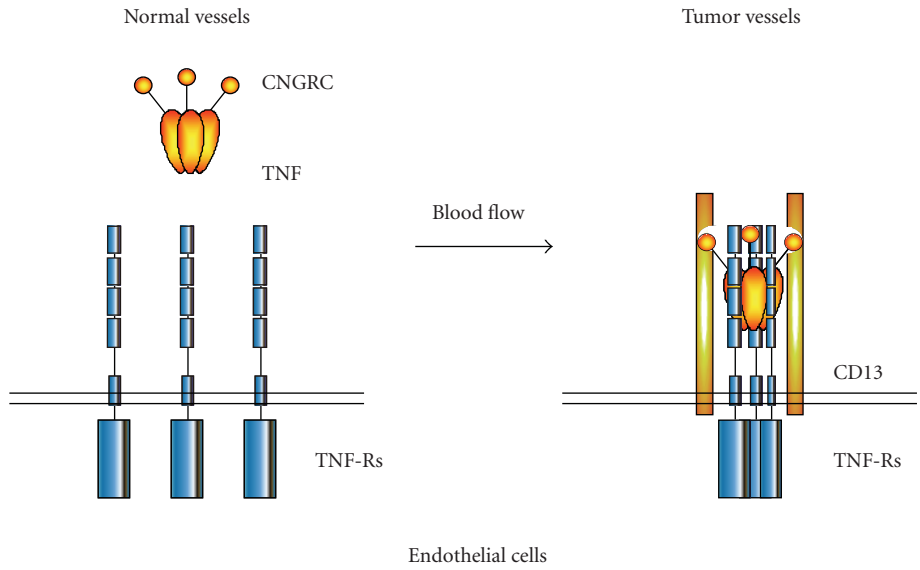


Figure 3: Proposed mechanism of the tumor homing properties of NGR-TNF. Ultra low dose NGR-TNF can interact more efficiently with CD13-positive tumor vessel, compared to CD13-negative normal vessels, by virtue of high avidity interactions of the CNGRC domain with CD13 and of the TNF domain with TNF receptors (TNF-Rs), thereby triggering local activation of endothelial cells, and inducing leukocyte adhesion molecules, cytokine secretion, procoagulant activity, and apoptosis.

activity in several tumor types and also in NSCLC [63–67]. In fact, Miller et al. tested the feasibility of lenalidomide at a dose escalated from 5 to 10 to 25 mg/day in 20 patients with solid tumors refractory to standard treatment [64]. Moderate dose-dependent and reversible haematological toxicity was observed. One partial response and three stable diseases were documented; of these patients three had NSCLC diagnosis. This study recommended 25 mg/day, orally, of lenalidomide as single agent for 4 weeks followed by 2-week rest period. Similarly, Kalmadi et al. explored safety and tolerability of lenalidomide in association with docetaxel and carboplatin in 14 patients with advanced solid tumors [67]. No treatment related deaths or irreversible toxicities were recorded. Five patients achieved partial response; 5 out of 9 patients had NSCLC. Docetaxel (60 mg/m^2) and carboplatin (AUC 6) on day 1 with lenalidomide 5 mg daily orally for 2 weeks of a 21 day cycle was the maximum tolerated dose without the use of prophylactic growth factors. Clinically, lenalidomide shows a different and more manageable toxicity profile compared to thalidomide, causing greater haematological toxicity (neutropenia and thrombocytopenia) but much less neurological toxicity [49].

4. NGR-TNF

NGR-TNF is an engineered TNF derivative with improved neovasculature homing properties. This drug, developed and initially tested at our Institute, is made of tumor necrosis factor α (TNF) fused to CNGRC, a peptide ligand of aminopeptidase N (CD13) overexpressed in tumor neovasculature [68–70]. A schematic representation of this drug and of the potential mechanism underlying its improved avidity for tumor blood vessels is shown in Figure 3. Experiments in animal models have shown that NGR-TNF, because of its tumor vasculature homing properties, is endowed of greater therapeutic activity and lower toxicity than TNF, enabling systemic administration of therapeutic doses [68, 71]. NGR-TNF can promote antitumor responses primarily by damaging the tumor vasculature. This provides the rationale for using NGR-TNF as a single agent. In addition, it has been demonstrated that ultra-low doses of NGR-TNF (picograms/mouse) are sufficient to alter permeability in tumor vessels of tumor-bearing mice and improve the penetration of various chemotherapeutic drugs in tumor tissues, including melphalan, doxorubicin, cisplatin, gemcitabine, and paclitaxel [71–73]. Thus, NGR-TNF may have a dual pharmacological effects, acting both as a vascular damaging agent and as an enhancer of chemotherapy. In principle, NGR-TNF could be exploited either to improve the penetration of conventional doses of chemotherapeutic drugs in tumors, favoring their local antitumor and immune adjuvant effects, or to reduce the dose of chemotherapeutic drugs and their toxicity, including that against cells of the immune system. This combined strategy might also benefit from the ability of TNF to promote anti-tumor immune responses [74, 75]. Interestingly, targeted delivery of TNF alone or in association with chemotherapy has been shown to cure tumors in animal models and to induce protective immunity [68]. This suggests that the immune response, and in particular T-cell dependent mechanisms, represent an important arm of NGR-TNF activity. TNF targeted to vessels might also enhance the production of endothelial immuneregulating cytokines or chemokines and/or upregulate endothelial adhesion molecules, favoring extravasation of immune cells, and improving the ability of the immune

system to cope with residual tumor cells [76]. A schematic representation of NGR-TNF mechanism of action is shown in Figure 3.

Various Phase I and Phase II clinical studies have been undertaken with NGR-TNF in solid tumors showing manageable toxicity profile and evidence of disease control, particularly in hepatocarcinoma, pleural mesothelioma, and colorectal cancer [77–80]. At our institution, we are currently testing low-dose NGR-TNF both as a single agent and in combination with chemotherapeutic agents, such as antracyclines and cisplatin, in several solid tumors, such as pleural mesothelioma, NSCLC and small cell lung cancer. Further studies are warranted to confirm NGR-TNF as a treatment option for NSCLC patients.

5. Conclusions

Standard treatment options for NSCLC patients, who are mostly diagnosed in advanced stage of disease, often lead to disappointing results. Immunotherapy is a promising approach in several tumors and preliminary but promising data are arising also for lung cancer. Despite initial attempts to treat NSCLC with immunomodulatory agents were unsuccessful, the development of new drugs endowed of immunomodulatory and antivascular activity have stimulated further clinical studies. The first agent we focused on (PF-3512676) is an example of an immunomodulatory agent with modest antivascular activity. Unfortunately, also this drug eventually turned out to be ineffective in large phase III trials for advanced NSCLC. However, the promising early findings, in terms of efficacy and toxicity, obtained with lenalidomide and NGR-TNF, that is, two immunomodulatory agents endowed with strong antivascular activity, may suggest that these compounds could play a role in the treatment of NSCLC, both as single agents and in combination with chemotherapeutic drugs. These combinations could be the key to move a step forward in improving prognosis in NSCLC patients.

Acknowledgment

This paper was supported in part by Associazione Italiana per la Ricerca sul Cancro (AIRC), FIRB and Alleanza Contro il Cancro (ACC) of Italy.

References

[1] American Cancer Society, *Cancer Facts and Figures 2005*, American Cancer Society, Atlanta, Ga, USA.

[2] D. Ferrigno and G. Buccheri, "Second-line chemotherapy for recurrent non-small cell lung cancer: do new agents make a difference?" *Lung Cancer*, vol. 29, no. 2, pp. 91–104, 2000.

[3] P. C. Hoffman, A. M. Mauer, and E. E. Vokes, "Lung cancer," *The Lancet*, vol. 355, no. 9202, pp. 479–485, 2000.

[4] D. O'Mahony, S. Kummar, and M. E. Gutierrez, "Non-small-cell lung cancer vaccine therapy: a concise review," *Journal of Clinical Oncology*, vol. 23, no. 35, pp. 9022–9028, 2005.

[5] M. Tishler and Y. Shoenfeld, "BCG immunotherapy—from pathophysiology to clinical practice," *Expert Opinion on Drug Safety*, vol. 5, no. 2, pp. 225–229, 2006.

[6] L. Apetoh, F. Ghiringhelli, A. Tesniere, et al., "The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy," *Immunological Reviews*, vol. 220, no. 1, pp. 47–59, 2007.

[7] K. Chen, J. Huang, W. Gong, P. Iribarren, N. M. Dunlop, and J. M. Wang, "Toll-like receptors in inflammation, infection and cancer," *International Immunopharmacology*, vol. 7, no. 10, pp. 1271–1285, 2007.

[8] H. Hemmi, O. Takeuchi, T. Kawai, et al., "A Toll-like receptor recognizes bacterial DNA," *Nature*, vol. 408, no. 6813, pp. 740–745, 2000.

[9] S. Rakoff-Nahoum, J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov, "Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis," *Cell*, vol. 118, no. 2, pp. 229–241, 2004.

[10] J. Vollmer and A. M. Krieg, "Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists," *Advanced Drug Delivery Reviews*, vol. 61, no. 3, pp. 195–204, 2009.

[11] A. Cafaro, F. Titti, C. Fracasso, et al., "Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P)," *Vaccine*, vol. 19, no. 20–22, pp. 2862–2877, 2001.

[12] A. F. Carpentier, J. Xie, K. Mokhtari, and J.-Y. Delattre, "Successful treatment of intracranial gliomas in rat by oligodeoxynucleotides containing CpG motifs," *Clinical Cancer Research*, vol. 6, no. 6, pp. 2469–2473, 2000.

[13] A. Dalpke, S. Zimmermann, and K. Heeg, "CpG DNA in the prevention and treatment of infections," *Bio Drugs*, vol. 16, no. 6, pp. 419–431, 2002.

[14] I. Sabroe, L. C. Parker, A. G. Wilson, M. K. B. Whyte, and S. K. Dower, "Toll-like receptors: their role in allergy and non-allergic inflammatory disease," *Clinical and Experimental Allergy*, vol. 32, no. 7, pp. 984–989, 2002.

[15] J. Van Uden and E. Raz, "Immunostimulatory DNA and applications to allergic disease," *Journal of Allergy and Clinical Immunology*, vol. 104, no. 5, pp. 902–910, 1999.

[16] H. Wang, E. Rayburn, and R. Zhang, "Synthetic oligodeoxynucleotides containing deoxycytidyl-deoxyguanosine dinucleotides (CpG ODNs) and modified analogs as novel anticancer therapeutics," *Current Pharmaceutical Design*, vol. 11, no. 22, pp. 2889–2907, 2005.

[17] D. M. Klinman, A.-K. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg, "CpG motifs present in bacterial DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 7, pp. 2879–2883, 1996.

[18] D. M. Klinman, "Immunotherapeutic uses of CpG oligodeoxynucleotides," *Nature Reviews Immunology*, vol. 4, no. 4, pp. 249–258, 2004.

[19] A. M. Krieg, "CpG motifs in bacterial DNA and their immune effects," *Annual Review of Immunology*, vol. 20, pp. 709–760, 2002.

[20] F. Takeshita, I. Gursel, K. J. Ishii, K. Suzuki, M. Gursel, and D. M. Klinman, "Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9," *Seminars in Immunology*, vol. 16, no. 1, pp. 17–22, 2004.

[21] H. Wang, E. Rayburn, and R. Zhang, "Synthetic oligodeoxynucleotides containing deoxycytidyl-deoxyguanosine dinucleotides (CpG ODNs) and modified analogs as novel anticancer therapeutics," *Current Pharmaceutical Design*, vol. 11, no. 22, pp. 2889–2907, 2005.

[22] E. R. Kandimalla, L. Bhagat, Y. Li, et al., "Immunomodulatory oligonucleotides containing a cytosine-phosphate-2′-deoxy-7-deazaguanosine motif as potent Toll-like receptor 9 agonists," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 19, pp. 6925–6930, 2005.

[23] E. R. Kandimalla, L. Bhagat, F.-G. Zhu, et al., "A dinucleotide motif in oligonucleotides shows potent immunomodulatory activity and overrides species-specific recognition observed with CpG motif," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 14303–14308, 2003.

[24] Y. Li, E. R. Kandimalla, D. Yu, and S. Agrawal, "Oligodeoxynucleotides containing synthetic immunostimulatory motifs augment potent Th1 immune responses to HBsAg in mice," *International Immunopharmacology*, vol. 5, no. 6, pp. 981–991, 2005.

[25] L. Bhagat, F.-G. Zhu, D. Yu, et al., "CpG penta- and hexadeoxyribonucleotides as potent immunomodulatory agents," *Biochemical and Biophysical Research Communications*, vol. 300, no. 4, pp. 853–861, 2003.

[26] E. R. Kandimalla, L. Bhagat, D. Wang, et al., "Divergent synthetic nucleotide motif recognition pattern: design and development of potent immunomodulatory oligodeoxyribonucleotide agents with distinct cytokine induction profiles," *Nucleic Acids Research*, vol. 31, no. 9, pp. 2393–2400, 2003.

[27] D. Yu, F.-G. Zhu, L. Bhagat, et al., "Potent CpG oligonucleotides containing phosphodiester linkages: in vitro and in vivo immunostimulatory properties," *Biochemical and Biophysical Research Communications*, vol. 297, no. 1, pp. 83–90, 2002.

[28] Y. M. Murad, T. M. Clay, H. K. Lyerly, and M. A. Morse, "CPG-7909 (PF-3512676, ProMune): Toll-like receptor-9 agonist in cancer therapy," *Expert Opinion on Biological Therapy*, vol. 7, no. 8, pp. 1257–1266, 2007.

[29] K. Chen, J. Huang, W. Gong, P. Iribarren, N. M. Dunlop, and J. M. Wang, "Toll-like receptors in inflammation, infection and cancer," *International Immunopharmacology*, vol. 7, no. 10, pp. 1271–1285, 2007.

[30] "Drugs R D," *International Immunopharmacology*, vol. 7, no. 5, pp. 312–316, 2006.

[31] A. L. Angiolillo, C. Sgadari, and G. Tosato, "A role for the interferon-inducible protein 10 in inhibition of angiogenesis by interleukin-12," *Annals of the New York Academy of Sciences*, vol. 795, pp. 158–167, 1996.

[32] R. S. Geary, J. M. Leeds, S. P. Henry, D. K. Monteith, and A. A. Levin, "Antisense oligonucleotide inhibitors for the treatment of cancer. Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides," *Anti-Cancer Drug Design*, vol. 12, no. 5, pp. 383–393, 1997.

[33] M. A. Hofmann, C. Kors, H. Audring, P. Walden, W. Sterry, and U. Trefzer, "Phase 1 evaluation of intralesionally injected TLR9-agonist PF-3512676 in patients with basal cell carcinoma or metastatic melanoma," *Journal of Immunotherapy*, vol. 31, no. 5, pp. 520–527, 2008.

[34] J. A. Thompson, T. Kuzel, B. J. Drucker, W. Urba, and R. Bukowski, "Safety and efficacy of PF-3512676 for the treatment of stage IV renal cell carcinoma: an open-label, multicenter phase I/II study," *Clinical Genitourinary Cancer*, vol. 7, no. 3, pp. E58–E65, 2009.

[35] J. P. Leonard, B. K. Link, C. Emmanouilides, et al., "Phase I trial of Toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non-Hodgkin's lymphoma," *Clinical Cancer Research*, vol. 13, no. 20, pp. 6168–6174, 2007.

[36] B. K. Link, Z. K. Ballas, D. Weisdorf, et al., "Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma," *Journal of Immunotherapy*, vol. 29, no. 5, pp. 558–568, 2006.

[37] P. A. Bradbury and F. A. Shepherd, "Immunotherapy for lung cancer," *Journal of Thoracic Oncology*, vol. 3, no. 6, pp. S164–S170, 2008.

[38] K. Yamada, M. Nakao, C. Fukuyama, et al., "Phase I study of TLR9 agonist PF-3512676 in combination with carboplatin and paclitaxel in patients with advanced non-small-cell lung cancer," *Cancer Science*, vol. 101, no. 1, pp. 188–195, 2010.

[39] G. Leichman, D. Gravenor, D. Woytowitz, et al., "CPG 7909, a TLR9 agonist, added to first line taxane/platinum for advanced non-small cell lung cancer, a randomized, controlled phase II study," *Journal of Clinical Oncology*, vol. 23, no. 16S, 2005, abstract 7036.

[40] C. Manegold, D. Gravenor, D. Woytowitz, et al., "Randomized phase II trial of a Toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 26, no. 24, pp. 3979–3986, 2008.

[41] D. O'Mahony, S. Kummar, and M. E. Gutierrez, "Non-small-cell lung cancer vaccine therapy: a concise review," *Journal of Clinical Oncology*, vol. 23, no. 35, pp. 9022–9028, 2005.

[42] V. Hirsh, M. Boyer, R. Rosell, et al., "Randomized phase III trial of paclitaxel/carboplatin with or without PF-3512676 as first line treatment of advanced non-small cell lung cancer (NSCLC)," *Journal of Clinical Oncology*, vol. 26, supplement, 2008, abstract no. 8016.

[43] C. Manegold, N. Thatcher, R. J. Benner, et al., "Randomized phase III trial of gemcitabine/cisplatin with or without PF-3512676 as first line treatment of advanced non-small cell lung cancer (NSCLC)," *Journal of Clinical Oncology*, vol. 26, 2008, abstract no. 8017.

[44] G. W. Mellin and M. Katzenstein, "The saga of thalidomide. Neuropathy to embryopathy, with case reports of congenital anomalies," *The New England Journal of Medicine*, vol. 267, pp. 1184–1192, 1962.

[45] F. O. Kelsey, "Thalidomide update: regulatory aspects," *Teratology*, vol. 38, no. 3, pp. 221–226, 1988.

[46] R. J. D'Amato, M. S. Loughnan, E. Flynn, and J. Folkman, "Thalidomide is an inhibitor of angiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 9, pp. 4082–4085, 1994.

[47] B. M. Kenyon, F. Browne, and R. J. D'Amato, "Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization," *Experimental Eye Research*, vol. 64, no. 6, pp. 971–978, 1997.

[48] Press Release, "Thalomid® sNDA Granted FDA Approval For Treatment of Newly Diagnosed Multiple Myeloma," 2007.

[49] K. C. Anderson, "Lenalidomide and thalidomide: mechanisms of action—similarities and differences," *Seminars in Hematology*, vol. 42, supplement 4, pp. S3–S8, 2005.

[50] A. A. Chanan-Khan and B. D. Cheson, "Lenalidomide for the treatment of B-cell malignancies," *Journal of Clinical Oncology*, vol. 26, no. 9, pp. 1544–1552, 2008.

[51] G. W. Muller, R. Chen, S.-Y. Huang, et al., "Amino-substituted thalidomide analogs: potent inhibitors of TNF-alpha production," *Bioorganic and Medicinal Chemistry Letters*, vol. 9, no. 11, pp. 1625–1630, 1999.

[52] S. Niwayama, B. E. Turk, and J. O. Liu, "Potent inhibition of tumor necrosis factor-alpha production by tetrafluorothalidomide and tetrafluorophthalimides," *Journal of Medicinal Chemistry*, vol. 39, no. 16, pp. 3044–3045, 1996.

[53] L. G. Corral, P. A. J. Haslett, G. W. Muller, et al., "Differential cytokine modulation and T cell activation by two distinct classes of thalidomide analogues that are potent inhibitors of TNF-alpha," *Journal of Immunology*, vol. 163, no. 1, pp. 380–386, 1999.

[54] J. B. Bartlett, K. Dredge, and A. G. Dalgleish, "The evolution of thalidomide and its IMiD derivatives as anticancer agents," *Nature Reviews Cancer*, vol. 4, no. 4, pp. 314–322, 2004.

[55] L. G. Corral and G. Kaplan, "Immunomodulation by thalidomide and thalidomide analogues," *Annals of the Rheumatic Diseases*, vol. 58, supplement 1, pp. I107–I113, 1999.

[56] C. Galustian, B. Meyer, M.-C. Labarthe, et al., "The anticancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells," *Cancer Immunology Immunotherapy*, vol. 58, no. 7, pp. 1033–1045, 2009.

[57] K. Dredge, J. B. Marriott, C. D. Macdonald, et al., "Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects," *British Journal of Cancer*, vol. 87, no. 10, pp. 1166–1172, 2002.

[58] D. Chauhan, A. V. Singh, B. Ciccarelli, P. G. Richardson, M. A. Palladino, and K. C. Anderson, "Combination of novel proteasome inhibitor NPI-0052 and lenalidomide trigger in vitro and in vivo synergistic cytotoxicity in multiple myeloma," *Blood*, vol. 115, no. 4, pp. 834–845, 2010.

[59] L. Lu, F. Payvandi, L. Wu, et al., "The anti-cancer drug lenalidomide inhibits angiogenesis and metastasis via multiple inhibitory effects on endothelial cell function in normoxic and hypoxic conditions," *Microvascular Research*, vol. 77, no. 2, pp. 78–86, 2009.

[60] D. P. Mangiameli, J. A. Blansfield, S. Kachala, et al., "Combination therapy targeting the tumor microenvironment is effective in a model of human ocular melanoma," *Journal of Translational Medicine*, vol. 5, article 38, 2007.

[61] D. Gupta, S. P. Treon, Y. Shima, et al., "Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications," *Leukemia*, vol. 15, no. 12, pp. 1950–1961, 2001.

[62] S. Lentzsch, M. S. Rogers, R. LeBlanc, et al., "S-3-aminophthalimido-glutarimide inhibits angiogenesis and growth of B-cell neoplasias in mice," *Cancer Research*, vol. 62, no. 8, pp. 2300–2305, 2002.

[63] T. Eisen, U. Trefzer, A. Hamilton, et al., "Results of a multicenter, randomized, double-blind phase 2/3 study of lenalidomide in the treatment of pretreated relapsed or refractory metastatic malignant melanoma," *Cancer*, vol. 116, no. 1, pp. 146–154, 2010.

[64] A. A. Miller, D. Case, M. Harmon, et al., "Phase I study of lenalidomide in solid tumors," *Journal of Thoracic Oncology*, vol. 2, no. 5, pp. 445–449, 2007.

[65] W. L. Dahut, J. B. Aragon-Ching, S. Woo, et al., "Phase I study of oral lenalidomide in patients with refractory metastatic cancer," *Journal of Clinical Pharmacology*, vol. 49, no. 6, pp. 650–660, 2009.

[66] S. L. Sanborn, J. Gibbons, S. Krishnamurthi, et al., "Phase I trial of docetaxel given every 3 weeks and daily lenalidomide in patients with advanced solid tumors," *Investigational New Drugs*, vol. 27, no. 5, pp. 453–460, 2009.

[67] S. Kalmadi, M. Davis, A. Dowlati, et al., "Phase I trial of three-weekly docetaxel, carboplatin and oral lenalidomide (Revlimid) in patients with advanced solid tumors," *Investigational New Drugs*, vol. 25, no. 3, pp. 211–216, 2007.

[68] F. Curnis, A. Sacchi, L. Borgna, F. Magni, A. Gasparri, and A. Corti, "Enhancement of tumor necrosis factor alpha antitumor immunotherapeutic properties by targeted delivery to aminopeptidase N (CD13)," *Nature Biotechnology*, vol. 18, no. 11, pp. 1185–1190, 2000.

[69] F. Curnis, G. Arrigoni, A. Sacchi, et al., "Differential binding of drugs containing the NGR motif to CD13 isoforms in tumor vessels, epithelia, and myeloid cells," *Cancer Research*, vol. 62, no. 3, pp. 867–874, 2002.

[70] P. Di Matteo, F. Curnis, R. Longhi, et al., "Immunogenic and structural properties of the Asn-Gly-Arg (NGR) tumor neovasculature-homing motif," *Molecular Immunology*, vol. 43, no. 10, pp. 1509–1518, 2006.

[71] F. Curnis, A. Sacchi, and A. Corti, "Improving chemotherapeutic drug penetration in tumors by vascular targeting and barrier alteration," *Journal of Clinical Investigation*, vol. 110, no. 4, pp. 475–482, 2002.

[72] A. Corti and M. Ponzoni, "Tumor vascular targeting with tumor necrosis factor alpha and chemotherapeutic drugs," *Annals of the New York Academy of Sciences*, vol. 1028, pp. 104–112, 2004.

[73] A. Sacchi, A. Gasparri, C. Gallo-Stampino, S. Toma, F. Curnis, and A. Corti, "Synergistic antitumor activity of cisplatin, paclitaxel, and gemcitabine with tumor vasculature-targeted tumor necrosis factor-alpha," *Clinical Cancer Research*, vol. 12, no. 1, pp. 175–182, 2006.

[74] E. L. Balza, L. Mortara, F. Sassi, et al., "Targeted delivery of tumor necrosis factor-alpha to tumor vessels induces a therapeutic T cell-mediated immune response that protects the host against syngeneic tumors of different histologic origin," *Clinical Cancer Research*, vol. 12, no. 8, pp. 2575–2582, 2006.

[75] M. A. Palladino, M. R. Shalaby, S. M. Kramer, et al., "Characterization of the antitumor activities of human tumor necrosis factor-alpha and the comparison with other cytokines: induction of tumor-specific immunity," *Journal of Immunology*, vol. 138, no. 11, pp. 4023–4032, 1987.

[76] M. Bellone, A. Mondino, and A. Corti, "Vascular targeting, chemotherapy and active immunotherapy: teaming up to attack cancer," *Trends in Immunology*, vol. 29, no. 5, pp. 235–241, 2008.

[77] E. Bennicelli, A. Santoro, L. Rimassa, et al., "Phase II study of NGR-HTNF, a novel vascular targeting agent in colorectal cancer patients refractory to standard regimens," *Annals of Oncology*, vol. 19, supplement 9, 2008, abstract no. H16, ix77.

[78] G. Citterio, A. Santoro, T. Pressiani, et al., "Safety and antitumor activity of NGR-HTNF, a selective vascular targeting agent, administered at low dose in pretreated patients with hepatocellular carcinoma: preliminary results of a phase II trial," *Annals of Oncology*, vol. 19, supplement 8, 2008, abstract no. 546P, viii178.

[79] V. Gregorc, P. Zucali, G. Ceresoli, et al., "Phase II trial of NGR-HTNF, a novel vascular targeting agent, in malignant pleural

mesothelioma," *Annals of Oncology*, vol. 19, supplement 9, 2008, abstract no. F8, ix57.

[80] H. van Laarhoven, W. Fiedler, I. M. Desar, et al., "Phase I and DCE-MRI evaluation of NGR-TNF, a novel vascular targeting agent, in patients with solid tumors (EORTC 16041)," *Journal of Clinical Oncology*, vol. 26, supplement, 2008, abstract no. 3521.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 272170, 7 pages
doi:10.1155/2010/272170

Review Article

Angiogenesis: From Chronic Liver Inflammation to Hepatocellular Carcinoma

Paloma Sanz-Cameno,[1,2,3] María Trapero-Marugán,[2,3] María Chaparro,[2,3] Evan Anthony Jones,[2] and Ricardo Moreno-Otero[2,3]

[1] *Laboratory of Investigation, Molecular Biology Unit, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, 28006-Madrid, Spain*
[2] *Hepatology Unit, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, 28006 Madrid, Spain*
[3] *CIBERehd, Instituto de Salud Carlos III, 28006 Madrid, Spain*

Correspondence should be addressed to Ricardo Moreno-Otero, rmoreno.hlpr@salud.madrid.org

Received 18 November 2009; Revised 24 February 2010; Accepted 16 March 2010

Academic Editor: Arkadiusz Dudek

Recently, new information relating to the potential relevance of chronic hepatic inflammation to the development and progression of hepatocellular carcinoma (HCC) has been generated. Persistent hepatocellular injury alters the homeostatic balance within the liver; deregulation of the expression of factors involved in wound healing may lead to the evolution of dysplastic lesions into transformed nodules. Progression of such nodules depends directly on the development and organization of a vascular network, which provides the nutritional and oxygen requirements to an expanding nodular mass. Angiogenic stimulation promotes intense structural and functional changes in liver architecture and physiology, in particular, it facilitates transformation of dysplasia to nodular lesions with carcinogenic potential. HCC depends on the growth and spreading of vessels throughout the tumor. Because these vascular phenomena correlate with disease progression and prognosis, therapeutic strategies are being developed that focus on precluding vascular expansion in these tumors. Accordingly, an in-depth study of factors that promote and support pathological angiogenesis in chronic hepatic diseases may provide insights into methods of preventing the development of HCC and/or stimulating the regression of established HCC.

1. Introduction

In recent years an appreciable worldwide increase in the incidence of tumors, such as hepatocellular carcinoma (HCC), has occurred in association with changes in socioeconomic development, which include potentially relevant changes in lifestyles [1]. Development of HCC is known to be triggered by factors that lead to chronic hepatic injury and deregulation of the normal process of wound healing, which promote persistent stimulation of profibrotic and proangiogenic processes that lead to significant structural changes in the liver and functional changes in hepatic physiology [2].

Formation of new blood vessels (angiogenesis) is closely related to the development of HCC. Indeed, HCC is a highly vascularized tumor, a feature that has implications for investigative procedures applied for its detection [3]. Among features of the vasculature of the liver, not found in other tissues, are the hepatic sinusoids, characteristics of which include the presence of hepatic sinusoidal endothelial cells (LSECs) that possess distinctive fenestrations and pericytes or hepatic stellate cells (HSCs); sinusoids have the ability to synthesize liver-specific factors, such as angiopoietin-like 3 (Angpl3). Angiogenesis in HCC depends on the same fundamental principles of activation, proliferation and migration of endothelial cells that occur in other tumors and diseases in which enhanced angiogenesis occurs [4].

Neoplastic tissue requires a supply of oxygen and nutrients. Thus, avascular solid tumors only grow to a certain size and then undergo regression, if their metabolic demands are not met [5]. For continued growth, it is necessary for a tumor to orchestrate the formation of a functioning system

of blood vessels, which allows the delivery of metabolites (including growth factors) and cells (immunological cells and other cellular precursors) to the tumor environment. This process involves neo-vascularization of the tumor or tumor angiogenic switch [6, 7]. Once the requirements for tumor growth are met as a result of angiogenesis, the tumor mass then becomes restricted by surrounding normal tissue, which degenerates and is replaced by tumor tissue. These phenomena stimulate further immune-driven angiogenesis; formation of more blood vessels tends to ameliorate tissue damage. Moreover, spatiotemporal deregulation of pathological angiogenesis may lead to newly formed vessels having abnormal architecture and function; the frequently immature and anomalous characteristics of tumor vasculature tend to facilitate the spread of tumor cells [8]. If disseminated tumor cells become located in other tissues, the whole process of tumorigenesis may reoccur with the result that secondary tumors are generated (Figure 1).

2. Factors That Stimulate Angiogenesis

Hypoxia and inflammation are the main factors that stimulate angiogenesis. Hypoxia promotes angiogenesis as a consequence of signalling mediated by hypoxia-inducible transcription factors [9]. Inflammation increases vascular permeability and promotes chemokine-mediated recruitment of monocytes, macrophages, platelets, mast cells and other leukocytes that can synthesize angiogenic cytokines and growth factors [10, 11].

Specifically, hypoxia impairs the hydroxylation of HIF-1alpha and hence, reduces its subsequent degradation by proteasome. Consequently, HIF-1alpha accumulates in the cytosol, thereby facilitating its interaction with beta subunits and translocation to the nucleus, with the consequent modulation of the expression of many angiogenesis-promoting genes that contain hypoxia-responsive elements (HREs) (Figure 2).

Furthermore, tissue injury triggers an immune response, in which immune cells in peripheral blood extravasate into the damaged tissue, where they mediate restoration of tissue homeostasis. However, if the injury and associated inflammation persist, continuing endothelial activation would promote the infiltration of immune factors and cells into the focus of inflammation; this process may stimulate the proliferation and migration of endothelial cells, the prerequisites for the generation of new blood vessels (Figure 3) [12]. Thus, a close relationship between chronic inflammation, angiogenesis and cancer, recently the subject of several reviews [13–17], would be expected.

3. Phases of Angiogenesis

The formation of new functional blood vessels depends on precise regulation of the molecular effectors that promote the different processes involved [13, 14].

Endothelial cell budding is facilitated by vasodilatation, loosening of interendothelial contacts and leakage from pre-existing vessels. These phenomena allow extravasation of plasma proteins that, together with ECM components, facilitate the laying down of a provisional scaffold for migrating endothelial cells (EC). Nitric oxide (NO), the angiogenic properties of which have been well characterized [18–20], is the main factor responsible for vasodilatation. Vascular endothelial growth factor (VEGF) increases vascular permeability. The basement membrane (mainly collagen type IV and laminin) and the ECM (collagen type I and elastin) must undergo degradation to allow subsequent migration and proliferation of EC. This process is mediated by specific proteinases, which include plasminogen activator. Proteolysis of ECM leads to the exposure of cryptic epitopes and release of ECM-embedded factors that promote migration and proliferation of EC [21, 22].

ECs proliferate in response to growth factors secreted by themselves or by surrounding cells, such as hepatic stellate cells, leukocytes, hepatocytes and Kupffer cells. The most thoroughly characterized of these growth factors is VEGF, which is a multifunctional protein that binds to two tyrosine kinase receptors that are designated kinase insert domain receptor (KDR) and fms-like tyrosine kinase receptor (Flt-1) [23]. VEGF, whose promoter contains hypoxia-inducible factor-responsive elements (HREs), plays a crucial role virtually in all pathological situations in which angiogenesis occurs. The therapeutic potential of strategies aimed at blocking its mechanism of action is currently being evaluated [24].

A VEGF-induced increase in vascular permeability facilitates interchange of metabolites and cells between peripheral blood and surrounding tissues. The vasculature constitutes a structural and functional barrier to the transfer of cells and solutes, which may traverse vascular endothelium by paracellular and/or transcellular routes [25]. The former route implies the rupture of intercellular junctions, while the latter route involves the organization of caveolae or vacuolar vesicles to generate an intracellular conduit.

VEGF can increase the permeability of the endothelium by enhancing both routes of transfer across vascular endothelium. Signalling mediated by VEGFR2 promotes the phosphorylation of vascular endothelial cadherin (VE-Cadh), which impairs its binding to the actin cytoskeleton and, hence, leads to deterioration of intercellular junctions. In addition, this phenomenon is enhanced by VEGF-mediated dissociation of the VE-PTP phosphatase of VE-Cadh. Furthermore, the increase of VE-Cadh endocytosis, as well as the improvement in pathways of transcytosis mediated by VEGF, may account for associated vascular integrity and modulation of permeability [26]. As the delivery of antiangiogenic and antitumor drugs to subendothelial tumor tissue is promoted by increased vascular permeability, methods of modulating the transcellular transfer of drugs are being studied in depth with the aim of enhancing the efficacy of pharmacotherapies for cancer.

In addition to VEGF, proliferation of EC may be stimulated by other growth factors, such as acidic and basic fibroblast growth factors (aFGF, and bFGF), hepatocyte growth factor (HGF) and transforming growth factor (TGF) [27–29]. An orderly proliferation of EC leads to the

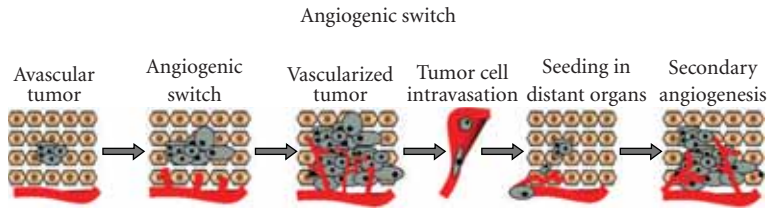
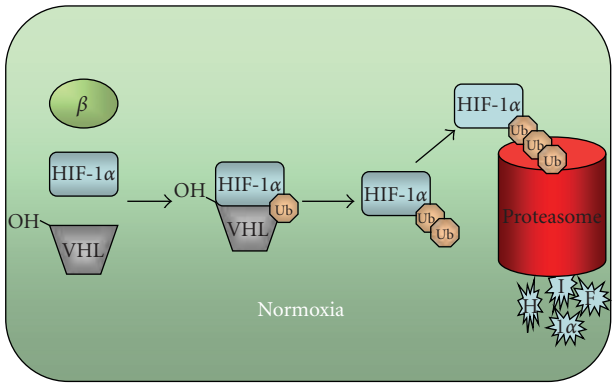
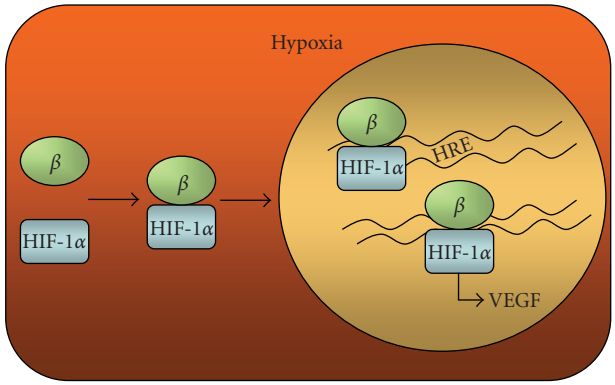


Figure 1: Angiogenic switch: transformed cells proliferate as an avascular nodule until they reach a certain size. Angiogenic switch enables exponential tumor growth and facilitates dissemination of tumor cells to secondary locations, where pathological angiogenesis may again be initiated.



(a)



(b)

Figure 2: Oxygen-dependent regulation of HIF-1α: in normal oxidative conditions, HIF-1α is hydroxylated and it becomes ubiquitinated by VHL. Subsequently, HIF-1α is degraded by the proteasome. In contrast, low oxygen tension leads to stabilization of HIF-1 and its interaction with beta subunit, which triggers translocation to the nucleus and modulation of the transcription of diverse genes that are involved in the response to hypoxia.

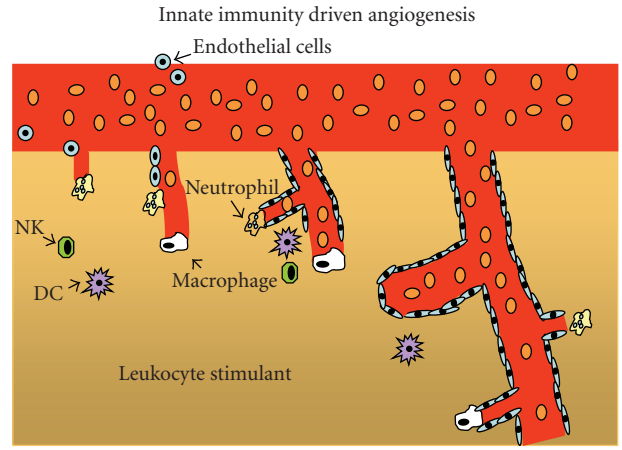


Figure 3: Innate immunity-driven angiogenesis: immune cells, mostly neutrophils and monocyte-macrophages, mediate initial tunnel formation in certain models of angiogenesis. Other myeloid cell types, such as dendritic cells (DCs) and natural killer cells (NK), produce angiogenic factors that attract endothelial cells that become essential components of developing blood vessels. Adapted from Noonan et al., 2008.

formation of a lumen [30, 31]. A structured 3-dimensional network of vessels of uniform size then develops, with its organization being regulated by mechanisms that involve signalling pathways for determination of branching and formation of basement membrane and ECM components, as well as migration of cells and their differentiation. Maturation of nascent vessels requires recruitment of pericytes, and formation of a new basement membrane and ECM to provide structural stabilization [31]. Both physical forces and specific different molecules contribute to these processes (Figure 4).

Additionally to the induction of proliferation of EC, effective angiogenesis also requires stabilization of nascent blood vessels, and formation of interendothelial cell junctions and lumens. Angiopoietin-1 (Ang-1) stabilizes nascent vessels by binding to the Tie-2 receptor, thereby modulating junctional molecules [32] and facilitating communication between EC and mural cells [33]. However, an excess of Ang-1 increases stiffness and inhibits branching of vessels. Angiopoietin-2 (Ang-2) may mediate opposing effects: in the absence of VEGF, it antagonizes Ang-1, thereby destabilizing vessels, inducing death of EC and leading to regression of vessels; in the presence of VEGF, however, it facilitates the branching of vessels [33, 34].

Zhang et al. [35] evaluated the expression of angiogenic factors in specimens of HCC and nonneoplastic hepatic tissue. They found a higher expression of Ang-2, and lower expression Ang-1 in HCC than in nonneoplastic tissue, suggesting that these molecules may play a role in angiogenesis associated with carcinogenesis and progression of

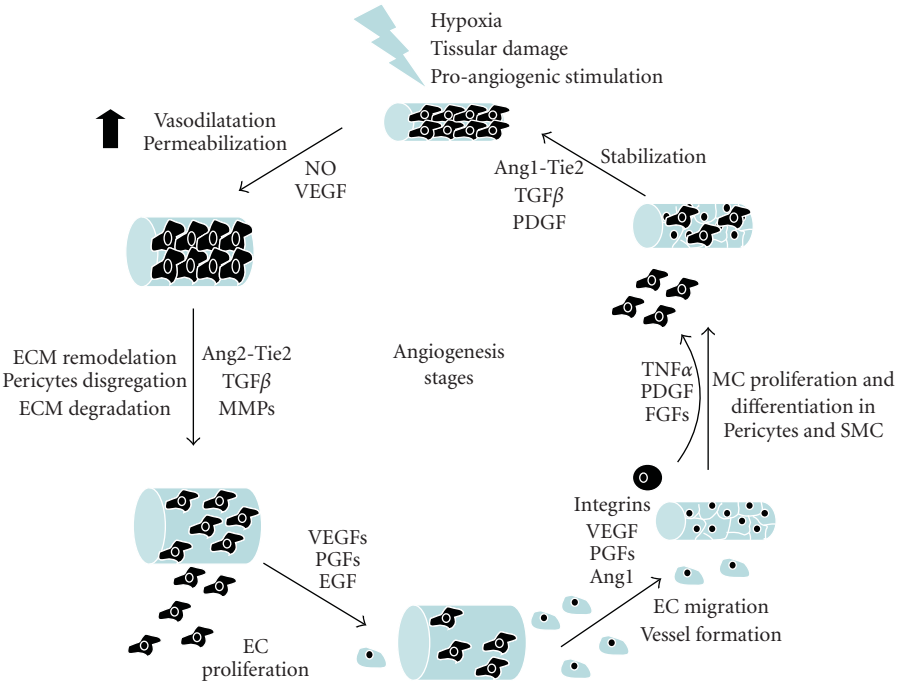


Figure 4: Stages of angiogenesis: angiogenic stimuli, by promoting production of NO and VEGF, lead to dilatation and increased permeability of blood vessels. Subsequently, other factors, particularly Ang-1, TGF-β, and MMPs, facilitate degradation of basement membrane and extracellular matrix and detachment of pericytes. Consequently, angiogenic stimuli facilitate proliferation and migration of endothelial cells. In addition, mesenchymal cells proliferate and differentiate into pericytes and smooth muscle cells that contribute to the stabilization of the new blood vessels.

HCC. Pappeti and Herman have reported that pathological angiogenesis associated with tumors is different from that associated with physiological processes [36]. Under normal conditions vascular inactivity and stabilization are mediated by Ang-1, Ang-2, and Tie-2; in pathological angiogenesis an abnormal Ang-2/Ang-1 ratio, in the presence of VEGF, plays a critical role in the transformation of noncancerous liver tissue to HCC by initiating early neovascularization. Vajkoczy et al. [37] reported that the earliest stage of tumor development is initiated by VEGF, VEGF receptor-2 and Ang-2 interacting with host vessels. Therefore, augmented expression of Ang-2, and downregulation of Ang-1, acting via the Tie2 receptor in the presence of VEGF, play important roles in initiating early neovascularization and transformation of noncancerous hepatic tissue to HCC.

4. Physiological Angiogenesis

Experimental animal models of liver regeneration after partial hepatectomy have enabled the development of functional sinusoids to be studied. Resection of two thirds of the total liver mass results in stimulation of quiescent hepatocytes, and activation of numerous transcription factors that modulate many genes involved in the proliferation of hepatocytes [38–40]. An exponential increase in the number of hepatocytes leads to definitive architectural and functional changes in the liver; avascular clusters of cells are generated that induce the differentiation and subsequent replication of the other types of hepatic cells, notably liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and other EC precursors mobilized from the bone marrow. All of these different types of cell migrate and interact with the emerging mass of hepatocytes; they augment the metabolic functions, including energy metabolism, of hepatocytes and provide structural support of the maturing hepatic parenchyma. Subsequently, fullyfunctional sinusoids are generated about six to eight days after hepatectomy [41, 42].

5. Pathological Hepatic Angiogenesis

While physiological hepatic angiogenesis during liver regeneration leads to the formation of new functional sinusoids, pathological angiogenesis, which occurs in many chronic liver diseases, is characterized by the appearance of capillarized vascular structures [16, 43] (Figure 5). Such anomalous intrahepatic vascular organization impairs the physiological interplay between sinusoids and parenchymal cells. Hepatic injury is exacerbated by enhanced activation of immunological responses and generation of more intense hypoxic areas.

Most chronic liver diseases are characterized by diffuse, chronic processes of inflammation, necrosis and fibrosis [2]. Altered mechanisms of chronic wound healing in response to persistent liver injury lead to an imbalance in the expression of many cytokines, growth factors and

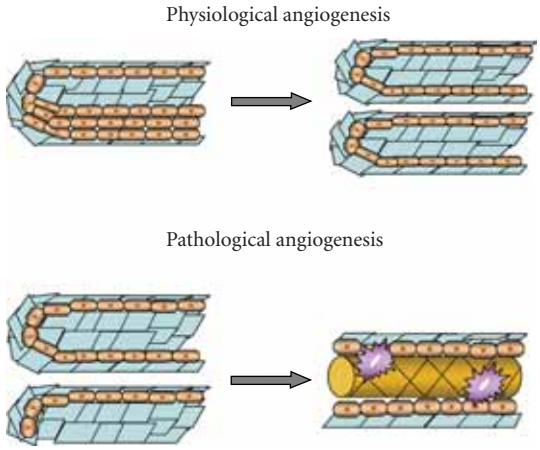


Figure 5: Hepatic angiogenesis: during liver regeneration, newly formed sinusoids have normal architecture consistent with their physiological roles. In contrast, pathological hepatic angiogenesis leads to the capillarization of hepatic sinusoids with the result that the structure of sinusoids becomes distorted and characteristic endothelial fenestrations are lost.

metalloproteinases (MMPs), which deregulate pathways of physiological angiogenesis and, hence, lead to structural and functional hepatic disorganization [44]. These phenomena induce accumulation of an excess of extracellular matrix, the composition of which becomes altered by the deposition of fibrillar collagen (type I) rather than physiological sinusoidal collagen (type IV). Subsequently, the characteristic fenestrations of LSEC become blocked, and the physiological interchange of oxygen and metabolic factors is hampered. The deposition of fibrous tissue increases resistance to blood flow and, consequently, impairs the delivery of oxygen to the parenchyma; the resulting tissue hypoxia exacerbates tissue injury.

Our group described the existence of abnormal vascular architecture in the liver of patients with autoimmune hepatitis and primary biliary cirrhosis; these intrahepatic structural changes were associated with an increase in the expression of VEGF and angiopoietins [45]. Similar changes in VEGF and angiopoietins have been reported in the serum and liver tissue of patients with other chronic liver diseases, including chronic hepatitis B and, more recently, chronic hepatitis C [34, 46].

The development of HCC has long been related to chronic hepatocellular injury. Malignant transformation of hepatocytes may take place in regenerative nodules that undergo dysplastic changes. However, a potential contribution of hepatocytic precursors cannot be dismissed. Uncontrolled proliferation of hepatocytes generates hypoxic hepatic nodules that promote the stimulation of mechanisms that mediate the angiogenic switch, a phenomenon that has both diagnostic and therapeutic relevance [47–49].

In addition to the development of pharmacologic interventions directed at modulating many of the signalling routes involved in cell proliferation, survival and invasion [50], the hypervascular characteristics of HCC have also encouraged therapeutic approaches that focus on inhibiting progression of the vascular network present in incipient malignant nodules and the distinctive tumor vasculature in established HCC [51, 52]. In many advanced malignancies, effective delivery of therapeutic drugs to the tumor is limited by specific complex features of tumor tissue. Recently, delivery of pharmacologic agents to malignant nodules has been enhanced by adopting measures that facilitate the transcellular route of molecular trafficking [53].

In summary, the relevance of chronic hepatocellular injury to the development of HCC has been emphasized. In particular, the significance of angiogenesis in the progression of chronic hepatic disease to hepatic malignancies has been highlighted. A role for deregulation of different stages of angiogenesis in the establishment of advanced tumours and their subsequent dissemination to other tissues has become recognized. Accordingly, the in-depth study of regulatory mechanisms that mediate angiogenesis in pathological conditions would appear to be indicated. One specific aim of such an initiative would be to augment the efficacy of currently available therapies for chronic (inflammatory) hepatic disease and HCC.

Acknowledgments

This work was supported by the Grants SAF 2007/63674 (to Ricardo Moreno-Otero) and Mutua Madrileña (to Paloma Sanz-Cameno and to Ricardo Moreno-Otero) CIBERehd is supported by Instituto de Salud Carlos III, Madrid, Spain.

References

[1] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *Ca: A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[2] J. Bruix and M. Sherman, "Management of hepatocellular carcinoma," *Hepatology*, vol. 42, no. 5, pp. 1208–1236, 2005.

[3] A. Forner, R. Vilana, C. Ayuso, et al., "Diagnosis of hepatic nodules 20 mm or smaller in cirrhosis: prospective validation of the noninvasive diagnostic criteria for hepatocellular carcinoma," *Hepatology*, vol. 47, no. 1, pp. 97–104, 2008.

[4] D. Semela and J.-F. Dufour, "Angiogenesis and hepatocellular carcinoma," *Journal of Hepatology*, vol. 41, no. 5, pp. 864–880, 2004.

[5] H. H. Marti, "Angiogenesis–a self-adapting principle in hypoxia," *EXS*, no. 94, pp. 163–180, 2005.

[6] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[7] G. Bergers and L. E. Benjamin, "Tumorigenesis and the angiogenic switch," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 401–410, 2003.

[8] P. Friedl and K. Wolf, "Tumour-cell invasion and migration: diversity and escape mechanisms," *Nature Reviews Cancer*, vol. 3, no. 5, pp. 362–374, 2003.

[9] C. W. Pugh and P. J. Ratcliffe, "Regulation of angiogenesis by hypoxia: role of the HIF system," *Nature Medicine*, vol. 9, no. 6, pp. 677–684, 2003.

[10] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.

[11] K. J. Simpson, N. C. Henderson, C. L. Bone-Larson, N. W. Lukacs, C. M. Hogaboam, and S. L. Kunkel, "Chemokines in the pathogenesis of liver disease: so many players with poorly defined roles," *Clinical Science*, vol. 104, no. 1, pp. 47–63, 2003.

[12] D. M. Noonan, A. De Lerma Barbaro, N. Vannini, L. Mortara, and A. Albini, "Inflammation, inflammatory cells and angiogenesis: decisions and indecisions," *Cancer and Metastasis Reviews*, vol. 27, no. 1, pp. 31–40, 2008.

[13] P. Carmeliet, "Mechanisms of angiogenesis and arteriogenesis," *Nature Medicine*, vol. 6, no. 4, pp. 389–395, 2000.

[14] P. Carmeliet, "Angiogenesis in health and disease," *Nature Medicine*, vol. 9, no. 6, pp. 653–660, 2003.

[15] M. Chaparro, P. Sanz-Cameno, M. Trapero-Marugán, L. García-Buey, and R. Moreno-Otero, "Mechanisms of angiogenesis in chronic inflammatory liver disease," *Annals of Hepatology*, vol. 6, no. 4, pp. 208–213, 2007.

[16] C. García-Monzón, F. Sánchez-Madrid, L. García-Buey, A. García-Arroyo, A. García-Sánchez, and R. Moreno-Otero, "Vascular adhesion molecule expression in viral chronic hepatitis: evidence of neoangiogenesis in portal tracts," *Gastroenterology*, vol. 108, no. 1, pp. 231–241, 1995.

[17] J. Medina, A. G. Arroyo, F. Sánchez-Madrid, and R. Moreno-Otero, "Angiogenesis in chronic inflammatory liver disease," *Hepatology*, vol. 39, no. 5, pp. 1185–1195, 2004.

[18] P. Majano, E. Lara-Pezzi, M. López-Cabrera, A. Apolinario, R. Moreno-Otero, and C. García-Monzón, "Hepatitis B virus X protein transactivates inducible nitric oxide synthase gene promoter through the proximal nuclear factor κB-binding site: evidence that cytoplasmic location of X protein is essential for gene transactivation," *Hepatology*, vol. 34, no. 6, pp. 1218–1224, 2001.

[19] P. L. Majano, C. García-Monzón, M. López-Cabrera, et al., "Inducible nitric oxide synthase expression in chronic viral hepatitis. Evidence for a virus-induced gene upregulation," *Journal of Clinical Investigation*, vol. 101, no. 7, pp. 1343–1352, 1998.

[20] T. Murohara, T. Asahara, M. Silver, et al., "Nitric oxide synthase modulates angiogenesis in response to tissue ischemia," *Journal of Clinical Investigation*, vol. 101, no. 11, pp. 2567–2578, 1998.

[21] N. Hiraoka, E. Allen, I. J. Apel, M. R. Gyetko, and S. J. Weiss, "Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins," *Cell*, vol. 95, no. 3, pp. 365–377, 1998.

[22] C. Jackson, "Matrix metalloproteinases and angiogenesis," *Current Opinion in Nephrology and Hypertension*, vol. 11, no. 3, pp. 295–299, 2002.

[23] N. Ferrara, H.-P. Gerber, and J. LeCouter, "The biology of VEGF and its receptors," *Nature Medicine*, vol. 9, no. 6, pp. 669–676, 2003.

[24] J.-S. Silvestre, R. Tamarat, T. G. Ebrahimian, et al., "Vascular endothelial growth factor-B promotes in vivo angiogenesis," *Circulation Research*, vol. 93, no. 2, pp. 114–123, 2003.

[25] E. S. Wittchen, "Endothelial signaling in paracellular and transcellular leukocyte transmigration," *Frontiers in Bioscience*, vol. 14, pp. 2522–2545, 2009.

[26] E. Dejana, F. Orsenigo, and M. G. Lampugnani, "The role of adherens junctions and VE-cadherin in the control of vascular permeability," *Journal of Cell Science*, vol. 121, no. 13, pp. 2115–2122, 2008.

[27] J. Medina, L. Caveda, P. Sanz-Cameno, et al., "Hepatocyte growth factor activates endothelial proangiogenic mechanisms relevant in chronic hepatitis C-associated neoangiogenesis," *Journal of Hepatology*, vol. 38, no. 5, pp. 660–667, 2003.

[28] X. Salcedo, J. Medina, P. Sanz-Cameno, et al., "The potential of angiogenesis soluble markers in chronic hepatitis C," *Hepatology*, vol. 42, no. 3, pp. 696–701, 2005.

[29] G. D. Yancopoulos, S. Davis, N. W. Gale, J. S. Rudge, S. J. Wiegand, and J. Holash, "Vascular-specific growth factors and blood vessel formation," *Nature*, vol. 407, no. 6801, pp. 242–248, 2000.

[30] R. K. Jain, "Molecular regulation of vessel maturation," *Nature Medicine*, vol. 9, no. 6, pp. 685–693, 2003.

[31] R. Kalluri, "Basement membranes: structure, assembly and role in tumour angiogenesis," *Nature Reviews Cancer*, vol. 3, no. 6, pp. 422–433, 2003.

[32] G. Thurston, J. S. Rudge, E. Ioffe, et al., "Angiopoietin-1 protects the adult vasculature against plasma leakage," *Nature Medicine*, vol. 6, no. 4, pp. 460–463, 2000.

[33] T. R. Carlson, Y. Feng, P. C. Maisonpierre, M. Mrksich, and A. O. Morla, "Direct cell adhesion to the angiopoietins mediated by integrins," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 26516–26525, 2001.

[34] P. Sanz-Cameno, S. Martín-Vílchez, E. Lara-Pezzi, et al., "Hepatitis B virus promotes angiopoietin-2 expression in liver tissue: role of HBV X protein," *American Journal of Pathology*, vol. 169, pp. 1215–1222, 2006.

[35] Z.-L. Zhang, Z.-S. Liu, and Q. Sun, "Expression of angiopoietins, Tie2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma," *World Journal of Gastroenterology*, vol. 12, no. 26, pp. 4241–4245, 2006.

[36] M. Papetti and I. M. Herman, "Mechanisms of normal and tumor-derived angiogenesis," *American Journal of Physiology*, vol. 282, pp. C947–C970, 2002.

[37] P. Vajkoczy, M. Farhadi, A. Gaumann, et al., "Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2," *Journal of Clinical Investigation*, vol. 109, no. 6, pp. 777–785, 2002.

[38] A. K. Greene, S. Wiener, M. Puder, et al., "Endothelial-directed hepatic regeneration after partial hepatectomy," *Annals of Surgery*, vol. 237, no. 4, pp. 530–535, 2003.

[39] G. K. Michalopoulos, "Liver regeneration," *Journal of Cellular Physiology*, pp. 286–300, 2007.

[40] K. E. Wack, M. A. Ross, V. Zegarra, L. R. Sysko, S. C. Watkins, and D. B. Stolz, "Sinusoidal ultrastructure evaluated during the revascularization of regenerating rat liver," *Hepatology*, vol. 33, no. 2, pp. 363–378, 2001.

[41] M. A. Ross, C. M. Sander, T. B. Kleeb, S. C. Watkins, and D. B. Stolz, "Spatiotemporal expression of angiogenesis growth factor receptors during the revascularization of regenerating rat liver," *Hepatology*, vol. 34, no. 6, pp. 1135–1148, 2001.

[42] T. Sato, O. N. El-Assal, T. Ono, A. Yamanoi, D. Kumar Dhar, and N. Nagasue, "Sinusoidal endothelial cell proliferation and expression of angiopoietin/Tie family in regenerating rat liver," *Journal of Hepatology*, vol. 34, no. 5, pp. 690–698, 2001.

[43] J. Medina, L. García-Buey, and R. Moreno-Otero, "Review article: immunopathogenetic and therapeutic aspects of autoimmune hepatitis," *Alimentary Pharmacology and Therapeutics*, vol. 17, no. 1, pp. 1–16, 2003.

[44] S. S. Thorgeirsson and J. W. Grisham, "Molecular pathogenesis of human hepatocellular carcinoma," *Nature Genetics*, vol. 31, no. 4, pp. 339–346, 2002.

[45] J. Medina, P. Sanz-Cameno, L. García-Buey, S. Martín-Vílchez, M. López-Cabrera, and R. Moreno-Otero, "Evidence of angiogenesis in primary biliary cirrhosis: an immunohistochemical descriptive study," *Journal of Hepatology*, vol. 42, no. 1, pp. 124–131, 2005.

[46] P. Sanz-Cameno, S. Martin-Vilchez, Y. Rodriguez-Munoz, et al., "Intrahepatic angiopoietin-2 protein expression modulation by hepatitis c virus: MAPK, PI3K and reactive oxygen species (ROS) implication," *Journal of Hepatology*, vol. 48, supplement 2, pp. S230–S231, 2008.

[47] M. Borzio, S. Fargion, F. Borzio, et al., "Impact of large regenerative, low grade and high grade dysplastic nodules in hepatocellular carcinoma development," *Journal of Hepatology*, vol. 39, no. 2, pp. 208–214, 2003.

[48] L. R. Roberts and G. J. Gores, "Hepatocellular carcinoma: molecular pathways and new therapeutic targets," *Seminars in Liver Disease*, vol. 25, no. 2, pp. 212–225, 2005.

[49] T. Takayama, M. Makuuchi, S. Hirohashi, et al., "Malignant transformation of adenomatous hyperplasia to hepatocellular carcinoma," *The Lancet*, vol. 336, no. 8724, pp. 1150–1153, 1990.

[50] D. Hanahan and R. A. Weinberg, "The hallmarks of cancer," *Cell*, vol. 100, no. 1, pp. 57–70, 2000.

[51] M. Fernández, D. Semela, J. Bruix, I. Colle, M. Pinzani, and J. Bosch, "Angiogenesis in liver disease," *Journal of Hepatology*, vol. 50, no. 3, pp. 604–620, 2009.

[52] Z. F. Yang and R. T. P. Poon, "Vascular changes in hepatocellular carcinoma," *Anatomical Record*, vol. 291, no. 6, pp. 721–734, 2008.

[53] M. Simionescu, D. Popov, and A. Sima, "Endothelial transcytosis in health and disease," *Cell and Tissue Research*, vol. 335, no. 1, pp. 27–40, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 468725, 8 pages
doi:10.1155/2010/468725

Review Article

Angiogenesis and Lymphangiogenesis of Gastric Cancer

Yasuhiko Kitadai

Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

Correspondence should be addressed to Yasuhiko Kitadai, kitadai@hiroshima-u.ac.jp

Received 26 October 2009; Accepted 8 February 2010

Academic Editor: Kalpna Gupta

Tumor angiogenesis is the result of an imbalance between positive and negative angiogenic factors released by tumor and host cells into the microenvironment of the neoplastic tissue. The stroma constitutes a large part of most solid tumors, and cancer-stromal cell interactions contribute functionally to tumor growth and metastasis. Activated fibroblasts and macrophages in tumor stroma play important roles in angiogenesis and tumor progression. In gastric cancer, tumor cells and stromal cells produce various angiogenic factors, including vascular endothelial growth factor, interleukin-8, platelet-derived endothelial cell growth factor, and angiopoietin. In addition, *Helicobacter pylori* infection increases tumor cell expression of metastasis-related genes including those encoding several angiogenic factors. We review the current understanding of molecular mechanisms involved in angiogenesis and lymphangiogenesis of human gastric cancer.

1. Introduction

Gastric cancer is the world's second leading cause of cancer death [1]. In Asian countries such as Korea and China, gastric cancer is the leading cause of cancer death. Conventional therapies for advanced-stage gastric cancer include surgery, chemotherapy, and radiotherapy, but the prognosis for advanced-stage disease remains poor. Novel therapeutic strategies are needed, but their development depends on understanding cancer biology, especially changes that occur on the molecular level. A large number of genetic and epigenetic alterations in oncogenes and tumor suppressor genes as well as genetic instability determine the multistep process of gastric carcinogenesis [2]. In addition, the molecular events that characterize gastric cancer differ, depending on the histologic type, whether intestinal- or diffuse-type gastric cancer [2].

Tumor tissue, including gastric cancer, consists of both tumor cells and stromal cells. Tumor growth and metastasis are determined not only by tumor cells themselves but also by stromal cells. Recent studies have shown that interactions between tumor cells and activated stromal cells create a unique microenvironment that is crucial for tumor growth and metastasis (Figure 1) [3, 4]. The organ-specific microenvironment can influence the growth, vascularization, invasion, and metastasis of human neoplasms [5].

Angiogenesis and lymphangiogenesis are both essential for tumor growth and metastasis. Increased vascularity enhances the growth of primary neoplasms by supplying nutrients and oxygen, and it provides an avenue for hematogenous metastasis [6, 7]. Weidner et al. [8] first reported a direct correlation between the incidence of metastasis and the number and density of blood vessels in invasive breast cancers. Similar studies have confirmed this correlation in gastrointestinal cancers [9–12]. Induction of angiogenesis is mediated by a variety of molecules released by tumor cells as well as host stromal cells [6, 7]. Clinical prognosis depends on whether lymph node metastasis has occurred. The growth of lymphatic vessels (lymphangiogenesis) in the tumor periphery correlates with lymphatic metastasis in cases of gastric cancer [13, 14]. Lymphangiogenesis is regulated by members of the vascular endothelial growth factor (VEGF) family and their receptors. Herein, we discuss the role of angiogenic and lymphangiogenic fact-ors in the growth and metastasis of human gastric cancer.

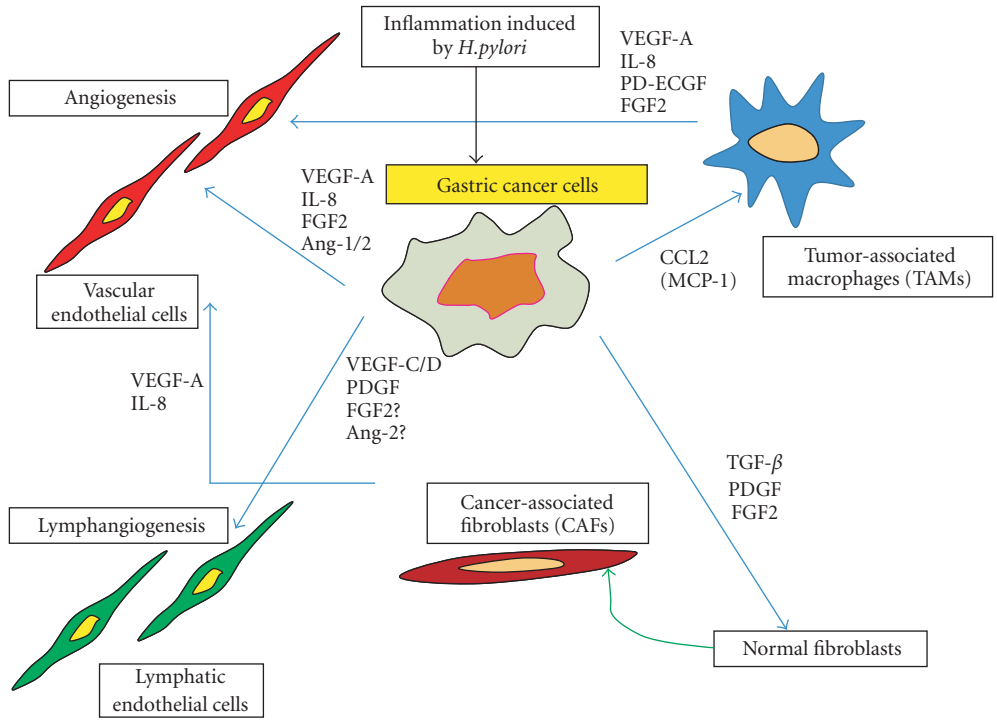


Figure 1: Interaction between gastric cancer cells and stromal cells influences angiogenesis and lymphangiogenesis through various angiogenic factors and cytokines.

2. Tumor Angiogenesis in Gastric Cancer

2.1. VEGF-A. Gastric cancer cells produce various angiogenic factors. Of these, VEGF (now termed VEGF-A) is considered one of the strongest promoters of angiogenesis of gastrointestinal tumors [15]. VEGF-A is released by cancer cells. Fibroblasts and inflammatory cells in tumor stroma are also sources of host-derived VEGF-A [16]. VEGF-A, also known as vascular permeability factor, is a secreted protein that may, in addition, play a pivotal role in hyperpermeability of the vessels [17]. Several groups of investigators have reported a correlation between VEGF-A expression and microvessel density (MVD) of human gastric cancer [11, 18, 19]. VEGF-A-positive tumors have been shown to have a poorer prognosis than that of VEGF-A-negative tumors [10, 12, 20].

The prognosis of gastric cancer depends on both histologic type and disease stage [21]. Intestinal-type gastric cancer tends to metastasize to the liver in a hematogenous manner. In contrast, diffuse-type gastric cancer is more invasive; dissemination is predominantly peritoneal. Factors responsible for liver metastasis and peritoneal dissemination have not yet been identified, however, we have found that the angiogenic phenotype differs between intestinal-type and diffuse-type gastric cancers [11, 19]. In comparison to diffuse-type gastric cancer, the intestinal-type is more dependent on angiogenesis. Intestinal-, but not diffuse-type, tumors have been shown to express high levels of VEGF-A, and the level of VEGF-A expression correlates significantly with vessel count [11, 19]. In contrast, fibroblast growth factor (FGF)-2 expression is higher in diffuse-type tumors, especially scirrhous-type tumors [22]. These findings suggest that VEGF-A promotes angiogenesis and progression of human gastric cancers, especially those of the intestinal-type.

Peripheral blood VEGF-A levels, that is, serum and/or plasma concentrations of VEGF-A, have been examined in patients with malignant disease. Ohta et al. [23] examined VEGF-A levels in peripheral blood and the tumor drainage vein and then evaluated these in relation to clinicopathologic features of gastric cancer. They found that the peripheral blood plasma VEGF-A level is increased in patients with venous invasion and that the increase correlates with lymph node metastasis. The level of VEGF-A in plasma from peripheral veins is a sensitive marker for the progression of gastric cancer.

2.2. Interleukin (IL)-8. IL-8 is a multifunctional cytokine that can stimulate division of endothelial cells. IL-8 can induce migration of some tumor cells [24] and has been implicated in the induction of angiogenesis in such diverse diseases as psoriasis and rheumatoid arthritis and in some malignant diseases. IL-8 is a known angiogenic factor present in human lung cancer [25, 26] and is also produced by melanomas [27] and bladder [28] and prostate [29] cancers. We examined expression of IL-8 in human gastric cancer and found that most tumor tissues express IL-8 at levels higher than those in the corresponding normal mucosa [29, 30]. The IL-8 mRNA level in neoplasms correlates strongly with vascularization, suggesting that IL-8 produced by tumor cells regulates neovascularization. To provide evidence for

a causal role of IL-8 in angiogenesis and tumorigenicity of human gastric cancer, we introduced the IL-8 gene into several human gastric cancer cell lines. Gastric cancer cells transfected with the IL-8 gene and injected orthotopically into the gastric wall of nude mice produce fast-growing, highly vascular neoplasms [31].

Gastric cancer cells express not only IL-8 but also IL-8 receptor A (CXCR1) and IL-8 receptor B (CXCR2) [32]. In vitro treatment of human gastric cancer cells (MKN-1 cells) with exogenous IL-8 enhances the expression of epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMP)-9, VEGF-A, and IL-8 mRNAs. In contrast, such treatment decreases expression of E-cadherin mRNA. IL-8 treatment increases the invasive capacity of gastric cancer cells, and IL-8 expression is associated with MMP-9 activity. Collectively, these findings indicate that human gastric cancer cells express receptors for IL-8 and that IL-8 may have autocrine/paracrine roles in the progressive growth of human gastric cancer.

The prognosis for patients with gastric cancer expressing high levels of IL-8 and VEGF-A is significantly poorer than that for patients whose tumors express low levels [20]. A high level of IL-8 in the drainage vein of gastric cancer is associated significantly with a relatively short disease-free survival period [33].

2.3. Platelet-Derived Endothelial Cell Growth Factor (PD-ECGF). PD-ECGF, an endothelial cell mitogen that was initially purified to homogeneity from human platelets, has chemotactic activity for endothelial cells in vitro and is angiogenic in vivo [34]. PD-ECGF was shown to be identical to thymidine phosphorylase, an enzyme involved in pyrimidine nucleoside metabolism [35]. PD-ECGF expression is elevated in several types of solid tumor including colon cancer [36, 37]. We reported that PD-ECGF is associated with angiogenesis of human colon cancer [38]. PD-ECGF is expressed at high levels in vascular tumors that express low levels of VEGF-A [38]. In such colon cancers, the major source of PD-ECGF is the infiltrating cells. A positive association between PD-ECGF expression and MVD has also been reported for human gastric cancer [39–41]. In human gastric cancer, PD-ECGF is expressed more frequently in infiltrating cells than in tumor epithelium [39]. An association exists between PD-ECGF expression by infiltrating cells, VEGF-A expression by tumor epithelium, and vessel counts in intestinal-type gastric cancer but not in diffuse-type gastric cancer [39].

2.4. Angiopoietin. The angiopoietin family growth factors have been identified as ligands for Tie-2. Angiopoietin-1 activates Tie-2, leading to receptor autophosphorylation upon binding, and it simulates endothelial cell migration in vitro and contributes to blood vessel stabilization by recruitment of pericytes [42]. Angiopoietin-2 is a natural antagonist for Tie-2 receptor [43]. As such, it antagonizes angiopoietin-1-vessel maturation and regulates blood vessel growth, regression, or sprouting, depending on the presence of VEGF [43]. VEGF-A/VEGFR2 is mainly involved in the initiation of angiogenesis, whereas the angiopoietin/Tie2 system is related to remodeling and maturation of vessels [44]. Angiopoietin-1 and -2 are reported to be highly expressed in human gastric cancer [45, 46]. Inhibition of angiopoietin-1 by antisense expression vector was shown to reduce tumorigenesis and angiogenesis of gastric cancer xenografts in nude mice [47]. Production of angiopoietin-2 also contributes to tumor angiogenesis of gastric cancer in the presence of VEGF by induction of proteases in endothelial cells [48].

3. Tumor Lymphangiogenesis in Gastric Cancer

The VEGF family includes VEGF-A, -B, -C, -D, -E, and -F and placental growth factor (PlGF) [49]. VEGF-C and VEGF-D are ligands for VEGF receptor (VEGFR)-3 and VEGFR-2. VEGFR-3 is a tyrosine kinase receptor, that is, expressed predominantly in the endothelium of lymphatic vessels [50]. Skobe et al. [51] described VEGF-C as a lymphangiogenic factor that can selectively induce hyperplasia of the lymphatic vasculature. A significant correlation between lymph node metastasis and VEGF-C expression has been reported in gastric cancer [13, 14]. Onogawa et al. [52] examined expression of VEGF-C and VEGF-D by immunohistochemistry in 140 archival surgical specimens of submucosally invasive gastric cancer. VEGF-C immunoreactivity was associated with lymphatic invasion, lymph node metastasis, and increased MVD. There was no association between VEGF-D immunoreactivity and clinicopathologic features. These results suggest that VEGF-C is a dominant regulator of lymphangiogenesis in early-stage human gastric cancer.

VEGFRs are expressed by a wide variety of cancer cell lines. VEGF-A and VEGFR-1/2 are coexpressed in a number of cancers, including cancers of the breast [53], prostate [54], colon [55], and pancreas [56, 57], suggesting that VEGF-A directly influences tumor cell growth via an autocrine mechanism. VEGFR-3 has also been detected on several types of malignant cells, although the significance of such expression remains unclear. We recently examined the expression and function of VEGFR-3 in gastric cancer cells [58]. In vitro treatment of gastric cancer cell line KKLS, which expresses VEGFR-3, with the ligand of this receptor, VEGF-C, stimulated cell proliferation and increased expression of mRNAs encoding cyclin D1, PlGF, and autocrine motility factor [58]. Thus, VEGF-C may act in both an autocrine fashion and paracrine fashion to promote angiogenesis and further the growth of human gastric cancer.

Other growth factors are reported to be lymphangiogenic, such as FGF-2 [59] and platelet-derived growth factor (PDGF)-BB [60]. The lymphangiogenic effect of FGF-2 appears to be indirect, that is, it occurs via VEGF-C or -D. We recently found that gastric cancer cells produce PDGF-BB and that dilated lymphatics in the tumor periphery express PDGF receptor-β (Kodama et al., unpublished data), suggesting that PDGF-BB is a regulator of lymphangiogenesis in gastric cancer. Angiopoietin-2 is crucial for establishing the lymphatic vasculature. VEGF-C/VEGFR-3 signaling is a key primary proliferation pathway for lymphatic vessels, whereas angiopoietin-2 is importantin later remodeling stages [61].

The importance of FGF-2, PDGF-BB, and angiopoietin-2 for lymphatic metastasis of human gastric cancer is still unknown.

4. Tumor-Stromal Cell Interaction in Tumor Angiogenesis

Tumor stroma consists of activated fibroblasts (myofibroblasts), smooth muscle cells, endothelial cells, and inflammatory cells, including macrophages. Macrophages migrating to tumor stroma are called tumor-associated macrophages (TAMs). The role of TAMs in tumor progression is complicated. Although activated macrophages may have antitumor activity, tumor cells are reported to escape the antitumor activity of TAMs [62]. It has become clear that TAMs are active players in the process of tumor progression and invasion. Indeed, removal of macrophages by genetic mutation has been shown to reduce tumor progression and metastasis [63]. One important characteristic of macrophages is the potential for angiogenic activity. Activated macrophages produce various factors that induce angiogenesis in wound repair [64] and in chronic inflammatory diseases [65, 66]. Upon activation by cancer cells, TAMs can release diverse growth factors, proteolytic enzymes, and cytokines. In clinical studies, high numbers of TAMs have been shown to correlate with high vessel density and tumor progression [67–69]. We previously reported that TAM infiltration into tumor tissue correlates significantly with tumor vascularity in human esophageal and gastric cancers [67, 68]. Ishigami et al. [69] also found direct associations between the degree of TAM infiltration and depth of tumor invasion, nodal status, and clinical stage in cases of gastric cancer. Macrophage recruitment is mediated by a variety of chemoattractants, including monocyte chemoattractant protein-1 (MCP-1/CCL2) and macrophage inflammatory protein 1α (MIP-1α/CCL3). Of the CC chemokines, MCP-1 is one of the most potent [70]. We found that MCP-1 produced by tumor cells is associated significantly with macrophage infiltration and malignant behavior, such as angiogenesis, tumor invasion, and lymphatic infiltration [67, 68]. Transfection of the MCP-1 gene into gastric cancer cells was shown to cause strong infiltration of macrophages into tumors and enhanced tumorigenicity and metastatic potential in a mouse orthotopic implantation model [71]. Because activated macrophages produce VEGF-A, IL-8, FGF-2, and PD-ECGF, MCP-1 expressed by gastric cancer cells plays a role in angiogenesis via recruitment and activation of macrophages.

Activated fibroblasts in cancer stroma are prominent modifiers of tumor progression and are therefore called cancer-associated fibroblasts (CAFs) [72, 73]. CAFs have gene expression profiles that are distinct from those of normal fibroblasts [74], and they acquire a modified phenotype, similar to that of fibroblasts associated with wound healing. Tumor tissue contains abundant growth factors, cytokines, and matrix-remodeling proteins; thus, tumors are likened to wounds that never heal [75]. Although the mechanisms that regulate activation of fibroblasts in tumors are not fully understood, PDGF, transforming growth factor-β, and FGF-2 are known to be partly involved [72, 73, 76]. We previously reported that CAFs and pericytes express PDGF receptor, and targeting the PDGF receptor on stromal cells inhibits growth and metastasis of human colon cancer [76, 77]. Therefore, CAFs might serve as novel therapeutic targets in cancer patients.

5. *Helicobacter pylori (H. pylori)* Stimulates Angiogenesis of Gastric Cancer

A recent advance in understanding the pathogenesis of gastric cancer is recognition of the role of *H. pylori* in gastric carcinogenesis. *H. pylori* infection is thought to contribute significantly to the pathogenesis of atrophic gastritis, intestinal metaplasia, and peptic ulcer. Epidemiologic studies have indicated that infection with *H. pylori* is a risk factor for gastric cancer, and in 1994 the WHO/IARC classified this bacterium as a definite biologic carcinogen [78]. In addition, inoculation of the stomach of Mongolian gerbils with *H. pylori* was shown to be associated with the occurrence of chronic gastritis, intestinal metaplasia, and adenocarcinoma [79, 80]. A recent study showed that *H. pylori* penetrates normal, metaplastic, and neoplastic epithelia to cause a strong immune-inflammatory response and promote gastric carcinogenesis [81]. *H. pylori* is a potent activator of nuclear factor-kB (NF-kB) in gastric epithelial cells. Activation of NF-kB by *H. pylori* infection induces a variety of cytokines, angiogenic factors, MMPs, and adhesion molecules [82, 83]. The relation between *H. pylori* infection and angiogenesis has been studied increasingly over the past few years. We reported previously that *H. pylori*-infected gastric cancer patients show greater tumor vascularity than that of gastric cancer patients after *H. pylori* eradication [84], suggesting that *H. pylori* infection influences angiogenesis of gastric cancer. Some studies suggested that the cagA-positive *H. pylori* strain plays an important role in tissue remodeling, angiogenesis, cancer invasion, and metastasis [85–87]. Crabtree et al. [85] reported that *H. pylori* infection induces IL-8 production by gastric epithelium. We found that coculture of gastric cancer cells with *H. pylori* induces expression of mRNAs encoding IL-8, VEGF-A, angiogenin, urokinase-type plasminogen activator, and MMP-9 by gastric cancer cells [86]. Wu et al. [87] also reported that *H. pylori* influences expression of VEGF-A and MMP-9 and promotes gastric cell invasion via COX-2- and NF-kB-mediated pathways. Expression of COX-2 was found to correlate significantly with VEGF expression and MVD in gastric cancer [88, 89].

6. Antiangiogenic Therapy Against Gastric Cancer

A novel category of anticancer drugs, "molecular-targeted drugs", has become available. Angiogenesis is considered one of the most important molecular targets for anticancer therapy because it is essential for tumor growth and metastasis. VEGF is one of the most potent angiogenic factors and is expressed in almost all human solid tumors

investigated, such as colorectal, esophageal, gastric, lung, breast, renal, and ovarian cancers [9, 12, 15, 90]. In these cancers, expression of VEGF correlates with advanced stage disease and poor prognosis. Therefore, inhibiting VEGF is a rational strategy for treating cancer [91]. Bevacizumab is a humanized monoclonal antibody that targets VEGF. Significant prolonged survival has been reported in patients with metastatic colorectal cancer treated with bevacizumab in combination with cytotoxic chemotherapy [92]. Similar improvement were observed in patients with breast and non-small cell lung cancers. A randomized trial evaluating the efficacy of this agent in patients with gastric cancer (the AVAGAST study) is now being conducted internationally; Japan and Korea are included [93]. Several other strategies targeting the VEGF signaling pathway have been developed, including use of soluble receptors binding directly to VEGF ligand, anti-VEGFR antibodies, and VEGR tyrosine kinase inhibitors. These next-generation targeted agents are being evaluated in early clinical studies [91, 93].

7. Future Perspectives

Tumor cells are genetically unstable and biologically heterogeneous, and these are the principal causes of the failure of systemic chemotherapies. It has been believed that endothelial cells and CAFs in tumor stroma are genetically stable and that these cells will not become drug resistant in response to antivascular therapy. However, recent studies showed that endothelial cells in certain tumor vessels are aneuploid and that they express neoplastic markers [94]. Recently, investigators have come to appreciate the significance of other cell types, such as pericytes and vascular smooth muscle cells, that make up vascular structures and are essential for the function and survival of endothelial cells. The structure and function of vessels differ in different tissues and in tumors at different sites. Inhibition of activated stromal cell components including TAMs and CAFs may effectively alter the tumor microenvironment involved in tumor angiogenesis and progression. Understanding the cellular and molecular mechanisms that regulate vascularization of tumors may facilitate development of effective antivascular therapies.

References

[1] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *Ca: A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[2] M. G. Smith, G. L. Hold, E. Tahara, and E. M. El-Omar, "Cellular and molecular aspects of gastric cancer," *World Journal of Gastroenterology*, vol. 12, no. 18, pp. 2979–2990, 2006.

[3] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[4] T. L. Whiteside, "The tumor microenvironment and its role in promoting tumor growth," *Oncogene*, vol. 27, no. 45, pp. 5904–5912, 2008.

[5] I. J. Fidler, "Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture," *Cancer Research*, vol. 50, no. 19, pp. 6130–6138, 1990.

[6] J. Folkman, "How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial award lecture," *Cancer Research*, vol. 46, no. 2, pp. 467–473, 1986.

[7] J. Folkman, "What is the evidence that tumors are angiogenesis dependent?" *Journal of the National Cancer Institute*, vol. 82, no. 1, pp. 4–6, 1990.

[8] N. Weidner, J. P. Semple, W. R. Welch, and J. Folkman, "Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma," *The New England Journal of Medicine*, vol. 324, no. 1, pp. 1–8, 1991.

[9] Y. Takahashi, Y. Kitadai, C. D. Bucana, K. R. Cleary, and L. M. Ellis, "Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer," *Cancer Research*, vol. 55, no. 18, pp. 3964–3968, 1995.

[10] N. Tanigawa, H. Amaya, M. Matsumura, et al., "Extent of tumor vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas," *Cancer Research*, vol. 56, no. 11, pp. 2671–2676, 1996.

[11] Y. Takahashi, K. R. Cleary, M. Mai, Y. Kitadai, C. D. Bucana, and L. M. Ellis, "Significance of vessel count and vascular endothelial growth factor and its receptor (KDR) in intestinal-type gastric cancer," *Clinical Cancer Research*, vol. 2, no. 10, pp. 1679–1684, 1996.

[12] K. Maeda, Y.-S. Chung, Y. Ogawa, et al., "Prognostic value of vascular endothelial growth factor expression in gastric carcinoma," *Cancer*, vol. 77, no. 5, pp. 858–863, 1996.

[13] T. Amioka, Y. Kitadai, S. Tanaka, et al., "Vascular endothelial growth factor-C expression predicts lymph node metastasis of human gastric carcinomas invading the submucosa," *European Journal of Cancer*, vol. 38, no. 10, pp. 1413–1419, 2002.

[14] Y. Yonemura, Y. Endo, H. Fujita, et al., "Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer," *Clinical Cancer Research*, vol. 5, no. 7, pp. 1823–1829, 1999.

[15] G. D. Yancopoulos, S. Davis, N. W. Gale, J.S. Rudge, S. J. Wiegand, and J. Holash, "Vascular-specific growth factors and blood vessel formation," *Nature*, vol. 407, no. 6801, pp. 242–248, 2000.

[16] D. Fukumura, R. Xavier, T. Sugiura, et al., "Tumor induction of VEGF promoter activity in stromal cells," *Cell*, vol. 94, no. 6, pp. 715–725, 1998.

[17] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, 1983.

[18] L. F. Brown, B. Berse, R. W. Jackman, et al., "Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract," *Cancer Research*, vol. 53, no. 18, pp. 4727–4735, 1993.

[19] S. Yamamoto, W. Yasui, Y. Kitadai, et al., "Expression of vascular endothelial growth factor in human gastric carcinomas," *Pathology International*, vol. 48, no. 7, pp. 499–506, 1998.

[20] S. Kido, Y. Kitadai, N. Hattori, et al., "Interleukin 8 and vascular endothelial growth factor—prognostic factors in human gastric carcinomas?" *European Journal of Cancer*, vol. 37, no. 12, pp. 1482–1487, 2001.

[21] I. Duarte and O. Llanos, "Patterns of metastases in intestinal and diffuse types of carcinoma of the stomach," *Human Pathology*, vol. 12, no. 3, pp. 237–242, 1981.

[22] H. Tanimoto, K. Yoshida, H. Yokozaki, et al., "Expression of basic fibroblast growth factor in human gastric carcinomas," *Virchows Archiv B*, vol. 61, no. 4, pp. 263–267, 1991.

[23] M. Ohta, H. Konno, T. Tanaka, et al., "The significance of circulating vascular endothelial growth factor (VEGF) protein in gastric cancer," *Cancer Letters*, vol. 192, no. 2, pp. 215–225, 2003.

[24] J. M. Wang, G. Taraboletti, K. Matsushima, J. Van Damme, and A. Mantovani, "Induction of haptotactic migration of melanoma cells by neutrophil activating protein/interleukin-8," *Biochemical and Biophysical Research Communications*, vol. 169, no. 1, pp. 165–170, 1990.

[25] R. M. Strieter, P. J. Polverini, D. A. Arenberg, et al., "Role of C-X-C chemokines as regulators of angiogenesis in lung cancer," *Journal of Leukocyte Biology*, vol. 57, no. 5, pp. 752–762, 1995.

[26] D. R. Smith, P. J. Polverini, S. L. Kunkel, et al., "Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma," *Journal of Experimental Medicine*, vol. 179, no. 5, pp. 1409–1415, 1994.

[27] R. K. Singh, M. Gutman, R. Radinsky, C. D. Bucana, and I. J. Fidler, "Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice," *Cancer Research*, vol. 54, no. 12, pp. 3242–3247, 1994.

[28] M. Tachibana, A. Miyakawa, J. Nakashima, et al., "Constitutive production of multiple cytokines and a human chorionic gonadotrophin β-subunit by a human bladder cancer cell line (KU-19-19): possible demonstration of totipotential differentiation," *British Journal of Cancer*, vol. 76, no. 2, pp. 163–174, 1997.

[29] G. F. Greene, Y. Kitadai, C. A. Pettaway, A. C. von Eschenbach, C. D. Bucana, and I. J. Fidler, "Correlation of metastasis-related gene expression with metastatic potential in human prostate carcinoma cells implanted in nude mice using an in situ messenger RNA hybridization technique," *American Journal of Pathology*, vol. 150, no. 5, pp. 1571–1582, 1997.

[30] Y. Kitadai, K. Haruma, K. Sumii, et al., "Expression of interleukin-8 correlates with vascularity in human gastric carcinomas," *American Journal of Pathology*, vol. 152, no. 1, pp. 93–100, 1998.

[31] Y. Kitadai, Y. Takahashi, K. Haruma, et al., "Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice," *British Journal of Cancer*, vol. 81, no. 4, pp. 647–653, 1999.

[32] Y. Kitadai, K. Haruma, N. Mukaida, et al., "Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8," *Clinical Cancer Research*, vol. 6, no. 7, pp. 2735–2740, 2000.

[33] H. Konno, M. Ohta, M. Baba, S. Suzuki, and S. Nakamura, "The role of circulating IL-8 and VEGF protein in the progression of gastric cancer," *Cancer Science*, vol. 94, no. 8, pp. 735–740, 2003.

[34] F. Ishikawa, K. Miyazono, U. Hellman, et al., "Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor," *Nature*, vol. 338, no. 6216, pp. 557–562, 1989.

[35] A. Moghaddam and R. Bicknell, "Expression of platelet-derived endothelial cell growth factor in Escherichia coli and confirmation of its thymidine phosphorylase activity," *Biochemistry*, vol. 31, no. 48, pp. 12141–12146, 1992.

[36] K. Reynolds, F. Farzaneh, W. P. Collins, et al., "Association of ovarian malignancy with expression of platelet-derived endothelial cell growth factor," *Journal of the National Cancer Institute*, vol. 86, no. 16, pp. 1234–1238, 1994.

[37] M. Toi, K. Inada, S. Hoshina, H. Suzuki, S. Kondo, and T. Tominaga, "Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human," *Clinical Cancer Research*, vol. 1, no. 9, pp. 961–964, 1995.

[38] Y. Takahashi, C. D. Bucana, W. Liu, et al., "Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: role of infiltrating cells," *Journal of the National Cancer Institute*, vol. 88, no. 16, pp. 1146–1151, 1996.

[39] Y. Takahashi, C. D. Bucana, Y. Akagi, et al., "Significance of platelet-derived endothelial cell growth factor in the angiogenesis of human gastric cancer," *Clinical Cancer Research*, vol. 4, no. 2, pp. 429–434, 1998.

[40] K. Maeda, Y.-S. Chung, Y. Ogawa, et al., "Thymidine phosphorylase/platelet-derived endothelial cell growth factor expression associated with hepatic metastasis in gastric carcinoma," *British Journal of Cancer*, vol. 73, no. 8, pp. 884–888, 1996.

[41] H. Saito, S. Tsujitani, S. Oka, et al., "The expression of thymidine phosphorylase correlates with angiogenesis and the efficacy of chemotherapy using fluorouracil derivatives in advanced gastric carcinoma," *British Journal of Cancer*, vol. 81, no. 3, pp. 484–489, 1999.

[42] S. Davis, T. H. Aldrich, P. F. Jones, et al., "Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning," *Cell*, vol. 87, no. 7, pp. 1161–1169, 1996.

[43] P. C. Maisonpierre, C. Suri, P. F. Jones, et al., "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55–60, 1997.

[44] T. N. Sato, Y. Tozawa, U. Deutsch, et al., "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation," *Nature*, vol. 376, no. 6535, pp. 70–74, 1995.

[45] J. Wang, K. Wu, D. Zhang, et al., "Expressions and clinical significances of angiopoietin-1, -2 and Tie2 in human gastric cancer," *Biochemical and Biophysical Research Communications*, vol. 337, no. 1, pp. 386–393, 2005.

[46] T. Nakayama, A. Yoshizaki, N. Kawahara, et al., "Expression of Tie-1 and 2 receptors, and angiopoietin-1, 2 and 4 in gastric carcinoma; immunohistochemical analyses and correlation with clinicopathological factors," *Histopathology*, vol. 44, no. 3, pp. 232–239, 2004.

[47] J. Wang, K.-C. Wu, D.-X. Zhang, and D.-M. Fan, "Antisense angiopoietin-1 inhibits tumorigenesis and angiogenesis of gastric cancer," *World Journal of Gastroenterology*, vol. 12, no. 15, pp. 2450–2454, 2006.

[48] T. Etoh, H. Inoue, S. Tanaka, G. F. Barnard, S. Kitano, and M. Mori, "Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases," *Cancer Research*, vol. 61, no. 5, pp. 2145–2153, 2001.

[49] Z. K. Otrock, J. A. Makarem, and A. I. Shamseddine, "Vascular endothelial growth factor family of ligands and receptors: review," *Blood Cells, Molecules, and Diseases*, vol. 38, no. 3, pp. 258–268, 2007.

[50] A. Kaipainen, J. Korhonen, T. Mustonen, et al., "Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 8, pp. 3566–3570, 1995.

[51] M. Skobe, T. Hawighorst, D. G. Jackson, et al., "Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis," *Nature Medicine*, vol. 7, no. 2, pp. 192–198, 2001.

[52] S. Onogawa, Y. Kitadai, T. Amioka, et al., "Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in early gastric carcinoma: correlation with clinicopathological parameters," *Cancer Letters*, vol. 226, no. 1, pp. 85–90, 2005.

[53] J. S. de Jong, P. J. van Diest, P. van der Valk, and J. P. A. Baak, "Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: an inventory in search of autocrine and paracrine loops," *Journal of Pathology*, vol. 184, no. 1, pp. 44–52, 1998.

[54] M. E. Harper, E. Glynne-Jones, L. Goddard, V. J. Thurston, and K. Griffiths, "Vascular endothelial growth factor (VEGF) expression in prostatic tumours and its relationship to neuroendocrine cells," *British Journal of Cancer*, vol. 74, no. 6, pp. 910–916, 1996.

[55] T. Kuwai, T. Nakamura, S.-J. Kim, et al., "Intratumoral heterogeneity for expression of tyrosine kinase growth factor receptors in human colon cancer surgical specimens and orthotopic tumors," *American Journal of Pathology*, vol. 172, no. 2, pp. 358–366, 2008.

[56] J. Itakura, T. Ishiwata, B. Shen, M. Kornmann, and M. Korc, "Concomitant over-expression of vascular endothelial growth factor and its receptors in pancreatic cancer," *International Journal of Cancer*, vol. 85, no. 1, pp. 27–34, 2000.

[57] Z. von Marschall, T. Cramer, and M. Höcker, "De novo expression of vascular endothelial growth factor in human pancreatic cancer: evidence for an autocrine mitogenic loop," *Gastroenterology*, vol. 119, no. 5, pp. 1358–1372, 2000.

[58] M. Kodama, Y. Kitadai, M. Tanaka, et al., "Vascular endothelial growth factor C stimulates progression of human gastric cancer via both autocrine and paracrine mechanisms," *Clinical Cancer Research*, vol. 14, no. 22, pp. 7205–7214, 2008.

[59] H. Kubo, R. Cao, E. Bräkenhielm, T. Mäkinen, Y. Cao, and K. Alitalo, "Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 13, pp. 8868–8873, 2002.

[60] R. Cao, M. A. Björndahl, P. Religa, et al., "PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis," *Cancer Cell*, vol. 6, no. 4, pp. 333–345, 2004.

[61] N. W. Gale, G. Thurston, S. F. Hackett, et al., "Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1," *Developmental Cell*, vol. 3, no. 3, pp. 411–423, 2002.

[62] C. E. Lewis and J. W. Pollard, "Distinct role of macrophages in different tumor microenvironments," *Cancer Research*, vol. 66, no. 2, pp. 605–612, 2006.

[63] J. Condeelis and J. W. Pollard, "Macrophages: obligate partners for tumor cell migration, invasion, and metastasis," *Cell*, vol. 124, no. 2, pp. 263–266, 2006.

[64] S. J. Leibovich and D. M. Wiseman, "Macrophages, wound repair and angiogenesis," *Progress in Clinical and Biological Research*, vol. 266, pp. 131–145, 1988.

[65] A. E. Koch, P. J. Polverini, and S. J. Leibovich, "Stimulation of neovascularization by human rheumatoid synovial tissue macrophages," *Arthritis and Rheumatism*, vol. 29, no. 4, pp. 471–479, 1986.

[66] J. E. Wolf Jr., "Angiogenesis in normal and psoriatic skin," *Laboratory Investigation*, vol. 61, no. 2, pp. 139–142, 1989.

[67] M. Ohta, Y. Kitadai, S. Tanaka, et al., "Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas," *International Journal of Cancer*, vol. 102, no. 3, pp. 220–224, 2002.

[68] M. Ohta, Y. Kitadai, S. Tanaka, et al., "Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human gastric carcinomas," *International Journal of Oncology*, vol. 22, no. 4, pp. 773–778, 2003.

[69] S. Ishigami, S. Natsugoe, K. Tokuda, et al., "Tumor-associated macrophage (TAM) infiltration in gastric cancer," *Anticancer Research*, vol. 23, no. 5, pp. 4079–4083, 2003.

[70] M. E. Fuentes, S. K. Durham, M. R. Swerdel, et al., "Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1," *Journal of Immunology*, vol. 155, no. 12, pp. 5769–5776, 1995.

[71] T. Kuroda, Y. Kitadai, S. Tanaka, et al., "Monocyte chemoattractant protein-1 transfection induces angiogenesis and tumorigenesis of gastric carcinoma in nude mice via macrophage recruitment," *Clinical Cancer Research*, vol. 11, no. 21, pp. 7629–7636, 2005.

[72] A. Orimo, P. B. Gupta, D. C. Sgroi, et al., "Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion," *Cell*, vol. 121, no. 3, pp. 335–348, 2005.

[73] O. De Wever and M. Mareel, "Role of tissue stroma in cancer cell invasion," *Journal of Pathology*, vol. 200, no. 4, pp. 429–447, 2003.

[74] M. Allinen, R. Beroukhim, L. Cai, et al., "Molecular characterization of the tumor microenvironment in breast cancer," *Cancer Cell*, vol. 6, no. 1, pp. 17–32, 2004.

[75] H. F. Dvorak, "Tumors: wounds that do not heal: similarities between tumor stroma generation and wound healing," *The New England Journal of Medicine*, vol. 315, no. 26, pp. 1650–1659, 1986.

[76] Y. Kitadai, T. Sasaki, T. Kuwai, et al., "Expression of activated platelet-derived growth factor receptor in stromal cells of human colon carcinomas is associated with metastatic potential," *International Journal of Cancer*, vol. 119, no. 11, pp. 2567–2574, 2006.

[77] Y. Kitadai, T. Sasaki, T. Kuwai, T. Nakamura, C. D. Bucana, and I. J. Fidler, "Targeting the expression of platelet-derived growth factor receptor by reactive stroma inhibits growth and metastasis of human colon carcinoma," *American Journal of Pathology*, vol. 169, no. 6, pp. 2054–2065, 2006.

[78] Y. Kitadai, T. Sasaki, T. Kuwai, T. Nakamura, C. D. Bucana, and I. J. Fidler, "Schistosomes, liver flukes and *Helicobacter pylori*. IARC working group on the evaluation of carcinogenic risks to humans. Lyon, 7–14 June 1994," *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, vol. 61, pp. 1–241, 1994.

[79] T. Watanabe, M. Tada, H. Nagi, S. Sasaki, and M. Nakao, "*Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils," *Gastroenterology*, vol. 115, no. 3, pp. 642–648, 1998.

[80] S. Honda, T. Fujioka, M. Tokieda, R. Satoh, A. Nishizono, and M. Nasu, "Development of *Helicobacter pylori*-induced gastric carcinoma in mongolian gerbils," *Cancer Research*, vol. 58, no. 19, pp. 4255–4259, 1998.

[81] V. Necchi, M. E. Candusso, F. Tava, et al., "Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by *Helicobacter pylori*," *Gastroenterology*, vol. 132, no. 3, pp. 1009–1023, 2007.

[82] S. A. Sharma, M. K. R. Tummuru, M. J. Blaser, and L. D. Kerr, "Activation of IL-8 gene expression by *Helicobacter pylori* is regulated by transcription factor nuclear factor-κB in gastric epithelial cells," *Journal of Immunology*, vol. 160, no. 5, pp. 2401–2407, 1998.

[83] R. A. Hatz, G. Rieder, M. Stolte, et al., "Pattern of adhesion molecule expression on vascular endothelium in *Helicobacter pylori*-associated antral gastritis," *Gastroenterology*, vol. 112, no. 6, pp. 1908–1919, 1997.

[84] A. Sasaki, Y. Kitadai, M. Ito, et al., "*Helicobacter pylori* infection influences tumor growth of human gastric carcinomas," *Scandinavian Journal of Gastroenterology*, vol. 38, no. 2, pp. 153–158, 2003.

[85] J. E. Crabtree, J. I. Wyatt, L. K. Trejdosiewicz, et al., "Interleukin-8 expression in *Helicobacter pylori* infected, normal, and neoplastic gastroduodenal mucosa," *Journal of Clinical Pathology*, vol. 47, no. 1, pp. 61–66, 1994.

[86] Y. Kitadai, A. Sasaki, M. Ito, et al., "*Helicobacter pylori* infection influences expression of genes related to angiogenesis and invasion in human gastric carcinoma cells," *Biochemical and Biophysical Research Communications*, vol. 311, no. 4, pp. 809–814, 2003.

[87] C.-Y. Wu, C.-J. Wang, C.-C. Tseng, et al., "*Helicobacter pylori* promote gastric cancer cells invasion through a NF-κB and COX-2-mediated pathway," *World Journal of Gastroenterology*, vol. 11, no. 21, pp. 3197–3203, 2005.

[88] Y.-E. Joo, J.-S. Rew, Y.-H. Seo, et al., "Cyclooxygenase-2 overexpression correlates with vascular endothelial growth factor expression and tumor angiogenesis in gastric cancer," *Journal of Clinical Gastroenterology*, vol. 37, no. 1, pp. 28–33, 2003.

[89] K. Uefuji, T. Ichikura, and H. Mochizuki, "Cyclooxygenase-2 expression is related to prostaglandin biosynthesis and angiogenesis in human gastric cancer," *Clinical Cancer Research*, vol. 6, no. 1, pp. 135–138, 2000.

[90] Y. Kitadai, K. Haruma, T. Tokutomi, et al., "Significance of vessel count and vascular endothelial growth factor in human esophageal carcinomas," *Clinical Cancer Research*, vol. 4, no. 9, pp. 2195–2200, 1998.

[91] J. Iwasaki and S.-I. Nihira, "Anti-angiogenic therapy against gastrointestinal tract cancers," *Japanese Journal of Clinical Oncology*, vol. 39, no. 9, pp. 543–551, 2009.

[92] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *The New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[93] A. Ohtsu, "Chemotherapy for metastatic gastric cancer: past, present, and future," *Journal of Gastroenterology*, vol. 43, no. 4, pp. 256–264, 2008.

[94] B. Streubel, A. Chott, D. Huber, et al., "Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas," *The New England Journal of Medicine*, vol. 351, no. 3, pp. 250–259, 2004.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 928628, 14 pages
doi:10.1155/2010/928628

Research Article

Inhibition of Melanoma Angiogenesis by Telomere Homolog Oligonucleotides

Christina Coleman,[1] Danielle Levine,[1] Raj Kishore,[2] Gangjian Qin,[2] Tina Thorne,[2] Erin Lambers,[2] Sharath P. Sasi,[3] Mina Yaar,[1] Barbara A. Gilchrest,[1] and David A. Goukassian[1,3]

[1] *Department of Dermatology, Boston University School of Medicine, Boston, MA 02118, USA*
[2] *Feinberg Cardiovascular Research Institute, Feinberg School of Medicine, Chicago, IL 60611, USA*
[3] *Division of Cardiovascular Research, Department of Medicine, Center of Cancer Systems Biology, Caritas St. Elizabeth's Medical Center of Boston, MA 02135, USA*

Correspondence should be addressed to David A. Goukassian, dgoukass@bu.edu, david.goukassian@tufts.edu

Received 13 November 2009; Revised 6 May 2010; Accepted 7 May 2010

Academic Editor: Arkadiusz Dudek

Telomere homolog oligonucleotides (T-oligos) activate an innate telomere-based program that leads to multiple anticancer effects. T-oligos act at telomeres to initiate signaling through the Werner protein and ATM kinase. We wanted to determine if T-oligos have antiangiogenic effects. We found that T-oligo-treated human melanoma (MM-AN) cells had decreased expression of vascular endothelial growth factor (VEGF), VEGF receptor 2, angiopoeitin-1 and -2 and decreased VEGF secretion. T-oligos activated the transcription factor E2F1 and inhibited the activity of the angiogenic transcription factor, HIF-1α. T-oligos inhibited EC tubulogenesis and total tumor microvascular density matrix invasion by MM-AN cells and ECs in vitro. In melanoma SCID xenografts, two systemic T-oligo injections decreased by 60% ($P < .004$) total tumor microvascular density and the functional vessels density by 80% ($P < .002$). These findings suggest that restriction of tumor angiogenesis is among the host's innate telomere-based anticancer responses and provide further evidence that T-oligos may offer a powerful new approach for melanoma treatment.

1. Introduction

Angiogenesis, the formation of new blood vessels, is essential for tumor growth and metastasis [1] and inhibition of angiogenesis is an important new approach for therapy of many cancers [1, 2]. A principal regulator of angiogenesis is vascular endothelial growth factor (VEGF) [3]. The VEGF family is comprised of at least five genes (VEGF A through E), of which the most potent activators of angiogenesis are VEGF-A and VEGF-B [4]. Analysis of the VEGF-A (termed VEGF here and elsewhere in the text) gene promoter region has revealed numerous potential transcriptional activator sites [4]. One of the best studied stimuli for VEGF synthesis and secretion is hypoxia, which acts by upregulating hypoxia inducible factor-1α (HIF-1α) [5]. Inhibition of HIF-1α may therefore decrease angiogenesis by reducing VEGF levels and potentially other proangiogenic factors, such as angiopoeitin-1 and -2 (Ang-1 and Ang-2) [6–8], basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF), all well known regulators of angiogenesis [1].

The role of E2F1 in apoptosis is well recognized. It can act in concert with p53 or independent of p53 to induce apoptosis [9]. T-oligos, telomere homolog oligonucleotides, activate p53 and E2F1, resulting in apoptosis [10]. However, in p53 null cells like malignant melanoma AN cells (MM-AN), apoptosis is induced by the p53 homolog p73 [11], presumably acting coordinately with E2F1. Moreover, E2F1 has also been reported to inhibit angiogenesis [9, 12]. While E2F1 decreases VEGF production in fibroblasts through p53 activation [12], its effect on angiogenesis in cells that lack functional p53, such as MM-AN cells has not been studied [11, 13].

Several lines of evidence suggest that normal cells have an integrated program of genome-protective responses, functionally analogous to the bacterial SOS response [14], that is based in the telomeres and appears to be abrogated in malignancy [15]. Telomeres, the ends of chromosomes, are maintained in a loop configuration by insertion of the single-stranded 3′ overhang into the proximal telomere duplex [16]. Disruption of this loop structure by removal of the principal binding protein TRF2 (telomere repeat binding factor 2) leads to exposure and digestion of the overhang and activation of ATM (ataxia telangiectasia mutated) and its effector protein p53, followed by apoptosis or senescence, depending on cell type [16]. Knockdown of another telomere-associated protein, the protection of telomeres-1 (POT-1), also expected to expose the TTAGGG telomere repeat sequence, activates ATR (ataxia telangiectasia and Rad3-related), leading to similar downstream effects [16, 17]. Moreover, treatment of malignant cells with RNAi to knockdown the expression of TER, the RNA subunit of telomerase, rapidly alters the expression of many genes in a pattern predicted to reduce cancer cell proliferation and invasiveness [18], then leads to apoptosis in a time course far too rapid to be attributable to the expected loss of telomerase activity and consequent telomere shortening, suggesting that other telomere-based effects are responsible [18].

Our laboratory has described several anticancer properties of oligonucleotides homologous to the telomere repeat sequence TTAGGG (T-oligos) [10, 11, 13, 15, 19–28]. T-oligos provided to cultured cells rapidly accumulate in the nucleus and mediate DNA damage responses without digestion of the telomere overhang or other detectable effects on genomic DNA [10, 13, 15, 19, 23, 25, 26, 29]. T-oligos activate the ATM kinase [21, 27], upregulate and activate p53 [30, 31], as well as upregulate and/or activate its homolog p73, E2F1, $p16^{INK4a}$, p33, p27, and p95/Nbs1, and phosphorylate the histone variant protein H2AX [11, 13, 21, 22, 24, 25]. In addition, T-oligos promote differentiation of melanoma cells and downregulate the inhibitor of apoptosis protein IAP/livin in these cells [13]. T-oligo effects require WRN [22], the protein mutated in the progeroid cancer-prone Werner syndrome [32], and are associated with formation of classic DNA damage foci at telomeres [22]. In combination, these signaling cascades result in induction of apoptosis, autophagy, and/or senescence selectively in cancer cells [13, 19, 27, 28]; while in normal cells they lead to transient cell cycle arrest, increased DNA repair capacity and adaptive differentiation [19, 21, 23, 25–27, 30]. Complementary, unrelated, or scrambled oligonucleotides comparably accumulate in the nucleus, but do not cause DNA damage-like signaling or affect growth, differentiation or survival of malignant cells [11, 13, 15, 19, 21, 24, 27, 28].

Because the T-oligo-induced transcription factors p53, p73, and E2F1 are known to affect endothelial cell (EC) survival, differentiation, and proliferation during tumor angiogenesis [12, 33, 34] and because blocking angiogenesis would be an additional anticancer mechanism of action for T-oligos, we asked whether T-oligo treatment inhibits tumor angiogenesis. We now report that T-oligo inhibits angiogenesis in the aggressive human melanoma cell line MM-AN, derived from a metastatic melanoma [35], by decreasing production and secretion of proangiogenic factors in both tumor cells and ECs. As well, T-oligo treatment decreases the number of total and functional (perfused) vessels in flank tumors of MM-AN cells in SCID mice after two systemic injections.

2. Materials and Methods

2.1. Cell Cultures. Human microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs) were obtained at passage 2 and used by passage 4–6. Cells were maintained in EGM-2 medium with 2% FBS plus growth factors (bullet kit) (Cambrex Bio Sciences, Walkersville, MD). Human melanoma MM-AN and EP cells [35] were cultured in modified Eagle's medium (MEM) (Mediatech, Inc., Herndon, VA) supplemented with fetal bovine serum (FBS 2%), calf serum (CS 8%), and antibiotic-antimicotic. Human breast adenocarcinoma (MCF-7) and ovarian adenocarcinoma (OVCAR3) cells were cultured and maintained according to ATCC recommendations. All cells were incubated at 37° with 5% CO_2.

2.2. Oligonucleotides. We used a 16-base 100% telomere homolog with the sequence 5′-GTTAGGGTTAGGGTTA-3′ and phosphodiester backbone. The oligonucleotide was synthesized by Midland Certified Reagent (Midland, TX) and then diluted from a 2 mM stock in medium to obtain a final concentration of 40 μM.

2.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). MM-AN, EP, MCF-7, and OVCAR3 cells were grown in their corresponding media and treated once with diluent or 40 μM T-oligo. For qRT-PCR, we performed a reverse transcription reaction then amplified specific genes as described [36]. mRNA expression levels were assessed for E2F1, ANG-1, and ANG-2. The sequences used were as follows: E2F1 forward: CGGTGTCGTCGACCTGAACT, E2F1 reverse: AGGACGTTGGTGATGTCATAGATG, E2F1 probe: TGCCGAGGTGCTGAAGGTGCAG; ANG-1 forward: CAGAAAACAGTGGGAGAAGATATAACC, ANG-1 reverse: TGCCATCGTGTTCTGGAAGA, ANG-1 probe: CAACATGGGCAATGTGCCTACACTTTC; ANG-2 forward: GGCTGGGCAATGAGTTTGTC, ANG-2 reverse: CCCAGTCCTTCAGCTGGATCT; ANG-2 probe: ACCGGTCAGCACCGCTACGTGC.

2.4. Electromobility Shift Assay (EMSA). Cells were treated with diluent or 40 μM T-oligo and harvested 0, 1, 16, and 32 hours after treatment. EMSAs were carried out as described [10] using a total of 5 μg of nuclear protein per lane. The assay was performed using consensus sequences of E2F1 and HIF-1α transcription factors (Santa Cruz Biotechnology, CA). The specificity of the bands was confirmed by using either ×25 or ×50 excess of cold probe as competitor and mutant oligos as control. As an additional negative control, nuclear extracts were incubated with a specific competing

E2F1 antibody before adding the radioactive (^{32}P-labelled) consensus oligonucleotides.

2.5. Western Blot Analysis. Cells were treated with T-oligos, harvested at various times, snap-frozen, and stored at −70°C. Total cellular protein was isolated and 50 μg of total protein was processed for western blot analysis as described [10]. Antibody reactions were performed with the following antibodies: anti-E2F1 (Neomarkers, Inc., Fremont, CA), anti-VEGF-A (sc-507, Santa Cruz Biotechnology, Inc, Santa Cruz, CA) that recognizes all VEGF-A isoforms, and anti-VEGF-R2 (a kind gift from Dr. Nader Rahimi, Departments of Ophthalmology and Biochemistry, Boston University School of Medicine).

2.6. VEGF Enzyme-Linked Immunosorbent Assay (ELISA). The Human VEGF Immunoassay kit (Quantikine, R&D Systems, Minneapolis, MN) was used to compare the release of VEGF into the medium by ECs and MM-AN cells. Each cell type was cultured in its appropriate medium and treated with either diluent or T-oligo (40 μM). The conditioned medium was collected at 24, 48, and 72 hours after treatment and frozen at −70°C until it processed for ELISA. The assay was performed according to the manufacturer's protocol (R&D Systems). The plate was read using the Tecan Spectra model 96 Well Microplate Reader (MTX Lab Systems, Vienna, VA).

2.7. Tubulogenesis Assay. 200 μL of Matrigel basement membrane matrix (phenol-red free) (BD Biosciences, Bedford, MA) was added per 4-well chamber slide and allowed to solidify for 1 hour at 37°C. HUVECs (50,000 cells) were then added to each chamber in 500 μL of medium as described [37]. Cells were treated at the time of plating with diluent, 40 μM, 80 μM, or 120 μM of T-oligo. Some of the cells treated with 40 μM of T-oligo were retreated a second time at 2 hours postplating, at the time tube-formation could first be detected by phase microscopy. Some cells that were treated twice with 40 μM of T-oligo received a third treatment 6 hours after plating. Tube-formation was observed and photographed at numerous time points starting from 2 hours and up to 24 hours after treatment. Of note, after tubular structures formed in control wells, they stayed intact up to 72 hours (data not shown). Image-Tool (The University of Texas Health Science Center in San Antonio, San Antonio, TX) software was used to quantify the length of tubular structures.

2.8. Invasion Assay. The BioCoat Matrigel Invasion Chamber (BD Biosciences, Bedford, MA) was used for this assay. MM-AN cells were treated with diluent or 40 μM T-oligo and media were collected after 72 hours, based on ELISA data (MM-AN conditioned media). Conditioned MM-AN medium was then used as the chemoattractant for the assay. ECs and MM-AN cells were grown on the inserts (upper chambers) and allowed to invade through the Matrigel and attach to the membrane as described in the manufacturer's protocol. Invasion was assessed after 22 hours by staining with Diff-Quick (Fisher, Atlanta, GA) bisected membranes from the bottom of the chambers (containing the invading cells) as described in the manufacturer's protocol. Of note, we believe that any effect on ECs or MM-AN invasion cannot be attributed to residual active T-oligos in the conditioned medium, diffusing into the upper chamber and killing MM-AN cells, because T-oligos are rapidly degraded in serum-containing medium. With a measured half-life for a 12-base telomere homolog of 4–6 hours [38], the 16-base T-oligo would thus have been present in the medium after 72 hours of conditioning at an estimated $(1/2)^{12}$ or 10^7-fold less than the initial therapeutic concentration.

2.9. SCID Melanoma Flank Xenograft Model. All studies were approved by Boston University's IACUC Committee. 2×10^6 MM-AN cells were injected subcutaneously into the flank of 6 week old SCID mice (Fox-Chase Cancer Center, Philadelphia, PA). Mice (5-6 per treatment group) were injected via tail vein with T-oligo or diluent alone when tumors were first palpable (2-3 mm diameter). Tumor sizes were recorded every 2-3 days throughout the experiment using electronic callipers and all animals were sacrificed 4 weeks after tumor inoculation. The diagnosis of melanoma was confirmed histologically on sections of each nodule cut through the center of the clinical tumor.

2.10. Quantification of Tumor Microvessel Density. All studies were approved by Boston University's IACUC Committee. 2×10^6 MM-AN cells were injected subcutaneously into the flank of 6 week old SCID mice (Fox-Chase Cancer Center, Philadelphia, PA). Then mice (5-6 per treatment group) were injected via the tail vein with T-oligo or diluent alone (60 nmoles/injection, 15 mg/kg) when tumors were first palpable (2-3 mm diameter). Microvascular density (MVD) in bisected tumors were assessed 24 hours after two systemic injections of T-oligos by using two EC-specific markers CD31 (PECAM-1) [12] and *Bandeurea simplicifolia* (BS)-1 lectin conjugated to Rhodamine (Vector Laboratories, Burlingame, CA) [39]. To measure functional MVD in the tumor tissue, 30 minutes before sacrifice a set of mice (5 per treatment group) were anesthetized and were perfused with 0.5 mg (in 100 μl of isotonic solution) of Rhodamine-conjugated BS-1 lectin as described [40, 41]. To measure total MVD, 6 μm cross-sections of bisected tumor tissue (of the same mice perfused with BS-1 lectin) were stained with CD31 primary antibody followed by FITC-labelled secondary antibody as described [12]. Samples were photographed using a multicolour fluorescence microscope (Nikon, Nikon Instruments Inc, Melville, NY), and analyzed using a digital image analysis system (Nikon). The diagnosis of melanoma was confirmed histologically in tumor sections of each nodule.

2.11. Statistical Analysis. ANOVA with post hoc analysis by Scheffe and Bonferroni-Dunn and unpaired t-Test were performed using StatView (SAS Institute, Inc., Version 5.0). Statistical significance was established at $P < .05$.

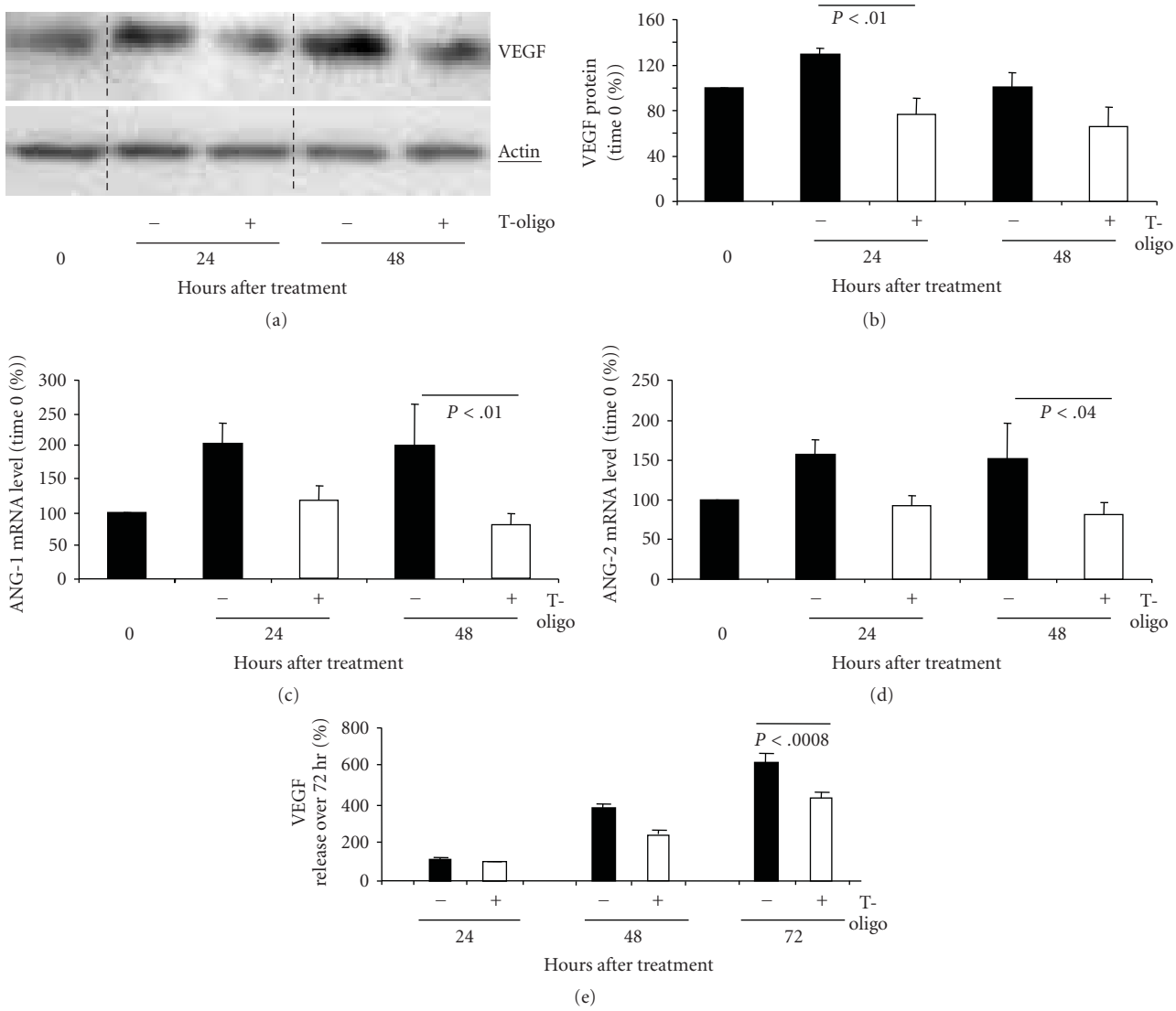


Figure 1: T-oligo treatment down-regulates expression of VEGF, Ang-1, Ang-2 and decreases VEGF production in MM-AN cells. MM-AN cells were treated once at time 0 with either 40 μM T-oligo or diluent alone. (a) Western blot analysis of VEGF protein level. (b) Densitometric analysis of VEGF protein levels (after loading adjustment against actin expression) represented as a percent of time 0 level. Graphs represent pooled data (mean ± SEM) from three independent experiments. (c) and (d) Quantitative real time-PCR (qRT-PCR) of MM-AN cells treated with either 40 μM T-oligo or diluent alone. Results are presented as percent of time 0 (set at 100%) and examined over 48 hours for both control and T-oligo-treated cells. ANG-1 gene expression. (c) ANG-2 gene expression. (d) These experiments are repeated twice with similar results. (e) MM-AN cells were treated with 40 μM T-oligo or diluent alone. The culture medium was collected after 24, 48, and 72 hours. Cumulative VEGF protein released into the medium was measured by ELISA. Results represent data pooled from triplicate dishes for each time point and treatment condition. Changes over time are calculated as percent of the 24 hours values (set at 100%).

3. Results

3.1. T-oligo Treatment Decreases the Expression and Level of Angiogenic Factors and Reduces VEGF Release into MM-AN Cell Medium. MM-AN cells were treated once with T-oligo or diluent alone, provided in fresh medium, and processed for western blot analysis. Compared to diluent, T-oligo decreased VEGF protein level at 24 hours ($P < .01$) by ~41% and at 48 hours (P = NS) by ~32% (Figures 1(a) and 1(b)). In addition, after 48 hours T-oligo treatment reduced ANG-1 mRNA ($P < .01$) by ~60% (Figure 1(c)) and ANG-2 mRNA ($P < .04$) by ~45% (Figure 1(d)). Similar decreases in VEGF (Supplemental Figures 1(a)–1(c) and ANG-1 (Supplemental Figures 2(a)–2(c)) mRNA were seen in a second human melanoma line EP, as well as in other malignant cell types.

Paired dishes of MM-AN and ECs were treated with 40 μM T-oligo or diluent alone once. Medium was collected after 24, 48 and 72 hours and VEGF level in the medium was measured by ELISA. In ECs (HMVEC and HUVEC), there was a ~50%–75% variable and statistically insignificant decrease in VEGF release into the medium over time in both diluent- and T-oligo-treated cells (data not shown).

In contrast, total VEGF release from MM-AN cells in diluent-treated dishes rose progressively over 72 hours to ~600% (of 24-hours VEGF levels), while VEGF release in T-oligo-treated dishes was decreased ($P < .0008$) by ~30% over the 72-hours experiment after a single dosing at time 0 (Figure 1(e)). Thus, T-oligo not only decreased VEGF protein expression in tumor cells but also the release of this potent angiogenic factor into the surrounding culture medium.

3.2. T-oligo Upregulates E2F1 Gene Expression, Protein Level, and DNA Binding Activity and Decreases the DNA Binding Activity of the Angiogenic Transcription Factor HIF-1α. E2F1 mRNA expression was increased at 24 hours ($P < .0001$) by ~41% and at 48 hours ($P < .0001$) by ~100% in T-oligo-treated MM-AN cells compared to control (Figure 2(a)). Similar increases in E2F1 mRNA were seen in a second human melanoma line EP, as well as in other malignant cell types (Supplemental Figures 3(a)–3(c)). T-oligo treatment also increased E2F1 protein level at 24 hours to ~218% and at 48 hours to ~286% ($P < .02$) of control values (Figures 2(b) and 2(c)). In addition, there was a doubling in E2F1 DNA binding activity 32 hours after treatment compared to diluent-treated cells (Figures 2(d) and 2(e), compare lane 3 versus 4).

MM-AN cells were treated once with T-oligo or diluent alone and nuclear proteins were harvested up to 32 hours after treatment to evaluate DNA binding activities of the angiogenic transcription factor HIF-1α. There was ~80%–95% decrease in HIF-1α DNA binding activity at 1 and 16 hours after treatments (Figures 2(f) and 2(g), lanes 3 versus 4, and 5 versus 6, respectively). These data suggest that T-oligo-mediated inhibition in DNA binding activity of HIF-1α transcription factor contributes to decreased expression of the angiogenic factors, VEGF and ANG-1, whose promoters contain binding sites for HIF-1α [42].

3.3. T-oligo Inhibits Matrigel Invasion by Melanoma Cells and ECs. We used MM-AN medium collected 72 hours after addition of T-oligo or diluent alone (MM-AN conditioned media: T-oligo CM or diluent CM) as the chemoattractant for an in vitro invasion assay [43]. MM-AN and HMVECs were both evaluated for invasion through Matrigel to determine if T-oligo treatment reduces the chemoattractant properties of MM-AN cells. As recommended by the manufacturer of the Matrigel invasion kit, after 22 hours of incubation the invading cells were fixed, stained and counted for each membrane. MM-AN cells plated above T-oligo CM had a ~96% decrease in invasion ($P < .03$) through Matrigel (Figures 3(a) and 3(b)). HMVECs plated on inserts coated with Matrigel and exposed to T-oligo CM had a ~40% reduction in Matrigel invasion compared to controls, but this did not reach statistical significance ($P < .08$) (Figures 3(c) and 3(d)). These findings demonstrate that T-oligos reduce the migration/invasion of MM-AN cells towards chemoattractant stimuli and, possibly, to a lesser degree affect HMVECs. This is consistent with the observation that MM-AN cells elaborate factors that promote migration and invasion of tumor cells, like VEGF and ANG-1, and that these factor(s) are reduced as a result of T-oligo treatment.

3.4. T-oligo Treatment Decreases the Expression of VEGF and VEGFR-2 in Endothelial Cells. Because VEGF signaling through VEGFR-2 is principally responsible for EC survival, proliferation, migration, and angiogenesis [44–47], we examined the effect of T-oligo treatment on these proteins. HMVECs and HUVECs were grown and treated with 40 μM T-oligo or diluent alone. Compared to diluent-treated control cells there was a decrease ($P < .004$) of ~86% in VEGF expression at 24 hours and an insignificant decrease of ~50% at 48 hours in T-oligo-treated samples (Figures 4(a)–4(c)). In addition, in T-oligo-treated HMVECs and HUVECs there were ~29% (P = NS) and ~59% ($P < .04$) reduction in the protein level of VEGFR-2 at 24 and 48 hours, respectively (Figures 4(d)–4(f)). Thus, T-oligo-mediated antiangiogenic effects on ECs may be, in part, due to inhibition of VEGF-VEGR2 axis.

3.5. T-oligo Inhibits EC Function In Vitro in Tubulogenesis Assay. HMVECs were plated on Matrigel in 4-well chamber slides in serum-containing medium and treated with diluent alone or increasing concentrations of T-oligo. Tube-like structure formation was maximal in controls at 22 hours and formal comparisons were made after 2–22 hours, as customary for this assay [39]. By 22 hours, in cells treated once with either 40 μM or 80 μM T-oligo there was a ~19% reduction in average tube length compared to cells treated with diluent alone (P = NS) (Figures 5(a) and 5(b)). Cells treated with two separate doses of 40 μM T-oligo at plating and 2 hours after plating showed a ~35% reduction in average tube length ($P < .03$). Cells treated once at plating with 120 μM T-oligo showed a ~58% reduction in average tube length compared to the diluent-treated cells ($P < .001$). A reduction of ~83% ($P < .0001$) in tube length was observed in cells treated with 3 separate doses of 40 μM T-oligo (40 μM ×3) added to the medium at the time of plating and then at 2 and 6 hours (Figures 5(a) and 5(b)). These results indicate that T-oligo inhibits EC function in a manner dependent on the dose and frequency of administration, with the caveat that the very large amount of oligos administered, rather that specific telomere-based T-oligo initiated signalling may have contributed to the effect observed. In our future experiments, we plan to examine the effect of large and/or fractioned T-oligo doses on survival of ECs.

3.6. Systemic Administration of T-oligo Reduces Tumor Angiogenesis and Vessel Patency In Vivo in a SCID Mouse Xenograft Model. In melanoma tumors inoculated into SCID mice, we determined the effect of systemic T-oligo injection on melanoma angiogenesis by evaluating functional and total vessel density in tumor tissue (5 mice per group). When tumors became palpable (2-3 mm in diameter, day 5 to 14 after inoculation) we injected T-oligo (60 nmoles/injection, 15 mg/kg) via the tail vein (IV) and injected again after

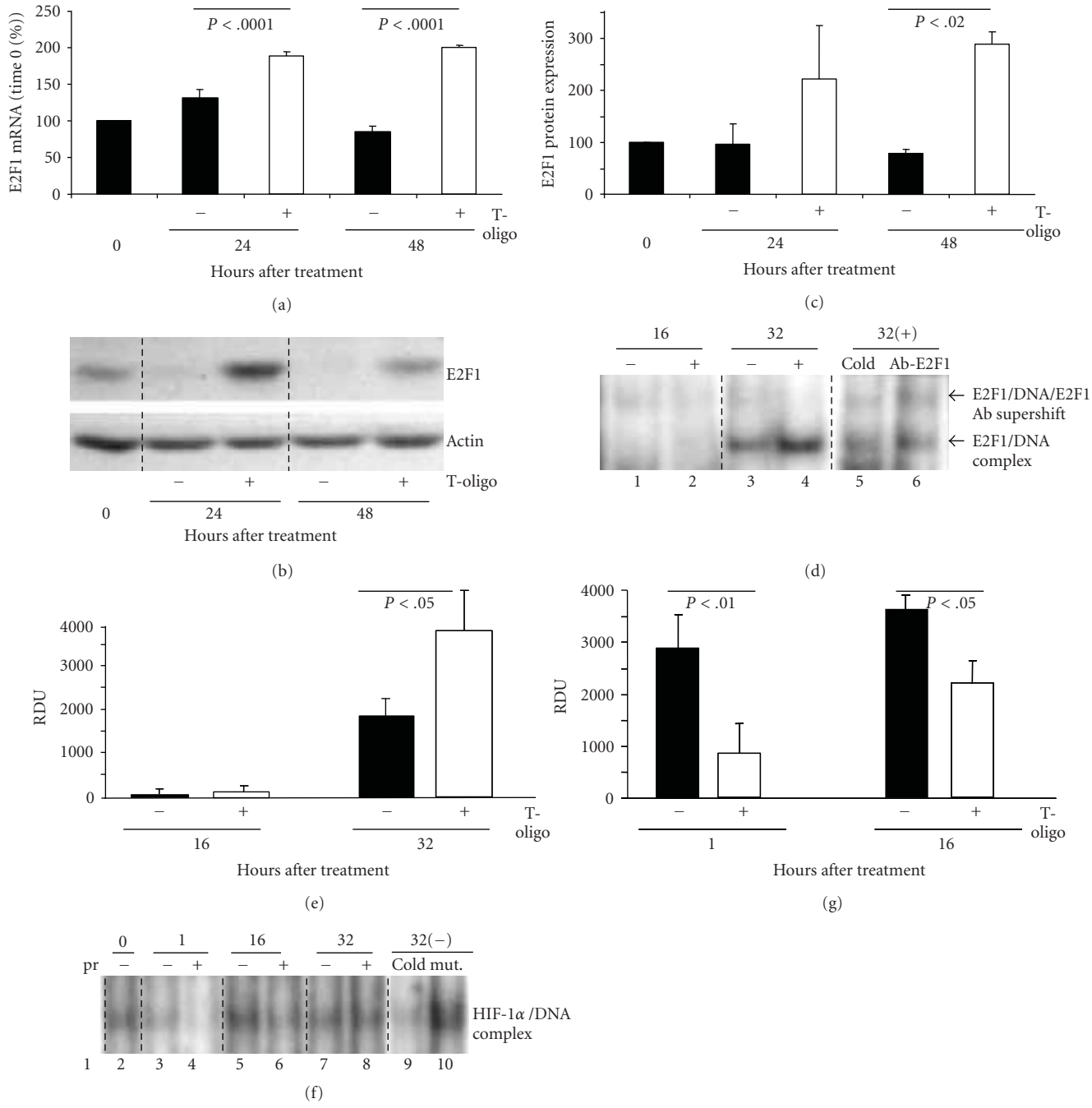


Figure 2: T-oligo treatment increases E2F1 expression/activity and decreases HIF-1α DNA binding activity in MM-AN cells. Cells were treated with 40 μM T-oligo or diluent alone and harvested at various times. (a) The pellets were examined by qRT-PCR for E2F1 mRNA level, shown as a percent of time 0 levels (mean ± SEM) for 2 separate experiments in triplicate. (b) The pellets were also examined by western blot analysis for E2F1 protein levels. Actin expression was used as an internal loading control. (c) Densitometric analysis of E2F1 protein expression after loading adjustment by actin, represented as a percent of time 0 levels. Results are pooled data (mean ± SEM) from three independent experiments. (d) The DNA binding activity of E2F1 was analyzed by EMSA. No difference in E2F1 DNA binding activity was detected between the treatment groups at 16 hours (lane 1 versus 2) but E2F1 DNA binding activity doubled in T-oligo treated cells at 32 hours (lane 3 versus 4). Specificity of bands was confirmed by preincubating the nuclear protein of T-oligo-treated cells harvested at 32 hours with ×25 cold probe (lane 4 versus 5) and by supershift of E2F1 protein/DNA and E2F1 competing antibody complex (lane 4 versus 6). (e) Quantification of the band intensity of DNA binding activity is represented as relative density untis (RDU) for both treatment groups at 16 and 32 hours after treatment. E2F1 EMSA was repeated 2 times with similar results. (f) Nuclear protein was isolated from cells and processed for electromobility shift assay (EMSA) for evaluation of HIF-1α DNA binding activity. Specificity of the bands was confirmed by preincubating the nuclear protein extract of cells treated with diluent for 32 hours with ×25 cold probe (not labelled with ^{32}P) and mutant HIF-1α consensus sequence for 20 minutes before incubating the nuclear extracts with ^{32}P-labeled consensus oligonucleotides. HIF-1α EMSA was repeated 2 times with similar results. (g) Densitometric analysis of the protein/DNA complex bands for HIF-1α is graphed as relative densitometric units (RDU).

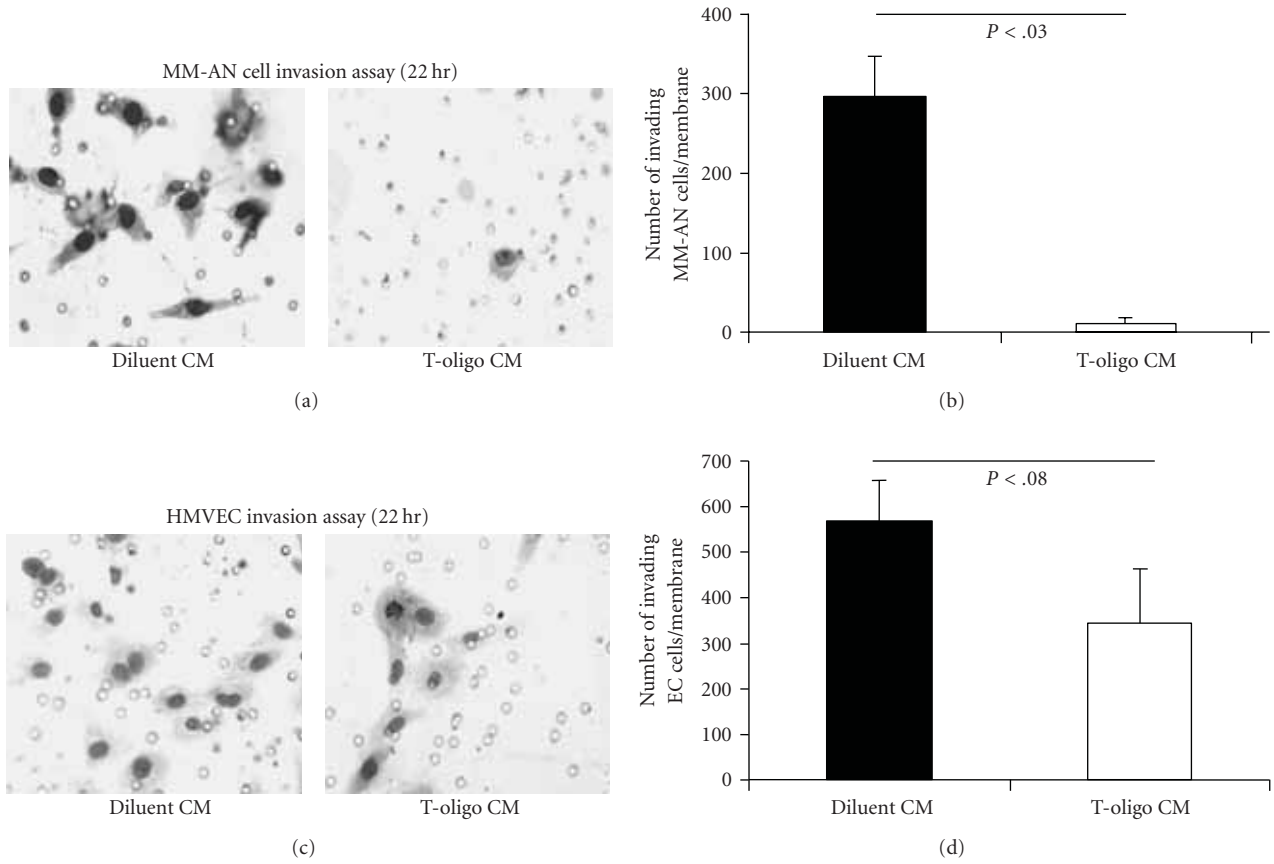


Figure 3: T-oligo treatment inhibits Matrigel invasion by MM-AN cells and HMVECs. MM-AN cells were treated with 40 μM T-oligo or diluent alone. The culture medium was collected after 72 hours. The conditioned medium harvested after 72 hours was used as the chemoattractant for the invasion assay for MM-AN and HMVECs. MM-AN cells were plated on the inserts and allowed 22 hours to move through the pores on the membrane in the bottom of the inserts toward the medium in the lower chamber, interpreted as invasion of the gel. The experimental inserts had a layer of Matrigel, whereas control inserts (not shown) did not. After 22 hours cells that moved through the pores in the membrane were fixed, stained and photographed. (a) Representative images for MM-AN cells are shown. Small open circles are the pores in the membrane, not cells. (b) The total number of cells was counted for 3 membranes for each treatment condition and graphed as a number of cells (mean ± SEM) for both treatment groups. The assay was repeated twice with identical results. (c) Representative images for HMVECs treated as described for MM-AN above are shown. Small open circles are the pores in the membrane, not cells. (d) The total number of HMVECs were counted for 3 membranes for each treatment condition and graphed as an average number of cells for each treatment group (mean ± SD). The assay was repeated twice with identical results. Reductions approached but did not reach statistical significance.

6 hours, then harvested tumors 24 hours after the second T-oligo injections. Representative images of tumor cross-sections were immunostained to identify all vessels as well as functional (patent) vessels 24 hours after T-oligo injection (Figure 6(a)). Compared to control-injected mice there was more than ~80% decrease ($P < .002$) in functional vessels in T-oligo-injected mice (Figure 6(b)). There was also more than ~60% decrease ($P < .004$) in total vessels in T-oligo-injected mice (Figure 6(c)). These data corroborate our in vitro findings (Figures 4 and 5) and demonstrate that two systemic administrations of T-oligo reduce tumor angiogenesis and vessel patency in vivo (Figures 6(a)–6(c)).

To determine the effect of systemic T-oligo treatment on melanoma growth, in SCID xenograft model MM-AN cells were injected subcutaneously in the flank. When tumors became palpable (2-3 mm in diameter, day 5 to 14), the mice received daily systemic injections of T-oligo (60 nmoles/injection, 15 mg/kg BID) or vehicle for 5 days only. In control animals, tumors increased in volume from 6.96 ± 2.9 mm^3 on day 6 to 88.04 ± 19.72 mm^3 on day 27 (Figure 6(d), blue line). In contrast, in T-oligo treated animals, whose tumors were the same size as in controls on day 6 (6.48 ± 2.57) there was very little tumor growth through day 15 (6.48 ± 2.57 versus 9.92 ± 3.8 mm^3) and thereafter a slower and statistically insignificant increase in volume to 29.65 ± 10.47 mm^3 by day 27 (Figure 6(d), pink line). Thereby, in T-oligo-treated mice tumor growth was inhibited ($P < .003$) by ~53% after ~4 weeks, when the experiment was terminated. These data demonstrate that systemic administration of T-oligo for only 5 days has a persistent inhibitory effect on melanoma growth. We have

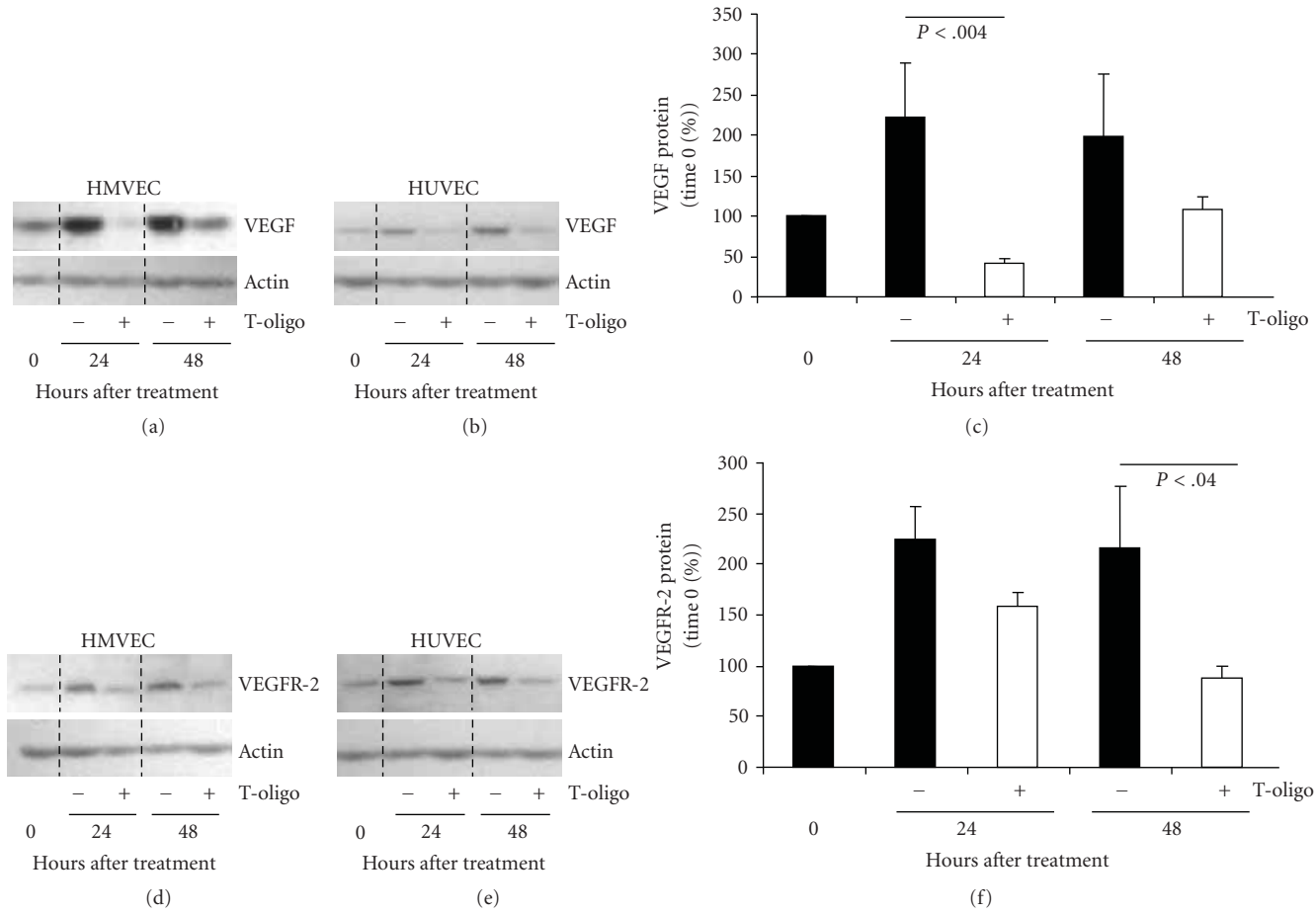


Figure 4: T-oligo decreases VEGF and VEGFR-2 protein levels in normal endothelial cells. HMVEC and HUVEC were treated with 40 μM T-oligo or diluent alone and harvested for western blot analysis over 48 hours. (a) VEGF protein expression in HMVEC. Here and elsewhere actin expression was used to adjust the loading. (b) VEGF protein expression in HUVEC. (c) Combined densitometric analysis of VEGF expression in HMVEC and HUVEC as a percent of time 0 levels, after loading adjustment. (d) VEGFR-2 protein expression in HMVEC. (e) VEGFR-2 protein expression and HUVEC, and (f) Combined densitometric analysis, as in (c).

also evaluated T-oligos toxicity in several internal organs of SCID mice 24 hours after the last IV injections (15 mg/kg BID for 5 days). No systemic toxicity was observed in bone marrow, liver, intestines, brain, lungs and kidneys of T-oligo-injected mice (Figures 7(a)–7(f)).

4. Discussion

Tumor angiogenesis is essential for tumor growth and metastasis [1]. Without active angiogenesis tumor diameter rarely exceeds 2-3 mm [1, 48]. Angiogenesis is mediated through release of angiogenic factors by tumor cells and cells in the tumor stroma and microenvironment including, but not limited to, endothelial cells [48]. We now report that telomere homolog oligonucleotides (T-oligos) decrease the synthesis and release of angiogenic factors by ECs and melanoma cells, inhibit EC tubulogenesis and impede melanoma cells and ECs from invading matrix (Matrigel). In addition, systemic administration of T-oligos decreases tumor vascularity in vivo.

EC migration, proliferation and differentiation are all essential processes for tumor angiogenesis [1]. EC proliferation, in vitro tubulogenesis, and survival are all known to be stimulated in large part by VEGF [49]. Decreased VEGF levels or inhibition of receptor activation in ECs often correlate with decrease in tumor size and metastatic potential [50]. VEGF binds to the extracellular domain of the VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), inducing receptor dimerization and activation of tyrosine kinases by autophosphorylation, leading to angiogenesis, increased vascular permeability, and EC proliferation and survival [49]. It is generally accepted that VEGFR-2 is the major mediator of these effects [51]. We found that T-oligo decreases the expression of VEGFR-2 by HMVEC and HUVEC (Figures 4(c) and 4(f)). Other investigators reported that receptor tyrosine kinase inhibitors (TKIs) such as sunitinib and dasatinib reduce signaling through the RAF/MEK/ERK pathway that is activated by ligand binding to angiogenic receptors like VEGFR-2, PDGFR-β, FH-3 and c-kit [52], indirectly inhibiting tumor growth by affecting tumor angiogenesis [52]. By reducing VEGF-R2 level, T-oligos appear to have similar effects.

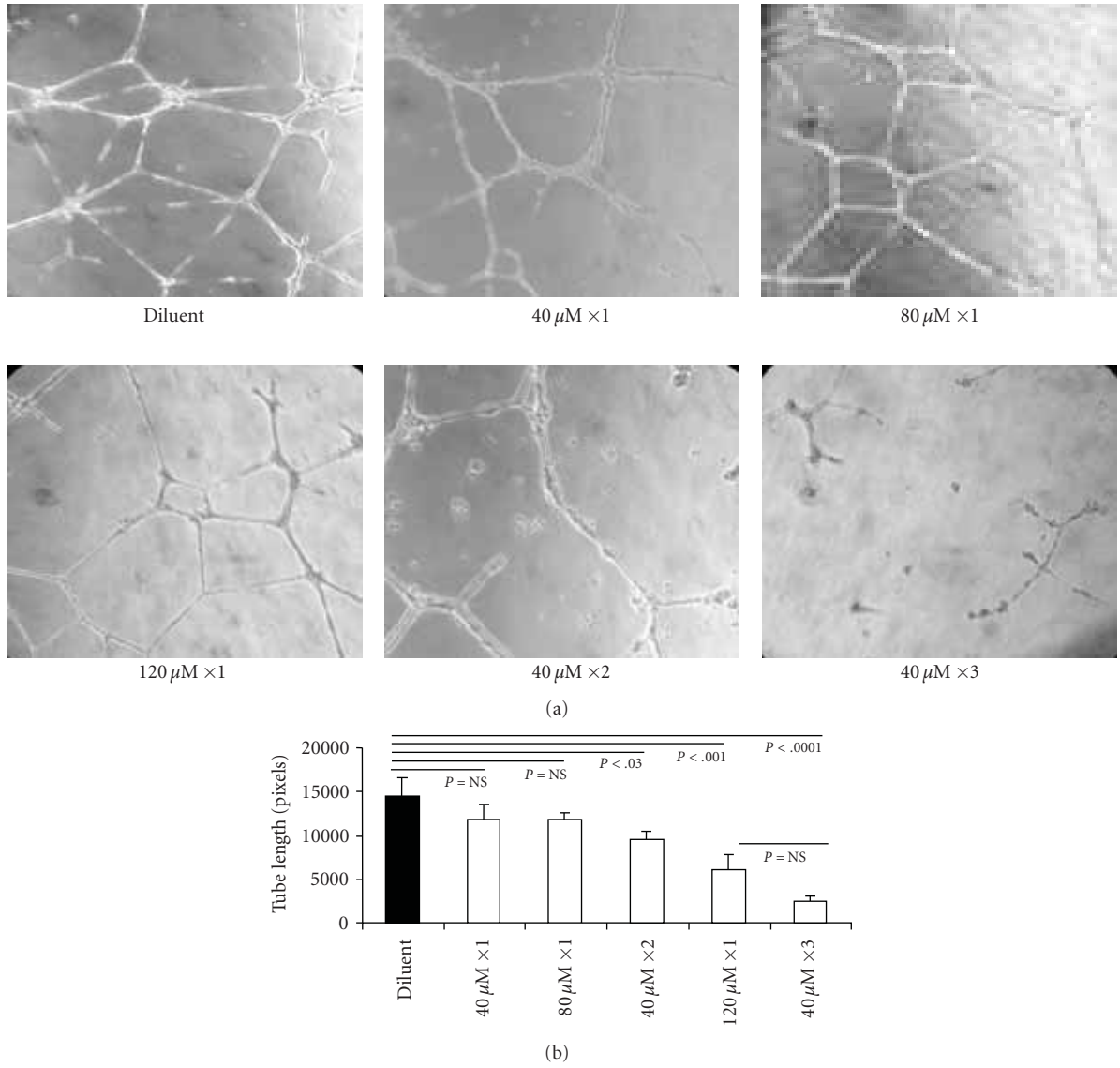


Figure 5: T-oligo treatment inhibits EC tubulogenesis in vitro. HMVEC cells were plated on Matrigel in four-well chamber slides and treated in triplicate with T-oligo or diluent alone, as described in the text. (a) All representative images are taken 22 hours after plating cells into chambers, the time of biggest differences among treatment conditions. (b) The length of tube-like structures was quantified as total average tube lengths per visual field from 3 separate chambers for each treatment condition. The differences in the length of tube-like structures were quantified (in pixels) in at least 3–5 representative photographs per chamber/treatment condition using computer-assisted image analysis.

VEGF also induces leakage within tumor vessels, allowing tumor cells to infiltrate blood vessels and migrate into the blood stream [53]. Hence, changes in angiogenic factors even early in tumor formation can affect metastasis and spread [53] and inhibiting VEGF production by T-oligos would be expected to reduce the metastatic potential of tumor cells [53]. Additionally, increased blood vessel permeability within the tumor may interfere with adequate delivery and retention of chemotherapeutic agents [54, 55]. Indeed, certain antiangiogenic agents that prevent tumor vessel leakage (a phenomenon called "vessel normalization") were shown to enhance the delivery of chemotherapeutic agents into tumors [56]. Thus, combination treatment with antiangiogenic factors together with conventional chemotherapeutic agents may be superior to using the latter alone. Furthermore, because VEGF is likely required for migration and recruitment of ECs, T-oligo-me diated VEGF reduction would also likely decrease the number of blood vessels in the tumor [1, 6]. Our present findings suggest that T-oligos may induce potent antiangiogenic effects, enhancing their attractiveness as a therapeutic option for cancer.

In addition to VEGF and its receptors, angiopoietin 1 and 2 (ANG-1 and ANG-2) and their tyrosine kinase receptor Tie-2 have been identified as major players in the processes of growth and remodelling of tumor vasculature [57, 58]. ANG-1 activates the Tie-2 signalling pathway [58]. Although

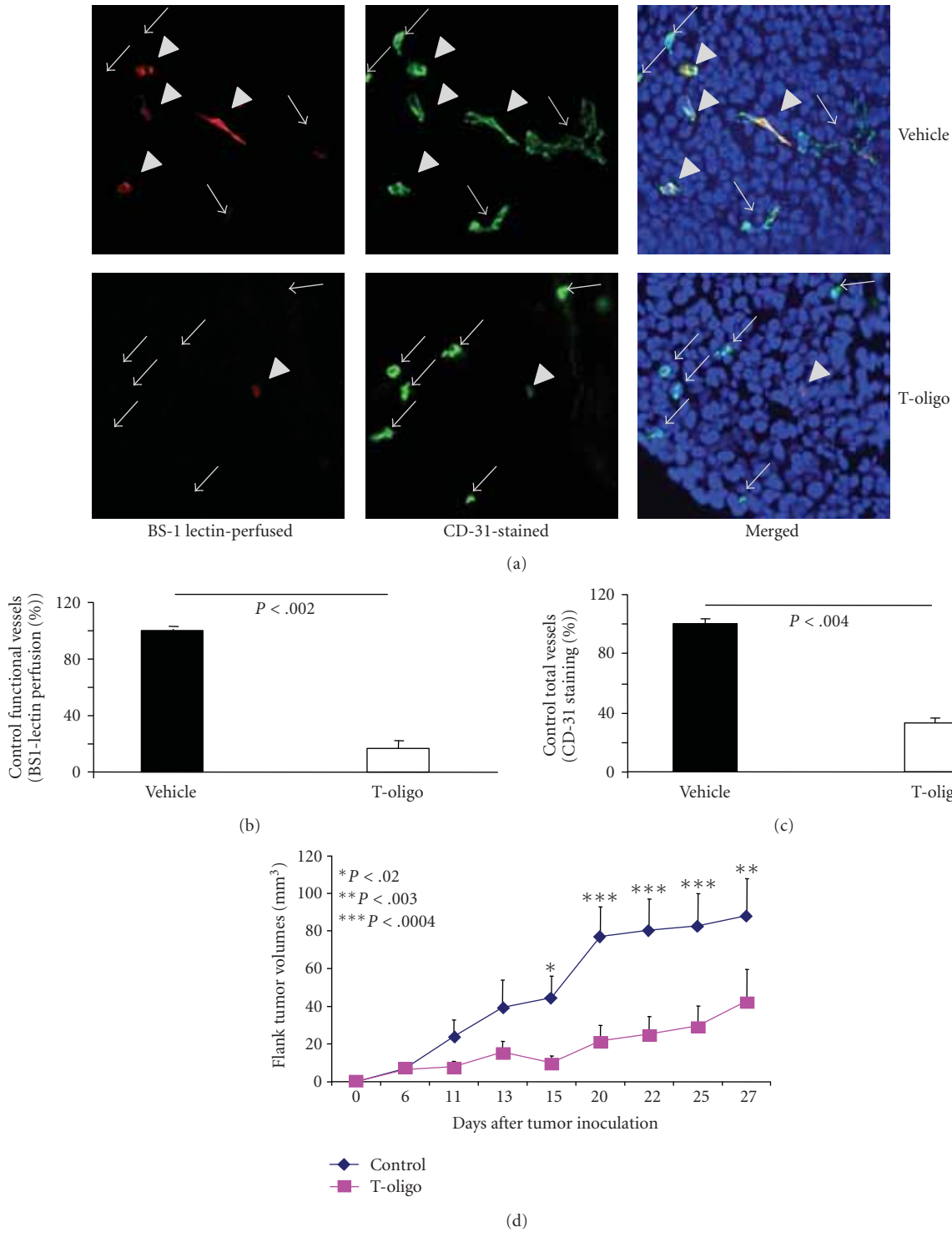


FIGURE 6: T-oligo decrease tumor angiogenesis and melanoma tumor volumes in mouse SCID xenografts. (a) Representative images of 6 μm tumor cross-sections immunostained with CD31 (green) and TopRo-3 (blue-nuclei) and perfused in vivo with BS-1 lectin (red), to determine tumor microvascular density (MVD) per high power field (HPF) ×40 magnification. Both functional and total vessels were examined in 5 mice/group. Arrows indicate CD31 (+) vessels that are considered nonfunctional (not perfused) whereas arrowheads indicate double (+) BS-1 lectin/CD31 vessels that are considered functional (perfused in vivo). (b) Percent functional vessels (red-BS-1 lectin staining) in T-oligo injected mice, taking MVD in vehicle injected mice as 100%. (c) Percent total vessels (green-CD31 staining) in T-oligo injected mice, taking MVD in vehicle injected mice as 100%. (d) SCID mice were injected with MMAN cells into the flank. T-oligo or vehicle was injected daily for up to 5 days when tumors were first palpable (2-3 mm diameter). Average tumor volume/animal was recorded over 4-weeks in 5-6 mice/group.

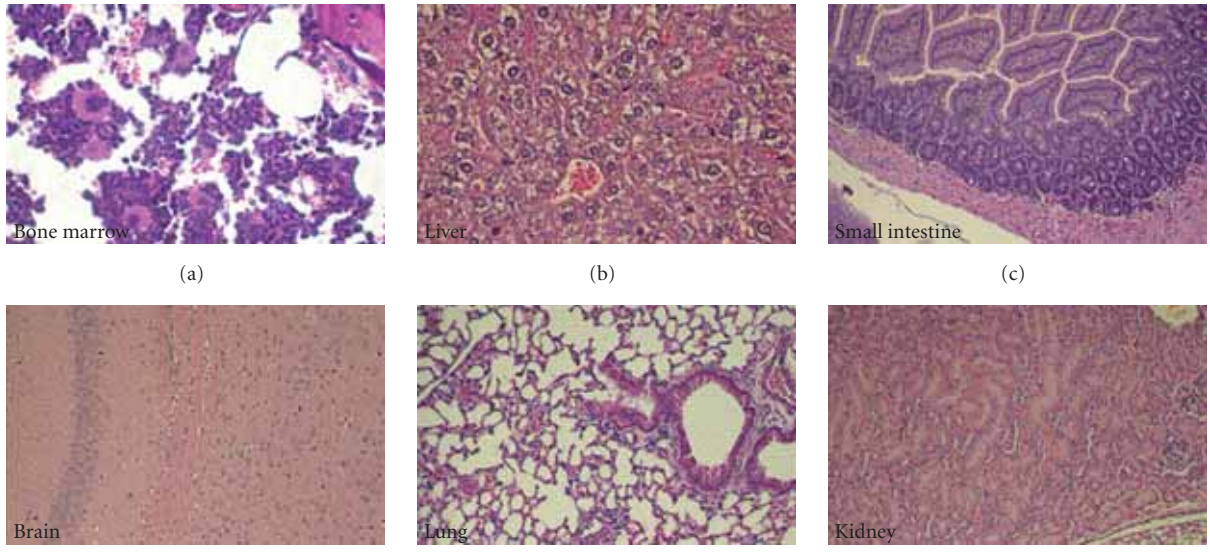


Figure 7: Evaluation of T-oligos toxicity in internal organs of SCID mice 24 hours after the last IV injection (15 mg/kg BID for 5 days). (a) Bone marrow, displaying a mixture of myeloid and erythroid precursor cells as well as plasma cells. Scattered megakeryocytes are also present. There is no evidence of bone marrow suppression or toxicity. (b) Liver lobule with a central vein surrounded by hepatocytes. The cells display a fixation artifact but otherwise appear normal. There is no evidence of cellular necrosis or apoptosis. (c) Jejunal mucosa displaying normal arrangement of villi lined by tall columnar cells. Both the mucosa and the submucosa appear normal. Fragments of normal pancreatic acinar tissue are seen in the bottom left of the image. (d) Section of the brain showing normal brain architecture with typical neuronal ganglia and scattered small dark glial cells in a pink neuropil background. (e) Normal lung tissue displaying multiple alveoli as well as bronchioles lined with cuboidal epithelial lining. (f) The kidney displays two normal glomeruli that are surrounded by tubules with cuboidal epithelium.

under certain conditions ANG-2 may inhibit ANG-1 effect, in an in vivo mouse model for melanoma [59] and in glioma cells [60] increased expression of ANG-2 is thought to stimulate angiogenesis. Another angiogenic factor, HIF-1α, a transcription factor that is activated by hypoxia, exerts its effect by upregulating VEGF levels. We found that T-oligo treatment inhibits HIF-1α activity and ANG-1 and ANG-2 expression in melanoma cell line and decreases VEGF synthesis secretion in these cells, strongly suggesting that T-oligo-mediated effects on tumor angiogenesis are transcriptional and ultimately affect several angiogenic molecules.

Our laboratory has previously shown that T-oligo increases p73 level in the p53 null MM-AN cells and that blocking p73 expression by RNAi decreases T-oligo-induced apoptosis in these cells [11]. Like p53, p73 is known to inhibit angiogenesis, primarily through VEGF down-regulation [34]. Therefore, we assume that in MM-AN cells T-oligo decreases VEGF production, in addition to its effect on HIF-1α, by activating p73 through induction of E2F1. However, in cells with functional p53, we assume that T-oligo-induced p53 and p73 would cooperate to inhibit angiogenesis. Furthermore, E2F1 is known to induce apoptosis in part by its effect on p73. Thus, induction of E2F1 by T-oligos [11] also contributes to tumor cell apoptosis. E2F1 is reported to be upregulated by active (phosphorylated) ATM [61]. We have previously shown that T-oligo treatment activates (phosphorylates) ATM [21, 27]. Moreover, the E2F1 promoter contains binding sites for E2F1 [62]. We therefore suggest that T-oligo regulates E2F1 first via ATM-mediated phosphorylation of E2F1 [61] and that E2F1 then further transcriptionally upregulates E2F1 by binding to its own promoter. Indeed, we show that T-oligo increases E2F1 mRNA and protein levels as well as its DNA binding activity in human MM-AN melanoma cells, further confirming our previous finding that E2F1contributes to T-oligo effects [11].

The T-oligos used in the present experiments have physiologic readily hydrolysable phosphodiester linkage, unlike for example antisense DNA in which phosphorothioate or other nonhydrolyzable linkage is employed to increase the molecule's half-life (T 1/2). Hydrolyzable linkage is required for initiation of T-oligo signalling [22] and, despite the known short T 1/2, approximately 4–6 hours in serum-containing medium for a 12-base 100% telomere homolog [38], nevertheless this allows for the cellular responses observed in the present and previous experiments to evolve over 3–5 days [11, 13, 19, 21, 22, 24–28]. This may reflect the fact that at least in vitro T-oligos rapidly enter the nucleus [11, 22, 24, 27] and that, once in the nucleus, such oligos have a far longer T 1/2 [63]. The efficacy of these presumptively short-lived T-oligos may also reflect the likelihood that, after interaction of the oligos with the Werner protein and formation of DNA damage-like foci at telomeres [22], signalling through the DNA damage response pathways may continue even if the T-oligos have been hydrolyzed, at least through 48 hours at which time the DNA damage foci can still be observed by immunohistochemistry [22].

In earlier studies, we have shown that T-oligos inhibit tumor growth in SCID mouse models by inducing cell cycle arrest, differentiation, apoptosis, and senescence [13, 15, 27]. We now show that T-oligos also inhibit angiogenesis. Angiogenesis inhibition encompassing both ECs and melanoma cells, in combination with other T-oligo-mediated antitumor effects [11, 13, 22, 25, 27], likely combine to significantly decrease melanoma burden in established SCID mouse models, as reported in our earlier publications [13, 27]. These multiple diverse responses are all mediated through activation and/or upregulation of DNA damage response proteins. They are thus reminiscent of the less complex but very well characterized bacterial SOS response, a genome-protective mechanism that enhances survival of prokaryotic organisms in the face of DNA damage [14]. The telomere-based DNA damage-like signalling initiated by T-oligos may be viewed as an evolutionarily perfected parallel mechanism in mammalian cells that addresses the threat to genomic integrity posed by malignant transformation [64].

In summary, the present paper indicates that T-oligos exert multiple antiangiogenic effects. These data reinforce prior evidence that T-oligo therapy may offer a powerful new approach to treatment of human primary melanoma and possibly other human malignancies, with several conceptual advantages over the currently lionized targeted therapy approach [64].

Conflict of Interest

Aspects of this paper are patent-protected and licensed to SemaCo Inc., a for-profit company. Dr Gilchrest and Dr. Yaar are shareholders in SemaCo.

Acknowledgments

C. Coleman and D. Levine contributed equally to the paper. C. Coleman is currently affiliated with the University of New England and Danielle Levine, M.D., is dermatology resident at Harvard Medical School. This work was supported by grants from the NIH CA10515 (to BAG) and American Skin Association (to DL).

References

[1] J. Folkman, "Angiogenesis," *Annual Review of Medicine*, vol. 57, pp. 1–18, 2006.

[2] N. Weidner, "New paradigm for vessel intravasation by tumor cells," *American Journal of Pathology*, vol. 160, no. 6, pp. 1937–1939, 2002.

[3] C.-H. Chen, W. Jiang, D. P. Via et al., "Oxidized low-density lipoproteins inhibit endothelial cell proliferation by suppressing basic fibroblast growth factor expression," *Circulation*, vol. 101, no. 2, pp. 171–177, 2000.

[4] D. Bradbury, D. Clarke, C. Seedhouse, L. Corbettt, J. Stocks, and A. Knox, "Vascular endothelial growth factor induction by prostaglandin E2 in human airway smooth muscle cells is mediated by E prostanoid EP 2/EP4 receptors and SP-1 transcription factor binding sites," *Journal of Biological Chemistry*, vol. 280, no. 34, pp. 29993–30000, 2005.

[5] G. Pagès and J. Pouysségur, "Transcriptional regulation of the Vascular Endothelial Growth Factor gene—a concert of activating factors," *Cardiovascular Research*, vol. 65, no. 3, pp. 564–573, 2005.

[6] C. R. Tait and P. F. Jones, "Angiopoietins in tumours: the angiogenic switch," *Journal of Pathology*, vol. 204, no. 1, pp. 1–10, 2004.

[7] Y. S. Park, N. H. Kim, and I. Jo, "Hypoxia and vascular endothelial growth factor acutely up-regulate angiopoietin-1 and Tie2 mRNA in bovine retinal pericytes," *Microvascular Research*, vol. 65, no. 2, pp. 125–131, 2003.

[8] P. S. Ray, T. Estrada-Hernandez, H. Sasaki, L. Zhu, and N. Maulik, "Early effects of hypoxia/reoxygenation on VEGF, Ang-1, Ang-2 and their receptors in the rat myocardium: implications for myocardial angiogenesis," *Molecular and Cellular Biochemistry*, vol. 213, no. 1-2, pp. 145–153, 2000.

[9] J. Stanelle, T. Stiewe, C. C. Theseling, M. Peter, and B. M. Pützer, "Gene expression changes in response to E2F1 activation," *Nucleic Acids Research*, vol. 30, no. 8, pp. 1859–1867, 2002.

[10] V. Marwaha, Y.-H. Chen, E. Helms et al., "T-oligo treatment decreases constitutive and UVB-induced COX-2 levels through p53- and NFκB-dependent repression of the COX-2 promoter," *Journal of Biological Chemistry*, vol. 280, no. 37, pp. 32379–32388, 2005.

[11] M. S. Eller, N. Puri, I. M. Hadshiew, S. S. Venna, and B. A. Gilchrest, "Induction of apoptosis by telomere 3′ overhang-specific DNA," *Experimental Cell Research*, vol. 276, no. 2, pp. 185–193, 2002.

[12] G. Qin, R. Kishore, C. M. Dolan et al., "Cell cycle regulator E2F1 modulates angiogenesis via p53-dependent transcriptional control of VEGF," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 29, pp. 11015–11020, 2006.

[13] N. Puri, M. S. Eller, H. R. Byers, S. Dykstra, J. Kubera, and B. A. Gilchrest, "Telomere-based DNA damage responses: a new approach to melanoma," *FASEB Journal*, vol. 18, no. 12, pp. 1373–1381, 2004.

[14] M. Radman, "SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis," *Basic Life Sciences*, vol. 5, pp. 355–367, 1975.

[15] B. A. Gilchrest and M. S. Eller, "The tale of the telomere: implications for prevention and treatment of skin cancers," *The Journal of Investigative Dermatology. Symposium Proceedings*, vol. 10, no. 2, pp. 124–130, 2005.

[16] T. De Lange, "Protection of mammalian telomeres," *Oncogene*, vol. 21, no. 4, pp. 532–540, 2002.

[17] E. L. Denchi and T. De Lange, "Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1," *Nature*, vol. 448, no. 7157, pp. 1068–1071, 2007.

[18] S. Li, J. E. Rosenberg, A. A. Donjacour et al., "Rapid inhibition of cancer cell growth induced by lentiviral delivery and expression of mutant-template telomerase RNA and anti-telomerase short-interfering RNA," *Cancer Research*, vol. 64, no. 14, pp. 4833–4840, 2004.

[19] H. Aoki, E. Iwado, M. S. Eller et al., "Telomere 3′ overhang-specific DNA oligonucleotides induce autophagy in malignant glioma cells," *FASEB Journal*, vol. 21, no. 11, pp. 2918–2930, 2007.

[20] S. Arad, N. Konnikov, D. A. Goukassian, and B. A. Gilchrest, "T-oligos augment UV-induced protective responses in human skin," *The FASEB Journal*, vol. 20, no. 11, pp. 1895–1897, 2006.

[21] M. S. Eller, G.-Z. Li, R. Firoozabadi, N. Puri, and B. A. Gilchrest, "Induction of a p95/Nbs1-mediated S phase checkpoint by telomere 3′ overhang specific DNA," *FASEB Journal*, vol. 17, no. 2, pp. 152–162, 2003.

[22] M. S. Eller, X. Liao, S. Liu et al., "A role for WRN in telomere-based DNA damage responses," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 41, pp. 15073–15078, 2006.

[23] D. A. Goukassian, S. Bagheri, L. el-Keeb, M. S. Eller, and B. A. Gilchrest, "DNA oligonucleotide treatment corrects the age-associated decline in DNA repair capacity," *The FASEB Journal*, vol. 16, no. 7, pp. 754–756, 2002.

[24] G.-Z. Li, M. S. Eller, R. Firoozabadi, and B. A. Gilchrest, "Evidence that exposure of the telomere 3′ overhang sequence induces senescence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 2, pp. 527–531, 2003.

[25] G.-Z. Li, M. S. Eller, K. Hanna, and B. A. Gilchrest, "Signaling pathway requirements for induction of senescence by telomere homolog oligonucleotides," *Experimental Cell Research*, vol. 301, no. 2, pp. 189–200, 2004.

[26] N. Ohashi, M. Yaar, M. S. Eller, F. Truzzi, and B. A. Gilchrest, "Features that determine telomere homolog oligonucleotide-induced therapeutic DNA damage-like responses in cancer cells," *Journal of Cellular Physiology*, vol. 210, no. 3, pp. 582–595, 2007.

[27] M. Yaar, M. S. Eller, I. Panova et al., "Telomeric DNA induces apoptosis and senescence of human breast carcinoma cells," *Breast Cancer Research*, vol. 9, no. 1, article R13, 2007.

[28] H. O. Longe, P. B. Romesser, A. M. Rankin et al., "Telomere homolog oligonucleotides induce apoptosis in malignant but not in normal lymphoid cells: mechanism and therapeutic potential," *International Journal of Cancer*, vol. 124, no. 2, pp. 473–482, 2009.

[29] I. M. Hadshiew, M. S. Eller, F. P. Gasparro, and B. A. Gilchrest, "Stimulation of melanogenesis by DNA oligonucleotides: effect of size, sequence and 5′ phosphorylation," *Journal of Dermatological Science*, vol. 25, no. 2, pp. 127–138, 2001.

[30] M. S. Eller, T. Maeda, C. Magnoni, D. Atwal, and B. A. Gilchrest, "Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 23, pp. 12627–12632, 1997.

[31] D. A. Goukassian, M. S. Eller, M. Yaar, and B. A. Gilchrest, "Thymidine dinucleotide mimics the effect of solar simulated irradiation on p53 and p53-regulated proteins," *Journal of Investigative Dermatology*, vol. 112, no. 1, pp. 25–31, 1999.

[32] P. L. Opresko, M. Otterlei, J. Graakjær et al., "The werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2," *Molecular Cell*, vol. 14, no. 6, pp. 763–774, 2004.

[33] R. Kishore, C. Luedemann, E. Bord, D. Goukassian, and D. W. Losordo, "Tumor necrosis factor-mediated E2F1 suppression in endothelial cells: differential requirement of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signal transduction pathways," *Circulation Research*, vol. 93, no. 10, pp. 932–940, 2003.

[34] B. Salimath, D. Marmé, and G. Finkenzeller, "Expression of the vascular endothelial growth factor gene is inhibited by p73," *Oncogene*, vol. 19, no. 31, pp. 3470–3476, 2000.

[35] H. R. Byers, T. Etoh, K. W. Lee, M. C. Mihm Jr., and S. Gattoni-Celli, "Organ-specific metastases in immunodeficient mice injected with human melanoma cells: a quantitative pathological analysis," *Melanoma Research*, vol. 3, no. 4, pp. 247–253, 1993.

[36] K. F. Kusano, R. Pola, T. Murayama, et al., "Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling," *Nature Medicine*, vol. 11, no. 11, pp. 1197–1204, 2005.

[37] D. A. Goukassian, G. Qin, C. Dolan et al., "Tumor necrosis factor-α receptor p75 is required in ischemia-induced neovascularization," *Circulation*, vol. 115, no. 6, pp. 752–762, 2007.

[38] W. E. Wright, D. Brasiskyte, M. A. Piatyszek, and J. W. Shay, "Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids," *EMBO Journal*, vol. 15, no. 7, pp. 1734–1741, 1996.

[39] D. Goukassian, A. Diez-Juan, T. Asahara et al., "Overexpression of $p27^{Kip1}$ by doxycycline-regulated adenoviral vectors inhibits endothelial cell proliferation and migration and impairs angiogenesis," *FASEB Journal*, vol. 15, no. 11, pp. 1877–1885, 2001.

[40] J. Alroy, V. Goyal, and E. Skutelsky, "Lectin histochemistry of mammalian endothelium," *Histochemistry*, vol. 86, no. 6, pp. 603–607, 1987.

[41] T. Asahara, D. Chen, T. Takahashi et al., "Tie2 receptor ligands, angiopoietin-1 and angiopoietin-2, modulate VEGF- induced postnatal neovascularization," *Circulation Research*, vol. 83, no. 3, pp. 233–240, 1998.

[42] B. Enholm, K. Paavonen, A. Ristimäki et al., "Comparison of VEGF, VEGF-B, VEGF-C and Ang-1 mRNA regulation by serum, growth factors, oncoproteins and hypoxia," *Oncogene*, vol. 14, no. 20, pp. 2475–2483, 1997.

[43] A. Albini, R. Benelli, D. M. Noonan, and C. Brigati, "The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes," *International Journal of Developmental Biology*, vol. 48, no. 5-6, pp. 563–571, 2004.

[44] S. Dias, K. Hattori, B. Heissig et al., "Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 19, pp. 10857–10862, 2001.

[45] B. Li, E. E. Sharpe, A. B. Maupin et al., "VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization," *The FASEB Journal*, vol. 20, no. 9, pp. 1495–1497, 2006.

[46] R. Zhang, Y. Xu, N. Ekman et al., "Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway," *Journal of Biological Chemistry*, vol. 278, no. 51, pp. 51267–51276, 2003.

[47] M. Detmar, L. F. Brown, K. P. Claffey et al., "Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis," *Journal of Experimental Medicine*, vol. 180, no. 3, pp. 1141–1146, 1994.

[48] C.-C. Liu, Z. Shen, H.-F. Kung, and M. C. M. Lin, "Cancer gene therapy targeting angiogenesis: an updated review," *World Journal of Gastroenterology*, vol. 12, no. 43, pp. 6941–6948, 2006.

[49] Z. K. Otrock, J. A. Makarem, and A. I. Shamseddine, "Vascular endothelial growth factor family of ligands and receptors: review," *Blood Cells, Molecules, and Diseases*, vol. 38, no. 3, pp. 258–268, 2007.

[50] J. Folkman, "Antiangiogenesis in cancer therapy—endostatin and its mechanisms of action," *Experimental Cell Research*, vol. 312, no. 5, pp. 594–607, 2006.

[51] K. Holmes, O. L. Roberts, A. M. Thomas, and M. J. Cross, "Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition," *Cellular Signalling*, vol. 19, no. 10, pp. 2003–2012, 2007.

[52] H. Zhong and J. P. Bowen, "Molecular design and clinical development of VEGFR kinase inhibitors," *Current Topics in Medicinal Chemistry*, vol. 7, no. 14, pp. 1379–1393, 2007.

[53] H.-P. Gerber and N. Ferrara, "Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies," *Cancer Research*, vol. 65, no. 3, pp. 671–680, 2005.

[54] V. F. Casneuf, P. Demetter, T. Boterberg, L. Delrue, M. Peeters, and N. Van Damme, "Antiangiogenic versus cytotoxic therapeutic approaches in a mouse model of pancreatic cancer: an experimental study with a multitarget tyrosine kinase inhibitor (sunitinib), gemcitabine and radiotherapy," *Oncology Reports*, vol. 22, no. 1, pp. 105–113, 2009.

[55] R. T. Tong, Y. Boucher, S. V. Kozin, F. Winkler, D. J. Hicklin, and R. K. Jain, "Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors," *Cancer Research*, vol. 64, no. 11, pp. 3731–3736, 2004.

[56] R. K. Jain, "Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy," *Science*, vol. 307, no. 5706, pp. 58–62, 2005.

[57] P. C. Maisonpierre, C. Suri, P. F. Jones et al., "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55–60, 1997.

[58] S. Davis, T. H. Aldrich, P. F. Jones et al., "Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning," *Cell*, vol. 87, no. 7, pp. 1161–1169, 1996.

[59] J. Pomyje, J. H. Živný, T. Stopka, J. Šimák, H. Vaňková, and E. Nečas, "Angiopoietin-1, angiopoietin-2 and Tie-2 in tumour and non-tumour tissues during growth of experimental melanoma," *Melanoma Research*, vol. 11, no. 6, pp. 639–643, 2001.

[60] K. Koga, T. Todaka, M. Morioka et al., "Expression of angiopoietin-2 in human glioma cells and its role for angiogenesis," *Cancer Research*, vol. 61, no. 16, pp. 6248–6254, 2001.

[61] W.-C. Lin, F.-T. Lin, and J. R. Nevins, "Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation," *Genes and Development*, vol. 15, no. 14, pp. 1833–1844, 2001.

[62] C. Y. Lin, Y. C. Liang, and B. Y.-M. Yung, "Nucleophosmin/B23 regulates transcriptional activation of E2F1 via modulating the promoter binding of NF-κB, E2F1 and pRB," *Cellular Signalling*, vol. 18, no. 11, pp. 2041–2048, 2006.

[63] Z. Cao, C.-C. Huang, and W. Tan, "Nuclease resistance of telomere-like oligonucleotides monitored in live cells by fluorescence anisotropy imaging," *Analytical Chemistry*, vol. 78, no. 5, pp. 1478–1484, 2006.

[64] B. A. Gilchrest and M. S. Eller, "Cancer therapeutics: smart and smarter," *Drugs of the Future*, vol. 34, no. 3, pp. 205–216, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 351908, 11 pages
doi:10.1155/2010/351908

Review Article

Molecular Therapeutic Targets for Glioma Angiogenesis

Shingo Takano,[1] Toshiharu Yamashita,[2] and Osamu Ohneda[2]

[1] *Department of Neurosurgery, Institute of Clinical Medicine, University of Tsukuba, Tsukuba Ibaraki 305-8575, Japan*
[2] *Regenerative Medicine/Stem Cell Biology, Institute of Basic Medical Science, University of Tsukuba, Tsukuba Ibaraki 305-8575, Japan*

Correspondence should be addressed to Shingo Takano, shingo4@md.tsukuba.ac.jp

Received 28 October 2009; Revised 9 December 2009; Accepted 27 January 2010

Academic Editor: Arkadiusz Dudek

Due to the prominent angiogenesis that occurs in malignant glioma, antiangiogenic therapy has been attempted. There have been several molecular targets that are specific to malignant gliomas, as well as more broadly in systemic cancers. In this review, I will focus on some topics related to molecular therapeutic targets for glioma angiogenesis. First, important angiogenic factors that could be considered molecular targets are VEGF, VEGF-induced proteins on endothelial cells, tissue factor, osteopontin, $\alpha_v\beta_3$ integrin, and thymidine phosphorylase as well as endogenous inhibitors, soluble Flt1, and thrombospondin 1. Second, hypoxic areas are also decreased by metronomic CPT11 treatment as well as temozolomide. Third, glioma-derived endothelial cells that are genetically and functionally distinct from normal endothelial cells should be targeted, for example, with SDF-1 and CXCR7 chemokine. Fourth, endothelial progenitor cells (EPCs) likely contribute towards glioma angiogenesis in the brain and could be useful as a drug delivery tool. Finally, blockade of delta-like 4 (Dll4) results in a nonfunctioning vasculature and could be another important target distinct from VEGF.

1. Introduction

Malignant gliomas tend to be highly vascularized and contain hypoxic regions. Therefore, an antiangiogenic strategy is promising for malignant gliomas [1, 2]. In this review, I focus on some molecular therapeutic targets for glioma angiogenesis: (1) angiogenic factors, (2) hypoxia, (3) glioma-derived endothelial cells, and (4) resistance to antiangiogenic therapy. The problem of how to treat patients who fail to respond to antiangiogenic therapy remains a challenge, and the mechanisms of resistance are being studied. The potential mechanisms by which tumor cells can evade antiangiogenic therapy include upregulation of non-VEGF-mediated pathways of angiogenesis, recruitment of bone marrow-derived cells, increased pericyte coverage, and increased use of preexistent vasculature by invasion [3].

2. Angiogenic Growth Factors in Glioblastoma

Vascular endothelial growth factor (VEGF) is a major angiogenic factor in glioblastoma [4]. VEGF is localized within tumor cytoplasm and endothelium (Figure 1). VEGF is predominantly located in the perinecrotic area, which is referred to as the pseudopallisading area and appears to be hypoxic. By contrast, there are few VEGF positive cells in low-grade astrocytomas and no VEGF positive cells in the normal brain. We initially demonstrated increased expression of VEGF in malignant glioma tissues, with both ELISA (Figure 2) and immunohistochemistry. We also demonstrated high VEGF protein concentrations in the cyst fluid from glioma, but VEGF was not detectable in the serum.

VEGF-related angiogenic factors have been also clearly demonstrated in glioma tissues by RT-PCR and immunohistochemistry. Tissue factor is highly expressed in malignant gliomas associated with VEGF expression (Figure 3) [5]. Also, osteopontin and $\alpha_v\beta_3$ integrin, which are also induced by VEGF in endothelium, are predominantly expressed in tumor endothelium. Another angiogenic factor, thymidine phosphorylase, is also only expressed in malignant gliomas, but not in low-grade glioma or normal brain (Figure 4) [6].

Endogenous angiogenesis inhibitors are important molecules in the delicate balance of angiogenic potential in tumors. The soluble form of the VEGF receptor 1 (sFlt-1) is a measurable, potent, and specific VEGF inhibitor. The

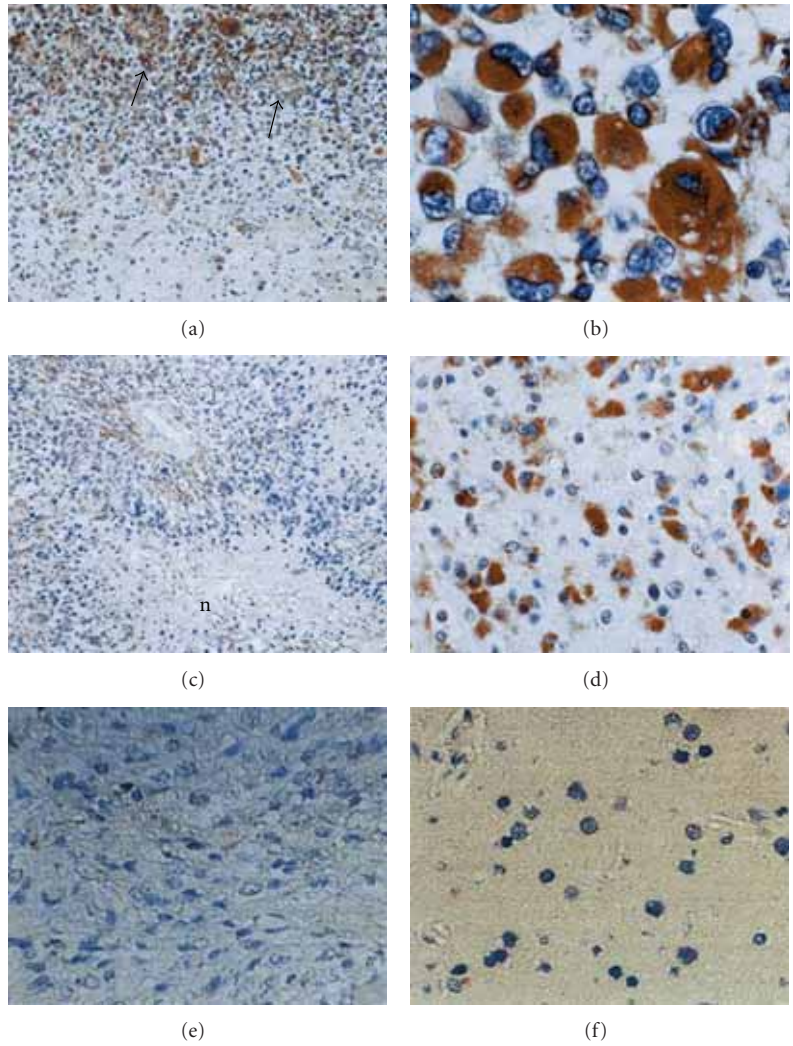


Figure 1: VEGF localization in gliomas. (a)–(c) Glioblastoma. VEGF localizes in the cytoplasm of the tumor cells and tumor capillary around the necrosis and the tumor periphery. (d) Anaplastic astroctytoma. (e) Diffuse astrocytoma. (f) Normal brain. Original magnification (a, c) ×50, (b, d, e, f) ×200.

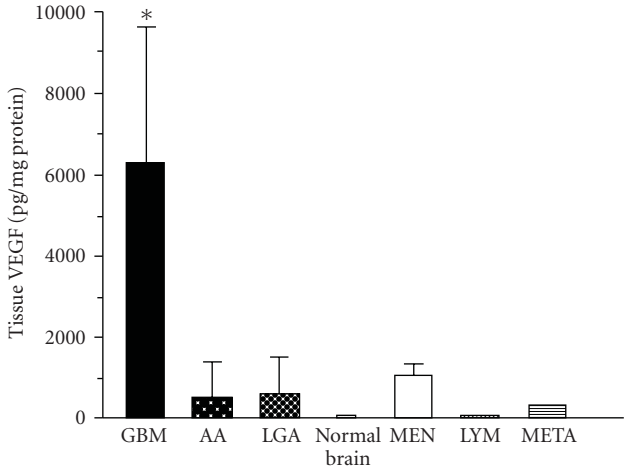


Figure 2: VEGF concentration in the various brain tumors.

concentrations of sFlt-1 and VEGF have been measured in glioma tissues by ELISA. A VEGF/sFlt-1 ratio greater than 1 is a worse prognostic factor in glioblastomas (Figure 5) [7]. The significance of the VEGF/sFlt-1 ratio as a prognostic factor is greater than for the VEGF concentration alone, suggesting that the angiogenic balance between angiogenic factor and its inhibitor is important in tumor angiogenesis. Experimentally, transfection of human glioma cells with sFlt-1 demonstrated low expression of VEGF mRNA compared to transfection with an empty vector. The tumor growth of these sFlt-1 transfectants was inhibited, but the inhibitory activity was limited [7]. Another endogenous angiogenic inhibitor, thrombospondin1 (TSP1), was introduced into human U87 glioma cells by transfection (Figure 6). The glioma growth of the TSP1 transfectant was significantly inhibited compared to those of parent and vector-alone transfectants (Figure 7) [7]. These clinical and experimental data support the importance of angiogenic balance as a key

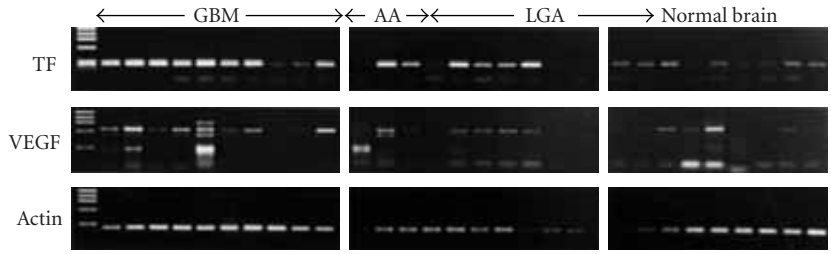


Figure 3: Tissue factor and VEGF mRNA expression in human glioma samples. Tissue factor expression was frequent and highly observed in glioblastomas associating with VEGF expression.

Table 1: Inhibition of angiogenesis in the chick chorioallantoic membrane assay.

Suramin (μg/disk)	Embryos evaluated (positive/total)	% of inhibition
0	0/10	0
30	0/7	0
250	2/8	25
500	5/10	50
1000	12/17	71
1500	7/7	100

factor in antiangiogenic therapy. This is likely because these endogenous angiogenesis inhibitors are upregulated in malignant gliomas, as a consequence of the upregulation of angiogenic factors.

The general growth factor receptor inhibitor, suramin, was investigated for its antiangiogenic action [8]. Suramin inhibited physiologic angiogenesis in a dose-dependent manner, based on the chorioallantoic membrane (CAM) assay (Table 1). Suramin inhibited the bFGF-induced endothelial expression of urokinase-type plasminogen-activator (uPA) using gelatin zymogram (Figure 8). uPA is closely related to the initiating step of angiogenesis, degradation of the extracellular matrix. Using a rat intracranial C6 glioma model, suramin inhibited Ki67 labeling of the tumor endothelium (Figure 9) [9]. All of these data suggest that suramin can inhibit physiological and tumor angiogenesis at multiple levels.

Recently, the anti-VEGF antibody, bavacizumab, has been used in the treatment of glioblastoma [10]. The growth inhibitory effect is dramatic, especially when determined with MRI enhancement and MRI perfusion study [11]. Interestingly, ACNU (1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-cholroethyl)-3-nitrosourea hydrochloride) chemotherapeutic agents resulted in upregulation of VEGF mRNA in glioma cells (Figure 10) [12]. A similar effect on upregulation of VEGF has been demonstrated by irradiation of glioma cells. Thus, the combination of VEGF antagonism, that is, VEGF antibody in the initial glioma therapy, is a reasonable strategy in ACNU chemotherapy and in radiation therapy (Figure 11). The VEGF antibody is attractive for attacking tumor stem cells, as a new strategy to combat glioblastomas, because the VEGF antibody could inhibit maintenance of glioma stem cells by destroying glioma vascular niche [13, 14], in contrast to the effects of radiation or other chemotherapeutics. However, the antiangiogenic antiglioma effect is transient (Figure 12). Congruent with the results obtained in orthotropic mouse models of GBM, four recent clinical studies have implicated proinvasive adaptation in humans, as observed by MRI, in a subset of GBM patients who developed multifocal or diffuse recurrence of the tumor during a course of anti-VEGF therapy with bevacizumab, as in our case [15–17]. Thus, there is a clear need for alternative strategies after bevacizumab failure.

3. Hypoxia in Gliomas

One of the mechanisms of resistance to angiogenic treatment is the presence of hypoxic regions in glioma tissues. Hypoxia-inducible factor 1α (HIF1α) is induced by hypoxia and is upstream of VEGF mRNA expression. Immunohistochemical expression of HIF-1α clearly correlated with the degree of glioma malignancies and predicted survival among patients with malignant gliomas (Figure 13) and the degree of necrosis on MRI (data not shown). Therefore, HIF-1α has been the focus of antiangiogenic treatment [18]. Downregulation of HIF-1α in glioma cells using siRNA resulted in growth inhibition and an angiosuppressive effect on glioma growth (unpublished data). Metronomic chemotherapy is a promising strategy to overcome resistance to antiangiogenic treatment [19]. We demonstrated that SN38, the active metabolite of CPT11, exhibited an antiangiogenic effect (Figure 14). SN38 inhibited HIF-1α and VEGF mRNA and protein expression of glioma cells in a dose- and time-dependent manner [20]. Metronomic CPT11 treatment of gliomas exhibited growth inhibitory effects without systemic toxicity, that is, through comparison of body weight loss that was not observed by conventional CPT11 treatment. Tumor tissues treated with metronomic CPT11 exhibited decreased expression of HIF-1α protein and pimonidazole expression, which were indicative of areas of hypoxia by immunohistochemistry (Figure 15). A recent advance in glioma chemotherapy is the discovery of temozolomide. Temozolomide is a powerful chemotherapeutic agent that prolongs overall survival of initial glioblastoma by up to 2.5 months [21]. More recently, the feasibility of bevacizumab with radiation therapy and temozolomide

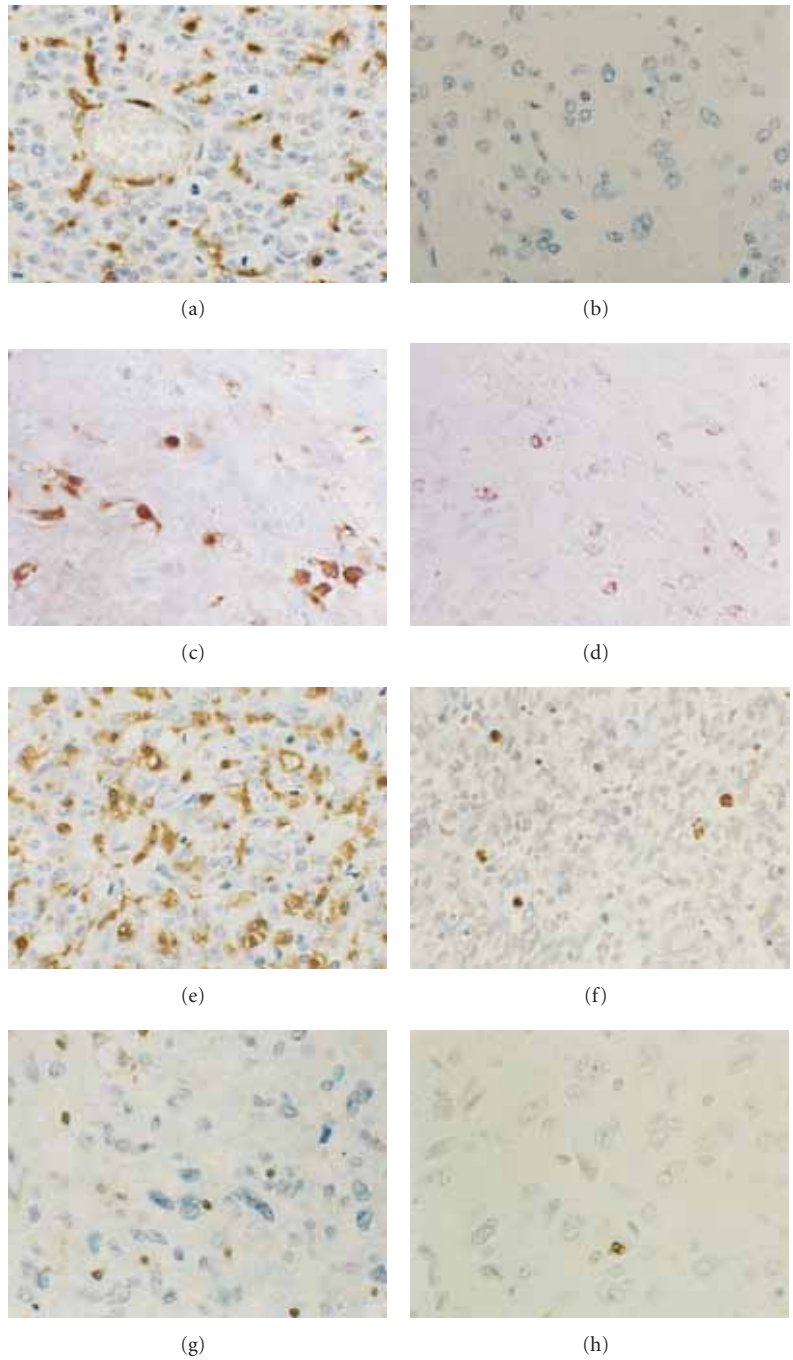


Figure 4: Thymidine phosphorylase immunohistochemistry in human gliomas. Glioblastoma shows intense immunoreaction for thymidine phosphorylase both in tumor and endothelial cells (a). Diffuse astrocytoma shows no expression (b). Some of the tymidine phosphorylase positive cells (c) are macrophages ((d) serial section of (c)). Thymidine phosphorylase positive glioblastoma (e) reveals a high apoptotic index ((f) serial section of (e)), while Thymidine phosphorylase negative glioblastoma (g) reveals a low apoptotic index ((h) serial section of (g)). Original magnification ×200.

in newly diagnosed high-grade gliomas has been reported [22, 23]. Interestingly, temozolomide has an inhibitory effect on HIF-1α expression and endothelial cell tube formation [24]. The metronomic temozolomide treatment is reasonable and clinical results have been demonstrated [25, 26].

4. Glioma-Derived Endothelial Cells

Many studies focusing on tumor angiogenesis and endothelial biology are based on established normal cells lines that is, human umbilical vein endothelial cells (HUVECs). Whether or not tumor endothelial cells and normal endothelial cells

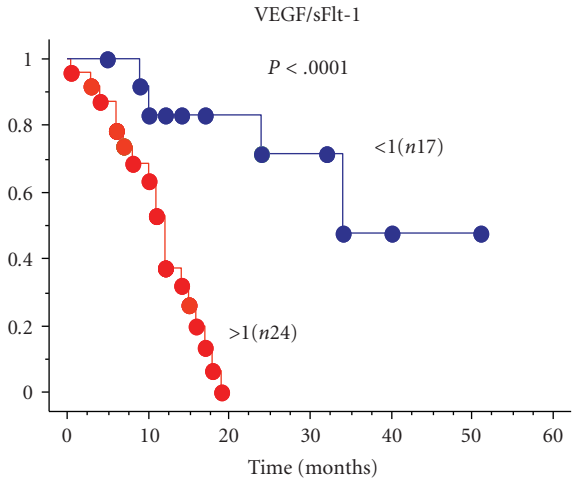


Figure 5: Malignant glioma survival by VEGF/sFlt-1 ratio.

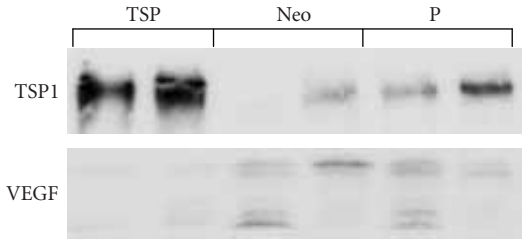


Figure 6: Characterization of thrombospondin-1 transfected U87. Thrombospondin-1 expression was markedly elevated in the transfectant (TSP) compared to vector alone (Neo) and parent U87 (P). Also VEGF expression was decreased in transfectant (TSP).

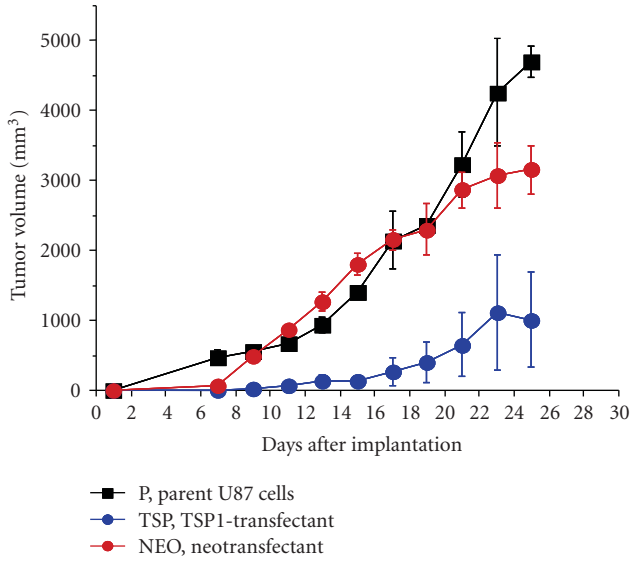


Figure 7: Inhibition of glioma growth by thrombospondin-1 transfection. The glioma growth is significantly inhibited by thrombospondin-1 transfectant (TSP-1 transfectant) compared to parent U87 and vector alone (Neotransfectant).

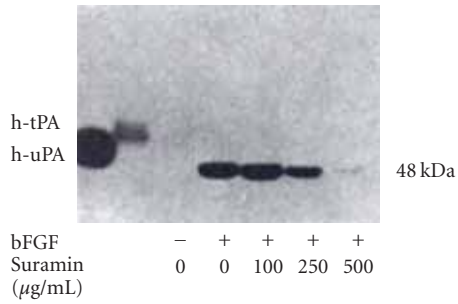


Figure 8: Suramin inhibition of bFGF induced endothelial cell urokinase type plasminogen activator activity on gelatin zymogram.

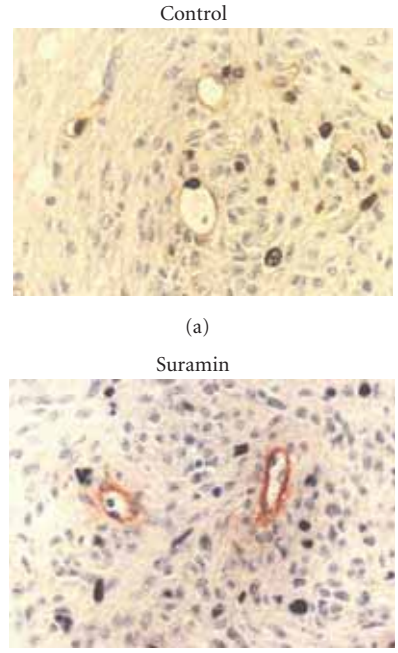


Figure 9: Suramin inhibition of tumor endothelial cell Ki67 labeling.

are genetically and functionally identical remains controversial. Comparisons between tumor-derived and normal ECs have been made for a variety of systemic tumors [27]. They have shown that tumor endothelium exhibits a phenotype of activated ECs, as reflected in the high expression of angiogenic molecules, that is, VEGFR, the angiopoietin receptor Tie2, and the adhesion molecules ICAM-1, E-selectin, and CD44. In recent publications, researchers have suggested that the tumor-associated ECs derived from GBM tissues have different phenotypic and functional properties compared to normal ECs [28, 29]; these differences may result in less effective antiangiogenic therapy if the target molecules are only expressed in normal blood vessels. Moreover, these publications have not mentioned the potential for interactions between tumor cells and tumor-derived endothelial cells. We isolated tumor endothelial cells from human glioblastoma samples using flow cytometry, cultured them,

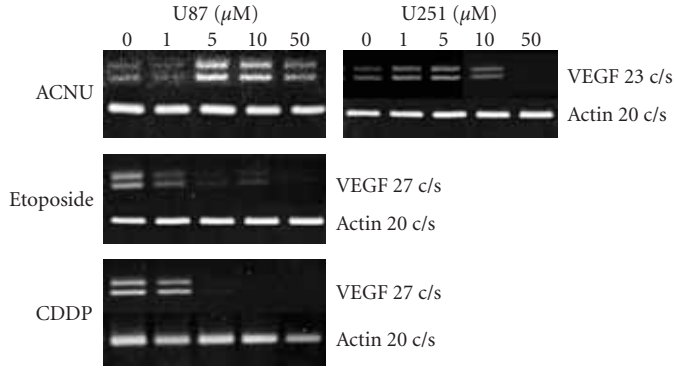


Figure 10: Elevation of VEGF mRNA expression by ACNU treatment, but not etoposide and CDDP treatment, in human glioma cells (U87 and U251).

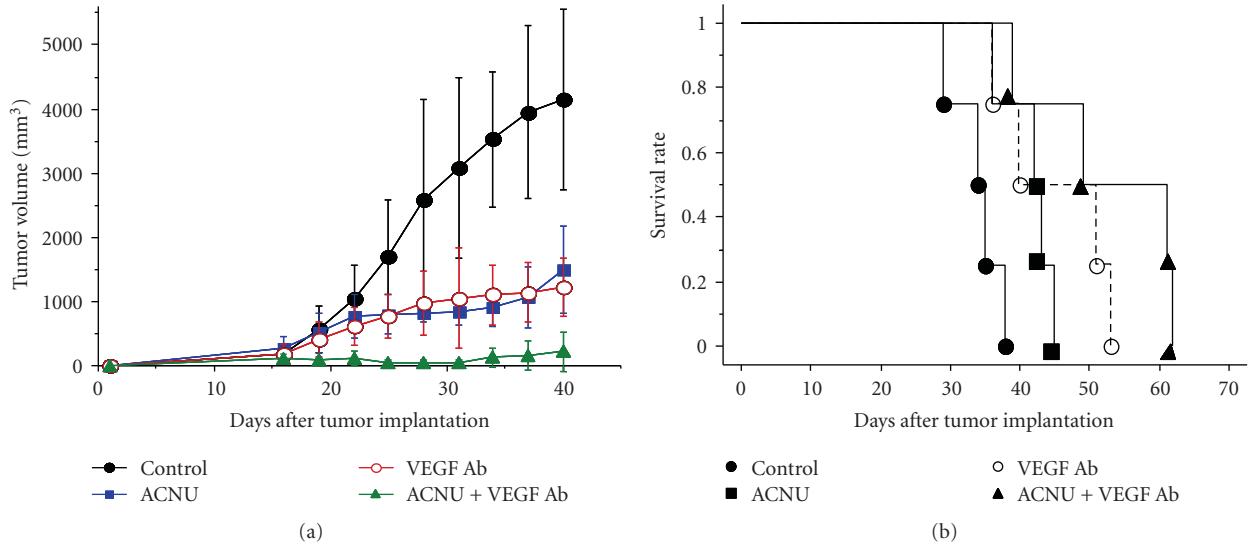


Figure 11: Glioma growth inhibition by VEGF antibody, ACNU, and the combination of both treatments with U87 subcutaneous (a) and intracranial (b) model.

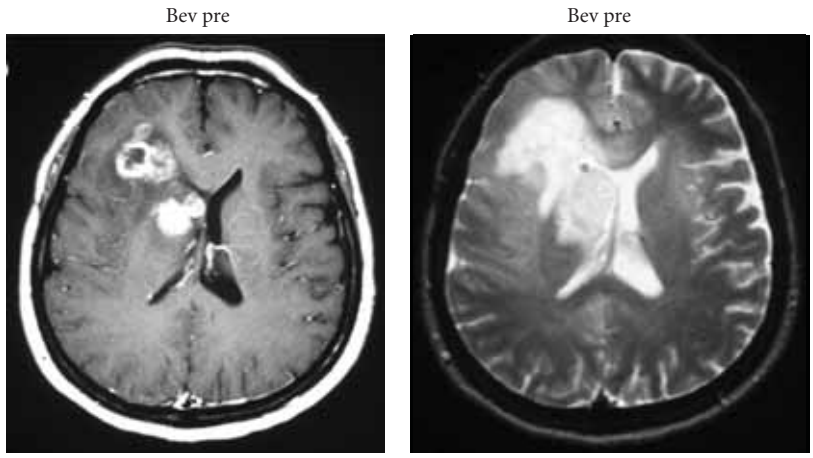


Figure 12: Continued.

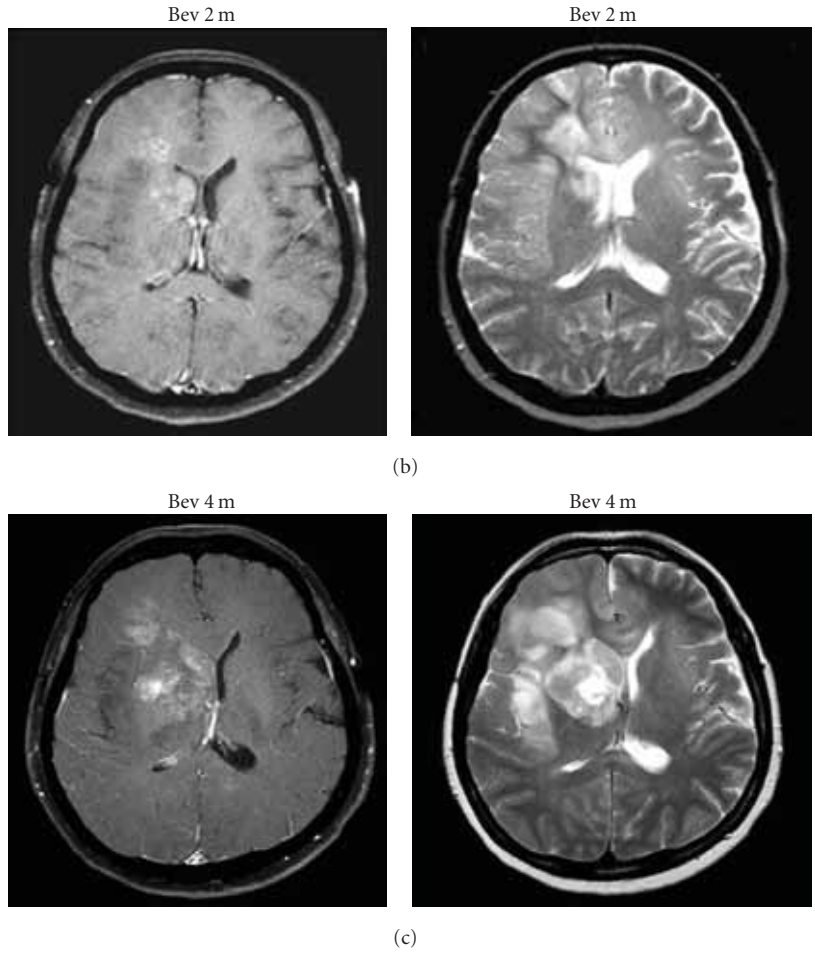


FIGURE 12: Clinical course of a case with recurrent malignant glioma with bevacizumab and CPT11 treatment. After 3 cycles enhanced tumor and perifocal edema is markedly diminished (Bev 2 m). However, after 6 cycles T2 high intense tumors regrow with minimal enhancement (Bev 4 m).

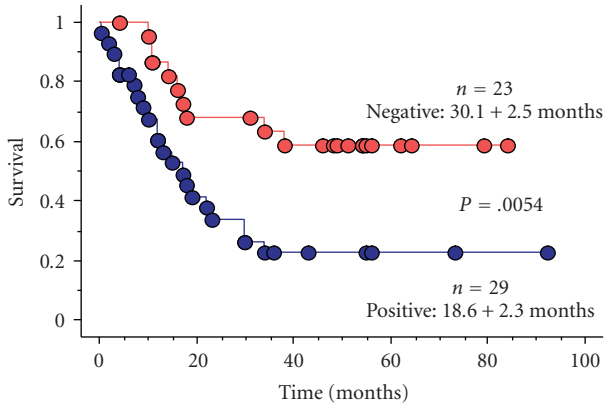


FIGURE 13: Malignant glioma survival with hypoxia inducible factor 1α (HIF-1α) expression on immunohistochemistry. HIF-1α expression is a negative prognostic factor.

and analyzed the genetic differences between these cell types and HUVEC regarding the mRNA and protein expression of angiogenic factors and chemokines. Glioblastoma-derived endothelial cells (GBMECs) exhibited high expression of VEGF, SDF-1, and CXCR7 mRNA compared to HUVEC, and GBMECs exhibited no expression of CXCR4 mRNA (Figure 16, unpublished data). We are now investigating functional differences between GBMECs and HEUVEC as well as the interaction between GBMECs and glioma cells using a coculture system. To obtain successful results with antiangiogenic therapy, tumor endothelial cells should be targeted in the future.

5. The Role of Endothelial Progenitor Cells on Tumor Angiogenesis

Another important mechanism of resistance to antiangiogenic treatment is related to EPCs. EPCs are introduced into tumor angiogenesis by tumor stimuli from the bone marrow. We investigated the role of EPCs on glioma angiogenesis. C6 glioma cells (5×10^6 cells) were stereotactically implanted into the brain. After 7 days, EPCs (3×10^5 cells) that were harvested from umbilical cord blood [30] were intravenously injected via the tail vein. Seven days after the

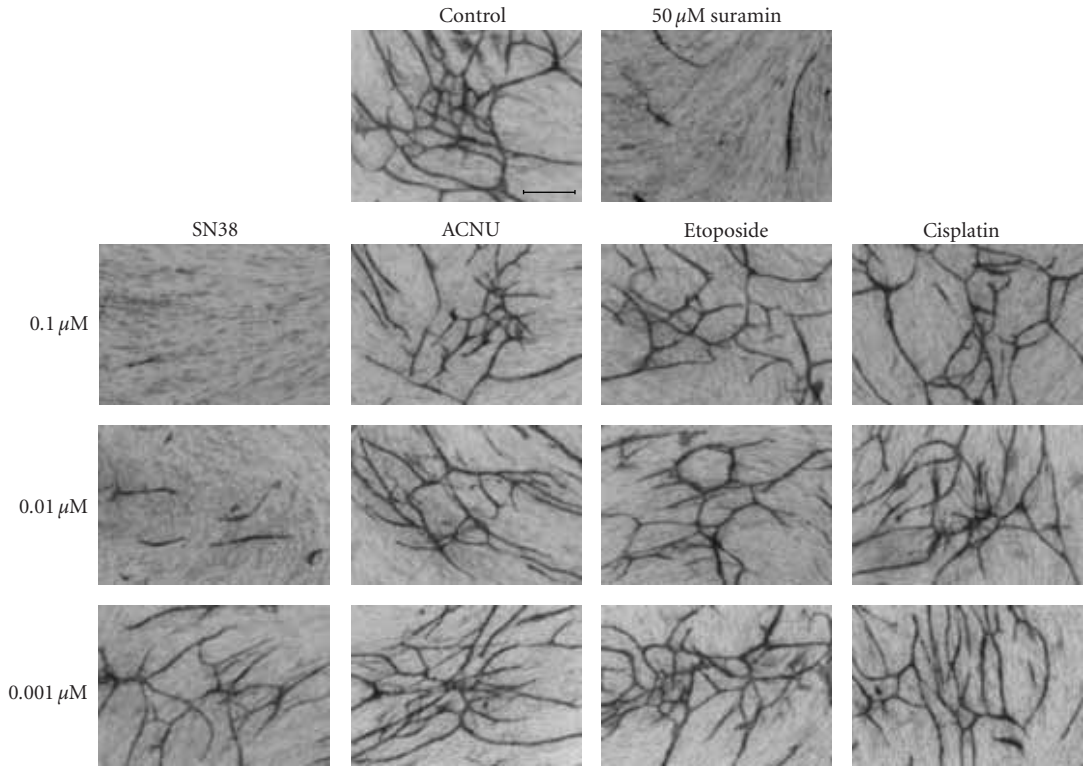


Figure 14: Antiangiogenic effect of SN38, active metabolite of CPT11. Low dose of SN38 (0.01 and 0.1 μM) inhibited tube formation of HUVEC.

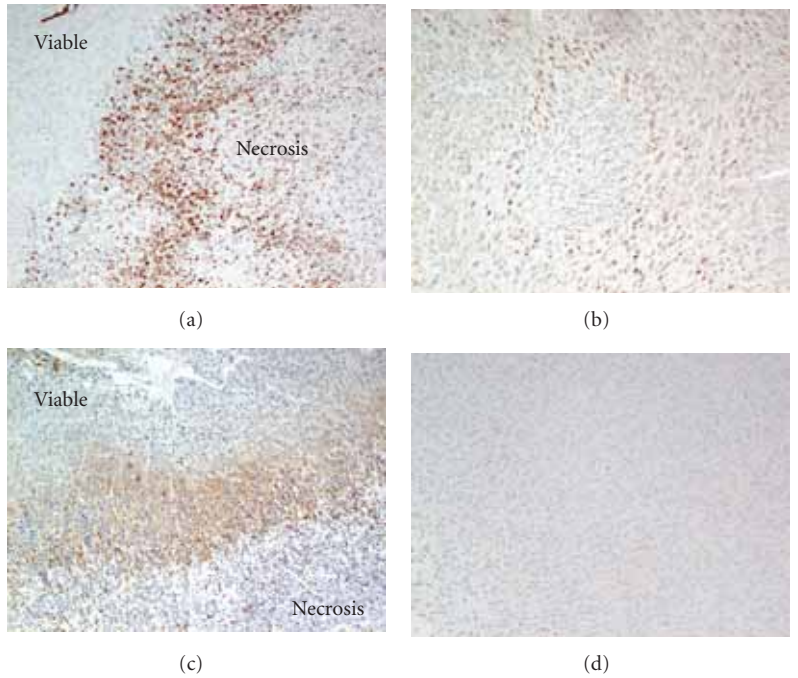


Figure 15: Hypoxia inducible factor 1α expression and hypoxic area with (b, d) and without (a, c) metronomic CPT11 treatment. HIF-1α expression and hypoxic area around the necrosis of glioma tissue decreased with treatment.

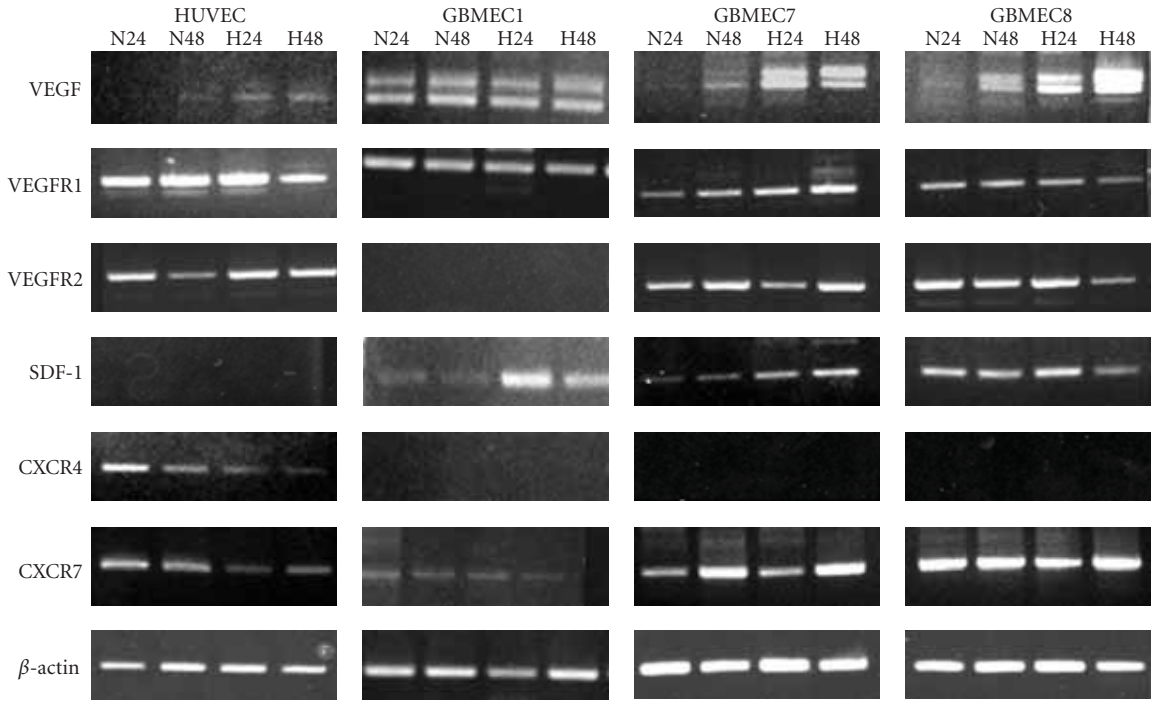


Figure 16: Angiogenic factor and chemokine expression in HUVEC and glioblastoma derived endothelial cells (GBMECs). GBMECs show high expression of VEGF, SDF-1, and CXCR7 compared to HUVEC and no expression of CXCR4.

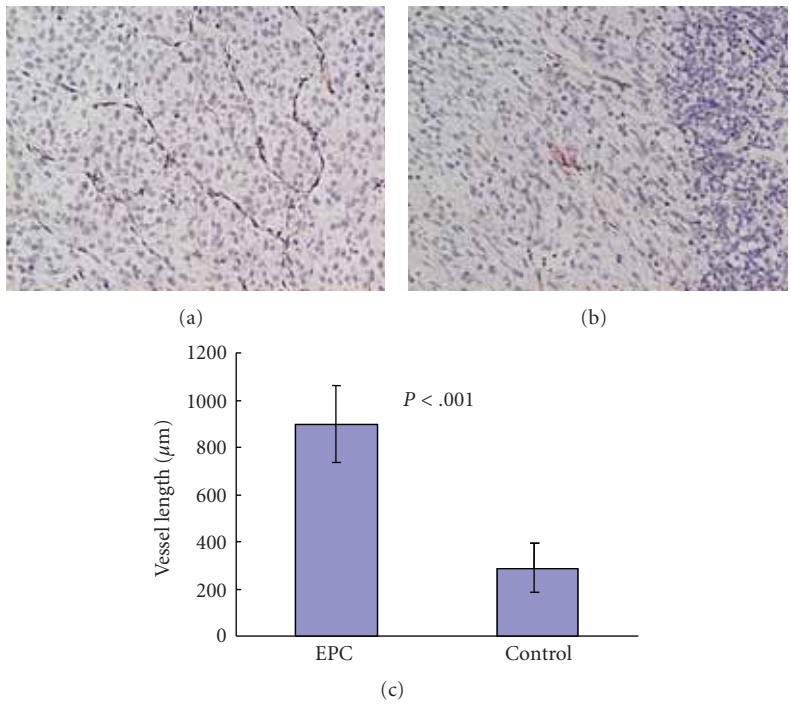


Figure 17: Rich vascular network with EPC-injected glioma. The vessel length of EPC-injected tumor (a) is significantly longer than those of control (b).

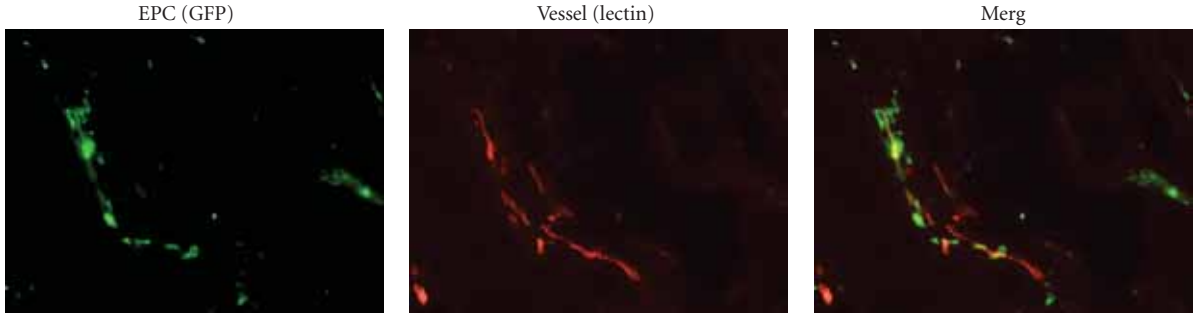


Figure 18: EPC homing to glioma vasculature. 11 days after injection of GFP labeled EPCs, EPCs localize lectin labeled glioma vasculature.

EPC injection, the rats were sacrificed and the C6 gliomas in the brain were fixed and stained with CD31. EPC-injected C6 glioma demonstrated a large narrow vascular network. The vessel length is significantly longer than EPCs in an uninjected tumor (Figure 17). Fluoroscopy demonstrated that GFP-labeled EPCs localized along with lectin-labeled tumor vessels (Figure 18). This result suggests that EPC could induce homing to the glioma vasculature and that this characteristic of tumor vasculature homing is useful when considering EPCs as drug delivery tools. If the EPCs contain angiogenesis inhibitors, the angiogenesis inhibitor is automatically delivered to the tumor vasculature. To date, this new strategy has not been published.

6. Molecular Targets of Glioma Angiogenesis in Future

Finally, the question remains, what are the current possible target molecules for glioma angiogenesis? Norden et al. [31] reported some molecules other than VEGF. Among them, delta-like 4 (Dll4) remains promising, because the mechanisms of angiosuppression are quite different to those of VEGF and the role of Dll4 is reciprocal to VEGF [32, 33]. Remarkably, Dll4 and VEGF are the only known genes for which loss of a single allele results in embryonic lethality due to failure to form a functional vasculature. Dll4 is exclusively expressed by endothelial cells; therefore, this ligand is a potential therapeutic target. Although blocking Dll4 appears to promote angiogenesis, the neovasculature is functionally abnormal and it cannot support tumor cell survival [34]. Preclinical studies have shown that blockade of Dll4 was effective in inhibiting the growth of tumors that are resistant to VEGF inhibition [32]. Whereas most of current antiangiogenesis approaches act through the reduction or elimination of tumor blood vessels, Dll4 blockade results in the formation of a nonfunctional vasculature that is unable to support tumor growth. This paradoxical strategy for targeting tumors will be the focus of intense research for years ahead.

Furthermore, the expression of recombinant toxic proteins that specifically target tumor endothelium appears to be promising [35]. Fusion proteins directed against urokinase-type plasminogen-activator receptor (uPAR) may be appropriate for targeting endothelial cells in the tumor vasculature compared with normal endothelium, as uPAR may be preferentially expressed in proliferating endothelium. The efficacy of protein DTAT13 that was synthesized to target uPAR on the neovasculature and uPAR- and interleukin-13-expressing glioblastoma cells has been demonstrated on glioma growth in vitro and in vivo [36].

7. Conclusion

Anti-angiogenesis therapy for malignant gliomas is promising by not only inhibiting angiogenesis but also through alteration of the tumor microenvironment, that is, the tumor vascular niche. Moreover, various combinations of strategies including the development of new molecular targets have been investigated. Overcoming resistance to antiangiogenic therapy with minimal side effects should be considered.

References

[1] R. S. Kerbel, "Tumor angiogenesis," *The New England Journal of Medicine*, vol. 358, no. 19, pp. 2039–2049, 2008.

[2] P. Y. Wen and S. Kesari, "Malignant gliomas in adults," *The New England Journal of Medicine*, vol. 359, no. 5, pp. 492–507, 2008.

[3] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[4] S. Takano, Y. Yoshii, S. Kondo, et al., "Concentration of vascular endothelial growth factor in the serum and tumor tissue of brain tumor patients," *Cancer Research*, vol. 56, no. 9, pp. 2185–2190, 1996.

[5] S. Takano, K. Tsuboi, Y. Tomono, Y. Mitsui, and T. Nose, "Tissue factor, osteopontin, $\alpha_v\beta_3$ integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression," *British Journal of Cancer*, vol. 82, no. 12, pp. 1967–1973, 2000.

[6] S. Takano, K. Tsuboi, A. Matsumura, Y. Tomono, Y. Mitsui, and T. Nose, "Expression of the angiogenic factor thymidine phosphorylase in human astrocytic tumors," *Journal of Cancer Research and Clinical Oncology*, vol. 126, no. 3, pp. 145–152, 2000.

[7] S. Takano, H. Kamiyama, K. Tsuboi, and A. Matsumura, "Angiogenesis and antiangiogenic therapy for malignant

gliomas," *Brain Tumor Pathology*, vol. 21, no. 2, pp. 69–73, 2004.

[8] S. Takano, S. Gately, M. E. Neville, et al., "Suramin, an anticancer and angiosuppressive agent, inhibits endothelial cell binding of basic fibroblast growth factor, migration, proliferation, and induction of urokinase-type plasminogen activator," *Cancer Research*, vol. 54, no. 10, pp. 2654–2660, 1994.

[9] S. Takano, S. Gately, H. Engelhard, A. M. C. Tsanaclis, and S. Brem, "Suramin inhibits glioma cell proliferation in vitro and in the brain," *Journal of Neuro-Oncology*, vol. 21, no. 3, pp. 189–201, 1994.

[10] J. J. Vredenburgh, A. Desjardins, J. E. Herndon II, et al., "Bevacizumab plus irinotecan in recurrent glioblastoma multiforme," *Journal of Clinical Oncology*, vol. 25, no. 30, pp. 4722–4729, 2007.

[11] S. Ananthnarayan, J. Bahng, J. Roring, et al., "Time course of imaging changes of GBM during extended bevacizumab treatment," *Journal of Neuro-Oncology*, vol. 88, no. 3, pp. 339–347, 2008.

[12] S. Takano, K. Tsuboi, A. Matsumura, and T. Nose, "Therapeutic combination of anti-VEGF antibody and ACNU chemotherapy: effects on tumor growth and angiogenesis in human glioblastoma xenografts," *Neuro-Oncology*, vol. 5, no. 1, pp. 1–7, 2003.

[13] C. Folkins, S. Man, P. Xu, et al., "Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor-like cell fraction in glioma xenograft tumors," *Cancer Research*, vol. 67, no. 8, pp. 3560–3564, 2007.

[14] R. J. Gilbertson and J. N. Rich, "Making a tumour's bed: glioblastoma stem cells and the vascular niche," *Nature Reviews Cancer*, vol. 7, no. 10, pp. 733–736, 2007.

[15] A. Narayana, J. G. Golfinos, I. Fischer, et al., "Feasibility of using bevacizumab with radiation therapy and temozolomide in newly diagnosed high-grade glioma," *International Journal of Radiation Oncology Biology Physics*, vol. 72, no. 2, pp. 383–389, 2008.

[16] A. D. Norden, G. S. Young, K. Setayesh, et al., "Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence," *Neurology*, vol. 70, no. 10, pp. 779–787, 2008.

[17] R. M. Zuniga, R. Torcuator, R. Jain, et al., "Efficacy, safety and patterns of response and recurrence in patients with recurrent high-grade gliomas treated with bevacizumab plus irinotecan," *Journal of Neuro-Oncology*, vol. 91, no. 3, pp. 329–336, 2009.

[18] S. Sathornsumetee, Y. Cao, J. E. Marcello, et al., "Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan," *Journal of Clinical Oncology*, vol. 26, no. 2, pp. 271–278, 2008.

[19] D. Hanahan, G. Bergers, and E. Bergsland, "Less is, more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice," *Journal of Clinical Investigation*, vol. 105, no. 8, pp. 1045–1047, 2000.

[20] H. Kamiyama, S. Takano, K. Tsuboi, and A. Matsumura, "Anti-angiogenic effects of SN38 (active metabolite of irinotecan): inhibition of hypoxia-inducible factor 1 alpha (HIF-1a)/vascular endothelial growth factor (VEGF) expression of glioma and growth of endothelial cells," *Journal of Cancer Research and Clinical Oncology*, vol. 131, no. 4, pp. 205–213, 2005.

[21] R. Stupp, W. P. Mason, M. J. van den Bent, et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *The New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.

[22] A. Lai, E. Filka, B. McGibbon, et al., "Phase II pilot study of bevacizumab in combination with temozolomide and regional radiation therapy for up-front treatment of patients with newly diagnosed glioblastoma multiforme: interim analysis of safety and tolerability," *International Journal of Radiation Oncology Biology Physics*, vol. 71, no. 5, pp. 1372–1380, 2008.

[23] A. Narayana, P. Kelly, J. Golfinos, et al., "Antiangiogenic therapy using bevacizumab in recurrent high-grade glioma: impact on local control and patient survival," *Journal of Neurosurgery*, vol. 110, no. 1, pp. 173–180, 2009.

[24] V. Mathieu, N. de Neve, M. le Mercier, et al., "Combining bevacizumab with temozolomide increases the antitumor efficacy of temozolomide in a human glioblastoma orthotopic xenograft model," *Neoplasia*, vol. 10, no. 12, pp. 1383–1392, 2008.

[25] S. Baruchel, M. Diezi, D. Hargrave, et al., "Safety and pharmacokinetics of temozolomide using a dose-escalation, metronomic schedule in recurrent paediatric brain tumours," *European Journal of Cancer*, vol. 42, no. 14, pp. 2335–2342, 2006.

[26] D. S. Kong, J. I. Lee, W. S. Kim, et al., "A pilot study of metronomic temozolomide treatment in patients with recurrent temozolomide-refractory glioblastoma," *Oncology Reports*, vol. 16, no. 5, pp. 1117–1121, 2006.

[27] B. Croix, C. Rago, V. Velculescu, et al., "Genes expressed in human tumor endothelium," *Science*, vol. 289, no. 5482, pp. 1197–1202, 2000.

[28] C. Charalambous, F. M. Hofman, and T. C. Chen, "Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells," *Journal of Neurosurgery*, vol. 102, no. 4, pp. 699–705, 2005.

[29] S. Miebach, S. Grau, V. Hummel, P. Rieckmann, J.-C. Tonn, and R. H. Goldbrunner, "Isolation and culture of microvascular endothelial cells from gliomas of different WHO grades," *Journal of Neuro-Oncology*, vol. 76, no. 1, pp. 39–48, 2006.

[30] M. Nagano, T. Yamashita, H. Hamada, et al., "Identification of functional endothelial progenitor cells suitable for the treatment of ischemic tissue using human umbilical cord blood," *Blood*, vol. 110, no. 1, pp. 151–160, 2007.

[31] A. D. Norden, J. Drappatz, and P. Y. Wen, "Novel anti-angiogenic therapies for malignant gliomas," *The Lancet Neurology*, vol. 7, no. 12, pp. 1152–1160, 2008.

[32] I. Noguera-Troise, C. Daly, N. J. Papadopoulos, et al., "Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis," *Nature*, vol. 444, no. 7122, pp. 1032–1037, 2006.

[33] J. Ridgway, G. Zhang, Y. Wu, et al., "Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis," *Nature*, vol. 444, no. 7122, pp. 1083–1087, 2006.

[34] M. Yan and G. D. Plowman, "Delta-like 4/notch signaling and its therapeutic implications," *Clinical Cancer Research*, vol. 13, no. 24, pp. 7243–7246, 2007.

[35] D. A. Vallera, C. Li, N. Jin, A. Panoskaltsis-Mortari, and W. A. Hall, "Targeting Urokinase-type plasminogen activator receptor on human glioblastoma tumors with diphtheria toxin fusion protein DTAT," *Journal of the National Cancer Institute*, vol. 94, no. 8, pp. 597–606, 2002.

[36] W. A. Hall and D. A. Vallera, "Efficacy of antiangiogenic targeted toxins against glioblastoma multiforma," *Neurosurg Focus*, vol. 20, no. 4, pp. 1–6, 2006.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 689018, 14 pages
doi:10.1155/2010/689018

Review Article

Progress on Antiangiogenic Therapy for Patients with Malignant Glioma

Manmeet S. Ahluwalia[1,2] and Candece L. Gladson[1,3]

[1] *Brain Tumor Neuro-Oncology Center, Neurological Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA*
[2] *Department of Solid Tumor Oncology, Taussig Cancer Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA*
[3] *Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA*

Correspondence should be addressed to Candece L. Gladson, gladsoc@ccf.org

Received 21 November 2009; Revised 25 January 2010; Accepted 11 February 2010

Academic Editor: Arkadiusz Dudek

Glioblastoma (GBM) is the most common primary brain tumor occurring in America. Despite recent advances in therapeutics, the prognosis for patients with newly diagnosed GBM remains dismal. As these tumors characteristically show evidence of angiogenesis (neovascularization) there has been great interest in developing anti-angiogenic therapeutic strategies for the treatment of patients with this disease and some anti-angiogenic agents have now been used for the treatment of patients with malignant glioma tumors. Although the results of these clinical trials are promising in that they indicate an initial therapeutic response, the anti-angiogenic therapies tested to date have not changed the overall survival of patients with malignant glioma tumors. This is due, in large part, to the development of resistance to these therapies. Ongoing research into key features of the neovasculature in malignant glioma tumors, as well as the general angiogenesis process, is suggesting additional molecules that may be targeted and an improved response when both the neovasculature and the tumor cells are targeted. Prevention of the development of resistance may require the development of anti-angiogenic strategies that induce apoptosis or cell death of the neovasculature, as well as an improved understanding of the potential roles of circulating endothelial progenitor cells and vascular co-option by tumor cells, in the development of resistance.

1. Introduction

Malignant gliomas include WHO grade IV gliomas, also known as glioblastomas (GBM), and WHO grade III gliomas referred to as anaplastic gliomas (AG) (anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma). GBM is the most common primary brain tumor occurring in the United States of America; approximately 10,000 new cases are diagnosed each year [1, 2]. In this review, we will focus on the status of antiangiogenic therapy for GBM, as these tumors characteristically show evidence of angiogenesis (neovascularization) on histologic examination. Despite recent advances in therapeutics, the prognosis for patients with newly diagnosed GBM remains dismal; the median survival is 15 months when treated with the current standard of therapy, which is a combination of maximal surgical resection followed by concurrent chemoradiation and six months of adjuvant temozolomide [3]. Most patients with GBM develop tumor recurrence after the above therapy, and many centers are now treating these patients with bevacizumab (a monoclonal antibody to vascular endothelial growth factor (VEGF) that was recently approved by the FDA). Although clearly of benefit to some patients, the majority die within 6-9 months after initiation of anti-VEGF therapy [4–6]. Other antiangiogenic agents also have been examined in phase I or II clinical trials for patients with GBM, and promising results have emerged; however, a statistically significant increase in overall survival has not been reported to date. It is now becoming apparent that tumors also can act to enhance their vasculature through other mechanisms such as "co-option" of the existing vasculature. The contribution of these processes to tumor vascularization and their potential effects on anti-angiogenic therapies is an emerging field of great interest.

2. The Biology of Brain Tumor Vascularization

GBMs are among the most vascular tumors known and hence the tumor-associated vasculature is an attractive therapeutic target [7]. It is now well established that tumors can promote the formation of new vessels through the process of angiogenesis. It is thought that when a tumor reaches a certain size, the requirements for oxygen and nutrients lead to the growth of new blood vessels [8, 9]. The neovasculature that is formed in GBM never completely matures; however, this leads to an atypical vasculature that is constantly undergoing remodeling. There also is evidence to suggest that prior to the triggering of the process of formation of new vessels, tumor cells can obtain the necessary nutrients and oxygen by "co-opting" existing blood vessels [10]. This process appears to occur in very small tumors and appears to be dependent on the microenvironment in the specific organ and perhaps on the tumor type. In transplanted C6 rat gliomas in the rat brain co-option of existing blood vessels by tumor cells occurred initially when the tumors were several mm in diameter, and this was followed by vascular regression and ultimately by the development of a neovasculature [10]. Moreover, the process of vasculogenic mimicry [11], in which tumor cells function as blood vessel lining cells, may contribute to the blood supply in malignant tumors. Most of the research concerning tumor vasculature has focused on the mechanisms that promote the formation of new blood vessels through the process of angiogenesis and it is these mechanisms that have been targeted in the development of antiangiogenic therapies. Currently, relatively little is known concerning the mechanisms underlying co-option of blood vessels and vasculogenic mimicry, the effects of anti-angiogenic therapies on these processes, or the role of these processes in the activity of, or resistance to, the anti-angiogenic therapies that have been developed.

2.1. Angiogenic Growth Factors: Their Receptors and Function. The signaling of VEGF, a proangiogenic growth factor, is important for GBM angiogenesis and involves paracrine interactions between the glioma cells, and the inflammatory cells that secrete VEGF, and the tumor-associated endothelial cells (EC) that express receptors for VEGF (VEGFR) [9]. The VEGF gene family is composed of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the placental-derived growth factor (PlGF) [10–12]. Of these, family members VEGF-A, VEGF-B, and PlGF are involved in vascular angiogenesis, whereas VEGF-C and -D regulate lymphatic angiogenesis [9]. VEGF-A was originally discovered as a factor that induces vascular permeability, and has since been shown to be an important EC mitogen [13, 14]. It belongs to the platelet-derived growth factor (PDGF) superfamily and is located on chromosome 6 [15]. Once secreted by the tumor cells, stromal cells or ECs, VEGF can be tethered in the extracellular matrix (ECM) due to an association with proteoglycans or glycosaminoglycans [16, 17].

The main receptors for VEGF-A are VEGFR1 and VEGFR2 [15]. These receptors are upregulated on the ECs in GBM as compared to the ECs of normal brain [18]. VEGFR-2 is a major mediator of the mitogenic and angiogenic effects of VEGF through its activation of the phosphatidyl inositol 3-kinase (PI3K)/protein kinase B (AKT) and RAF-MAPK kinase-(MEK-) extracellular signal-regulated (ERK) MAP kinase pathways [15].

Basic fibroblast growth factor (bFGF) is another proangiogenic growth factor that is upregulated in GBM, in which it is expressed focally by tumor cells and also is expressed by the vasculature [19, 20]. The receptors for bFGF include FGFR1, FGFR2, and FGFR4. FGFR1 is upregulated in GBM and is expressed by both the tumor cells and the tumor ECs. FGFR4 is expressed only by the tumor cells, and FGFR2 is not expressed in the tumors but it is expressed in the normal brain [21, 22]. As is the case for VEGF, once secreted by the tumor cells, stromal cells or ECs, bFGF can be tethered in the ECM due to an association with proteoglycans or glycosaminoglycans [16, 17]. Binding of bFGF to its receptor results in activation of the protein kinase Cα (PKCα) pathway [23, 24] and the ERK pathway [23, 25].

In addition to VEGF and bFGF, several other proangiogenic molecules have been implicated in the initiation or amplification of angiogenesis in GBM including stem cell factor (SCF) and interleukin-8 (IL-8), hepatocyte growth factor and urokinase [26–30]. SCF mediates its signals through activation of the c-KIT receptor and may be of particular interest in GBM as its overexpression is associated with a shorter survival in patients with malignant glioma [31]. IL-8 is a chemokine that is synthesized by macrophages, ECs (where it is stored in the Weibel-Palade bodies), and other cell types (such as epithelial cells) [32, 33]. IL-8 is released from macrophages as a result of a host inflammatory response and activates ECs through the chemokine-1 and 2 receptors (CXCR1 and CXCR2) [34].

Finally, in normal blood vessels, angiopoietin-1 (Ang-1) is expressed by pericytes, binds the Tie2 tyrosine kinase receptor expressed on the associated EC and signals for survival and stabilization of the blood vessel [35, 36]. In GBM there is increased expression of both Ang-1 and its antagonist angiopoietin-2 (Ang-2); Ang-2 is expressed by the ECs, whereas Ang-1 is expressed by the tumor cells and is not expressed by the tumor blood vessels [37]. This has led investigators to speculate that Ang-2 may have a proangiogenic function in the neovasculature of tumors. Of note, Ang-2 expression has been implicated in the co-option process as described below [10].

2.2. Proteolytic Degradation of the Basement Membrane, Endothelial Cell Sprouting, and Tube Formation. Proteolytic degradation of the EC basement membrane by matrix metalloproteinases (MMPs) exposes the ECs to ECM proteins that can regulate angiogenesis and promote EC movement or sprouting [11, 28, 31]. These cell-ECM interactions occur through specific receptors and binding partners expressed on the EC surface, such as integrin cell adhesion receptors that recognize specific ECM molecules [9, 28, 34, 38, 39]. In addition, integrin receptors cooperate, collaborate, or cross-talk with growth factor receptors in a specific manner to enhance the signaling of both the integrin and the growth factor receptor. For example, in the chick chorioallantoic

membrane model of angiogenesis, integrins $\alpha v\beta 5$ and $\alpha v\beta 3$, which are expressed on the EC, bind ligands in the chorioallantoic membrane and promote EC survival, proliferation, and sprouting (migration) by cooperating with VEGFR2 and FGFR, respectively [39]. At the same time, activation of the platelet-derived growth factor (PDGF) receptor signaling pathway recruits pericytes to the new EC tube, where they deposit ECM proteins that aid in the formation of a new EC basement membrane, inducing the expression of fibronectin and nidogen-1 by the ECs [40–43]. Pericyte recruitment to the EC tube also promotes the upregulation of certain integrin receptors ($\alpha 6\beta 1$, $\alpha 3\beta 1$, $\alpha 1\beta 1$, and $\alpha 5\beta 1$) on the ECs that mediate the interaction of the EC with fibronectin, nidogen-1, and laminin in the new basement membrane [42, 43].

Pericytes are necessary for stabilization of the new EC tube. The finding that EC tubes lacking pericyte coverage become dilated [42] suggests that pericyte coverage of the EC tube is necessary for maintaining the appropriate vessel diameter. This concept is consistent with the dilated and tortuous blood vessels that are observed on histologic examination of many GBM tumors and suggests that this may be associated with the reduced pericyte coverage of blood vessels observed in these tumors [36, 44].

Endothelial progenitor cells (EPCs) from the bone marrow may also contribute to the neovasculature. EPCs are mobilized from the bone marrow by the cytokine stromal-derived factor-1α (SDF-1α) [45] that is expressed by angiogenic vessels in GBM, SDF1α binds to the G protein-coupled chemokine 4 receptor (CXCR4) expressed on circulating EPCs and also on tumor vessels [46]. A role for the SDF1α/CXCR4 signaling pathway in the development or maintenance of the GBM neovasculature is suggested by a report that demonstrated that the administration of a CXCR4 antagonist to an orthotopic xenograft mouse model of GBM inhibited tumor growth [47].

2.3. Abnormalities in the Neovasculature of GBM and Hypoxia. The failure of the GBM neovasculature to mature completely results in an atypical neovasculature that demonstrates excessive leakiness and lacks a normal blood brain barrier (BBB). Electron microscopic examination of the neovasculature in GBM has revealed that the tight junctions and adherens junctions (important contributors to the BBB) are abnormal and that the actin filaments associated with the junctions are disorganized [48]. These changes likely decrease the osmotic gradient between the vasculature and interstitium, elevating the interstitial fluid pressure in the tumor [49]. This has certain important clinical implications. First, the elevated interstitial fluid pressure can compromise drug delivery to the GBM. Second, the abnormalities in the neovasculature may enhance tumor cell access to the vasculature and aid in tumor cell migration and invasion along the EC basement membrane to previously uninvolved brain tissue.

In addition, the neovasculature in GBM demonstrates prominent thrombosis, promoting local hypoxia within the tumor. This local hypoxia is exacerbated by the rapid growth of these tumors, which frequently results in an extensive necrotic core that further accentuates the hypoxic microenvironment. Hypoxia can promote tumor angiogenesis through activation of the transcription factor hypoxia-inducible factor -1α (HIF-1α) which, in turn, enhances production of proangiogenic growth factors. Normally, the von Hippel-Lindau (VHL) molecule inhibits the function of HIF-1α but hypoxia destabilizes VHL causing it to dissociate from HIF-1α. This results in HIF-1α binding to the hypoxia response element (HRE) in the promoter of several proangiogenic factors (i.e., VEGF and SDF-1α) thereby initiating their transcription [45, 50]. A second HIF family member, HIF-2α, is also activated by hypoxia and likely plays a role in promoting tumor angiogenesis in GBM [51]. Li and colleagues [51] have recently demonstrated that HIF-2α is preferentially expressed in glioma stem cells, in comparison to nonstem tumor cells and normal neural progenitors. Their work showed that HIF2α, not HIF1α, is selectively activated in glioma stem cells by hypoxia, inducing the expression of VEGF. In tumor specimens, HIF2α colocalized with markers of cancer stem cells. Furthermore, targeting HIF in glioma stem cells inhibited self-renewal, proliferation, and survival in vitro, and attenuated the tumor initiation potential of glioma stem cells in mouse xenografts. Therefore, HIF2α likely represents a promising new therapeutic target in patients with GBM [51].

2.4. Role of Cancer Stem Cells in Tumor Angiogenesis. In recent years, a minor population of cells has been identified in GBM and other malignant tumors that has characteristics of tumor-initiating cells. These cells have been referred to as cancer stem cells (CSCs) and in the case of GBM are referred to as glioma stem cells [52–54]. There is still some debate regarding the appropriate definition of CSCs, but an emerging consensus holds that these cells are capable of self-renewal, sustained proliferation, and initiating tumor formation when injected in very low numbers into an immunocompromised mouse host [55]. Of particular relevance to this review, a small but growing number of papers have suggested that CSCs promote angiogenesis in tumors. For example, Bao and colleagues (2006) demonstrated that conditioned media from glioma stem cells significantly promoted EC migration, proliferation, and tube formation as compared to conditioned media from nonstem glioma cells [56]. Consistent with this observation, Calabrese and colleagues (2007) showed that ECs interact closely with brain tumor stem cells in the perivascular location (this has been termed a vascular niche) and secrete factors that maintain these cells in a stem cell-like state [57]. Investigators have speculated that this vascular niche is an important target for therapeutic intervention, as disruption of the niche microenvironment can ablate the growth of CSCs and arrest tumor growth [57, 58]. It should therefore be borne in mind that this is a potential mechanism by which anti-angiogenic drugs could inhibit brain tumor growth [57]. Most recently, Folkins and colleagues (2009) compared the angiogenesis in tumor xenografts from C6 glioma cells containing either a low or a high fraction of CSCs, and found that CSC-high xenograft tumors demonstrated an increased microvessel

density and blood perfusion, as well as inducing greater mobilization and recruitment of bone marrow-derived EPCs to the tumors [59]. Also, the CSC-high C6 cultures and xenograft tumors expressed higher levels of VEGF and SDF1, and blocking of these proangiogenic factors resulted in a reduction in the growth of the tumors compared to that observed with CSC low C6 cells [59]. These data suggest that CSCs do contribute to tumor angiogenesis by promoting both local EC activity and systemic angiogenic processes that involve the recruitment of bone marrow-derived EPC in a VEGF-and SDF1-dependent manner [59].

2.5. Role of Bone Marrow-Derived Progenitor Cells in Tumor Angiogenesis. There is evidence that the adult bone marrow plays a significant role in endothelial and lymphatic neovessel formation that supports tumor growth and invasion [60, 61]. As the brain lacks a lymphatic system, lymphatic neovessel formation is not relevant for GBM. The nonhematopoietic ($CD45^-$) bone marrow-derived population of cells that likely contribute to tumor angiogenesis, bone marrow-derived EPCs, may directly contribute to the EC layer by merging with the wall of a growing blood vessel and differentiating into ECs, thereby providing an alternative source of ECs [62]. The circulating EPCs originally reported by Asahara et al. (1997) (defined as $CD34^{+/}$ $VEGFR2^+$) were shown to be capable of differentiating into an EC phenotype that expressed EC markers in vitro, and to be capable of incorporating into neovessels at sites of ischemia [62]. Shaked and colleagues (2006) showed that administration of a vascular disrupting agent, such as combretastatin, or chemotherapeutic drugs, such as Paclitaxel or 5-fluorouracil, to a mouse model of cancer resulted in the recruitment of bone marrow-derived EPCs to the tumor as well as severe tumor necrosis [63]. Other studies have suggested that circulating EPCs may contribute to tumor neovascularization in mouse models of cancer [64].

It should be noted that the contribution of circulating EPCs to neoangiogenesis has been questioned and the markers that identify bone marrow-derived EPCs have been debated vigorously [65, 66]. The reported level of incorporation of circulating EPCs into new blood vessels varies considerably and ranges in different studies from 5% to 50% [53, 63, 65, 67]. In addition, technical challenges exist in identifying vessel-incorporated bone marrow-derived EPCs. Most recently, Rafat and colleagues (2009) suggested that in 12 patients with GBM higher numbers of circulating EPCs appeared to correlate with a significantly higher tumor vessel density, as compared to patients with lower numbers of circulating EPCs [68]; however, this observation needs to be validated in a larger study.

Bone marrow-derived hematopoietic cells have also been reported to contribute to tumor angiogenesis and invasion. Unlike the circulating EPCs discussed above, these cells are $CD45^+$, and they include myeloid progenitor cells identified as $GR1^+CD11b^+$ in the mouse (in the human $CD3^+$, $CD14^+$, $CD19^+$, $CD57^+$ and HLA-DR negative) [69], $CD11b^+F4/80^+$ tumor-associated macrophages (TAMs) [70], Tie2-expressing monocytes [71], $CXCR4^+VEGFR1^+$ hemangiocytes [72], bone marrow-derived circulating cells that comprise a heterogeneous population of myeloid cells identified as $CD45^+CD11b^+$ in the mouse and human [73], platelet-derived growth factor receptor $(PDGFR)^+$ pericyte progenitors [74], and vascular endothelial-(VE-) $cadherin^+$ $CD45^+$ leukocytes [75]. Supporting their potential importance in the response of the tumor to therapy, Shojaei and Ferrara reported that the recruitment of bone marrow-derived $GR1^+$ myeloid cells resulted in a tumor that was refractory to treatment with inhibitors of VEGF [76]. In general, the above $CD45^+$ bone marrow-derived cells are not thought to incorporate into the EC layer of the new vasculature; rather, some of the above cells are thought to incorporate into the tumor neovasculature as perivascular cells where they function to promote angiogenesis through paracrine mechanisms, such as local secretion of VEGF [73].

2.6. Co-Option of Existing Blood Vessels Occurs in Very Small Tumors. As discussed above, there is evidence to suggest that in small tumors, in which the neovasculature has not developed, the tumor cells obtain the necessary nutrients and oxygen needed for growth by co-opting existing blood vessels [10]. Of note, in transplanted C6 rat gliomas in the rat brain co-option of existing blood vessels by tumor cells occurred initially when the tumors were several mm in diameter, and this was followed by vascular regression and ultimately by the development of a neovasculature [10]. The same study also showed co-option of existing blood vessels with the propagation of rat mammary cancer cells in the rat brain and with the metastasis of Lewis lung cancer cells to the lung after intravenous injection. In each of these models, Ang-2 expression was upregulated at the co-opted blood vessels, suggesting a role for Ang-2 signaling in the co-option process [10]. Vessel co-option has been observed in several other tumors, including melanoma, ovarian carcinoma, and Kaposi sarcoma (reviewed in [11]).

Another mechanism contributing to the blood supply in malignant tumors is vasculogenic mimicry [11]. In vasculogenic mimicry, tumor cells function as blood vessel lining cells and this has been described in several types of malignant tumors, including melanoma [11].

3. Clinical Use of Antiangiogenic Agents

3.1. VEGF Inhibitors. *Bevacizumab* is a monoclonal antibody directed toward VEGF that has become the prototype of antiangiogenic agents in clinical use for treatment of GBM (see Table 1). In a phase II clinical trial, 68 patients with recurrent malignant glioma (33 AG and 35 GBM) were treated with bevacizumab and irinotecan in two cohorts (see Table 2). The combination therapy produced an impressive initial radiographic response rate of 57% for GBM and 61% for AG [5, 77]. This compared favorably with the benchmark response rate for temozolomide therapy at the first tumor recurrence, 5% for patients with recurrent GBM and 35% for patients with recurrent AG [78–80]. With the combination of bevacizumab and irinotecan the progression-free survival

Table 1: Examples of antiangiogenic agents in clinical trial for patients with high grade glioma.

Drug	Type	Targets
ABT-510	Thrombospondin-1 mimetic peptide	CD36 receptor
AMG 102	Monoclonal antibody	HGF/SF
Aflibercept	Soluble decoy receptor	VEGF-A,B, PlGF
Bevacizumab	Monoclonal antibody	VEGF-A
Brivanib	Tyrosine kinase inhibitor	FGFR, VEGFR2
Cediranib	Tyrosine kinase inhibitor	VEGFR1–3, PDGFRβ, c-Kit
Cilengitide	RGD synthetic peptide	Integrins $\alpha v\beta 3$, $\alpha v\beta 5$
CT-322	Fibronectin (adnectin)-based inhibitor	VEGFR1–3
Dasatinib	Tyrosine kinase inhibitor	PDGFRβ, Src, BCR-ABL, c-Kit, EphA2
Imatinib	Tyrosine kinase inhibitor	PDGFRβ, BCR-ABL, c-Kit
Lenalidomide	Immunomodulatory and anti-inflammatory	FGF pathway
Pazopanib (GW786034)	Tyrosine kinase inhibitor	VEGFR1–3, PDGFRβ, c-Kit
Sorafenib	Tyrosine kinase inhibitor	VEGFR2,3, BRAF, PDGFRβ, c-Kit, Ras, p38α
Sunitinib	Tyrosine kinase inhibitor	VEGFR2, PDGFRβ, Flt3, c-Kit
Tandutinib (MLN518)	Tyrosine kinase inhibitor	PDGFRβ, Flt3, c-Kit
Vandetanib (ZD6474)	Tyrosine kinase inhibitor	VEGFR2, EGFR, RET
Vatalanib (PTK787)	Tyrosine kinase inhibitor	VEGFR1–3, PDGFRβ, c-Kit
XL-184	Tyrosine kinase inhibitor	VEGFR2, Met, RET, c-Kit, Flt3, Tie-2

A more complete listing of anti-angiogenic agents in clinical trials for patients with high grade gliomas can be found at the National Institutes of Health website http://www.clinicaltrials.gov/ when searching for "glioma, brain cancer, glioblastoma, and angiogenesis".

at six months (PFS-6) was 43% for recurrent GBM patients and 59% for AG patients, and this was an improvement over the accepted PFS-6 standard of 15% for GBM patients and 31% for AG patients treated with temozolomide [79, 80]. Therapy with bevacizumab and irinotecan also resulted in neurological improvement and a reduction or discontinuation in the use of corticosteroid treatment in 31% of patients. The regimen was well tolerated; only one CNS hemorrhage was reported in 68 patients treated, eight patients were taken off of the study for thrombotic complications (four patients with pulmonary embolism (PE), two with deep vein thrombosis, one with thrombotic thrombocytopenic purpura, and one with thrombotic stroke), and two patients died (one with PE and one with a thrombotic stroke). Other side effects reported included, proteinuria, fatigue, and gastro-intestinal toxicity; these have been described with bevacizumab therapy for other types of cancer. Other prospective and retrospective studies have demonstrated initial radiographic response rates of between 35% and 50% with the combination of bevacizumab and cytotoxic chemotherapy [4, 81–83].

As irinotecan has limited activity as a single agent, a phase II randomized clinical trial was performed to evaluate the benefit of the addition of irinotecan to bevacizumab. In this clinical trial 167 patients with recurrent GBM received either bevacizumab alone or bevacizumab in combination with irinotecan; there was no statistically significant difference in the median overall survival (OS) for bevacizumab therapy alone (9.2 months) when compared to the combination bevacizumab and irinotecan therapy (8.7 months) [6]. A recent study evaluated the approach of bevacizumab monotherapy in patients with recurrent GBM followed by irinotecan combined with bevacizumab [84]. In this study, 17/48 patients (35%) achieved an initial radiographic response and the PFS-6 was reported to be 29%. Addition of irinotecan to patients who progress on bevacizumab monotherapy failed to produce an objective radiographic response in any of the 19 patients [84]. Bevacizumab is presently being evaluated in the upfront setting with temozolomide and radiation in two randomized phase III trials sponsored by the Radiation Therapy Oncology Group and Hoffmann-La Roche [85, 86].

Other VEGF/VEGFR-targeted inhibitors include, *Aflibercept*, a soluble VEGF decoy or hybrid receptor that consists of portions of VEGFR-1 and -2 fused to an immunoglobulin G1 Fc region [87–89] and *Cediranib* (AZD2171), a pan-VEGFR tyrosine kinase inhibitor with activity against PDGFR and c-KIT [90] (see Table 1).

4. Multikinase Receptors Inhibitors (MTKI)

A number of MTKIs have been studied in GBM patients (see Tables 1 and 2) including, *Imatinib* [95–99], *Sorafenib* [100–102], *Sunitinib* [103], *Vandetanib* (ZD6474) [104–107], *Vatalanib* (PTK787) [91, 108–114], and *XL184* [92, 115–117].

5. Other Anti-Angiogenic Agents

Other anti-angiogenic agents evaluated in GBM (see Tables 1 and 2) include, *Thalidomide* [93, 118–123], *Lenalidomide* [124, 125], *Tamoxifen* [126–129], *Enzastaurin* (LY317615) [130, 131], and the integrin inhibitor *Cilengitide* [94, 132, 133].

Table 2: Selected clinical trials in patients with recurrent high grade glioma

Agent	Phase	Diagnosis	Number of patients and Histology	Response Rate	PFS-6
Bev + Ir [5, 77]	II	Recurrent MG	68 (33 AG, 35 GBM)		43% GBM, 59% AG
Bev versus Bev + Ir [6]	II	Recurrent GBM	85 GBM (Bev) Vs 82 GBM (Bev +Ir)	RRR = 28% (Bev), RRR = 37% (Bev +Ir)	42% (Bev) versus 50% (Bev + Ir)
Aflibercept [89]	II	Recurrent MG	48 (16 AG, 32 GBM)	50% AG30% GBM	
Cediranib [90]	II	Recurrent GBM	16 GBM	56%	
Vatalanib [91]	I/II	Recurrent GBM	55 GBM	PR = 4%, SD = 56%	
XL184 [92]	II	Recurrent GBM	26 GBM	PR = 38%	
Thalidomide [93]	II	Recurrent MG	39 (14 AG, 25 GBM)	PR = 6%, MR = 6%, SD = 33%	
Cilengitide [94]	II	Recurrent GBM	81 GBM		16%

Abbreviations: Bev: bevacizumab, Ir: Irinotecan, PFS-6: progression free survival at 6 months, MG: malignant glioma, GBM: glioblastoma, AG: anaplastic glioma (includes anaplastic astrocytoma, anaplastic oligodendrogioma and anaplastic oligoastrocytoma), RR: response rate, RRR: radiological response rate, PR: partial response, SD: stable disease, MR: minor response, TMZ: temozolomide, and XRT: radiation.

6. Challenges in Evaluating the Response to Anti-Angiogenic Therapy with Imaging

The Macdonald Criteria have been used since 1990 to define response or progression in clinical trials of malignant glioma [134]. The Macdonald Criteria, based on WHO Criteria, utilizes measurement of the largest cross-sectional area of tumor on contrast-enhanced CT or MRI scan. Malignant gliomas can be irregular in shape, include large necrotic cavities, or be partially or completely nonenhancing, creating difficulty in accurately measuring the largest cross-sectional area on contrast-enhanced CT or MRI scan. In addition, the interpreter needs to take into account concurrent corticosteroid use and changes in neurological function. Thus, response evaluation based on finding a difference in the largest cross-sectional area of tumor on contrast-enhanced CT or MRI is even more difficult with anti-angiogenic therapy.

Clinical trials with drugs that modify signal transduction through the VEGF signaling pathway (e.g., bevacizumab, and cediranib) can produce a rapid decrease in enhancement after initiation of therapy [5, 135], resulting in an apparent high response rate. Some of the changes observed on contrast-enhanced CT or MRI scan result from a rapid normalization of abnormally permeable blood vessels and are not due to an antitumor effect. Anti-angiogenic therapy likely decreases vascular permeability and restores at least in part the integrity of the BBB, this leads to less contrast leakage from the vasculature. There is evidence that VEGF pathway-targeted anti-angiogenic drugs likely alter the image characteristics of enhancing tumor more effectively than of nonenhancing tumor. Hence the extent of reduction in contrast enhancement may not reflect true antitumor activity of the anti-angiogenic agent. Not infrequently, the radiographic image observed after anti-angiogenic therapy suggests a radiographic response that is more impressive than the clinical benefit derived from the therapy. The term "pseudoresponse" has been used to define the situation wherein the contrast-enhanced MRI suggests an antitumor effect that does not correlate with a true clinical benefit [136]. Therefore, treatment response based on radiographic images is probably not an optimal end point for clinical trials evaluating anti-angiogenic agents. The international Response Assessment in Neuro-Oncology (RANO) Working Group has developed new criteria for evaluating tumor response in malignant gliomas that take into account both enhancing and nonenhancing tumor (best visualized on T2 and fluid-attenuated inversion recovery (FLAIR) MRI sequences) images [136]. This working group proposes that increased T2-FLAIR signal likely reflects growing tumor, such as when it appears outside of the radiation field, it produces mass effect, or it involves the cortical ribbon, and when it occurs in the absence of other potential explanation.

7. Resistance to Anti-Angiogenic Therapy

Although the anti-angiogenic therapy of patients with malignant glioma has resulted in a small increase in the PFS-6, in general these agents have failed to produce a sustained clinical response. For example, patients with malignant glioma treated with a VEGF inhibitor have shown temporary improvements, seen as reduced edema on imaging or tumor stabilization on imaging; however, the tumors ultimately progress after a brief response. In patients with tumor progression during treatment with bevacizumab, the downhill clinical course is often rapid, and may be fueled by discontinuing the agent. In a retrospective analysis, patients with malignant glioma who were treated with a second line regimen containing bevacizumab after failure of treatment with an initial therapy combination of bevacizumab and a cytotoxic agent had a median PFS of only 37.5 days [137]. Shaked and colleagues showed that treatment withdrawal of a vascular disrupting agent, such as combrestatin, to a mouse model of cancer resulted in the development of an aggressive and angiogenic tumor [63]. Supporting the concept that malignant tumors ultimately develop resistance to therapy with a VEGF inhibitor, two recent studies (one of which

included a xenograft mouse model of GBM) suggested that treatment with a VEGF inhibitor alters the natural history of the tumor and promotes a highly invasive and metastatic phenotype [138, 139].

The anti-angiogenic therapy failures described above are thought to be due to the development of resistance to anti-angiogenic therapy. Resistance to anti-angiogenic therapy has been broadly classified as either adaptive or intrinsic [140]. Animal models of malignant glioma and of other malignant tumors have shown that tumors adapt to treatment with angiogenesis inhibitors by upregulating, or acquiring, an alternate mechanism(s) to sustain tumor growth, which has been termed "adaptive evasive resistance". This can occur even when the specific target of the anti-angiogenic agent remains successfully inhibited. Adaptive resistance along with intrinsic resistance is thought to be the reason for the progression of malignant tumors treated with anti-angiogenic therapy. In tumors that show an initial response to anti-angiogenic therapy, adaptive evasive resistance is thought to be the main mechanism for the development of resistance, that is, blockade of one proangiogenic growth factor can lead to upregulation of an alternate proangiogenic growth factor [141]. For example, in a study of GBM patients treated with the pan-VEGFR inhibitor cediranib (AZD 2171), blood levels of the proangiogenic factors bFGF and SDF1α were noted to be higher in patients at the time of tumor progression or relapse as compared to the levels observed during the phase in which the patients showed a response to cediranib therapy [142].

Recruitment of vascular progenitor cells from the bone marrow may aid in the process of adaptive evasive resistance. Certain anti-angiogenic therapies can cause regression of tumor vessels resulting in hypoxia and lead to the recruitment of various bone marrow-derived progenitor cells. Bone marrow-derived progenitor cells (including both vascular progenitor cells and vascular modulatory cells) can be recruited through hypoxia-induced HIF1α activation resulting in the expression of the downstream effector SDF1α [45, 143–146]. GBM tumors with low HIF1α levels contain fewer bone marrow-derived cells and exhibit lower levels of angiogenesis and tumor growth, as compared to GBM tumors with high HIF1α levels [146]. This suggests that the recruitment of bone marrow-derived progenitor cells may constitute a mechanism for the development of adaptive evasive resistance to anti-angiogenic therapy.

One reason for the initial response to bevacizumab therapy reported for some patients with GBM may be that the GBM neovasculature typically contains a reduced density of pericyte coverage [36]. Pericytes are important constituents of blood vessels in general and of tumor blood vessels, they provide prosurvival signals to ECs. ECs are thought to induce pericyte recruitment, thereby promoting their own survival. Two of the best characterized prosurvival signals for ECs are VEGF and the signals derived from pericyte association with ECs [36]. Tumor vessels lacking adequate pericyte coverage are more vulnerable to VEGF inhibition as they lack the pro-survival signal from pericytes [147]. Blood vessels covered with the normal density of pericytes probably survive therapy with a VEGF inhibitor as the pericytes can signal for EC survival or quiescence [148, 149].

Finally, the possibility of the development of an alternative mechanism, such as co-option, for tumor cells to acquire oxygen and nutrients must be considered in terms of resistance. Notably, in an orthotopic mouse model of GBM treated with a VEGFR selective kinase inhibitor or with a multitarget VEGFR kinase inhibitor, tumor progression (growth) ultimately occurred that was highly invasive. Perivascular tumor invasion similar to tumor co-option of blood vessels was observed at autopsy. Moreover, two other recent studies have suggested that tumor co-option of pre-existing blood vessels can support a more invasive tumor cell phenotype and tumor growth after VEGF- or VEGFR2-targeted therapy [150, 151]. As Ang-2 signaling has been implicated in mediating blood vessel co-option in the untreated early C6 rat glioma model of malignant glioma [10], human GBM tumors were immunostained for Ang-1 and Ang-2 and it was reported that Ang-2 expression was upregulated at tumor co-opted blood vessels. Whether the co-opted blood vessels that were observed post anti-angiogenic therapy also showed upregulation of Ang-2 remains to be determined.

The second mechanism of resistance to anti-angiogenic therapy that has been suggested is an intrinsic or inherent resistance [140]. This concept stems from the observation that a minority of patients do not appear to respond to anti-angiogenic therapy. It has been suggested that this may be due to the pre-existing activation of multiple proangiogenic signaling pathways or a pre-existing inflammatory cell infiltrate that provides a source of tumor VEGF resulting in vascular protection [140].

8. The Blood-Brain Barrier (BBB) and the Challenges of Drug Delivery

Drug concentrations within the central nervous system are dependent on multiple factors that include, the permeability of the agent across the BBB, the extent to which it is actively transported out of the brain, and the volume of distribution in the brain parenchyma [152]. The BBB is an anatomic-physiologic barrier that is formed by multiple components, including tight junctions between endothelial cells, pericytes, and the astrocytic foot processes. This barrier selectively allows entry of some substances, such as glucose, lipid-soluble molecules, and oxygen, while preventing entry of other substances. It is important to understand that although the BBB may be disrupted in some areas of a GBM, a substantial proportion of glioma cells can be located in areas with an intact BBB. The magnitude of tumor vascular permeability varies within these tumors, with the greatest permeability being found in the tumor core and a relatively intact BBB at the proliferating tumor edge. The presence of an intact BBB in some areas of the tumor and the presence of a partially functional BBB in other areas of the tumor can prevent the effective delivery of active therapeutic compounds.

The BBB expresses high levels of drug efflux pumps such as P-glycoprotein (P-gp), breast cancer-resistance protein (BCRP), and other multiple drug resistance proteins (MRPs) that actively remove chemotherapeutic drugs from the brain [153]. Efflux transport systems such as by P-gp and the MRPs at the brain capillary ECs may play a role in limiting the passage of therapeutic agents across the BBB [154]. For example, the brain distribution of the tyrosine kinase inhibitor imatinib is reduced by active efflux via the P-glycoprotein [155]. The BCRP drug efflux transport pump is expressed in a number of normal tissues, in addition to the BBB [156]. Modulation of drug transporters represents a new potential strategy to improve efficacy of targeted agents. Anti-angiogenic agents such bevacizumab that mainly target the EC on the luminal side of the vessel may not depend on the ability to cross the BBB; however, this remains a concern for tyrosine kinase inhibitors like imatinib.

9. Biomarkers of Angiogenesis

Currently, there are no validated biomarkers to monitor the progress or response to anti-angiogenic therapy in patients with malignant glioma or other cancers [157]. Two promising biomarkers for assessment of angiogenesis in malignant tumors are the number of circulating endothelial cells (CECs) and the number of circulating endothelial progenitor cells (EPCs). A growing body of literature suggests that the number of CECs and circulating EPCs is significantly elevated in patients with different types of cancer [65]. Furthermore, in a small number of preclinical animal studies successful antitumor response to anti-angiogenic therapy was correlated with changes in the number of CECs and circulating EPCs [158]. Rafat and colleagues have evaluated the number of circulating EPCs in the blood of patients with malignant glioma, and reported that they were higher in patients with GBM as compared to healthy volunteers [68]. Higher tumor blood vessel densities were noted in the patients with GBM having higher numbers of circulating EPCs as compared to those patients with lower numbers of circulating EPCs [68]. This supports the further evaluation of circulating EPCs as a novel biomarker for the assessment of angiogenesis and for the assessment of the response to anti-angiogenic therapy in patients with GBM.

Measurement of VEGF plasma levels may not be a generally useful biomarker of tumor angiogenesis. For example, baseline plasma VEGF levels are not correlated with survival outcome for patients with metastatic colorectal cancer or for patients with metastatic nonsmall cell lung cancer [159, 160]. As expected, the plasma level of VEGF in patients with GBM and brain metastases is elevated as compared to normal healthy volunteers. In patients with metastatic breast cancer higher plasma levels of VEGF were associated with shorter time of progression [161]. Thus, additional studies need to be performed to determine whether the measurement of plasma VEGF levels is useful in monitoring the response to anti-angiogenic therapy in general and to VEGF inhibitor therapy. As previously noted, blood levels of bFGF and SDF1α were noted to be higher in patients at the time of relapse as compared to levels observed in the response phase in patients with recurrent GBM treated with cediranib [142].

10. Unanswered Questions

Despite the remarkable progress in our understanding of the process of angiogenesis and the potential promise of targeting these processes for the treatment of GBM, several critical questions need to be addressed. These include questions regarding the fundamental processes involved in GBM vascularization and their role in the failure of therapy. Pressing questions in this category are: (1) are the GBM tumors in patients that have failed bevacizumab or other anti-angiogenic therapy avascular, or are the tumors co-opting the existing blood vessels to obtain the oxygen and nutrients needed?, (2) is the angiopoietin signaling pathway driving a blood vessel co-option process in human GBM that have failed therapy with a VEGF inhibitor or other anti-angiogenic agent?, and (3) do cancer stem cells (or glioma stem cells) promote angiogenesis in malignant gliomas and could we target them specifically with novel therapy?

There also is an urgent need to improve the ability to assess the effects of the anti-angiogenic therapies on angiogenesis (rather than tumor growth). Questions in this category include: (1) does the number of CECs and circulating EPCs in patients with tumors correlate with the tumor grade, and could their number be used to monitor anti-angiogenic therapy? (Phenotyping circulating EPCs with a comprehensive set of endothelial, progenitor, and hematopoietic markers should be performed to address the issue of what are the appropriate markers to be used to identify circulating EPCs in patients [65]), (2) what is the contribution of circulating EPCs to angiogenesis in untreated virgin tumors?, and (3) could a biomarker be identified that would aid in identifying adaptive evasive resistance (escape pathways) that should be targeted when a patient's tumor develops resistance to anti-angiogenic therapy?

11. Conclusions

Anti-angiogenic therapy appears to be a promising and novel approach for the treatment of malignant brain tumors. Clinical trials have shown improvement in the short-term progression-free survival. The response of patients with GBM to therapy with a VEGF inhibitor likely depends, at least in part, on whether the tumor neovasculature contains a normal density of pericytes, how capable the tumor is in co-opting pre-existing blood vessels, and whether previously co-opted blood vessels exist in the tumor. A better understanding of the mechanisms of resistance to anti-angiogenic therapy is needed such that we can improve our treatment strategies for these patients. The development and optimization of biomarkers to measure angiogenesis in tumors, such as quantitation of the numbers of CECs and circulating EPCs, will potentially help us identify patients that are responding to, or failing, anti-angiogenic therapy. Theoretically, these markers could also be used to identify the subset of patients that would benefit from anti-angiogenic

therapy and thereby individualizing therapy for patients with malignant tumors, such as GBM. In addition, new anti-angiogenic therapies that induce apoptosis of the ECs in the neovasculature are needed, as this type of anti-angiogenic therapy may be less likely to induce therapeutic resistance.

Acknowledgments

We thank Drs. Gene Barnett, Derek Raghavan, and Fiona Hunter for critical review of this manuscript. This work was supported by grants CA 109748 and CA 127620 from the National Institutes of Health-National Cancer Institute to CLG.

References

[1] P. Y. Wen and S. Kesari, "Malignant gliomas in adults," *New England Journal of Medicine*, vol. 359, no. 5, pp. 492–507, 2008.

[2] CBTRUS, "Primary brain tumors in the United States, 2000–2004," Statistical Report, Central Brain Tumor Registry of the United States, 2008.

[3] R. Stupp, W. P. Mason, M. J. van den Bent, et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.

[4] T. Y. Kang, T. Jin, H. Elinzano, and D. Peereboom, "Irinotecan and bevacizumab in progressive primary brain tumors, an evaluation of efficacy and safety," *Journal of Neuro-Oncology*, vol. 89, no. 1, pp. 113–118, 2008.

[5] J. J. Vredenburgh, A. Desjardins, J. E. Herndon II, et al., "Bevacizumab plus irinotecan in recurrent glioblastoma multiforme," *Journal of Clinical Oncology*, vol. 25, no. 30, pp. 4722–4729, 2007.

[6] H. S. Friedman, M. D. Prados, P. Y. Wen, et al., "Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma," *Journal of Clinical Oncology*, vol. 27, no. 28, pp. 4733–4740, 2009.

[7] P. Kleihues, D. N. Louis, B. W. Scheithauer, et al., "The WHO classification of tumors of the nervous system," *Journal of Neuropathology and Experimental Neurology*, vol. 61, no. 3, pp. 215–225, 2002.

[8] J. Folkman, "Antiangiogenesis in cancer therapy—endostatin and its mechanisms of action," *Experimental Cell Research*, vol. 312, no. 5, pp. 594–607, 2006.

[9] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[10] J. Holash, P. C. Maisonpierre, D. Compton, et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF," *Science*, vol. 284, no. 5422, pp. 1994–1998, 1999.

[11] F. Hillen and A. W. Griffioen, "Tumour vascularization: sprouting angiogenesis and beyond," *Cancer and Metastasis Reviews*, vol. 26, no. 3-4, pp. 489–502, 2007.

[12] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid," *Science*, vol. 219, no. 4587, pp. 983–985, 1983.

[13] P. J. Keck, S. D. Hauser, G. Krivi, et al., "Vascular permeability factor, an endothelial cell mitogen related to PDGF," *Science*, vol. 246, no. 4935, pp. 1309–1312, 1989.

[14] D. W. Leung, G. Cachianes, W.-J. Kuang, D. V. Goeddel, and N. Ferrara, "Vascular endothelial growth factor is a secreted angiogenic mitogen," *Science*, vol. 246, no. 4935, pp. 1306–1309, 1989.

[15] N. Ferrara, H.-P. Gerber, and J. LeCouter, "The biology of VEGF and its receptors," *Nature Medicine*, vol. 9, no. 6, pp. 669–676, 2003.

[16] J. M. Whitelock, A. D. Murdoch, R. V. Iozzo, and P. A. Underwood, "The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases," *Journal of Biological Chemistry*, vol. 271, no. 17, pp. 10079–10086, 1996.

[17] D. Qiao, K. Meyer, C. Mundhenke, S. A. Drew, and A. Friedl, "Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain endothelial cells: specific role for glypican-1 in glioma angiogenesis," *Journal of Biological Chemistry*, vol. 278, no. 18, pp. 16045–16053, 2003.

[18] A. N. Witmer, J. Dai, H. A. Weich, G. F. J. M. Vrensen, and R. O. Schlingemann, "Expression of vascular endothelial growth factor receptors 1, 2, and 3 in quiescent endothelia," *Journal of Histochemistry and Cytochemistry*, vol. 50, no. 6, pp. 767–777, 2002.

[19] R. S. Morrison, F. Yamaguchi, H. Saya, et al., "Basic fibroblast growth factor and fibroblast growth factor receptor I are implicated in the growth of human astrocytomas," *Journal of Neuro-Oncology*, vol. 18, no. 3, pp. 207–216, 1994.

[20] R. S. Morrison, F. Yamaguchi, J. M. Bruner, M. Tang, W. McKeehan, and M. S. Berger, "Fibroblast growth factor receptor gene expression and immunoreactivity are elevated in human glioblastoma multiforme," *Cancer Research*, vol. 54, no. 10, pp. 2794–2799, 1994.

[21] S. M. Yamada, S. Yamada, Y. Hayashi, H. Takahashi, A. Teramoto, and K. Matsumoto, "Fibroblast growth factor receptor (FGFR) 4 correlated with the malignancy of human astrocytomas," *Neurological Research*, vol. 24, no. 3, pp. 244–248, 2002.

[22] T. Ueba, J. A. Takahashi, M. Fukumoto, et al., "Expression of fibroblast growth factor receptor-1 in human glioma and meningioma tissues," *Neurosurgery*, vol. 34, no. 2, pp. 221–226, 1994.

[23] M. Simons, "Integrative signaling in angiogenesis," *Molecular and Cellular Biochemistry*, vol. 264, no. 1-2, pp. 99–102, 2004.

[24] G. Pintucci, D. Moscatelli, F. Saponara, et al., "Lack of ERK activation and cell migration in FGF-2-deficient endothelial cells," *The FASEB Journal*, vol. 16, no. 6, pp. 598–600, 2002.

[25] G. Gliki, C. Wheeler-Jones, and I. Zachary, "Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3′-kinase-mediated PKCδ phosphorylation: role of PKC in angiogenesis," *Cell Biology International*, vol. 26, no. 9, pp. 751–759, 2002.

[26] S. Karcher, H.-H. Steiner, R. Ahmadi, et al., "Different angiogenic phenotypes in primary and secondary glioblastomas," *International Journal of Cancer*, vol. 118, no. 9, pp. 2182–2189, 2006.

[27] S. S. Lakka, C. S. Gondi, D. H. Dinh, et al., "Specific interference of urokinase-type plasminogen activator receptor and matrix metalloproteinase-9 gene expression induced by double-stranded RNA results in decreased invasion, tumor growth, and angiogenesis in gliomas," *Journal of Biological Chemistry*, vol. 280, no. 23, pp. 21882–21892, 2005.

[28] S. S. Lakka, C. S. Gondi, and J. S. Rao, "Proteases and glioma angiogenesis," *Brain Pathology*, vol. 15, no. 4, pp. 327–341, 2005.

[29] S. M. Pulukuri, C. S. Gondi, S. S. Lakka, et al., "RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival, and tumorigenicity in vivo," *Journal of Biological Chemistry*, vol. 280, no. 43, pp. 36529–36540, 2005.

[30] C. S. Gondi, S. S. Lakka, D. H. Dinh, W. C. Olivero, M. Gujrati, and J. S. Rao, "Intraperitoneal injection of a hairpin RNA—expressing plasmid targeting urokinase-type Plasminogen Activator (uPA) receptor and uPA retards angiogenesis and inhibits intracranial tumor growth in nude mice," *Clinical Cancer Research*, vol. 13, no. 14, pp. 4051–4060, 2007.

[31] L. Sun, A.-M. Hui, Q. Su, et al., "Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain," *Cancer Cell*, vol. 9, no. 4, pp. 287–300, 2006.

[32] B. Wolff, A. R. Burns, J. Middleton, and A. Rot, "Endothelial cell 'memory' of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies," *Journal of Experimental Medicine*, vol. 188, no. 9, pp. 1757–1762, 1998.

[33] J. O. Utgaard, F. L. Jahnsen, A. Bakka, P. Brandtzaeg, and G. Haraldsen, "Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells," *Journal of Experimental Medicine*, vol. 188, no. 9, pp. 1751–1756, 1998.

[34] D. J. Brat, A. C. Bellail, and E. G. Van Meir, "The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis," *Neuro-Oncology*, vol. 7, no. 2, pp. 122–133, 2005.

[35] L. Eklund and B. R. Olsen, "Tie receptors and their angiopoietin ligands are context-dependent regulators of vascular remodeling," *Experimental Cell Research*, vol. 312, no. 5, pp. 630–641, 2006.

[36] G. Bergers and S. Song, "The role of pericytes in blood-vessel formation and maintenance," *Neuro-Oncology*, vol. 7, no. 4, pp. 452–464, 2005.

[37] D. Zagzag, R. Amirnovin, M. A. Greco, et al., "Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis," *Laboratory Investigation*, vol. 80, no. 6, pp. 837–849, 2000.

[38] C. M. Overall and O. Kleifeld, "Validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy," *Nature Reviews Cancer*, vol. 6, no. 3, pp. 227–239, 2006.

[39] J. D. Hood, R. Frausto, W. B. Kiosses, M. A. Schwartz, and D. A. Cheresh, "Differential αv integrin-mediated Ras-ERK signaling during two pathways of angiogenesis," *Journal of Cell Biology*, vol. 162, no. 5, pp. 933–943, 2003.

[40] B. Kaur, F. W. Khwaja, E. A. Severson, S. L. Matheny, D. J. Brat, and E. G. Van Meir, "Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis," *Neuro-Oncology*, vol. 7, no. 2, pp. 134–153, 2005.

[41] P. Baluk, S. Morikawa, A. Haskell, M. Mancuso, and D. M. McDonald, "Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors," *American Journal of Pathology*, vol. 163, no. 5, pp. 1801–1815, 2003.

[42] A. N. Stratman, K. M. Malotte, R. D. Mahan, M. J. Davis, and G. E. Davis, "Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation," *Blood*, vol. 114, no. 24, pp. 5091–5101, 2009.

[43] A. N. Stratman, W. B. Saunders, A. Sacharidou, et al., "Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices," *Blood*, vol. 114, no. 2, pp. 237–247, 2009.

[44] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[45] D. J. Ceradini, A. R. Kulkarni, M. J. Callaghan, et al., "Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1," *Nature Medicine*, vol. 10, no. 8, pp. 858–864, 2004.

[46] S. A. Rempel, S. Dudas, S. Ge, and J. A. Gutierrez, "Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma," *Clinical Cancer Research*, vol. 6, no. 1, pp. 102–111, 2000.

[47] J. B. Rubin, A. L. Kung, R. S. Klein, et al., "A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 23, pp. 13513–13518, 2003.

[48] G. Arismendi-Morillo and A. Castellano, "Tumoral microblood vessels and vascular microenvironment in human astrocytic tumors. A transmission electron microscopy study," *Journal of Neuro-Oncology*, vol. 73, no. 3, pp. 211–217, 2005.

[49] Y. Boucher, M. Leunig, and R. K. Jain, "Tumor angiogenesis and interstitial hypertension," *Cancer Research*, vol. 56, no. 18, pp. 4264–4266, 1996.

[50] B. Kaur, F. W. Khwaja, E. A. Severson, S. L. Matheny, D. J. Brat, and E. G. Van Meir, "Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis," *Neuro-Oncology*, vol. 7, no. 2, pp. 134–153, 2005.

[51] Z. Li, S. Bao, Q. Wu, et al., "Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells," *Cancer Cell*, vol. 15, no. 6, pp. 501–513, 2009.

[52] T. Lapidot, C. Sirard, J. Vormoor, et al., "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice," *Nature*, vol. 367, no. 6464, pp. 645–648, 1994.

[53] B. A. Peters, L. A. Diaz Jr., K. Polyak, et al., "Contribution of bone marrow-derived endothelial cells to human tumor vasculature," *Nature Medicine*, vol. 11, no. 3, pp. 261–262, 2005.

[54] M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, and M. F. Clarke, "Prospective identification of tumorigenic breast cancer cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 3983–3988, 2003.

[55] M. F. Clarke, J. E. Dick, P. B. Dirks, et al., "Cancer stem cells—perspectives on current status and future directions: AACR workshop on cancer stem cells," *Cancer Research*, vol. 66, no. 19, pp. 9339–9344, 2006.

[56] S. Bao, Q. Wu, S. Sathornsumetee, et al., "Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor," *Cancer Research*, vol. 66, no. 16, pp. 7843–7848, 2006.

[57] C. Calabrese, H. Poppleton, M. Kocak, et al., "A perivascular niche for brain tumor stem cells," *Cancer Cell*, vol. 11, no. 1, pp. 69–82, 2007.

[58] R. J. Gilbertson and J. N. Rich, "Making a tumour's bed: glioblastoma stem cells and the vascular niche," *Nature Reviews Cancer*, vol. 7, no. 10, pp. 733–736, 2007.

[59] C. Folkins, Y. Shaked, S. Man, et al., "Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1," *Cancer Research*, vol. 69, no. 18, pp. 7243–7251, 2009.

[60] H.-G. Kopp, C. A. Ramos, and S. Rafii, "Contribution of endothelial progenitors and proangiogenic hematopoietic cells to vascularization of tumor and ischemic tissue," *Current Opinion in Hematology*, vol. 13, no. 3, pp. 175–181, 2006.

[61] P. Carmeliet, "Angiogenesis in life, disease and medicine," *Nature*, vol. 438, no. 7070, pp. 932–936, 2005.

[62] T. Asahara, T. Murohara, A. Sullivan, et al., "Isolation of putative progenitor endothelial cells for angiogenesis," *Science*, vol. 275, no. 5302, pp. 964–967, 1997.

[63] Y. Shaked, A. Ciarrocchi, M. Franco, et al., "Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors," *Science*, vol. 313, no. 5794, pp. 1785–1787, 2006.

[64] D. G. Duda, K. S. Cohen, S. V. Kozin, et al., "Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors," *Blood*, vol. 107, no. 7, pp. 2774–2776, 2006.

[65] F. Bertolini, Y. Shaked, P. Mancuso, and R. S. Kerbel, "The multifaceted circulating endothelial cell in cancer: towards marker and target identification," *Nature Reviews Cancer*, vol. 6, no. 11, pp. 835–845, 2006.

[66] R. S. Kerbel, R. Benezra, D. C. Lyden, et al., "Endothelial progenitor cells are cellular hubs essential for neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition but not adenomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 34, p. E54, 2008.

[67] H. Spring, T. Schuler, B. Arnold, G. J. Hämmerling, and R. Ganss, "Chemokines direct endothelial progenitors into tumor neovessels," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 50, pp. 18111–18116, 2005.

[68] N. Rafat, G. C. Beck, J. Schulte, J. Tuettenbeg, and P. Vajkoczy, "Circulating endothelial progenitor cells in malignant gliomas," *Journal of Neurosurgery*, vol. 112, no. 1, pp. 43–49, 2010.

[69] L. Yang, J. Huang, X. Ren, et al., "Abrogation of TGFβ signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis," *Cancer Cell*, vol. 13, no. 1, pp. 23–35, 2008.

[70] J. W. Pollard, "Tumour-educated macrophages promote tumour progression and metastasis," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 71–78, 2004.

[71] M. De Palma, M. A. Venneri, R. Galli, et al., "Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors," *Cancer Cell*, vol. 8, no. 3, pp. 211–226, 2005.

[72] D. K. Jin, K. Shido, H.-G. Kopp, et al., "Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes," *Nature Medicine*, vol. 12, no. 5, pp. 557–567, 2006.

[73] M. Grunewald, I. Avraham, Y. Dor, et al., "VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells," *Cell*, vol. 124, no. 1, pp. 175–189, 2006.

[74] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ^+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[75] J. R. Conejo-Garcia, R. J. Buckanovich, F. Benencia, et al., "Vascular leukocytes contribute to tumor vascularization," *Blood*, vol. 105, no. 2, pp. 679–681, 2005.

[76] F. Shojaei and N. Ferrara, "Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells," *Cancer Research*, vol. 68, no. 14, pp. 5501–5504, 2008.

[77] J. J. Vredenburgh, A. Desjardins, J. E. Herndon II, et al., "Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma," *Clinical Cancer Research*, vol. 13, no. 4, pp. 1253–1259, 2007.

[78] E. T. Wong, K. R. Hess, M. J. Gleason, et al., "Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials," *Journal of Clinical Oncology*, vol. 17, no. 8, pp. 2572–2578, 1999.

[79] W. K. A. Yung, M. D. Prados, R. Yaya-Tur, et al., "Multicenter phase II trial of temozolomide in patients with anaplastic astrocytoma or anaplastic oligoastrocytoma at first relapse," *Journal of Clinical Oncology*, vol. 17, no. 9, pp. 2762–2771, 1999.

[80] W. K.A. Yung, R. E. Albright, J. Olson, et al., "A phase II study of temozolemide vs. procarbazine in patients with glioblastoma multiforme at first relapse," *British Journal of Cancer*, vol. 83, no. 5, pp. 588–593, 2000.

[81] W. Chen, S. Delaloye, D. H. S. Silverman, et al., "Predicting treatment response of malignant gliomas to bevacizumab and irinotecan by imaging proliferation with [^{18}F] fluorothymidine positron emission tomography: a pilot study," *Journal of Clinical Oncology*, vol. 25, no. 30, pp. 4714–4721, 2007.

[82] A. D. Norden, G. S. Young, K. Setayesh, et al., "Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence," *Neurology*, vol. 70, no. 10, pp. 779–787, 2008.

[83] S. Guiu, S. Taillibert, O. Chinot, et al., "Bevacizumab/Irinotecan. An active treatment for recurrent high grade gliomas: preliminary results of an ANOCEF Multicenter Study," *Revue Neurologique*, vol. 164, no. 6-7, pp. 588–594, 2008.

[84] T. N. Kreisl, L. Kim, K. Moore, et al., "Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma," *Journal of Clinical Oncology*, vol. 27, no. 5, pp. 740–745, 2009.

[85] http://www.rtog.org/members/protocols/0825/0825.pdf.

[86] http://clinicaltrials.gov/ct2/show/NCT00943826?term=bevacizumab+temozolomide+glioma&rank=17.

[87] J. Holash, S. Davis, N. Papadopoulos, et al., "VEGF-Trap: a VEGF blocker with potent antitumor effects," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11393–11398, 2002.

[88] J. Holash, S. Davis, N. Papadopoulos, et al., "VEGF-Trap: a VEGF blocker with potent antitumor effects," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11393–11398, 2002.

[89] J. F. De Groot, P. Y. Wen, K. Lamborn, et al., "Phase II single arm trial of aflibercept in patients with recurrent temozolomide-resistant glioblastoma: NABTC 0601," *Journal of Clinical Oncology*, vol. 26, p. 2020, 2008.

[90] T. T. Batchelor, A. G. Sorensen, E. di Tomaso, et al., "AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients," *Cancer Cell*, vol. 11, no. 1, pp. 83–95, 2007.

[91] C. Conrad, H. Friedman, D. Reardon, et al., "A phase I/II trial of single-agent PTK 787/ZK 222584 (PTK/ZK), a novel, oral angiogenesis inhibitor, in patients with recurrent glioblastoma multiforme (GBM)," *Journal of Clinical Oncology*, vol. 22, p. 1512, 2004.

[92] J. F. De Groot, M. Prados, and T. Urquhart, "A phase II study of XL184 in patients (pts) with progressive glioblastoma multiforme (GBM) in first or second relapse," *Journal of Clinical Oncology*, vol. 27, no. 15S, p. 2047, 2009.

[93] H. A. Fine, W. D. Figg, K. Jaeckle, et al., "Phase II trial of the antiangiogenic agent thalidomide in patients with recurrent high-grade gliomas," *Journal of Clinical Oncology*, vol. 18, no. 4, pp. 708–715, 2000.

[94] D. A. Reardon, K. L. Fink, T. Mikkelsen, et al., "Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme," *Journal of Clinical Oncology*, vol. 26, no. 34, pp. 5610–5617, 2008.

[95] T. Kilic, J. A. Alberta, P. R. Zdunek, et al., "Intracranial inhibition of platelet-derived growth factor-mediated glioblastoma cell growth by an orally active kinase inhibitor of the 2-phenylaminopyrimidine class," *Cancer Research*, vol. 60, no. 18, pp. 5143–5150, 2000.

[96] P. Y. Wen, W. K. A. Yung, K. R. Lamborn, et al., "Phase I/II study of imatinib mesylate for recurrent malignant gliomas: north American Brain Tumor Consortium Study 99–08," *Clinical Cancer Research*, vol. 12, no. 16, pp. 4899–4907, 2006.

[97] A. Desjardins, J. A. Quinn, J. J. Vredenburgh, et al., "Phase II study of imatinib mesylate and hydroxyurea for recurrent grade III malignant gliomas," *Journal of Neuro-Oncology*, vol. 83, no. 1, pp. 53–60, 2007.

[98] D. A. Reardon, M. J. Egorin, J. A. Quinn, et al., "Phase II study of imatinib mesylate plus hydroxyurea in adults with recurrent glioblastoma multiforme," *Journal of Clinical Oncology*, vol. 23, no. 36, pp. 9359–9368, 2005.

[99] D. A. Reardon, A. Desjardins, J. J. Vredenburgh, et al., "Safety and pharmacokinetics of dose-intensive imatinib mesylate plus temozolomide: phase 1 trial in adults with malignant glioma," *Neuro-Oncology*, vol. 10, no. 3, pp. 330–340, 2008.

[100] B. J. Scott, E. C. Quant, M. B. McNamara, P. A. Ryg, T. T. Batchelor, P. Y. Wen, "Bevacizumab salvage therapy following progression in high-grade glioma patients treated with VEGF receptor tyrosine kinase inhibitors," *Neuro-Oncology* [Epub ahead of print], 2010.

[101] E. P. Jane, D. R. Premkumar, and I. F. Pollack, "Coadministration of sorafenib with rottlerin potently inhibits cell proliferation and migration in human malignant glioma cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 3, pp. 1070–1080, 2006.

[102] C. Yu, B. B. Friday, J.-P. Lai, et al., "Cytotoxic synergy between the multikinase inhibitor sorafenib and the proteasome inhibitor bortezomib in vitro: induction of apoptosis through Akt and c-Jun NH_2-terminal kinase pathways," *Molecular Cancer Therapeutics*, vol. 5, no. 9, pp. 2378–2387, 2006.

[103] Q. Zhou and J. M. Gallo, "Differential effect of sunitinib on the distribution of temozolomide in an orthotopic glioma model," *Neuro-Oncology*, vol. 11, no. 3, pp. 301–310, 2009.

[104] S. Sathornsumetee and J. N. Rich, "Vandetanib, a novel multitargeted kinase inhibitor, in cancer therapy," *Drugs of Today*, vol. 42, no. 10, pp. 657–670, 2006.

[105] J. N. Rich, D. Loncar, S. Sathornsumetee, et al., "ZD6474, a vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor, inhibits growth of multiple primary central nervous system tumor types," p. 413.

[106] J. N. Rich, S. Sathornsumetee, S. T. Keir, et al., "ZD6474, a novel tyrosine kinase inhibitor of vascular endothelial growth factor receptor and epidermal growth factor receptor, inhibits tumor growth of multiple nervous system tumors," *Clinical Cancer Research*, vol. 11, no. 22, pp. 8145–8157, 2005.

[107] V. Damiano, D. Melisi, C. Bianco, et al., "Cooperative antitumor effect of multitargeted kinase inhibitor ZD6474 and ionizing radiation in glioblastoma," *Clinical Cancer Research*, vol. 11, no. 15, pp. 5639–5644, 2005.

[108] L. M. Jost, H.-P. Gschwind, T. Jalava, et al., "Metabolism and disposition of vatalanib (PTK787/ZK-222584) in cancer patients," *Drug Metabolism and Disposition*, vol. 34, no. 11, pp. 1817–1828, 2006.

[109] M. P. Saunders, R. Wilson, M. Peeters, et al., "Vandetanib with FOLFIRI in patients with advanced colorectal adenocarcinoma: results from an open-label, multicentre Phase i study," *Cancer Chemotherapy and Pharmacology*, vol. 64, no. 4, pp. 665–672, 2009.

[110] E. N. Scott, G. Meinhardt, C. Jacques, D. Laurent, and A. L. Thomas, "Vatalanib: the clinical development of a tyrosine kinase inhibitor of angiogenesis in solid tumours," *Expert Opinion on Investigational Drugs*, vol. 16, no. 3, pp. 367–379, 2007.

[111] A. L. Thomas, B. Morgan, M. A. Horsfield, et al., "Phase I study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of PTK787/ZK 222584 administered twice daily in patients with advanced cancer," *Journal of Clinical Oncology*, vol. 23, no. 18, pp. 4162–4171, 2005.

[112] D. Reardon, H. Friedman, N. Brada, et al., "A phase I/II trial of PTK787/ZK 222584 (PTK/ZK), a multi-VEGF receptor tyrosine kinase inhibitor, in combination with either temozolomide or lomustine for patients with recurrent glioblastoma multiforme (GBM)," *Neuro-Oncology*, vol. 6, p. 381, 2004.

[113] J. P. Kirkpatrick, J. N. Rich, J. J. Vredenburgh, et al., "Final report: phase I trial of imatinib mesylate, hydroxyurea, and vatalanib for patients with recurrent malignant glioma (MG)," *Journal of Clinical Oncology*, vol. 26, no. 15S, p. 2057, 2008.

[114] D. A. Reardon, M. J. Egorin, A. Desjardins, et al., "Phase I pharmacokinetic study of the vascular endothelial growth factor receptor tyrosine kinase inhibitor vatalanib (PTK787) plus imatinib and hydroxyurea for malignant glioma," *Cancer*, vol. 115, no. 10, pp. 2188–2198, 2009.

[115] R. Kurzrock, L. Camacho, D. Hong, et al., "A phase I dose-escalation study of the safety and pharmacokinetics of a XL184, a VEGFR and Met kinase inhibitor, administered orally to subjects with advanced malignancies," *European Journal of Cancer*, vol. 4, p. 405, 2006.

[116] R. Salgia, D. Hong, S. I. Sherman, et al., "A phase 1 dose-escalation study of the safety and pharmacokinetics (PK) of XL184, a VEGFR and MET kinase inhibitor, administered orally to patients (pts) with advanced malignancies," *Molecular Cancer Therapeutics*, vol. 6, p. 3385S, 2007.

[117] R. Abounader and J. Laterra, "Scatter factor/hepatocyte growth factor in brain tumor growth and angiogenesis," *Neuro-Oncology*, vol. 7, no. 4, pp. 436–451, 2005.

[118] R. J. D'Amato, M. S. Loughnan, E. Flynn, and J. Folkman, "Thalidomide is an inhibitor of angiogenesis," *Proceedings*

of the National Academy of Sciences of the United States of America, vol. 91, no. 9, pp. 4082–4085, 1994.

[119] S. C. Short, D. Traish, A. Dowe, F. Hines, M. Gore, and M. Brada, "Thalidomide as an anti-angiogenic agent in relapsed gliomas," *Journal of Neuro-Oncology*, vol. 51, no. 1, pp. 41–45, 2001.

[120] H. A. Fine, P. Y. Wen, E. A. Maher, et al., "Phase II trial of thalidomide and carmustine for patients with recurrent high-grade gliomas," *Journal of Clinical Oncology*, vol. 21, no. 12, pp. 2299–2304, 2003.

[121] G. M. Marx, N. Pavlakis, S. McCowatt, et al., "Phase II study of Thalidomide in the treatment of recurrent glioblastoma multiforme," *Journal of Neuro-Oncology*, vol. 54, no. 1, pp. 31–38, 2001.

[122] V. K. Puduvalli, P. Giglio, M. D. Groves, et al., "Phase II trial of irinotecan and thalidomide in adults with recurrent glioblastoma multiforme," *Neuro-Oncology*, vol. 10, no. 2, pp. 216–222, 2008.

[123] F. Baumann, M. Bjeljac, S. S. Kollias, et al., "Combined thalidomide and temozolomide treatment in patients with glioblastoma multiforme," *Journal of Neuro-Oncology*, vol. 67, no. 1-2, pp. 191–200, 2004.

[124] H. A. Fine, L. Kim, P. S. Albert, et al., "A phase I trial of lenalidomide in patients with recurrent primary central nervous system tumors," *Clinical Cancer Research*, vol. 13, no. 23, pp. 7101–7106, 2007.

[125] J. Drappatz, E. T. Wong, D. Schiff, et al., "A pilot safety study of lenalidomide and radiotherapy for patients with newly diagnosed glioblastoma multiforme," *International Journal of Radiation Oncology Biology Physics*, vol. 73, no. 1, pp. 222–227, 2009.

[126] A. B. Da Rocha, D. R. A. Mans, A. Regner, and G. Schwartsmann, "Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas?" *Oncologist*, vol. 7, no. 1, pp. 17–33, 2002.

[127] A. A. Brandes, M. Ermani, S. Turazzi, et al., "Procarbazine and high-dose tamoxifen as a second-line regimen in recurrent high-grade gliomas: a phase II study," *Journal of Clinical Oncology*, vol. 17, no. 2, pp. 645–650, 1999.

[128] A. M. Spence, R. A. Peterson, J. D. Scharnhorst, D. L. Silbergeld, and R. C. Rostomily, "Phase II study of concurrent continuous temozolomide (TMZ) and tamoxifen (TMX) for recurrent malignant astrocytic gliomas," *Journal of Neuro-Oncology*, vol. 70, no. 1, pp. 91–95, 2004.

[129] M. C. Chamberlain and P. A. Kormanik, "Salvage chemotherapy with tamoxifen for recurrent anaplastic astrocytomas," *Archives of Neurology*, vol. 56, no. 6, pp. 703–708, 1999.

[130] H. A. Fine, V. K. Puduvalli, M. C. Chamberlain, et al., "Enzastaurin (ENZ) versus lomustine (CCNU) in the treatment of recurrent, intracranial glioblastoma multiforme (GBM): a phase III study," *Journal of Clinical Oncology*, vol. 26, no. 25S, abstract 2005, 2008.

[131] J. R. Graff, A. M. McNulty, K. R. Hanna, et al., "The protein kinase Cβ-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts," *Cancer Research*, vol. 65, no. 16, pp. 7462–7469, 2005.

[132] C. J. Avraamides, B. Garmy-Susini, and J. A. Varner, "Integrins in angiogenesis and lymphangiogenesis," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 604–617, 2008.

[133] R. Stupp, R. Goldbrunner, and B. Neyns, "Mature results of a phase I/IIA trial of the integrin inhibitor cilengitide (EMD121974) added to standard concomitant and adjuvant temozolomide and radiotherapy (TMZ/RT) for newly diagnosed glioblastoma (GBM)," in *Proceedings of the 12th Annual Meeting of the Society for Neuro-Oncology*, 2007.

[134] D. R. Macdonald, T. L. Cascino, S. C. Schold Jr., and J. G. Cairncross, "Response criteria for phase II studies of supratentorial malignant glioma," *Journal of Clinical Oncology*, vol. 8, no. 7, pp. 1277–1280, 1990.

[135] T. T. Batchelor, D.G. Duda, and E. di Tomaso, "Normalization of tumor vasculature and alleviation of edema by AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor in glioblastoma patients," *International Journal of Radiation*, vol. 69, no. 3, supplement 1, pp. S50–S51, 2007.

[136] M. J. van den Bent, M. A. Vogelbaum, P. Y. Wen, D. R. Macdonald, and S. M. Chang, "End point assessment in gliomas: novel treatments limit usefulness of classical Macdonald's criteria," *Journal of Clinical Oncology*, vol. 27, no. 18, pp. 2905–2908, 2009.

[137] E. C. Quant, A. D. Norden, J. Drappatz, et al., "Role of a second chemotherapy in recurrent malignant glioma patients who progress on bevacizumab," *Neuro-Oncology*, vol. 11, no. 5, pp. 550–555, 2009.

[138] M. Paez-Ribes, E. Allen, J. Hudock, et al., "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[139] J. M. L. Ebos, C. R. Lee, W. Cruz-Munoz, G. A. Bjarnason, J. G. Christensen, and R. S. Kerbel, "Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis," *Cancer Cell*, vol. 15, no. 3, pp. 232–239, 2009.

[140] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[141] L. Ellis and D. J. Hicklin, "Pathways mediating resistance to vascular endothelial growth factor-targeted therapy," *Clinical Cancer Research*, vol. 14, no. 20, pp. 6371–6375, 2008.

[142] R. Abounader and J. Laterra, "Scatter factor/hepatocyte growth factor in brain tumor growth and angiogenesis," *Neuro-Oncology*, vol. 7, no. 4, pp. 436–451, 2005.

[143] I. Petit, D. Jin, and S. Rafii, "The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis," *Trends in Immunology*, vol. 28, no. 7, pp. 299–307, 2007.

[144] E. De Falco, D. Porcelli, A. R. Torella, et al., "SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells," *Blood*, vol. 104, no. 12, pp. 3472–3482, 2004.

[145] M. Aghi, K. S. Cohen, R. J. Klein, D. T. Scadden, and E. A. Chiocca, "Tumor stromal-derived factor-1 recruits vascular progenitors to mitotic neovasculature, where microenvironment influences their differentiated phenotypes," *Cancer Research*, vol. 66, no. 18, pp. 9054–9064, 2006.

[146] R. Du, K. V. Lu, C. Petritsch, et al., "HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion," *Cancer Cell*, vol. 13, no. 3, pp. 206–220, 2008.

[147] G. Bergers, S. Song, N. Meyer-Morse, E. Bergsland, and D. Hanahan, "Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1287–1295, 2003.

[148] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ^+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[149] D. C. Darland, L. J. Massingham, S. R. Smith, E. Piek, M. Saint-Geniez, and P. A. D'Amore, "Pericyte production of cell-associated VEGF is differentiation-dependent and is associated with endothelial survival," *Developmental Biology*, vol. 264, no. 1, pp. 275–288, 2003.

[150] P. Kunkel, U. Ulbricht, P. Bohlen, et al., "Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2," *Cancer Research*, vol. 61, no. 18, pp. 6624–6628, 2001.

[151] J. L. Rubenstein, J. Kim, T. Ozawa, et al., "Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption," *Neoplasia*, vol. 2, no. 4, pp. 306–314, 2000.

[152] L. L. Muldoon, C. Soussain, K. Jahnke, et al., "Chemotherapy delivery issues in central nervous system malignancy: a reality check," *Journal of Clinical Oncology*, vol. 25, no. 16, pp. 2295–2305, 2007.

[153] C. Calatozzolo, M. Gelati, E. Ciusani, et al., "Expression of drug resistance proteins Pgp, MRP1, MRP3, MRP5 AND GST-π in human glioma," *Journal of Neuro-Oncology*, vol. 74, no. 2, pp. 113–121, 2005.

[154] N. Giri, N. Shaik, G. Pan, et al., "Investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) on pharmacokinetics and central nervous system penetration of abacavir and zidovudine in the mouse," *Drug Metabolism and Disposition*, vol. 36, no. 8, pp. 1476–1484, 2008.

[155] H. Dai, P. Marbach, M. Lemaire, M. Hayes, and W. F. Elmquist, "Distribution of STI-571 to the brain is limited by P-glycoprotein-mediated efflux," *Journal of Pharmacology and Experimental Therapeutics*, vol. 304, no. 3, pp. 1085–1092, 2003.

[156] L. A. Doyle and D. D. Ross, "Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2)," *Oncogene*, vol. 22, no. 47, pp. 7340–7358, 2003.

[157] R. K. Jain, D. G. Duda, C. G. Willett, et al., "Biomarkers of response and resistance to antiangiogenic therapy," *Nature Reviews Clinical Oncology*, vol. 6, no. 6, pp. 327–338, 2009.

[158] L. G. Daenen, Y. Shaked, S. Man, et al., "Low-dose metronomic cyclophosphamide combined with vascular disrupting therapy induces potent antitumor activity in preclinical human tumor xenograft models," *Molecular Cancer Therapeutics*, vol. 8, no. 10, pp. 2872–2881, 2009.

[159] A. M. Jubb, H. I. Hurwitz, W. Bai, et al., "Impact of vascular endothelial growth factor-A expression, thrombospondin-2 expression, and microvessel density on the treatment effect of bevacizumab in metastatic colorectal cancer," *Journal of Clinical Oncology*, vol. 24, no. 2, pp. 217–227, 2006.

[160] A. Dowlati, R. Gray, A. B. Sandler, J. H. Schiller, and D. H. Johnson, "Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab—an Eastern Cooperative Oncology Group study," *Clinical Cancer Research*, vol. 14, no. 5, pp. 1407–1412, 2008.

[161] H. J. Burstein, Y.-H. Chen, L. M. Parker, et al., "VEGF as a marker for outcome among advanced breast cancer patients receiving anti-VEGF therapy with bevacizumab and vinorelbine chemotherapy," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7871–7877, 2008.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 251231, 16 pages
doi:10.1155/2010/251231

Review Article

Antiangiogenic Therapy and Mechanisms of Tumor Resistance in Malignant Glioma

Ruman Rahman,[1] Stuart Smith,[1] Cheryl Rahman,[2] and Richard Grundy[1]

[1] *Children's Brain Tumour Research Centre, Medical School, Queens Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK*
[2] *Department of Tissue Engineering, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, UK*

Correspondence should be addressed to Ruman Rahman, ruman.rahman@nottingham.ac.uk

Received 15 December 2009; Accepted 2 February 2010

Academic Editor: Arkadiusz Dudek

Despite advances in surgery, radiation therapy, and chemotherapeutics, patients with malignant glioma have a dismal prognosis. The formations of aberrant tumour vasculature and glioma cell invasion are major obstacles for effective treatment. Angiogenesis is a key event in the progression of malignant gliomas, a process involving endothelial cell proliferation, migration, reorganization of extracellular matrix and tube formation. Such processes are regulated by the homeostatic balance between proangiogenic and antiangiogenic factors, most notably vascular endothelial growth factors (VEGFs) produced by glioma cells. Current strategies targeting VEGF-VEGF receptor signal transduction pathways, though effective in normalizing abnormal tumor vasculature, eventually result in tumor resistance whereby a highly infiltrative and invasive phenotype may be adopted. Here we review recent anti-angiogenic therapy for malignant glioma and highlight implantable devices and nano/microparticles as next-generation methods for chemotherapeutic delivery. Intrinsic and adaptive modes of glioma resistance to anti-angiogenic therapy will be discussed with particular focus on the glioma stem cell paradigm.

1. Introduction

Glioblastoma multiforme (GBM) (WHO grade IV astrocytoma) is amongst the most vascular and aggressive of all solid tumors and continues to have an extremely poor prognosis. They are the most common primary brain tumor in adults, with about 4800 new cases each year in the United Kingdom (~17500 per year in the USA), comprising approximately one percent of all tumor diagnoses. The tumor is characterized on histological examination by poorly differentiated neoplastic astrocytes, with cellular polymorphism, nuclear atypia, mitotic activity, necrosis, vascular proliferation and thrombosis [1]. The tumors are highly infiltrative (though distant metastasis is rare) and inevitably recur, even after gross macroscopic surgical resection. However, studies have demonstrated that complete (>90%) resection is associated with survival benefit in both adults [2] and children [3]. Current first choice adjuvant therapy in adults subsequent to maximal surgery is radiation therapy (usually 60 Gy in 30 fractions) with concomitant temozolomide, an alkylating agent. This regime has shown a significant survival benefit [4], extending median survival from 12.1 to 14.6 months. Although a significant improvement, the long-term survival of these patients is still extremely limited with only 8% surviving for four years (compared to zero survivors in the radiotherapy only arm).

In childhood, low-grade astrocytomas are the most common tumor type with high-grade gliomas (HGG) making up 10%–15% of tumors diagnosed [5], a total of around 50–70 cases per year in the U.K. Paediatric central nervous system tumors however account for more expected life years lost than any other tumor group and are now the leading cause of cancer deaths in children, following improvements in survival rates for the leukemias. Treatment broadly follows adult regimes with surgery and radiotherapy being accompanied by alkylating agents. Comparatively, little research has been

performed in children regarding therapy and the molecular genetics of high-grade glioma, and although pediatric HGG may resemble adult GBM on histopathological criteria, there are significant differences both clinically and within the molecular biology of the tumors. Long-term survival is more frequent in children, especially those under three years old and it may be that radiotherapy can be avoided in this age group [6]. All modalities of treatment have potentially devastating side effects, often resulting in severe neurological disability. Pathways controlling the generation of new blood vessels (angiogenesis) are frequently implicated in both adult and pediatric tumors. Many genome wide studies have implicated proangiogenic pathways including vascular endothelial growth factor (VEGF) [7], epidermal growth factor (EGF) [8], and platelet-derived growth factor (PDGF) [9]. HGG are in general extremely vascular tumors with evidence of widespread production of new blood vessels. Efforts are currently underway to target these pathways therapeutically with the hope of developing effective novel treatments for HGG that are better tolerated than current cytotoxic chemotherapy.

It is clear that novel therapies are needed for this tumor to improve the current situation. Here we evaluate current antiangiogenic therapies (summarized in Table 1) and mechanisms of resistance for malignant glioma and consider the development of novel methods of drug delivery to overcome the problem of achieving therapeutic drug concentrations within the CNS caused by the impermeability of the blood brain barrier (BBB).

2. Glioma Angiogenesis and Invasion

Glioma vasculature formation occurs through two distinct processes. Glioma angiogenesis is a process involving the genesis of new blood vessels from rerouting and remodeling of preexisting vessels. Neoangiogenesis mainly develops in late embryonic development and during adulthood as a result of tissue demands [10]. Vasculogenesis (blood vessel arrangement) was classically considered an embryonic process but has since been identified in tumors as the *de novo* formation of primitive blood vessels by the differentiation of circulating bone marrow-derived endothelial progenitor cells [11]. Among solid tumors, glioblastoma multiforme displays the most angiogenic features and highest degree of vascular proliferation and endothelial cell hyperplasia [12]. Angiogenesis is thus a key pathologic event in glioblastoma tumors and is necessary for the progression of a localized neoplasm to a highly aggressive tumour. Moreover, malignant gliomas also require angiogenesis to establish a source of nutrients and oxygen and to eliminate cellular waste products [13].

2.1. The Molecular and Cellular Angiogenic Switch. Glioma angiogenesis is initiated when the homeostatic balance between pro-angiogenic and anti-angiogenic stimuli is disrupted in favor of the former, resulting in activation of pro-angiogenic signaling pathways. These stimuli are secreted by both cellular sources (glioma cells, endothelial cells and microglia) and environmental triggers (extracellular matrix (ECM), hypoxia) [14, 15]. The transition towards neo-angiogenesis is referred to as the "angiogenic switch" and is increasingly viewed as a rate-limiting secondary event in multistage carcinogenesis [16].

The initial stage in the formation of new blood vessels involves the breakdown of native vessels. Angiopoietin-1 (Ang-1) and its receptor Tie-2 are key components of this process and Ang-1 is increased in GBM tumor cells [17, 18]. Glioma cells first accumulate around existing cerebral blood vessels and lift off astrocytic process, leading to the disruption of normal contact between endothelial cells and the basement membrane [18]. Subsequently, these blood vessels become apoptotic and undergo involution. Vascular collapse ensues and results in the death of neighboring tumor cells and the formation of necrotic zones. Hypoxia arises in these regions resulting in expression of hypoxia inducible factor-1 (HIF-1). In GBMs, HIF-1α is primarily localized in pseudopalisading cells around areas of necrosis and in tumor cells infiltrating the brain at the tumor margin [19]. HIF-1α is a transcriptional master regulator that activates a plethora of genes, the protein products of which function to either increase oxygen availability or to allow metabolic adaptation to oxygen deprivation [20]. In this context, HIF-1α transcriptionally activates VEGF which in turn initiates and promotes glioma angiogenesis (see Mediators of glioma angiogenesis below) [21, 22].

Following regression of native blood vessels, the basement membrane and surrounding ECM are degraded to allow for endothelial cell invasion. The matrix-metalloproteinases (MMP)-2 and 9 are major factors for this event in brain tumor angiogenesis and MMP-2 and MMP-9 expression is associated with a poor outcome in glioma patients [23, 24]. Upon breakdown of the basement membrane, endothelial cells proliferate and migrate toward tumor cells that express pro-angiogenic factors. Activation of endothelial cells results in upregulation of cell surface adhesion/migration molecules, in particular $\alpha_v\beta_3$, $\alpha_5\beta_1$, and CD44 [25].

2.2. Pro-Angiogenic Mediators in Glioma

2.2.1. Vascular Endothelial Growth Factor. The VEGF family of growth factors and their receptors are the most important mediators of glioma angiogenesis. VEGF ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor) bind to and activate the VEGF receptor (VEGFR) tyrosine kinases (VEGFR-1 and VEGFR-2). Specifically, VEGF-A is upregulated in glioblastoma and regulates endothelial cell survival, proliferation, vascular permeability, and migration primarily via VEGFR-2 [26, 27]. VEGF-A is primarily induced by tissue hypoxia via the HIF-1α pathway. The hypoxic microenvironment leads to dissociation of von Hippel Lindau protein from HIF-1α, preventing its proteasomal-mediated degradation and permitting HIF-1α binding to hypoxia response elements in the promoter region of several pro-angiogenic factors, such as VEGF [28]. In addition to HIF-1α, a variety of growth factors can also upregulate VEGF expression, including transforming growth factor (TGF)-β, EGF, PDGF-B, and basic fibroblast growth factors

(FGF) [29–31]. The end result of VEGF signaling in the glioma neovascular niche is the production of immature highly permeable blood vessels with poor maintenance of the blood brain barrier and parenchymal edema [32]. VEGF also functions as a prosurvival factor for endothelia, mediated by suppression of p53, p21, p16, and p27, Bax pro-apoptotic protein and activation of phosphatidylinositol-3 kinase (PI3K)/Akt and Ras/mitogen activated protein kinase (MAPK) pathways [33, 34].

2.2.2. Fibroblast Growth Factor. Similar to VEGF, fibroblast growth factor (FGF) is expressed by glioma cells and their adjacent blood vessels [9]. FGF-receptor 1 is upregulated in endothelial cells while FGF-receptor 4 is expressed primarily in tumor cells [35, 36].

2.2.3. Platelet-Derived Growth Factor. In addition to endothelial cell migration, pericyte (mesenchymal-like cells, associated with the walls of small blood vessels) migration is an important part of the process of tumor vasculogenesis [37]. PDGF secretion by activated endothelial cells recruits pericytes to the site of newly formed vessels and contributes to the development of a new basement membrane [38]. Of the two PDGF tyrosine kinase receptors (PDGF-α and PDGF-β), PDGF-α is expressed in an autocrine manner in glioma cells, whereas PDGF-β is expressed in glioma endothelium and pericytes, particularly the latter, suggesting its importance in the migration of pericytes into newly formed blood vessels [39].

2.2.4. Tumor Necrosis Factor. Tumour necrosis factor (TNF)-α is a potent inflammatory cytokine found in malignant gliomas and other cells including reactive astrocytes. TNF-α induces tumor angiogenesis indirectly via the activation of other angiogenic factors, most notably VEGF [40]. Furthermore, VEGF is upregulated in human gliomas upon TNF-α treatment, mediated through the Sp1 transcription factor [41].

2.2.5. Integrins. Integrins are transmembrane receptor molecules that facilitate endothelial cell migration and invasion and specifically, integrin-$\alpha_v\beta_3$ correlates to glioma tumor grade and glioma cell proliferation [42, 43].

2.2.6. Matrix Metalloproteinases. MMP-2, and MMP-9 are highly expressed in astrocytomas and correlate with histological grade. Both proteins are detected in tumor and endothelial cells [44]. MMPs are involved in the proteolytic degradation of ECM components and facilitate cell motility during angiogenesis [45]. Upregulation of MMPs is required for the angiogenic effects of TGF-β and VEGF and MMP-2 and MMP-9 proteolytically cleave and activate TGF-β, thus promoting tumour invasion and angiogenesis [46, 47].

Table 1: Anti-angiogenic agents trialled in high-grade glioma and their respective targets.

Antibody Therapies	*Major Molecular Targets*
Bevacizumab	Free VEGF-A
IMC-3G3	PDGFR alpha
Ramucirumab	VEGFR-2
Nimotuzumab	EGFR
AMG-102	Hepatocyte Growth Factor
VEGF-trap	Decoy receptor for VEGF
Tyrosine Kinase Inhibitors	
Cediranib	VEGFR/PDGFR/c-Kit
Sorafenib	VEGFR/PDGFR/c-Kit/Raf
Sunitinib	VEGFR/PDGFR
AE788	EGFR/VEGFR
Erlotinib	EGFR
Imatinib	PDGFR/Bcr-abl/c-Kit
Dasatanib	Src kinases
Signal Pathway Inhibitors	
Enzastaurin	Protein Kinase C
Rapamycin/Temsirolimus	mTOR
Tipifarnib	Ras
Other Agents	
Thalidomide/Lenalidomide	NO/TNF alpha/IL-6
Celecoxib	Endostatin
Cilengitide	Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$
2-methoxyestradiol	HIF1α
Prinomastat	MMPs 2,9,13 and 14
SAHA(Vorinostat)	Histone deacetylase

2.2.7. AntiAngiogenic Mediators in Glioma. A number of anti-angiogenic factors have been described and are functionally relevant to tumor angiogenesis. Angiostatin is derived from degradation of plasminogen by proteases such as cathepsin D and MMPs and functions as an endogenous anti-angiogenic factor [48]. Murine models reveal that angiostatin impairs glioma angiogenesis and tumor growth through binding of $\alpha_v\beta_3$ on proliferating endothelial cells, resulting in apoptosis [49, 50].

The thrombospondins (TSPs) are another family of proteins that function as anti-angiogenic factors. TSP-1 is expressed on platelets, endothelial cells and smooth muscle cells in normal tissue [51]. TSP-1 reduces endothelial cell proliferation and induces apoptosis in vitro [52], TSP peptides derived from TSP-1 decrease glioma angiogenesis and tumor growth in mice [53].

Endostatin is a similar anti-angiogenic molecule, formed by proteolytic cleavage of collagen-18 in glioblastoma basement membrane by elastase, cathepsin-L and specific MMPs [54]. Endostatin-mediated angiogenesis blockade includes binding to $\alpha_5\beta_1$ integrin, inhibition of VEFGR-2, and decreased expression of the anti-apoptotic molecule, Bcl-2 [55].

2.3. Glioma Invasion. Animal models of glioma invasion fail to accurately mimic the invasiveness of human glioma

cells along white matter tracts. Alternative models such as matrigel invasion chambers and xenograft lines serially passaged in vivo have therefore been more informative for study. Glioma cell invasion requires four distinct processes: (i) detachment of invading cells from the primary tumor mass, involving destabilization and disorganization of cadherin-mediated junctions, downregulation of neural cell adhesion molecule and CD44 cleavage which anchors the primary mass to the ECM by the metalloproteinase ADAM; (ii) adhesion to the ECM, mediated by integrins, particularly $\alpha_v\beta_3$ which binds fibronectin in the ECM; (iii) degradation of the ECM by proteases such as MMP-2 and MMP-9; and (iv) cell motility and intracellular contractility, mediated by cytoplasmic mediators such as myosin [10]. Invasion along white matter tracts allows gliomas to extend at a microscopic level beyond surgical resection cavities or radiation treatment fields [56]. Glioma cells migrate in a similar fashion to nontransformed neural progenitors, whereby a prominent leading cytoplasmic process is followed by a burst of forward movement by the cell body. This raises the intriguing question of whether stem/progenitor-like cells in glioma have a causative role in glioma invasion, migration and metastases. Indeed glioma stem (or stem-like) cells are highly invasive and are able to invade across the corpus callosum along white matter tracts [57, 58]. Such stem cell models may contribute significantly to future glioma invasion models.

3. Anti-Angiogenic Therapies

3.1. Antibody Therapies. One of the most well-established anti-angiogenic therapies is bevacizumab (Avastin, Roche). This is an IgG1 monoclonal antibody against free VEGF-A in the circulation, to which it binds, preventing attachment to the VEGF receptor and activation of a pro-angiogenic stimulus. Bevacizumab was originally developed for use in metastatic colorectal and nonsmall cell lung cancers, and has subsequently also been approved by the European Medicines Agency for use in metastatic breast and kidney cancers [59]. In view of the high levels of new vessel formation in GBM, many groups have now used bevacizumab for the treatment of this tumor, often in combination with irinotecan (a topoisomerase inhibitor).

There are currently two phase III trials (NCI and Hoffman La Roche) recruiting newly diagnosed glioblastoma patients for double-blind placebo-controlled studies comparing surgery, radiotherapy and temozolomide with or without bevacizumab, results of which will be of huge interest.

Phase two trials in patients with recurrent disease have yielded very encouraging results [60] with 6-month progression free survival (PFS) rates of 46% and 6-month overall survival (OS) rate of 77% ($n = 35$ patients) for bevacizumab plus irinotecan in GBM [61]. The same group demonstrated 6-month PFS and OS of 55% and 79%, respectively, for the same agents in recurrent anaplastic astrocytoma (WHO grade III glioma) [62]. Radiological response rates were reported as 57%–63%. Similar response rates have also been observed by other groups in small scale series [63, 64]. As a comparator for this and other trials described, the six-month progression free survival for temozolomide as monotherapy in relapsed GBM has been reported as 21% [65].

Although initial response rates in the desperate situation of recurrent GBM are encouraging, it is clear that this response is temporary, and tumors eventually progress regardless. Attempts to modify bevacizumab regimes with second agents introduced at progression have not proved successful with 6-month PFS of 0%–2% [60, 66]. A particular concern with antibody therapy is its ability to cross the BBB. Clearly bevacizumab may be effective at reducing VEGF levels within the circulating volume, but it has not yet been demonstrated what effect it may have on paracrine VEGF pathways within the tumor and brain parenchyma itself, as it is probably unable to cross the BBB, although this barrier may be deficient in tumors.

Bevacizumab is in general well tolerated with few serious side effects, with approximately 10% of patients having to discontinue treatment in the trials to date. Serious side effects reported include intracerebral haemorrhage, bowel perforation, and thromboembolism. Predicting patients likely to respond to a particular therapy is an area likely to be of increasing significance as more therapies are developed, and more is known of the molecular biology of these tumors. One trial to date has reported that high immunohistochemical VEGF A expression was significantly associated with likelihood of radiographic response, but not overall survival [67]. VEGF single nucleotide polymorphisms may also be indicative of response rates [68].

It has been reported that recurrence after bevacizumab therapy is more likely to be diffuse and distant to the primary tumor location [64, 69]. One possible concern is that the therapy could be inducing increased cell migration within the tumor to escape from areas of hypoxia created by the drug. This diffuse infiltration may not be immediately obvious on conventional gadolinium-enhanced MRI and novel MRI techniques may be required to investigate this phenomenon [70]. It is unclear whether enlarging areas of T2 hyperintensity truly represent tumor invasion or simply increasing edema, with mismatch of clinical and radiological pictures [59].

A similar approach to bevacizumab is VEGF-Trap (Aflibercept—Sanofi/Regeneron). This is a fusion protein soluble decoy receptor with a high affinity for VEGF A [71]. It has been shown in animal glioma models to have significant antitumor activity [72], and phase I/II human clinical trials are underway (NCI).

Early stage trials (NCI) are being undertaken into other monoclonal antibodies directed against PDGFRα (IMC-3G3) or VEGFR-2 (ramucirumab), which seems to show promise in early trials in other malignancies [73]. A German randomised controlled trial of nimotuzumab (an EGFR receptor antibody) in newly diagnosed glioblastoma is ongoing. This antibody, along with the similar anti-EGFR antibody cetuximab, has been shown to radiosensitise glioma cells in a mouse model, with nimotuzumab having increased anti-angiogenic and antiproliferative effects [74]. AMG 102 (an antihepatocyte growth factor antibody) has

shown promising effects in vitro in combination with an anti-EGFR antibody [75].

3.2. Small Molecule Inhibitors. In recent years, there has been much interest in developing inhibitors of various components of the angiogenic pathway. Many of these compounds are of relatively low molecular weight, allowing improved penetration of the BBB and the cytoplasm or nucleus of cells. However, they may still be targeted by drug extrusion systems (e.g., PGP or MDR) and intracellular concentrations may be lowered as a result. In this way, precise molecular targets can be modulated, with the hope of efficacious anti-angiogenic therapy with minimal effect on normal nonangiogenic tissue. The compounds of this type are also usually able to be taken orally, with good tolerability in most trials to date. Agents have been or are in the development process for many molecular targets and they will be summarised here. Many of the small molecule inhibitors act on, for example, multiple molecularly related receptor tyrosine kinases. This may have some advantages in allowing targeting of entire angiogenic pathways, potentially more effective than simply targeting single growth factors or receptors.

3.3. Vascular Endothelial Growth Factor Inhibitors. In addition to antibody-based approaches targeting free VEGF, compounds have been developed to target the functioning of the VEGF receptor. An example of this class is cediranib (Recentin/AZD2171—Astra-Zeneca). This is an indole-ether quinazoline that inhibits tyrosine kinase receptors, particularly all subtypes of the VEGF receptor, and has some activity against the PDGF and c-Kit receptors. A phase II trial of cediranib showed a radiological response rate of 56%, with 6-month PFS of 26% in recurrent glioblastoma [59]. The same trial [76] also demonstrated that after just one day of cediranib treatment, magnetic resonance imaging can demonstrate changes in vascular permeability and flow using K^{trans} type techniques. Microvessel volume decreased back towards normal values and these changes were maintained until around the 56 day scan. When the authors combined measures of permeability, microvessel volume, and circulating IV collagen, the "vascular normalisation index" created was an excellent predictor of response and overall survival [77]. The same study also demonstrated reduction in the number of viable circulating endothelial cells and circulating progenitor cells which increased in number when the tumors progressed or relapsed. As well as decreasing new vessel formation, it is hypothesised that the process of normalisation of already formed vessels may enhance the effect of radiotherapy by reducing hypoxia and may enhance the delivery of other chemotherapeutic agents to the tumour. Strategies to extend this window of normalisation before the tumour begins to revert may need to be explored.

Sorafenib (Nexavar—Bayer/Onyx) inhibits a broad range of kinases including serine/threonine and receptor tyrosine kinases. Pathways known to be inhibited by this drug include VEGFR, PDGFR-β, c-Kit, and their downstream effectors C-Raf and B-Raf kinases. This in turn leads to decreased MEK 1, 2, ERK and MAPK activity, pathways implicated in cell proliferation as well as angiogenesis [78]. Sorafenib in combination with bortezomib [32] (a proteasome inhibitor) or rottlerin [79] (a protein kinase C inhibitor) has demonstrated efficiency against glioma cell lines, and several phase I/II trials are underway in both newly diagnosed and recurrent GBM. The drug has already been approved for use in renal cell carcinoma, and in common with other anti-angiogenic therapy, hypertension and fatigue are not uncommon side effects. Perhaps of greater concern is the risk of intracerebral haemorrhage which has been reported as higher than usual in patients with renal cell carcinoma cerebral metastases treated with VEGF inhibitors [80].

Sunitinib (Sutent—Pfizer) is an inhibitor of VEGF and PDGF-β receptors which has also been used with some success in metastatic renal cell carcinoma. It has demonstrated efficacy in reducing new blood vessel formation, with corresponding survival benefit in both subcutaneous and intracerebral murine glioma models [81] and is currently undergoing phase I/II trials in recurrent GBM in humans. Although a potent inhibitor of angiogenesis, and demonstrating beneficial effects in many animal models, some studies have demonstrated an apparent increase in metastatic behavior of tumors under certain situations, perhaps due to a preconditioning effect on the tumor microenvironment [82, 83]. These findings may echo the increased T2 signal change seen in GBM patients treated with VEGF inhibitors.

AEE788 (Novartis) is an inhibitor of EGFR, and at higher concentrations, VEGFR. This agent had also shown promise in mouse xenograft models when used in combination with everolimus [84], an mTOR pathway inhibitor related to rapamycin (sirolimus). Phase I/II clinical trials are underway currently in recurrent GBM, but further development may be suspended. Other agents being investigated include vatalanib and pazopanib, both VEGF inhibitors with some anti-PDGF activity.

3.4. Other Tyrosine Kinase Inhibitors. In addition to the VEGF inhibitors described with anti-PDGF activity, molecules have been developed specifically targeting the PDGF receptor and pathway. The most widely used of these is imatinib (Glivec—Novartis), a small molecule tyrosine kinase inhibitor active against PDGF, Bcr-abl, and c-Kit. Although well tolerated, single agent imatinib had very limited effect against high-grade glioma in adults, particularly in patients on anticonvulsants [85, 86]. Trials are now being undertaken to ascertain whether more effective results can be obtained by the use of imatinib in combination regimes, for example, with vatalanib [87] or temozolomide. Dasatanib, another multikinase inhibitor targeting Src kinases, is currently in early stage clinical trials and has shown promising antiglioma effect in vitro, particularly in combination with temozolomide [88]. NCI phase II studies of another multikinase inhibitor tandutinib are currently ongoing, one in combination with bevacizumab.

Erlotinib (Tarceva—OSI/Roche), is a selective EGFR antagonist that has been used in lung cancer. A phase II study

has shown very promising results with median survival of 19.3 months after diagnosis, compared to 14.1 months for historical controls [89], though a phase II trial of its use as monotherapy in recurrent GBM was disappointing [90]. Several further trials are ongoing, including in conjunction with bevacizumab. Gefitinib (Iressa—Astra Zeneca) is another selective EGFR antagonist that binds the ATP binding site of the EGF receptor. Disappointing results in the ISEL lung cancer trial [91] led to it being partially withdrawn in the United States, but hopes remain that a subset of patients with tumours containing appropriate EGF pathway mutations may have some benefit. Studies are ongoing in glioma, especially of its use in combination with other therapy, for example, everolimus [92], though a phase II trial showed limited benefit as monotherapy in recurrent GBM [93].

Other drugs developed to inhibit downstream kinases include enzastaurin (Eli Lilly) a selective protein kinase C β-inhibitor which suppresses phosphorylation of many targets including Akt, inhibiting development of xenografted glioma [94]. A phase I trial has showed some promising effects, although dosage was limited by thrombocytopenia and prolonged QT syndrome [95]. The mTOR pathway has also been targeted therapeutically, initially as immunomodulatory therapy for prevention of transplant rejection, but interest in its antineoplastic properties has increased in recent years. This pathway is of interest as it integrates multiple upstream cell signalling mechanisms to regulate protein synthesis, potentially allowing the effects of multiple growth factor receptors to be controlled. Rapamycin (Sirolimus—Wyeth) is a macrolide produced by the bacterium *Streptomyces hygroscopicus* that was originally isolated from soil samples from the island of Rapa Nui (Easter Island). It binds FKBP12 and this combination then inhibits the mTOR pathway. Rapamycin and its analogues temsirolimus and everolimus are currently undergoing phase I/II clinical trials, both as mono- and combination therapy. Temsirolimus has shown promise in published clinical trials, with extended time to progression [96].

3.5. Other Agents. Thalidomide (Celgene), a piperidinyl isoindole, has a broad spectrum of anti-angiogenic mechanisms, possibly including suppression of endothelial cell nitric oxide-induced migration, inhibition of TNFα and VEGF/IL-6 suppression [97]. In phase II clinical trials, the results have been somewhat mixed, with some groups reporting encouraging outcomes [98, 99], but others showing limited activity [100]. The addition of thalidomide and the cyclo-oxygenase-2 inhibitor celecoxib (shown to decrease angiogenesis in vitro through up regulation of the endogenous inhibitor endostatin) to temozolomide did not improve progression free survival [101]. The use of thalidomide can be limited by adverse effects such as thrombosis or peripheral neuropathy and analogues have been developed such as lenalidomide [102].

Cilengitide (EMD pharmaceuticals) is a cyclic arginine-glycine-aspartic acid peptide that selectively inhibits integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. In a phase II trial, the higher dose regime demonstrated a 6 month PFS of 15% in recurrent GBM [103] and trials are ongoing for its use in combination with temozolomide in newly diagnosed GBM. Other potential targets are the HIFs, which upregulate pro-angiogenic cellular factors in response to low-tissue oxygen partial pressure. The oestrogen derivative 2-methoxyestradiol (2ME2/Panzem—EntreMed) inhibits HIF1α mediated VEGF expression and directly downregulates HIF1α levels, as well as suppressing microtubule structure formation [97]. Clinical trials of 2ME2 in association with nanocrystal colloidal dispersion are ongoing. Research interest has also focused on attempts to inhibit matrix metalloproteinases (MMPs), important mediators of new vessel growth and tumour invasion. Prinomastat (Agouron) is a hydroxamate-based selective inhibitor of MMPs 2, 9, 13, and 14, which had mixed results in clinical trials in lung cancer [103], with trials in GBM completing at present. Farnesyl transferase inhibitors such as tipifarnib (Zarnestra—Johnson & Johnson) block the farnesylation of the Ras signalling molecule that is needed for it to fulfil its downstream effects in the signalling cascade that occurs after activation of growth factor receptors. Whilst evidence of benefit has been reported in one phase II trial [104], another found no benefit when given as preradiation sensitizer [105].

Histone deacetylase inhibitors (HDACi) are emerging as a promising class of anticancer agent, which act by alleviating transcriptionally silenced pathways in tumors, such as tumor suppressor pathways. HDACi have also been shown to possess antiinvasive and anti-angiogenic potential with SAHA/vorinostat currently undergoing phase II trials for recurrent GBM [106–108]. Although it is unclear whether these compounds have anti-angiogenic effects in glioma, we have revealed anti-proliferative and pro-apoptotic effects using the HDACi Trichoststin A in pediatric glioblastoma cells (R. Rahman, manuscript in preparation).

3.6. Pediatric Tumours. Very few trials of anti-angiogenic therapy have been performed in pediatric high-grade glioma. As previously stated, this is unfortunate as it is increasingly clear that it may not be possible to extrapolate results from adult trials to pediatric practice as genome wide studies indicate that there may be key differences in the molecular biology of HGG in different age groups.

Chemotherapy administered to children has often been performed in a metronomic fashion with the intention of targeting the tumor endothelium, and studies utilising thalidomide have been undertaken, with a subset of patients having prolonged PFS [109]. A phase I study of cilengitide demonstrated good response in patients completing the treatment and a phase II study is planned. Erlotinib has been evaluated in a phase I trial in children, adolescents, and young adults with newly diagnosed HGG [110], with evaluation proceeding. A phase I trial of imatinib in children with newly diagnosed brainstem and recurrent malignant gliomas showed some concerns with intratumoral haemorrhage and dose limiting toxicity in patients with brain stem glioma but seemed well tolerated in recurrent glioma [111]. Tipifarinib has been used in a phase I trial in brainstem glioma [112] and a phase II trial in several high-grade brain tumours

with good tolerability but little effect [90]. A phase I trial of semaxanib (SU5416) was terminated early due to the sponsor ceasing development of the drug, although prolonged disease stabilisation was seen in 25% of patients given the higher dosage [113].

4. Mechanisms of Glioma Anti-Angiogenic Resistance

Benefits of anti-angiogenic therapy both in preclinical settings and to patients are at best transitory, typically in the form of tumor stasis or shrinkage and in few cases, increased survival. Inevitably this period of clinical benefit (measured in weeks/months) is followed by restoration of tumor growth and progression [114]. Indeed, inhibiting VEGF does not appear to block tumour progression. Such resistance to anti-angiogenic therapy is counter-intuitive to the proposition that angiogenesis is essential for the progression of malignant glioma. Knowledge of the mechanistic basis governing anti-angiogenic resistance is required to fine-tune and better specify future treatment protocols using these drugs. The current status quo proposes two general modes of resistance to angiogenesis inhibitors, particularly those targeting VEGF and related pathways: adaptive (evasive) resistance, and intrinsic (preexisting) resistance (Figure 1) [115]. Multiple mechanisms are likely to underlie both modes of resistance.

4.1. Adaptive (Evasive) Resistance. An evolving hypothesis is that angiogenic tumors acquire the means to functionally evade the angiogenesis blockade induced by angiogenesis inhibitors [116–119]. Evasive resistance is indirect in so much as alternative means to sustain tumor growth are activated but the specific therapeutic target of the anti-angiogenic agent remains inhibited (Figure 1, top) [119]. Activation and/or upregulation of alternative pro-angiogenic signaling pathways may be one distinct adaptive mechanism, whereby substitution of a pro-angiogenic factor reestablishes neovascularization [120]. Evidence for FGF-dependent revascularization has come from a clinical investigation of glioblastoma patients being treated with the VEGFR inhibitor cediranib. After a measureable response phase, a relapse/progression phase was associated with higher blood levels of FGF2 compared to the same patients during the response phase [76]. Evaluation of the prevalence of this mechanism in human gliomas would be greatly facilitated by studying tissue obtained from patients undergoing re-resection subsequent to recurrence after anti-angiogenic therapy.

Recruitment of vascular progenitor cells and pro-angiogenic monocytes from the bone marrow is another distinct mechanism of resistance. Anti-angiogenic therapy-induced blood vessel regression may lead to hypoxia, creating conditions permissive for the recruitment of a heterogeneous population of bone marrow-derived monocytic cells that promote angiogenesis [115]. Specifically, these cells consist of endothelial and pericyte progenitors which differentiate into endothelial cells forming the inner lining of blood vessels, or pericytes that envelop blood vessels, respectively [121]. In GBM, HIF-1α recruits various pro-angiogenic bone marrow-derived CD45+ myeloid cells, and tumors lacking HIF-1α exhibit few such cells and are severely impaired in their angiogenic and tumor growth phenotypes [122]. These studies provide a mechanistic rationale for how hypoxic tension can create an environment that promotes neovascularization.

Although inhibition of VEGF signaling pathways causes vessel regression, a few thin vessels remain, densely and tightly covered with pericytes. Protective coating by pericytes presumably helps the tumor endothelium to survive and grow during the course of any anti-angiogenic therapy regime [121]. The contribution of this mode of resistance in GBM is undefined at present.

The switch to a condition of increased invasiveness without angiogenesis is another method of evasive adaptation. This phenotype was first described in orthotopic GBM mouse models, where neovascularization was blocked by genetically deleting VEGF and HIF-1α. GBM cells coopted normal blood vessels (perivascular invasion) to achieve the required vasculature in a dispersed fashion [123].

4.2. Intrinsic (Cellular) Resistance. A considerable minority of GBM patients tested in clinical trials for bevacizumab, sorafenib, and sunitinib failed to show even transitory clinical benefit [76]. As no period of tumor stasis was evident, these tumors are refractory to angiogenic therapy. It is plausible that the preexistence of FGF2 and other pro-angiogenic factors in late-stage GBM tumors could enable continued angiogenesis using redundant pathways during anti-angiogenic treatment.

Of particular interest is the glioma cell-type that is nonresponsive to anti-angiogenic therapy (and other chemotherapeutics). The stem cell paradigm for malignant gliomas presents tumour-initiating events as occurring within the genome of a cellular entity with intrinsic or acquired stem/progenitor cell-like properties. Glioma stem cells (GSC) have been reported to promote angiogenesis and vasculogenesis via increased expression of VEGF and stromal-derived factor 1 [124, 125]. Tumors enriched for GSCs showed increased vessel density, increased endothelial cell proliferation and tubule formation, increased endothelial progenitor mobilization, and recruitment of bone marrow-derived cells [125]. Although it may be counter-intuitive to expect GSCs, which by definition represent a very small minority of glioma tumor cells, to make a meaningful contribution to glioma angiogenesis, it is important to note that during early tumor-initiation or the seeding of a metastatic lesion, the GSC fraction would constitute a much greater proportion of the tumor mass. Therefore, it is conceivable that GSCs provide the necessary signals to trip the angiogenic switch early during the tumor growth of primary and/or metastatic tumors. Moreover, the hypoxic microenvironment has recently been shown to promote the expansion of GSC populations and promote a more stem-like phenotype in nonstem cell glioma populations [126, 127].

Regardless of whether the glioma cell of origin is a tissue-specific stem/progenitor cell or the specificity of

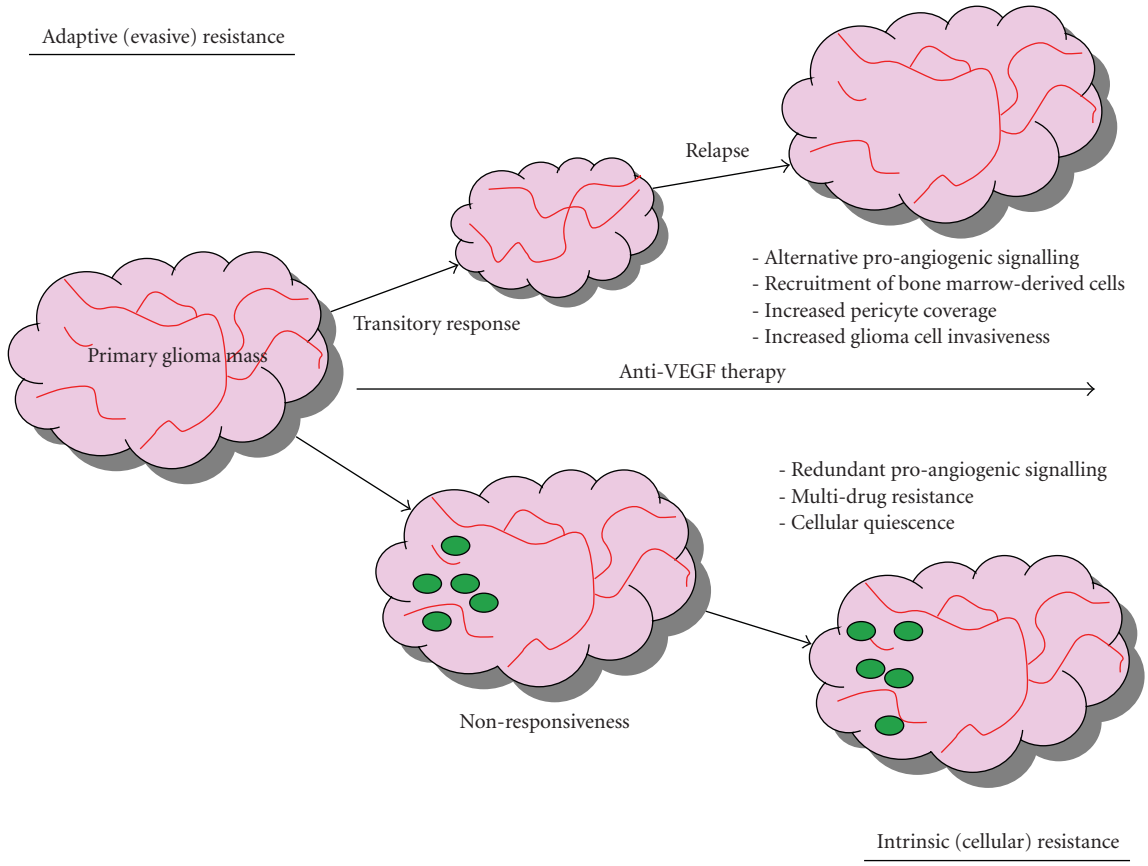


Figure 1: Mechanisms of glioma resistance to anti-angiogenic therapeutic modalities. *(Top) Adaptive (evasive) resistance.* After an initial transitory response phase, the tumor switches to mechanisms that induce neovascularization and renewed tumor growth and progression, thereby evading therapeutic blockade. These consist of pro-angiogenic factor substitution (typically dependence on FGF and angiopoietin signalling in cases of VEGF blockade), recruitment of endothelial cells and pericytes from the bon-marrow, protection of existing tumor blood vessels via increased pericyte coverage and increased tumor cell invasiveness whereby tumor cells invade adjacent normal tissue to achieve vascular sufficiency. *(Bottom) Intrinsic (cellular) resistance.* From the outset, some gliomas are nonresponsive to angiogenesis blockade. This may be accounted for by the preexistence of multiple redundant pro-angiogenic signals, which would allow for continued angiogenesis during anti-angiogenic insults. In addition, glioma stem cells (GSCs) (*green circles*) have been identified as key mediators of glioma angiogenesis and may share intrinsic cell survival and lifespan prolonging characteristics with normal tissue stem cells. Specifically, GSCs may reside in a noncycling quiescent state, thus blocking entry of drugs through the tumor cell membrane and may express relatively high levels of ABC-drug transporters, enabling a multi-drug resistance phenotype via efflux of drugs from the tumor cell. *Red lines, tumor blood vessels.*

cell surface antigens in delineating tumor-initiating glioma cells, the evidence that a subpopulation of glioma cells shares certain cardinal properties of stem cells and early progenitors (namely capacity for self-renewal and multilineage differentiation) provides a conceptual and technical framework in which to understand cellular resistance to therapies such as anti-angiogenic agents. It is logical to query whether surviving glioma cells intrinsically resistant to angiogenic therapy share characteristics with normal tissue stem cells which permit a long lifespan, such as cellular quiescence, expression of ATP-binding cassette (ABC) drug transporters, and increased DNA repair capacity.

Malignant gliomas that are resistant to chemotherapeutics often display a multidrug resistance phenotype due to reduced cellular drug accumulation through ABC membrane efflux pumps [128]. In addition, tissue stem cells generally reside in the G0 stage of the cell cycle and are only induced to activate self-renewal and differentiation programs when the respective tissue needs to be repopulated. It is possible that the expression of ABC transporters and the quiescent state of malignant GSCs may be key determinants of intrinsic nonresponsiveness to anti-angiogenic therapies, by the extrusion of agents from the cell, and by providing a barrier for entry of agents into the cell, respectively (Figure 1, bottom). In addition, studies have shown that GSCs promote radioresistance by preferential activation of the DNA damage response [124].

A number of caveats for these hypotheses emerge however. The expression profiles of drug resistance-related ABC transporters did not differ between primary and secondary glioblastomas and no correlation to recurrent tumors was evident [128]. Moreover, glioma cell populations sorted

for the expression of the ABCG2-transporter, revealed that both ABCG2+ and ABCG2− populations exhibited similar tumorigenicity [129]. Regarding the noncycling nature of GSCs, direct evidence for the prevalence of quiescence in glioma is lacking. Through studies of stem cell and proliferation marker expression coupled with telomerase enzymatic activity, we find evidence for lower proliferation levels in childhood ependymoma cell populations enriched for stem cells (R. Rahman, unpublished findings). However we cannot discriminate between a general reduced rate of proliferation in the population as a whole or to the existence of a quiescent subset within the total population. It remains to be elucidated whether the aforementioned modes of intrinsic GSCs are necessary and sufficient to achieve intrinsic resistance in malignant glioma. The relationship between the hypoxic glioma microenvironment and GSCs may also impact upon future therapy as it has recently been shown that HIF proteins are preferentially activated in GSCs compared to nontumor cells [130]. Better understanding in this respect will aid development of novel agents specifically targeting GSCs.

5. Future Therapeutic Directions

The question of how to prolong the sometimes excellent shortterm results of anti-angiogenic therapy is the subject of much investigation. Current trials are focusing on combination approaches using multiple therapies to target different pathways concurrently with the hope of preventing the use of different pathways to evade the inhibition of a single molecule. Novel agents are also underdevelopment which will target the previously discussed pathways more effectively, and also with the aim of controlling mechanisms not yet fully elucidated.

One promising new field that may yield effective means of controlling the angiogenic process is that of microRNAs. These are short (20–23 base pair) conserved sequences that are transcribed but not translated. First identified in *C. elegans* in the early 1990s, it is only in the last 10 years that their true importance has been recognized [56]. They act by binding complementary sequences on messenger RNA and interfering with (usually downregulating) the translation of the mRNA into protein. There are now several hundred recognised miRNA known to regulate human gene translation, with approximately 50% of cancer related genes having known miRNA regulators. High-grade gliomas have characteristic profiles of miRNA expression [131–133], and the differences between HGG, low-grade gliomas, and normal tissue may give important clues as to how these short RNA sequences control tumor growth and development, including angiogenesis [134]. In other brain tumors such as medulloblastoma [135], particular miRNAs have been shown to be closely linked to the molecular machinery driving the tumor. Various miRNA [136–138] have been shown to be over or underexpressed in adult HGG with changes in the particular miRNA identified profoundly influencing glioma growth. miRNA has been shown to be crucial to normal brain development [139, 140] and to the processes of blood vessel creation and angiogenesis in normal development [141] as well as in pathological processes such as tumors. It has also been shown in glioma cell lines that the upregulation of VEGF and other factors in response to hypoxia may be governed by miRNA expression levels [142].

Therapeutically, it has been demonstrated that the effect of a particular miRNA can be strongly inhibited by engineered oligonucleotide sequences, so-called "antagomirs", with consequent decreased levels of related protein synthesis [143]. It has been demonstrated in a murine liver cancer model that dramatic reduction in tumor burden can be achieved by reactivating expression of miR-26a which is down-regulated in liver cancer [144]. In human brain tumors it has been shown that miR-296 is elevated in tumor-related endothelial cells of new vessels and that this miRNA may govern growth factor receptor expression [145]. It seems likely that much work will focus on elucidating which are the key miRNAs in GBM, how to antagonise/upregulate them and on the delivery vectors that would be necessary to use them in clinical trials [146]. A recent in vitro report showed good activity against CD133+ glioma stem cells by a combination of imatinib and the miRNA 451 [147]. Complete dispersion of neurospheres was observed at low concentrations of the combined reagents.

5.1. Polymeric Controlled Release for Intracranial Drug Delivery. Over the past two decades, a variety of approaches to enhance intracranial chemotherapeutic drug delivery have been investigated. Local therapies (via injection) are diffusion limited and may not reach areas distant to the site of injection. One approach is polymeric-controlled release for direct delivery of agents to intracranial tumors. The rationale of such an approach is to improve upon the efficacy and reduce the debilitating side effects of current systemic chemotherapeutics. An attractive feature of biodegradable polymers is that they completely erode during drug delivery and are cleared from the body. The majority of biodegradable polymers undergo erosion simply by water permeating into the polymer matrix [148].

There are two main methods of utilising polymeric controlled release for intracranial drug delivery: implantable devices and nano- or microparticles for injection. Implantable biodegradable polymeric devices provide a practical means of localizing the chemotherapeutic agents specifically at the tumor site. An additional advantage of direct intracranial drug delivery is that the need for a chemotherapeutic agent to cross the BBB is eliminated.

The most common copolymer system used intracranially is polybis (p-carboxyphenoxy) propane-sebacic acid (p (CPP-SA)). This delivery system has been characterized for a variety of drugs and is in clinical use [149]. Many controlled release systems are based on an implantable wafer. The p(CPP-SA) wafer loaded with 1,3-*bis* (2-chloroethyl)-1-nitrosourea (BCNU), also known as carmustine, is available clinically as Gliadel [150]. Gliadel is implanted intracranially after surgical debulking of the tumor. It is commonly used for local delivery of BCNU to high-grade gliomas after resection and is associated with increased survival [151]; however drug diffusion from the site of implantation is limited.

Implantable poly(lactic-co-glycolic acid) (PLGA) matrices loaded with chemotherapeutics are currently under development [149]. In an analogous method to Gliadel, the use of biodegradable PLGA wafers containing BCNU has been investigated and research in this area is ongoing [152]. Biodegradable polymer matrices based on polymers of lactide and glycolide are a popular platform for local drug delivery. PLGA particles have been used as a controlled delivery system for proteins, drugs, cytokines, hormones, enzymes, vaccines, and chemotherapeutic agents [153–156]. The composition of the PLGA allows the control of the degradation rate and therefore the control of drug release kinetics [157].

In addition to controlled release via wafers, polymers can also be harnessed to aid delivery of chemotherapeutic drugs to the brain by injection of polymeric nano- or microparticles. To date, minimally invasive systemic delivery of drugs to the brain remains a challenge that has given rise to the development of new drug-targeting technologies. Many forms of systemic chemotherapy are excluded from the central nervous system by the BBB and the high systemic concentrations necessary to cross the BBB often lead to several side effects [158, 159]. The use of nano- or microparticles for drug delivery to the brain appears to be a promising option to overcome these problems.

The advantage of polymer particles lies in the route of administration. Nano- or microparticles may be injected stereotactically to any site in the brain due to their size and spherical shape [160]. This is less invasive than the implantation of polymeric wafers [149]. Recently, BCNU-loaded PLGA microspheres were developed in vitro for intracranial administration by cerebral stereotaxy, which could potentially be administered repeatedly [161]. The use of PLGA particles to deliver drugs other than BCNU is also being investigated. Benny and colleagues demonstrated the delivery of two endogenous anti-angiogenic inhibitors, hemopexin (PEX) and a fragment of platelet factor 4 (PF-4) (PF-4/CTF), in vitro and in vivo for human glioma therapy using PLGA microspheres, with glioma growth inhibition observed.

Over the last decade, coating polymeric particles with a surfactant has also been investigated to enhance intravenous delivery of chemotherapeutic drugs to the brain. As evidenced by a number of studies, poly(butyl cyanoacrylate) (PBCA) nanoparticles coated with polysorbate 80 (Tween 80) facilitate brain delivery of a number of drugs that are unable to cross the BBB in free form [162]. Polysorbate 80 also proved to be effective for brain delivery of different types of nanoparticles such as poly (alkyl cyanoacrylate) and solid lipid nanoparticles [163, 164]. Polysorbate 80-coated PBCA nanoparticles have been found to selectively adsorb certain plasma proteins from the blood. These proteins promote receptor-mediated endocytosis of the polymer particles by the endothelial cells forming the BBB, thus facilitating delivery of the nanoparticle-encapsulated drug to the brain [165].

The effectiveness of the polysorbate 80-coated PBCA nanoparticles for brain delivery was most clearly demonstrated by the high antitumor effect of nanoparticle-bound doxorubicin against intracranial glioblastoma in rats [166]. A different type of surfactant, poloxamer 188 (Pluronic F68), was also found to be effective for brain delivery by PBCA particles [167, 168]. Recently, Gelperina and colleagues showed that both polysorbate 80 and poloxamer 188 were also able to facilitate brain delivery for PLGA nanoparticles [169].

Harnessing polymeric controlled release in the form of implants and injectable particles is proving to be a promising area of investigation for intracranial drug delivery. Ongoing and future research into this innovative drug delivery method will strive to address the need for controllable intracranial drug delivery for glioma therapy.

6. Summary and Perspectives

Although the transitory efficacy of anti-angiogenic inhibitors such as those targeting VEGF signalling pathways are disappointing, with a temporary period of response followed by relapse, these results must be considered in the context of standard of care therapy for malignant glioma, in particular GBM. These strategies also typically exhibit an initial response followed by inevitable resistance and tumor progression. Also, the majority of patient trials have involved recurrent glioma tumors at a late-stage of disease progression. Studies of anti-angiogenic therapies in patients with newly diagnosed malignant glioma will help determine whether these agents have greater efficacy in an earlier setting.

Approaches to overcome anti-angiogenic resistance may include more potent anti-angiogenic agents, synergistic strategies with drugs that inhibit other relevant targets, or multi-targeted single agents that simultaneously inhibit several crucial targets. The optimum protocol for the use of anti-angiogenic therapy in combination with cytotoxic chemotherapy and radiation therapy is as yet unclear for gliomas and needs to be refined. The concept of a "window" for increased therapeutic effectiveness, opened by anti-angiogenic therapy through normalization of vasculature, is also being explored and may impact on regimes adopted in future trials. Combination therapy with drugs that target glioma invasion is a promising approach due to concerns that anti-angiogenic therapy may lead to infiltrative tumor growth. Furthermore, future modes of chemotherapeutic delivery such as stereotactic injection of nanoparticles and tumor site-specific implants concomitant with surgical resection may circumvent the inefficiency and reduce the toxicity of systemic drug delivery and lead to a more targeted and minimally invasive therapeutic approach to glioma.

Increasing evidence for the persistence of stem cell-like cells in glioma may shift therapeutic approaches to target these cell populations. With the elucidation of the role of GSCs in glioma angiogenesis, it is tempting to speculate that much of the observed intrinsic resistance and non-responsiveness to anti-angiogenic therapies is due to inherent properties of GSCs. We await a generation of cytotoxic agents that specifically target glioma cells with stem cell/progenitor properties. Combination strategies with

such compounds and anti-angiogenic drugs may be an exciting future avenue, where the latter mode of therapy chemosensitizes the tumor prior to a direct cytotoxic hit on GSCs.

A number of pertinent questions remain to be better understood, answers of which will influence future modes of therapy: are GSCs enriched in regions of hypoxia so prevalent in GBM tumors? Is the cell type resistant to anti-angiogenic therapy, the same cell type resistant to chemotherapy and/or radiation therapy? What distinguishes anti-angiogenic glioma cells with adaptive resistance and those with intrinsic resistance? Can the same glioma cell type within a given tumor exhibit both modes of anti-angiogenic resistance and what are the implications for therapy if so? What are the roles of bone marrow-derived cells and stromal cells in the process of anti-angiogenic resistance? Are GSCs involved in invasion, migration, and metastases and are such cells the same GSCs that initiated the primary tumor?

Research into glioma tumor biology will be required to identify novel targets amenable to anti-angiogenic therapy. In conjunction, the development of novel methods for chemotherapeutic delivery will continue to advance our understanding of targeting tumor angiogenesis with wider application for other solid tumors. Disruption of glioma neovascularisation not only remains an attractive method of intervention but provides a model to better overcome glioma resistance.

Acknowledgments

R. Rahman is funded by the Joseph Foote Foundation and S. Smith is funded by the Royal College of Surgeons of England.

References

[1] N. David, M. D. Louis, H. Ohgaki, O. D. Wiestler, and W. K. Cavenee, Eds., *WHO Classification of Tumours of the Central Nervous System*, IARC Press, 4th edition, 2007.

[2] W. Stummer, H.-J. Reulen, T. Meinel, et al., "Extent of resection and survival in glioblastoma multiforme: identification of and adjustment for bias," *Neurosurgery*, vol. 62, no. 3, pp. 564–576, 2008.

[3] J. H. Wisoff, J. M. Boyett, M. S. Berger, et al., "Current neurosurgical management and the impact of the extent of resection in the treatment of malignant gliomas of childhood: a report of the Children's Cancer Group Trial No. CCG-945," *Journal of Neurosurgery*, vol. 89, no. 1, pp. 52–59, 1998.

[4] R. Stupp, W. P. Mason, M. J. van den Bent, et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *The New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.

[5] I. F. Pollack, "Brain tumors in children," *The New England Journal of Medicine*, vol. 331, no. 22, pp. 1500–1507, 1994.

[6] R. G. Grundy, S. H. Wilne, K. J. Robinson, et al., "Primary postoperative chemotherapy without radiotherapy for treatment of brain tumours other than ependymoma in children under 3 years: results of the first UKCCSG/SIOP CNS 9204 trial," *European Journal of Cancer*, vol. 46, no. 1, pp. 120–133, 2010.

[7] Y. Liang, M. Diehn, N. Watson, et al., "Gene expression profiling reveals molecularly and clinically distinct subtypes of glioblastoma multiforme," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 16, pp. 5814–5819, 2005.

[8] R. McLendon, A. Friedman, D. Bigner, et al., "Comprehensive genomic characterization defines human glioblastoma genes and core pathways," *Nature*, vol. 455, no. 7216, pp. 1061–1068, 2008.

[9] S. Karcher, H.-H. Steiner, R. Ahmadi, et al., "Different angiogenic phenotypes in primary and secondary glioblastomas," *International Journal of Cancer*, vol. 118, no. 9, pp. 2182–2189, 2006.

[10] M. C. Tate and M. K. Aghi, "Biology of angiogenesis and invasion in glioma," *Neurotherapeutics*, vol. 6, no. 3, pp. 447–457, 2009.

[11] W. Risau and I. Flamme, "Vasculogenesis," *Annual Review of Cell and Developmental Biology*, vol. 11, pp. 73–91, 1995.

[12] S. Brem, R. Cotran, and J. Folkman, "Tumor angiogenesis: a quantitative method for histologic grading," *Journal of the National Cancer Institute*, vol. 48, no. 2, pp. 347–356, 1972.

[13] J. Folkman, "Angiogenesis," *Annual Review of Medicine*, vol. 57, pp. 1–18, 2006.

[14] J. M. Brown and W. R. Wilson, "Exploiting tumour hypoxia in cancer treatment," *Nature Reviews Cancer*, vol. 4, no. 6, pp. 437–447, 2004.

[15] D. Wang, J. C. Anderson, and C. L. Gladson, "The role of the extracellular matrix in angiogenesis in malignant glioma tumors," *Brain Pathology*, vol. 15, no. 4, pp. 318–326, 2005.

[16] D. Hanahan, G. Bergers, and E. Bergsland, "Less is, more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice," *Journal of Clinical Investigation*, vol. 105, no. 8, pp. 1045–1047, 2000.

[17] Y. Reiss, M. R. Machein, and K. H. Plate, "The role of angiopoietins during angiogenesis in gliomas," *Brain Pathology*, vol. 15, no. 4, pp. 311–317, 2005.

[18] D. Zagzag, R. Amirnovin, M. A. Greco, et al., "Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis," *Laboratory Investigation*, vol. 80, no. 6, pp. 837–849, 2000.

[19] D. Zagzag, H. Zhong, J. M. Scalzitti, E. Laughner, J. W. Simons, and G. L. Semenza, "Expression of hypoxia-inducible factor 1α in brain tumors: association with angiogenesis, invasion, and progression," *Cancer*, vol. 88, no. 11, pp. 2606–2618, 2000.

[20] G. L. Semenza, "Hypoxia-inducible factor 1: master regulator of O_2 homeostasis," *Current Opinion in Genetics and Development*, vol. 8, no. 5, pp. 588–594, 1998.

[21] K. H. Plate, G. Breier, H. A. Weich, H. D. Mennel, and W. Risau, "Vascular endothelial growth factor and glioma angiogenesis: coordinate induction of VEGF receptors, distribution of VEGF protein and possible in vivo regulatory mechanisms," *International Journal of Cancer*, vol. 59, no. 4, pp. 520–529, 1994.

[22] K. H. Plate, G. Breier, H. A. Weich, and W. Risau, "Vascular endothelial growth factor is a potential tumour angiogenssis factor in human gliomas in vivo," *Nature*, vol. 359, no. 6398, pp. 845–848, 1992.

[23] P. Guo, Y. Imanishi, F. C. Cackowski, et al., "Up-regulation of angiopoietin-2, matrix metalloprotease-2, membrane type 1 metalloprotease, and laminin 5 γ 2 correlates with the invasiveness of human glioma," *American Journal of Pathology*, vol. 166, no. 3, pp. 877–890, 2005.

[24] J. S. Rao, M. Yamamoto, S. Mohaman, et al., "Expression and localization of 92 kDa type IV collagenase/gelatinase B (MMP-9) in human gliomas," *Clinical and Experimental Metastasis*, vol. 14, no. 1, pp. 12–18, 1996.

[25] G. Serini, D. Valdembri, and F. Bussolino, "Integrins and angiogenesis: a sticky business," *Experimental Cell Research*, vol. 312, no. 5, pp. 651–658, 2006.

[26] P. Carmeliet, "VEGF gene therapy: stimulating angiogenesis or angioma-genesis?" *Nature Medicine*, vol. 6, no. 10, pp. 1102–1103, 2000.

[27] M. R. Machein and K. H. Plate, "VEGF in brain tumors," *Journal of Neuro-Oncology*, vol. 50, no. 1-2, pp. 109–120, 2000.

[28] M. Safran and W. G. Kaelin Jr., "HIF hydroxylation and the mammalian oxygen-sensing pathway," *Journal of Clinical Investigation*, vol. 111, no. 6, pp. 779–783, 2003.

[29] P. Guo, B. Hu, W. Gu, et al., "Platelet-derived growth factor-B enhances glioma angiogenesis by stimulating vascular endothelial growth factor expression in tumor endothelia and by promoting pericyte recruitment," *American Journal of Pathology*, vol. 162, no. 4, pp. 1083–1093, 2003.

[30] L. Pertovaara, A. Kaipainen, T. Mustonen, et al., "Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells," *Journal of Biological Chemistry*, vol. 269, no. 9, pp. 6271–6274, 1994.

[31] J.-C. Tsai, C. K. Goldman, and G. Y. Gillespie, "Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF," *Journal of Neurosurgery*, vol. 82, no. 5, pp. 864–873, 1995.

[32] R. K. Jain, E. di Tomaso, D. G. Duda, J. S. Loeffler, A. G. Sorensen, and T. T. Batchelor, "Angiogenesis in brain tumours," *Nature Reviews Neuroscience*, vol. 8, no. 8, pp. 610–622, 2007.

[33] H.-P. Gerber, V. Dixit, and N. Ferrara, "Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells," *Journal of Biological Chemistry*, vol. 273, no. 21, pp. 13313–13316, 1998.

[34] H.-P. Gerber, A. McMurtrey, J. Kowalski, et al., "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway: requirement for Flk-1/KDR activation," *Journal of Biological Chemistry*, vol. 273, no. 46, pp. 30336–30343, 1998.

[35] T. Ueba, J. A. Takahashi, M. Fukumoto, et al., "Expression of fibroblast growth factor receptor-1 in human glioma and meningioma tissues," *Neurosurgery*, vol. 34, no. 2, pp. 221–226, 1994.

[36] S. M. Yamada, S. Yamada, Y. Hayashi, H. Takahashi, A. Teramoto, and K. Matsumoto, "Fibroblast growth factor receptor (FGFR) 4 correlated with the malignancy of human astrocytomas," *Neurological Research*, vol. 24, no. 3, pp. 244–248, 2002.

[37] G. Bergers and S. Song, "The role of pericytes in blood-vessel formation and maintenance," *Neuro-Oncology*, vol. 7, no. 4, pp. 452–464, 2005.

[38] N. Ferrara, "VEGF as a therapeutic target in cancer," *Oncology*, vol. 69, supplement 3, pp. 11–16, 2005.

[39] W. G. Roberts, P. M. Whalen, E. Soderstrom, et al., "Antiangiogenic and antitumor activity of a selective PDGFR tyrosine kinase inhibitor, CP-673,451," *Cancer Research*, vol. 65, no. 3, pp. 957–966, 2005.

[40] M. R. Slowik, L. G. De Luca, W. Fiers, and J. S. Pober, "Tumor necrosis factor activates human endothelial cells through the p55 tumor necrosis factor receptor but the p75 receptor contributes to activation at low tumor necrosis factor concentration," *American Journal of Pathology*, vol. 143, no. 6, pp. 1724–1730, 1993.

[41] M. Ryuto, M. Ono, H. Izumi, et al., "Induction of vascular endothelial growth factor by tumor necrosis factor α in human glioma cells: possible roles of SP-1," *Journal of Biological Chemistry*, vol. 271, no. 45, pp. 28220–28228, 1996.

[42] G. M. D'Abaco and A. H. Kaye, "Integrins: molecular determinants of glioma invasion," *Journal of Clinical Neuroscience*, vol. 14, no. 11, pp. 1041–1048, 2007.

[43] S. Stromblad and D. A. Cheresh, "Integrins, angiogenesis and vascular cell survival," *Chemistry and Biology*, vol. 3, no. 11, pp. 881–885, 1996.

[44] P. A. Forsyth, H. Wong, T. D. Laing, et al., "Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas," *British Journal of Cancer*, vol. 79, no. 11-12, pp. 1828–1835, 1999.

[45] J. S. Rao, R. Sawaya, Z. L. Gokaslan, W. K. A. Yung, G. W. Goldstein, and J. Laterra, "Modulation of serine proteinases and metalloproteinases during morphogenic glial-endothelial interactions," *Journal of Neurochemistry*, vol. 66, no. 4, pp. 1657–1664, 1996.

[46] W. Wick, M. Platten, and M. Weller, "Glioma cell invasion: regulation of metalloproteinase activity by TGF-β," *Journal of Neuro-Oncology*, vol. 53, no. 2, pp. 177–185, 2001.

[47] Q. Yu and I. Stamenkovic, "Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis," *Genes and Development*, vol. 14, no. 2, pp. 163–176, 2000.

[48] J. C. Anderson, B. C. Mcfarland, and C. L. Gladson, "New molecular targets in angiogenic vessels of glioblastoma tumours," *Expert Reviews in Molecular Medicine*, vol. 10, article e23, 2008.

[49] Y.-A. Joe, Y.-K. Hong, D.-S. Chung, et al., "Inhibition of human malignant glioma growth in vivo by human recombinant plasminogen kringles 1–3," *International Journal of Cancer*, vol. 82, no. 5, pp. 694–699, 1999.

[50] M. Kirsch, J. Strasser, R. Allende, L. Bello, J. Zhang, and P. M. Black, "Angiostatin suppresses malignant glioma growth in vivo," *Cancer Research*, vol. 58, no. 20, pp. 4654–4659, 1998.

[51] J. C. Adams and J. Lawler, "The thrombospondins," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 6, pp. 961–968, 2004.

[52] R. Simantov and R. L. Silverstein, "CD36: a critical anti-angiogenic receptor," *Frontiers in Bioscience*, vol. 8, supplement 3, pp. s874–s882, 2003.

[53] J. C. Anderson, J. R. Grammer, W. Wang, et al., "ABT-510, a modified type 1 repeat peptide of thrombospondin, inhibits malignant glioma growth in vivo by inhibiting angiogenesis," *Cancer Biology and Therapy*, vol. 6, no. 3, pp. 454–462, 2007.

[54] H. M. Strik, M. Weller, B. Frank, et al., "Heat shock protein expression in human gliomas," *Anticancer Research*, vol. 20, no. 6 B, pp. 4457–4462, 2000.

[55] A. Sudhakar, H. Sugimoto, C. Yang, J. Lively, M. Zeisberg, and R. Kalluri, "Human tumstatin and human endostatin exhibit distinct antiangiogenic activities mediated by $\alpha v\beta$ and $\alpha 5\beta 1$ integrins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 8, pp. 4766–4771, 2003.

[56] A. Giese and M. Westphal, "Glioma invasion in the central nervous system," *Neurosurgery*, vol. 39, no. 2, pp. 235–252, 1996.

[57] A. Ernst, S. Hofmann, R. Ahmadi, et al., "Genomic and expression profiling of glioblastoma stem cell-like spheroid cultures identifies novel tumor-relevant genes associated with survival," *Clinical Cancer Research*, vol. 15, no. 21, pp. 6541–6550, 2009.

[58] D. A. Lim, S. Cha, M. C. Mayo, et al., "Relationship of glioblastoma multiforme to neural stem cell regions predicts invasive and multifocal tumor phenotype," *Neuro-Oncology*, vol. 9, no. 4, pp. 424–429, 2007.

[59] A. D. Norden, J. Drappatz, and P. Y. Wen, "Novel anti-angiogenic therapies for malignant gliomas," *The Lancet Neurology*, vol. 7, no. 12, pp. 1152–1160, 2008.

[60] T. N. Kreisl, L. Kim, K. Moore, et al., "Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma," *Journal of Clinical Oncology*, vol. 27, no. 5, pp. 740–745, 2009.

[61] J. J. Vredenburgh, A. Desjardins, J. E. Herndon II, et al., "Bevacizumab plus irinotecan in recurrent glioblastoma multiforme," *Journal of Clinical Oncology*, vol. 25, no. 30, pp. 4722–4729, 2007.

[62] A. Desjardins, D. A. Reardon, J. E. Herndon II, et al., "Bevacizumab plus irinotecan in recurrent WHO grade 3 malignant gliomas," *Clinical Cancer Research*, vol. 14, no. 21, pp. 7068–7073, 2008.

[63] P. H. Gutin, F. M. Iwamoto, K. Beal, et al., "Safety and efficacy of bevacizumab with hypofractionated stereotactic irradiation for recurrent malignant gliomas," *International Journal of Radiation Oncology Biology Physics*, vol. 75, no. 1, pp. 156–163, 2009.

[64] R. M. Zuniga, R. Torcuator, R. Jain, et al., "Efficacy, safety and patterns of response and recurrence in patients with recurrent high-grade gliomas treated with bevacizumab plus irinotecan," *Journal of Neuro-Oncology*, vol. 91, no. 3, pp. 329–336, 2009.

[65] W. K. A. Yung, R. E. Albright, J. Olson, et al., "A phase II study of temozolemide vs. procarbazine in patients with glioblastoma multiforme at first relapse," *British Journal of Cancer*, vol. 83, no. 5, pp. 588–593, 2000.

[66] E. C. Quant, A. D. Norden, J. Drappatz, et al., "Role of a second chemotherapy in recurrent malignant glioma patients who progress on bevacizumab," *Neuro-Oncology*, vol. 11, no. 5, pp. 550–555, 2009.

[67] S. Sathornsumetee, Y. Cao, J. E. Marcello, et al., "Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan," *Journal of Clinical Oncology*, vol. 26, no. 2, pp. 271–278, 2008.

[68] J. Y. Hsu and H. A. Wakelee, "Monoclonal antibodies targeting vascular endothelial growth factor: current status and future challenges in cancer therapy," *BioDrugs*, vol. 23, no. 5, pp. 289–304, 2009.

[69] A. D. Norden, G. S. Young, K. Setayesh, et al., "Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence," *Neurology*, vol. 70, no. 10, pp. 779–787, 2008.

[70] J.-P. Gagner, M. Law, I. Fischer, E. W. Newcomb, and D. Zagzag, "Angiogenesis in gliomas: imaging and experimental therapeutics," *Brain Pathology*, vol. 15, no. 4, pp. 342–363, 2005.

[71] J. Holash, S. Davis, N. Papadopoulos, et al., "VEGF-Trap: a VEGF blocker with potent antitumor effects," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 17, pp. 11393–11398, 2002.

[72] C. Gomez-Manzano, J. Holash, J. Fueyo, et al., "VEGF Trap induces antiglioma effect at different stages of disease," *Neuro-Oncology*, vol. 10, no. 6, pp. 940–945, 2008.

[73] Y. Krupitskaya and H. A. Wakelee, "Ramucirumab, a fully human mAb to the transmembrane signaling tyrosine kinase VEGFR-2 for the potential treatment of cancer," *Current Opinion in Investigational Drugs*, vol. 10, no. 6, pp. 597–605, 2009.

[74] A. Diaz Miqueli, J. Rolff, M. Lemm, I. Fichtner, R. Perez, and E. Montero, "Radiosensitisation of U87MG brain tumours by anti-epidermal growth factor receptor monoclonal antibodies," *British Journal of Cancer*, vol. 100, no. 6, pp. 950–958, 2009.

[75] V. Pillay, L. Allaf, A. L. Wilding, et al., "The plasticity of oncogene addiction: implications for targeted therapies directed to receptor tyrosine kinases," *Neoplasia*, vol. 11, no. 5, pp. 448–458, 2009.

[76] T. T. Batchelor, A. G. Sorensen, E. di Tomaso, et al., "AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients," *Cancer Cell*, vol. 11, no. 1, pp. 83–95, 2007.

[77] A. G. Sorensen, T. T. Batchelor, W.-T. Zhang, et al., "A "vascular normalization index" as potential mechanistic biomarker to predict survival after a single dose of cediranib in recurrent glioblastoma patients," *Cancer Research*, vol. 69, no. 13, pp. 5296–5300, 2009.

[78] B. Herrington and M. W. Kieran, "Small molecule inhibitors in children with malignant gliomas," *Pediatric Blood and Cancer*, vol. 53, no. 3, pp. 312–317, 2009.

[79] E. P. Jane, D. R. Premkumar, and I. F. Pollack, "Coadministration of sorafenib with rottlerin potently inhibits cell proliferation and migration in human malignant glioma cells," *Journal of Pharmacology and Experimental Therapeutics*, vol. 319, no. 3, pp. 1070–1080, 2006.

[80] D. Pouessel and S. Culine, "High frequency of intracerebral hemorrhage in metastatic renal carcinoma patients with brain metastases treated with tyrosine kinase inhibitors targeting the vascular endothelial growth factor receptor," *European Urology*, vol. 53, no. 2, pp. 376–381, 2008.

[81] S. de Boüard, P. Herlin, J. G. Christensen, et al., "Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma," *Neuro-Oncology*, vol. 9, no. 4, pp. 412–423, 2007.

[82] J. M. L. Ebos, C. R. Lee, W. Cruz-Munoz, G. A. Bjarnason, J. G. Christensen, and R. S. Kerbel, "Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis," *Cancer Cell*, vol. 15, no. 3, pp. 232–239, 2009.

[83] M. Pàez-Ribes, E. Allen, J. Hudock, et al., "Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis," *Cancer Cell*, vol. 15, no. 3, pp. 220–231, 2009.

[84] R. K. Goudar, Q. Shi, M. D. Hjelmeland, et al., "Combination therapy of inhibitors of epidermal growth factor receptor/vascular endothelial growth factor receptor 2 (AEE788) and the mammalian target of rapamycin (RAD001) offers improved glioblastoma tumor growth inhibition," *Molecular Cancer Therapeutics*, vol. 4, no. 1, pp. 101–112, 2005.

[85] E. Raymond, A. A. Brandes, C. Dittrich, et al., "Phase II study of imatinib in patients with recurrent gliomas of various histologies: a European organisation for research and treatment of cancer brain tumor group study," *Journal of Clinical Oncology*, vol. 26, no. 28, pp. 4659–4665, 2008.

[86] P. Y. Wen, W. K. A. Yung, K. R. Lamborn, et al., "Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08," *Clinical Cancer Research*, vol. 12, no. 16, pp. 4899–4907, 2006.

[87] D. A. Reardon, M. J. Egorin, A. Desjardins, et al., "Phase I pharmacokinetic study of the vascular endothelial growth factor receptor tyrosine kinase inhibitor vatalanib (PTK787) plus imatinib and hydroxyurea for malignant glioma," *Cancer*, vol. 115, no. 10, pp. 2188–2198, 2009.

[88] V. Milano, Y. Piao, T. Lafortune, and J. de Groot, "Dasatinib-induced autophagy is enhanced in combination with temozolomide in glioma," *Molecular Cancer Therapeutics*, vol. 8, no. 2, pp. 394–406, 2009.

[89] M. D. Prados, S. M. Chang, N. Butowski, et al., "Phase II study of erlotinib plus temozolomide during and after radiation therapy in patients with newly diagnosed glioblastoma multiforme or gliosarcoma," *Journal of Clinical Oncology*, vol. 27, no. 4, pp. 579–584, 2009.

[90] M. Fouladi, H. S. Nicholson, T. Zhou, et al., "A Phase II study of the farnesyl transferase inhibitor, tipifarnib, in children with recurrent or progressive high-grade glioma, medulloblastoma/primitive neuroectodermal tumor, or brainstem glioma: a children's oncology group study," *Cancer*, vol. 110, no. 11, pp. 2535–2541, 2007.

[91] N. Thatcher, A. Chang, P. Parikh, et al., "Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa Survival Evaluation in Lung Cancer)," *The Lancet*, vol. 366, no. 9496, pp. 1527–1537, 2005.

[92] T. N. Kreisl, A. B. Lassman, P. S. Mischel, et al., "A pilot study of everolimus and gefitinib in the treatment of recurrent glioblastoma (GBM)," *Journal of Neuro-Oncology*, vol. 92, no. 1, pp. 99–105, 2009.

[93] E. Franceschi, G. Cavallo, S. Lonardi, et al., "Gefitinib in patients with progressive high-grade gliomas: a multicentre phase II study by Gruppo Italiano Cooperativo di Neuro-Oncologia (GICNO)," *British Journal of Cancer*, vol. 96, no. 7, pp. 1047–1051, 2007.

[94] J. R. Graff, A. M. McNulty, K. R. Hanna, et al., "The protein kinase Cβ-selective inhibitor, Enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts," *Cancer Research*, vol. 65, no. 16, pp. 7462–7469, 2005.

[95] T. N. Kreisl, L. Kim, K. Moore, et al., "A phase I trial of enzastaurin in patients with recurrent gliomas," *Clinical Cancer Research*, vol. 15, no. 10, pp. 3617–3623, 2009.

[96] E. Galanis, J. C. Buckner, M. J. Maurer, et al., "Phase II trial of temsirolimus (CCI-779) in recurrent glioblastoma multiforme: a north central cancer treatment group study," *Journal of Clinical Oncology*, vol. 23, no. 23, pp. 5294–5304, 2005.

[97] M. L. H. Wong, A. Prawira, A. H. Kaye, and C. M. Hovens, "Tumour angiogenesis: its mechanism and therapeutic implications in malignant gliomas," *Journal of Clinical Neuroscience*, vol. 16, no. 9, pp. 1119–1130, 2009.

[98] H. A. Fine, P. Y. Wen, E. A. Maher, et al., "Phase II trial of thalidomide and carmustine for patients with recurrent high-grade gliomas," *Journal of Clinical Oncology*, vol. 21, no. 12, pp. 2299–2304, 2003.

[99] V. K. Puduvalli, P. Giglio, M. D. Groves, et al., "Phase II trial of irinotecan and thalidomide in adults with recurrent glioblastoma multiforme," *Neuro-Oncology*, vol. 10, no. 2, pp. 216–222, 2008.

[100] C. E. Fadul, L. S. Kingman, L. P. Meyer, et al., "A phase II study of thalidomide and irinotecan for treatment of glioblastoma multiforme," *Journal of Neuro-Oncology*, vol. 90, no. 2, pp. 229–235, 2008.

[101] S. Kesari, D. Schiff, J. W. Henson, et al., "Phase II study of temozolomide, thalidomide, and celecoxib for newly diagnosed glioblastoma in adults," *Neuro-Oncology*, vol. 10, no. 3, pp. 300–308, 2008.

[102] J. Drappatz, E. T. Wong, D. Schiff, et al., "A pilot safety study of lenalidomide and radiotherapy for patients with newly diagnosed glioblastoma multiforme," *International Journal of Radiation Oncology Biology Physics*, vol. 73, no. 1, pp. 222–227, 2009.

[103] D. Bissett, K. J. O'Byrne, J. von Pawel, et al., "Phase III study of matrix metalloproteinase inhibitor prinomastat in non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 23, no. 4, pp. 842–849, 2005.

[104] T. F. Cloughesy, P. Y. Wen, H. I. Robins, et al., "Phase II trial of tipifarnib in patients with recurrent malignant glioma either receiving or not receiving enzyme-inducing antiepileptic drugs: a North American Brain Tumor Consortium study," *Journal of Clinical Oncology*, vol. 24, no. 22, pp. 3651–3656, 2006.

[105] R. Lustig, T. Mikkelsen, G. Lesser, et al., "Phase II preradiation R115777 (tipifarnib) in newly diagnosed GBM with residual enhancing disease," *Neuro-Oncology*, vol. 10, no. 6, pp. 1004–1009, 2008.

[106] C. F. Deroanne, K. Bonjean, S. Servotte, et al., "Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling," *Oncogene*, vol. 21, no. 3, pp. 427–436, 2002.

[107] E. Galanis, K. A. Jaeckle, M. J. Maurer, et al., "Phase II trial of Vorinostat in recurrent glioblastoma multiforme: a north central cancer treatment group study," *Journal of Clinical Oncology*, vol. 27, no. 12, pp. 2052–2058, 2009.

[108] S. H. Kim, S. Ahn, J.-W. Han, et al., "Apicidin is a histone deacetylase inhibitor with anti-invasive and anti-angiogenic potentials," *Biochemical and Biophysical Research Communications*, vol. 315, no. 4, pp. 964–970, 2004.

[109] M. W. Kieran, C. D. Turner, J. B. Rubin, et al., "A feasibility trial of antiangiogenic (metronomic) chemotherapy in pediatric patients with recurrent or progressive cancer," *Journal of Pediatric Hematology/Oncology*, vol. 27, no. 11, pp. 573–581, 2005.

[110] A. Broniscer, S. J. Baker, C. F. Stewart, et al., "Phase I and pharmacokinetic studies of erlotinib administered concurrently with radiotherapy for children, adolescents, and young adults with high-grade glioma," *Clinical Cancer Research*, vol. 15, no. 2, pp. 701–707, 2009.

[111] I. F. Pollack, R. I. Jakacki, S. M. Blaney, et al., "Phase I trial of imatinib in children with newly diagnosed brainstem and recurrent malignant gliomas: a Pediatric Brain Tumor Consortium report," *Neuro-Oncology*, vol. 9, no. 2, pp. 145–160, 2007.

[112] D. A. Haas-Kogan, A. Banerjee, M. Kocak, et al., "Phase I trial of tipifarnib in children with newly diagnosed intrinsic diffuse brainstem glioma," *Neuro-Oncology*, vol. 10, no. 3, pp. 341–347, 2008.

[113] M. W. Kieran, J. G. Supko, D. Wallace, et al., "Phase I study of SU5416, a small molecule inhibitor of the vascular endothelial growth factor receptor (VEGFR) in refractory pediatric central nervous system tumors," *Pediatric Blood and Cancer*, vol. 52, no. 2, pp. 169–176, 2009.

[114] K. D. Miller, C. J. Sweeney, and G. W. Sledge Jr., "Can tumor angiogenesis be inhibited without resistance?" *EXS*, no. 94, pp. 95–112, 2005.

[115] G. Bergers and D. Hanahan, "Modes of resistance to anti-angiogenic therapy," *Nature Reviews Cancer*, vol. 8, no. 8, pp. 592–603, 2008.

[116] J. Glade Bender, E. M. Cooney, J. J. Kandel, and D. J. Yamashiro, "Vascular remodeling and clinical resistance to antiangiogenic cancer therapy," *Drug Resistance Updates*, vol. 7, no. 4-5, pp. 289–300, 2004.

[117] R. S. Kerbel, "Clinical trials of antiangiogenic drugs: opportunities, problems, and assessment of initial results," *Journal of Clinical Oncology*, vol. 19, supplement 18, pp. 45S–51S, 2001.

[118] P. Kunkel, U. Ulbricht, P. Bohlen, et al., "Inhibition of glioma angiogenesis and growth in vivo by systemic treatment with a monoclonal antibody against vascular endothelial growth factor receptor-2," *Cancer Research*, vol. 61, no. 18, pp. 6624–6628, 2001.

[119] J. L. Rubenstein, J. Kim, T. Ozawa, et al., "Anti-VEGF antibody treatment of glioblastoma prolongs survival but results in increased vascular cooption," *Neoplasia*, vol. 2, no. 4, pp. 306–314, 2000.

[120] A. K. Lucio-Eterovic, Y. Piao, and J. F. de Groot, "Mediators of glioblastoma resistance and invasion during antivascular endothelial growth factor therapy," *Clinical Cancer Research*, vol. 15, no. 14, pp. 4589–4599, 2009.

[121] S. Song, A. J. Ewald, W. Stallcup, Z. Werb, and G. Bergers, "PDGFRβ^+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival," *Nature Cell Biology*, vol. 7, no. 9, pp. 870–879, 2005.

[122] R. Du, K. V. Lu, C. Petritsch, et al., "HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion," *Cancer Cell*, vol. 13, no. 3, pp. 206–220, 2008.

[123] B. Blouw, H. Song, T. Tihan, et al., "The hypoxic response of tumors is dependent on their microenvironment," *Cancer Cell*, vol. 4, no. 2, pp. 133–146, 2003.

[124] S. Bao, Q. Wu, S. Sathornsumetee, et al., "Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor," *Cancer Research*, vol. 66, no. 16, pp. 7843–7848, 2006.

[125] C. Folkins, Y. Shaked, S. Man, et al., "Glioma tumor stem-like cells promote tumor angiogenesis and vasculogenesis via vascular endothelial growth factor and stromal-derived factor 1," *Cancer Research*, vol. 69, no. 18, pp. 7243–7251, 2009.

[126] J. M. Heddleston, Z. Li, R. E. McLendon, A. B. Hjelmeland, and J. N. Rich, "The hypoxic microenvironment maintains glioblastoma stem cells and promotes reprogramming towards a cancer stem cell phenotype," *Cell Cycle*, vol. 8, no. 20, pp. 3274–3284, 2009.

[127] A. Soeda, M. Park, D. Lee, et al., "Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α," *Oncogene*, vol. 28, no. 45, pp. 3949–3959, 2009.

[128] D. S. Tews, A. Nissen, C. Kulgen, and A. K. A. Gaumann, "Drug resistance-associated factors in primary and secondary glioblastomas and their precursor tumors," *Journal of Neuro-Oncology*, vol. 50, no. 3, pp. 227–237, 2000.

[129] L. Patrawala, T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, and D. G. Tang, "Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2$^+$ and ABCG2$^-$ cancer cells are similarly tumorigenic," *Cancer Research*, vol. 65, no. 14, pp. 6207–6219, 2005.

[130] Z. Li, S. Bao, Q. Wu, et al., "Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells," *Cancer Cell*, vol. 15, no. 6, pp. 501–513, 2009.

[131] J. T. Huse, C. Brennan, D. Hambardzumyan, et al., "The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo," *Genes and Development*, vol. 23, no. 11, pp. 1327–1337, 2009.

[132] T. Sasayama, M. Nishihara, T. Kondoh, K. Hosoda, and E. Kohmura, "MicroRNA-10b is overexpressed in malignant glioma and associated with tumor invasive factors, uPAR and RhoC," *International Journal of Cancer*, vol. 125, no. 6, pp. 1407–1413, 2009.

[133] H. Xia, Y. Qi, S. S. Ng, et al., "microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs," *Brain Research*, vol. 1269, no. C, pp. 158–165, 2009.

[134] S. Lawler and E. A. Chiocca, "Emerging functions of microRNAs in glioblastoma," *Journal of Neuro-Oncology*, vol. 92, no. 3, pp. 297–306, 2009.

[135] P. A. Northcott, A. Fernandez-L, J. P. Hagan, et al., "The miR-17/92 polycistron is up-regulated in sonic hedgehog-driven medulloblastomas and induced by N-myc in sonic hedgehog-treated cerebellar neural precursors," *Cancer Research*, vol. 69, no. 8, pp. 3249–3255, 2009.

[136] B. Kefas, J. Godlewski, L. Comeau, et al., "microRNA-7 inhibits the epidermal growth factor receptor and the akt pathway and is down-regulated in glioblastoma," *Cancer Research*, vol. 68, no. 10, pp. 3566–3572, 2008.

[137] L. Yunqing, F. Guessous, Z. Ying, et al., "MicroRNA-34a inhibits glioblastoma growth by targeting multiple oncogenes," *Cancer Research*, vol. 69, no. 19, pp. 7569–7576, 2009.

[138] T. Papagiannakopoulos, A. Shapiro, and K. S. Kosik, "MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells," *Cancer Research*, vol. 68, no. 19, pp. 8164–8172, 2008.

[139] R. Corbin, K. Olsson-Carter, and F. Slack, "The role of microRNAs in synaptic development and function," *BMB Reports*, vol. 42, no. 3, pp. 131–135, 2009.

[140] A. E. Erson and E. M. Petty, "MicroRNAs in development and disease," *Clinical Genetics*, vol. 74, no. 4, pp. 296–306, 2008.

[141] S. Wang, A. B. Aurora, B. A. Johnson, et al., "The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis," *Developmental Cell*, vol. 15, no. 2, pp. 261–271, 2008.

[142] Z. Hua, Q. Lv, W. Ye, et al., "Mirna-directed regulation of VEGF and other angiogenic under hypoxia," *PLoS One*, vol. 1, no. 1, article e116, 2006.

[143] J. Krützfeldt, N. Rajewsky, R. Braich, et al., "Silencing of microRNAs in vivo with 'antagomirs'," *Nature*, vol. 438, no. 7068, pp. 685–689, 2005.

[144] J. Kota, R. R. Chivukula, K. A. O'Donnell, et al., "Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model," *Cell*, vol. 137, no. 6, pp. 1005–1017, 2009.

[145] T. Würdinger, B. A. Tannous, O. Saydam, et al., "miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells," *Cancer Cell*, vol. 14, no. 5, pp. 382–393, 2008.

[146] B. D. Brown and L. Naldini, "Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications," *Nature Reviews Genetics*, vol. 10, no. 8, pp. 578–585, 2009.

[147] H. Gal, G. Pandi, A. A. Kanner, et al., "MIR-451 and Imatinib mesylate inhibit tumor growth of Glioblastoma stem cells," *Biochemical and Biophysical Research Communications*, vol. 376, no. 1, pp. 86–90, 2008.

[148] K. E. Uhrich, S. M. Cannizzaro, R. S. Langer, and K. M. Shakesheff, "Polymeric systems for controlled drug release," *Chemical Reviews*, vol. 99, no. 11, pp. 3181–3198, 1999.

[149] A. J. Sawyer, J. M. Piepmeier, and W. M. Saltzman, "New methods for direct delivery of chemotherapy for treating brain tumors," *Yale Journal of Biology and Medicine*, vol. 79, no. 3-4, pp. 141–152, 2006.

[150] A. B. Fleming and W. M. Saltzman, "Pharmacokinetics of the carmustine implant," *Clinical Pharmacokinetics*, vol. 41, no. 6, pp. 403–419, 2002.

[151] F. J. Attenello, D. Mukherjee, G. Datoo, et al., "Use of Gliadel (BCNU) wafer in the surgical treatment of malignant glioma: a 10-year institutional experience," *Annals of Surgical Oncology*, vol. 15, no. 10, pp. 2887–2893, 2008.

[152] H. Seong, T. K. An, G. Khang, S.-U. Choi, C. O. Lee, and H. B. Lee, "BCNU-loaded poly(D, L-lactide-co-glycolide) wafer and antitumor activity against XF-498 human CNS tumor cells in vitro," *International Journal of Pharmaceutics*, vol. 251, no. 1-2, pp. 1–12, 2003.

[153] M. J. Alonso, R. K. Gupta, C. Min, G. R. Siber, and R. Langer, "Biodegradable microspheres as controlled-release tetanus toxoid delivery systems," *Vaccine*, vol. 12, no. 4, pp. 299–306, 1994.

[154] J. Mullerad, S. Cohen, E. Voronov, and R. N. Apte, "Macrophage activation for the production of immunostimulatory cytokines by delivering interleukin 1 via biodegradable microspheres," *Cytokine*, vol. 12, no. 11, pp. 1683–1690, 2000.

[155] V.-G. Roullin, L. Lemaire, M.-C. Venier-Julienne, N. Faisant, F. Franconi, and J.-P. Benoit, "Release kinetics of 5-fluorouracil-loaded microspheres on an experimental rat glioma," *Anticancer Research*, vol. 23, no. 1A, pp. 21–25, 2003.

[156] A. Sanchez, R. K. Gupta, M. J. Alonso, G. R. Siber, and R. Langer, "Pulsed controlled-release system for potential use in vaccine delivery," *Journal of Pharmaceutical Sciences*, vol. 85, no. 6, pp. 547–552, 1996.

[157] J. M. Anderson and M. S. Shive, "Biodegradation and biocompatibility of PLA and PLGA microspheres," *Advanced Drug Delivery Reviews*, vol. 28, no. 1, pp. 5–24, 1997.

[158] D. R. Groothuis, "The blood-brain and blood-tumor barriers: a review of strategies for increasing drug delivery," *Neuro-Oncology*, vol. 2, no. 1, pp. 45–49, 2000.

[159] P. P. Wang, J. Frazier, and H. Brem, "Local drug delivery to the brain," *Advanced Drug Delivery Reviews*, vol. 54, no. 7, pp. 987–1013, 2002.

[160] O. Benny, M. Duvshani-Eshet, T. Cargioli, et al., "Continuous delivery of endogenous inhibitors from poly (lactic-co-glycolic acid) polymeric microspheres inhibits glioma tumor growth," *Clinical Cancer Research*, vol. 11, no. 2, pp. 768–776, 2005.

[161] M. E. Gil-Alegre, I. Gonzalez-Alvarez, L. Gutierrez-Pauls, and A. I. Torres-Suarez, "Three weeks release BCNU loaded hydrophilic-PLGA microspheres for interstitial chemotherapy: development and activity against human glioblastoma cells," *Journal of Microencapsulation*, vol. 25, no. 8, pp. 561–568, 2008.

[162] J. Kreuter, "Nanoparticulate systems for brain delivery of drugs," *Advanced Drug Delivery Reviews*, vol. 47, no. 1, pp. 65–81, 2001.

[163] P. Calvo, B. Gouritin, H. Chacun, et al., "Long-circulating pegylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery," *Pharmaceutical Research*, vol. 18, no. 8, pp. 1157–1166, 2001.

[164] T. M. Goppert and R. H. Muller, "Polysorbate-stabilized solid lipid nanoparticles as colloidal carriers for intravenous targeting of drugs to the brain: comparison of plasma protein adsorption patterns," *Journal of Drug Targeting*, vol. 13, no. 3, pp. 179–187, 2005.

[165] J. Kreuter, D. Shamenkov, V. Petrov, et al., "Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood-brain barrier," *Journal of Drug Targeting*, vol. 10, no. 4, pp. 317–325, 2002.

[166] S. C. J. Steiniger, J. Kreuter, A. S. Khalansky, et al., "Chemotherapy of glioblastoma in rats using doxorubicin-loaded nanoparticles," *International Journal of Cancer*, vol. 109, no. 5, pp. 759–767, 2004.

[167] A. Ambruosi, S. Gelperina, A. Khalansky, S. Tanski, A. Theisen, and J. Kreuter, "Influence of surfactants, polymer and doxorubicin loading on the anti-tumour effect of poly(butyl cyanoacrylate) nanoparticles in a rat glioma model," *Journal of Microencapsulation*, vol. 23, no. 5, pp. 582–592, 2006.

[168] B. Petri, A. Bootz, A. Khalansky, et al., "Chemotherapy of brain tumour using doxorubicin bound to surfactant-coated poly(butyl cyanoacrylate) nanoparticles: revisiting the role of surfactants," *Journal of Controlled Release*, vol. 117, no. 1, pp. 51–58, 2007.

[169] S. Gelperina, O. Maksimenko, A. Khalansky, et al., "Drug delivery to the brain using surfactant-coated poly(lactide-co-glycolide) nanoparticles: influence of the formulation parameters," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 74, no. 2, pp. 157–163, 2010.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 961243, 8 pages
doi:10.1155/2010/961243

Review Article

Ganglioside GM3 Is Antiangiogenic in Malignant Brain Cancer

Thomas N. Seyfried and Purna Mukherjee

Department of Biology, Boston College, Chestnut Hill, MA 02467, USA

Correspondence should be addressed to Thomas N. Seyfried, thomas.seyfried@bc.edu

Received 17 December 2009; Revised 12 March 2010; Accepted 10 April 2010

Academic Editor: Arkadiusz Dudek

Progression of malignant brain tumors is dependent upon vascularity and is associated with altered ganglioside composition and distribution. Evidence is reviewed showing that the simple monosialoganglioside, GM3, possesses powerful antiangiogenic action against the highly vascularized CT-2A mouse astrocytoma, which primarily expresses complex gangliosides. Brain tumors expressing high levels of GM3 are generally less vascularized and grow slower than tumors that express low levels of GM3. GM3 inhibits angiogenesis through autocrine and paracrine effects on vascular endothelial growth factor (VEGF) and associated receptors. GM3 should be a clinically useful compound for managing brain tumor angiogenesis.

1. Introduction

Malignant brain cancer persists as a catastrophic illness and is the second leading cause of cancer death in children [1–4]. The failure to effectively manage malignant brain cancer has been due in large part to the highly invasive nature of the disease and to the unique anatomical and metabolic environment of the brain, which prevents the large-scale resection of tumor tissue and impedes the delivery of therapeutic drugs. Invasion/metastasis involves the dissemination of tumor cells from the primary neoplasm to surrounding tissue and distant regions. In addition, the invasive cells establish a microenvironment facilitating colonization (angiogenesis and further proliferation), resulting in macroscopic malignant secondary tumors [5, 6]. Tumor cell invasion is correlated with tumor angiogenesis (vascularity), as prognosis is generally worse for brain tumors that are more vascular than for those that are less vascular [7–9]. Consequently, therapies that can simultaneously target both angiogenesis and invasion could provide effective longer-term management of malignant brain cancer.

2. Glycosphingolipids and Angiogenesis

Gangliosides are a family of cell surface-enriched glycosphingolipids that have long been implicated in tumorigenesis [10–12]. These molecules contain an oligosaccharide head group attached to a lipophilic ceramide, consisting of a sphingosine base and a long-chain fatty acid (Figure 1). The presence of sialic acid (N-acetylneuraminic acid, NeuAc) distinguishes the gangliosides from other glycosphingolipids. Gangliosides are anchored in the outer surface of plasma membranes through their ceramide moiety, which allows the head group to modulate numerous cell surface events such as growth, migration, adhesion, and signaling [12–15].

The structurally simple monosialoganglioside GM3 contains a single terminal sialic acid (Figure 1). N-acetylneuraminic acid is the predominant sialic acid species expressed in mammalian brain gangliosides [16, 17]. In contrast to N-acetylneuraminic acid, N-glycolylneuraminic acid is a predominant sialic acid species expressed in gangliosides from nonneural tissues of most nonhuman species (rodents, bovine, etc.) [17]. As humans lack the gene for the synthesis of N-glycolylneuraminic acid [18, 19], expression of N-glycolylneuraminic acid in gangliosides of human cells or tissues is attributed to contamination from exposure to nonhuman serum or from diet [17, 20, 21]. The involvement of gangliosides in angiogenesis is dependent on the intact molecules as neither asialo species nor sialic alone influence angiogenesis [22].

GM3 modulates the function of several receptors implicated with angiogenesis to include those for the insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (b-FGF), epidermal growth factor (EGF), platelet-derived

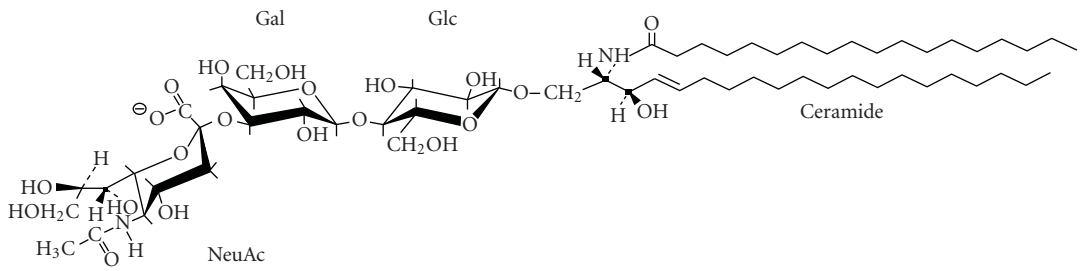


Figure 1: Structure of Ganglioside GM3 (NeuAc-alpha 2→ 3Gal-beta1 → 4Glc-beta1 → 1′ Ceramide) (from [14] with permission).

Table 1: Association of GM3 levels to the vascularity of experimental brain *tumors**.

	Ganglioside Distribution		Vascularity
Brain Tumors	GM3	Complex Gangliosides	
Mouse			
EPEN	High	Low	Low
CBT-1	High	Low	Low
CBT-3	High	Low	Low
CBT-4	Low	high	High
CT-2	Low	High	High
CT-2A	Low	High	High
Human			
U87MG	Low	High	High

*All mouse brain tumors were produced from implantation of 20-methylcholantherene into the ventricle (EPEN), the cerebrum (CT), or the cerebellum (CBT) of C57BL/6J mice as we previously described [43].

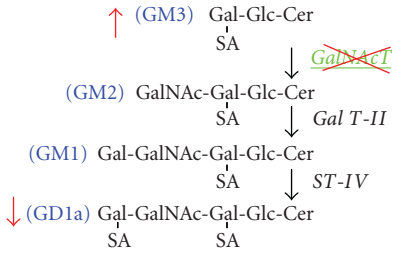


Figure 2: Pathway for the synthesis of ganglioside GM2 from GM3 by Gal*N*Ac-T. Gal*N*Ac-T adds a beta-linked *N*-acetylgalactosamine residue to the galactose of GM3 to form GM2, a key step required for the synthesis of complex gangliosides, GM2, GM1, and GD1a. Antisense targeting of the *GalNAc-T* gene reduces GD1a content, while increasing GM3 content [7].

growth factor (PDGF), vascular endothelial growth factor (VEGF), and cell adhesion molecules including the integrins [7, 12, 23–28]. GM3 also reduces proliferation and enhances apoptosis of rapidly proliferating neural stem cells [29]. Furthermore, Alessandri, Ziche, and coworkers originally found that several complex gangliosides (GM2, GM1, GD3, GD1a, GD1b, and GT1b) enhanced the action of angiogenic inducers, whereas ganglioside GM3 was inhibitory [30–32]. These observations suggest that GM3 could have therapeutic potential against tumor cell proliferation and angiogenesis.

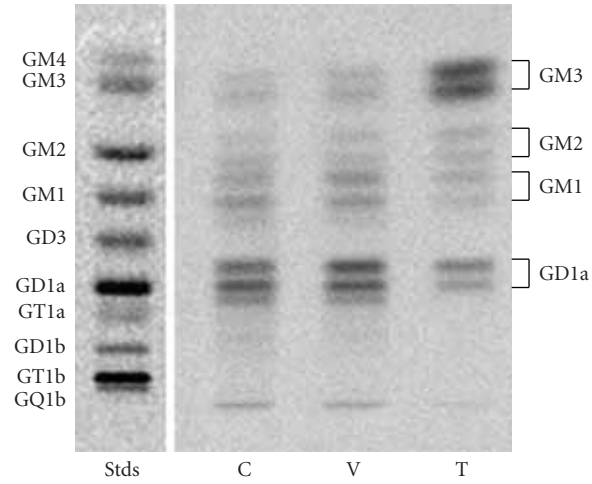


Figure 3: High-performance thin-layer chromatographic analysis of ganglioside distribution in the CT-2A astrocytoma: Control untransfected CT-2A (C), CT-2A transfected with empty vector alone (V), and CT-2A transfected with the antisense sequence to the *GalNAc-T* gene (T). Synthesized gangliosides appear as double bands due to ceramide structural heterogeneity. Analysis of synthesized gangliosides and standards (left lane) was as we described [7]. Knockdown of the *GalNAc-T* gene elevated GM3 content, while reducing GD1a content in the antisense T cells.

The ratio of GM3 to the proangiogenic gangliosides GD3 and GD1a (GM3/GD3; GM3/GD1a) is lower in more metastatic and aggressive tumors than that in less metastatic tumors [7, 33–35], suggesting that elevated expression of complex gangliosides enhances tumor malignancy. In contrast to most human glioma tumor tissues, which contain high levels of the pro-angiogenic ganglioside GD3 [36–41], GD3 is not heavily expressed in mouse brain tumors or in most cultured human brain tumor cells [17, 42–44]. Although GM3 is also expressed in malignant human brain tumors, we think that GM3 expression in these tumors might serve to regulate or to counteract the pro-angiogenic action of GD3 and other complex gangliosides.

3. Evidence Supporting the Anti-Angiogenic Action of GM3 in Brain Cancer

Table 1 summarizes data from our previous studies on the association of GM3 expression with the angiogenic

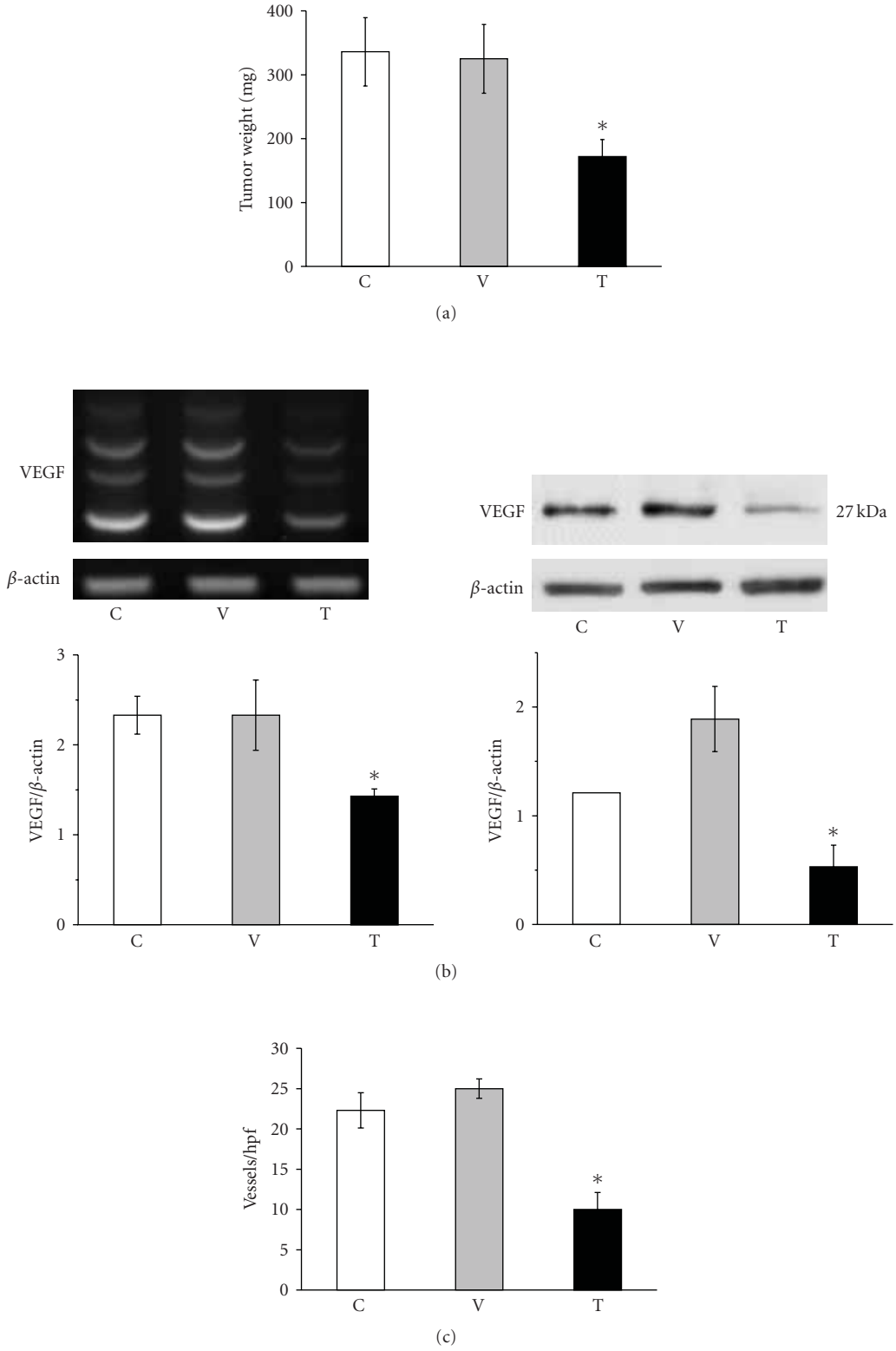


Figure 4: Ganglioside shift reduces growth, VEGF gene and protein expression, and vascularity in the CT-2A astrocytoma: Control untransfected CT-2A (C), CT-2A transfected with empty vector alone (V), and CT-2A transfected with the antisense sequence to the *GalNAc-T* gene (T). (a) The values are expressed as mean mg wet weight ± SE. *: significant compared to control C and V tumors at the $P < .01$ level. CT-2A ($n = 10$), CT-2A/V ($n = 12$), and CT-2A/TNG ($n = 14$) independent tumors. (b) RT-PCR and Western blot for VEGF. Other details are in [7]. (c) Vascularity determined using factor VIII-immunostained microvessels per ×200 field (hpf, high-powered field) from tissue sections as we described [7].

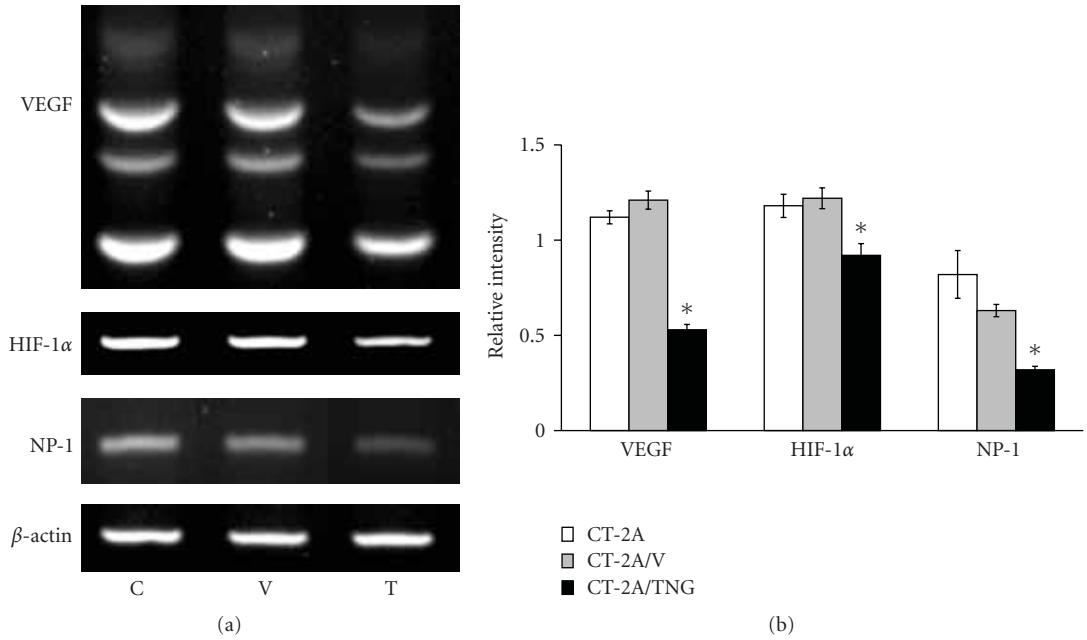


Figure 5: Ganglioside shift reduces VEGF, HIF-1α, and NP-1 gene expression in CT-2A- cultured cells: Control untransfected CT-2A (C), CT-2A transfected with empty vector alone (V), and CT-2A transfected with the anti-sense sequence to (a) the *GalNAc-T* gene (T). VEGF (multiple splice variants: 400–600 bp), HIF-1α (365 bp), and NP-1 (551 bp) amplification products were detected in each tumor cell line. Experimental conditions are as we described [7]. The gene to β-actin levels are expressed as the means of three independent samples ± SE.*: Significant compared to control C and V cells at (b) the $P < .01$.

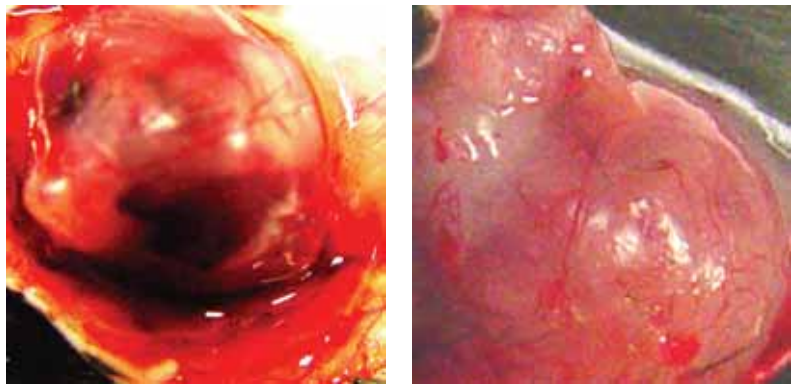


Figure 6: GM3 reduces CT-2A tumor vascularity when added to the tumor microenvironment. Small fragments of the CT-2A tumor were grown in Matrigel that contained either no GM3 (control) or GM3 (40 μM). The tumor was grown in Matrigel for approximately two weeks in the flank of the syngeneic host C57BL/6 mice according to our standard procedures [7, 34]. Florid vascularization and the number and size thrombotic vessels were noticeably less in the presence than in the absence of GM3. Similar results were found in two independent experiments.

properties of multiple experimental mouse and human brain tumor models [17, 44, 45]. This survey shows that brain tumors with high GM3 expression are less angiogenic (vascularized) than brain tumors with low GM3 expression. GM3 expression was also correlated with greater cell-cell adhesion and slower growth [14, 44]. We later showed that the gene-linked knockdown of GM3 expression in the experimental ependymoblastoma (EPEN) tumor, which contains GM3 as the only ganglioside, increased vascularity (angiogenesis) [34]. An opposite effect was observed in the highly angiogenic CT-2A astrocytoma when we upregulated GM3 expression (below). These and other findings led us to conclude that the ratio of GM3 to complex gangliosides (GM1, GD1a, GT1b) can influence the angiogenic properties

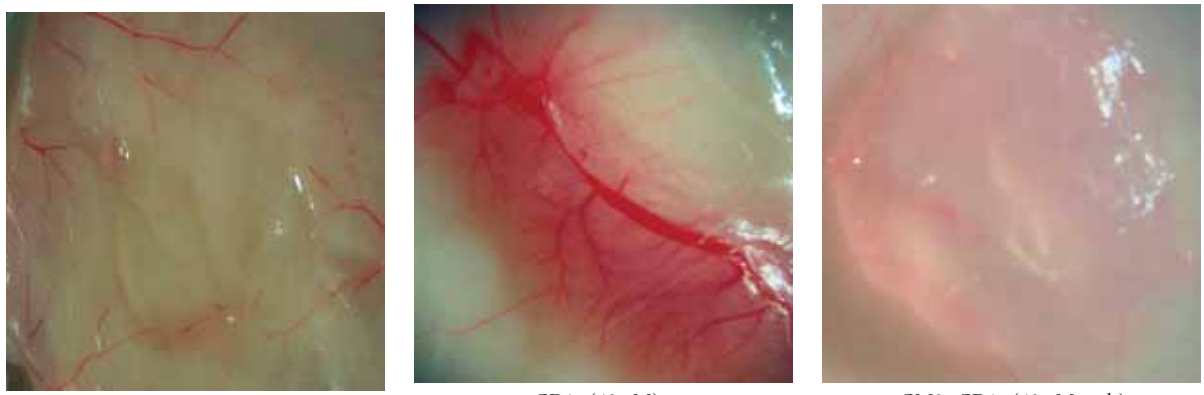


Figure 7: GM3 inhibits the pro-angiogenic effects of GD1a in the in vivo Matrigel assay. Matrigel alone (control) or containing GD1a or GD1a with GM3 was injected subcutaneously (s.c.) in SCID mice as we described [49]. Plugs were photographed (12.5×) on day 7 after Matrigel injection.

of a broad range of brain tumor types and are consistent with previous findings on the role of GM3 in other systems [22, 29, 32, 41, 46–49].

4. Anti-Angiogenic Action of GM3 in the CT-2A Astrocytoma

The CT-2A astrocytoma was produced following implantation of the chemical carcinogen, 20-methylcholanthrene, into the cerebral cortex of C57BL/6J mouse according to the procedures of Zimmerman and Arnold [44, 50]. The CT-2A tumor grows rapidly, is deficient in the phosphatase and tensin homologue/tuberous sclerosis complex 2, and is highly angiogenic [7, 51, 52]. We used an antisense construct to inhibit GalNAc-T expression in CT-2A cells as shown in (Figure 2). This caused a significant shift in ganglioside distribution, elevating GM3 content while reducing GD1a content (Figure 3).

The shift in ganglioside distribution significantly reduced growth, VEGF gene and protein expression, and blood vessel density in the orthotopically grown CT-2A tumors (Figure 4). Moreover, the shift in ganglioside distribution reduced gene expression for hypoxia inducible factor 1a (HIF-1α) and the VEGF coreceptor neruropilin-1 (NP-1) in the CT-2A cultured cells (Figure 5). This is interesting as HIF-1α is a transcription factor that regulates VEGF expression through the PI-3k/Akt signaling pathway [51, 53–55]. Viewed collectively, these data show that endogenous upregulation of GM3 reduces growth and angiogenesis in the rapidly growing and highly vascularized CT-2A mouse astrocytoma.

It was initially unclear, however, whether it was the elevation of GM3, the reduction of the pro-angiogenic ganglioside GD1a, or the change in GM3/GD1a ratio that was responsible for the reduction in CT-2A angiogenesis. It is well documented that gangliosides are shed from tumor cells into the microenvironment where stromal (endothelial) cells take them up to influence tumor progression [56–60]. Our most recent findings show that GM3, by itself, markedly reduces CT-2A vascularity when grown in the in vivo Matrigel model (Figure 6). These findings suggest that GM3 could be applied as a drug therapy directly to the tumor site and to surrounding areas following surgical tumor resection in humans. Alternatively, GM3 could be applied in liposomes as a pharmacotherapy for preformed tumors. Our findings in brain tumor cells are also consistent with previous findings in rabbit cornea showing that GM3 applied directly to tissue is anti-angiogenic [32]. Viewed, collectively, our findings indicate that GM3 has powerful anti-angiogenic action against the CT-2A astrocytoma when present in the microenvironment and can counteract the pro-angiogenic effects of complex gangliosides.

Further evidence for a direct anti-angiogenic role of GM3 came from our recent studies with human umbilical vein endothelial cells, HUVEC. We found that GM3, by itself, significantly suppresses VEGF-induced proliferation and migration of HUVEC [49]. Moreover, GM3 significantly blocks GD1a-induced angiogenesis in the in vivo Matrigel assay (Figure 7). GD1a is a complex ganglioside associated with enhanced angiogenesis [7, 61]. The suppression of VEGF receptor 2 and Akt phosphorylation underlies the anti-angiogenic effect of GM3 on HUVEC (Figure 8). Additionally, the EPEN tumor, which expresses only GM3, has few blood vessels relative to tumors that express complex gangliosides [44, 45]. Consistent with our findings, Chung and coworkers recently showed that GM3 could suppress angiogenesis through the inactivation of VEGF-induced signaling by direct interaction with VEGFR-2 [47]. GM3 treatment could also reduce in vivo vascularity in the Lewis lung carcinoma model [47], while van Cruijsen et al. showed that vascularity was less and patient survival was better for non-small cell lung carcinomas that contained more GM3 than less GM3 [62]. Hence, GM3 is anti-angiogenic through its inhibition of the proangiogenic actions of complex gangliosides as well as through its direct inhibition of endothelial cell growth.

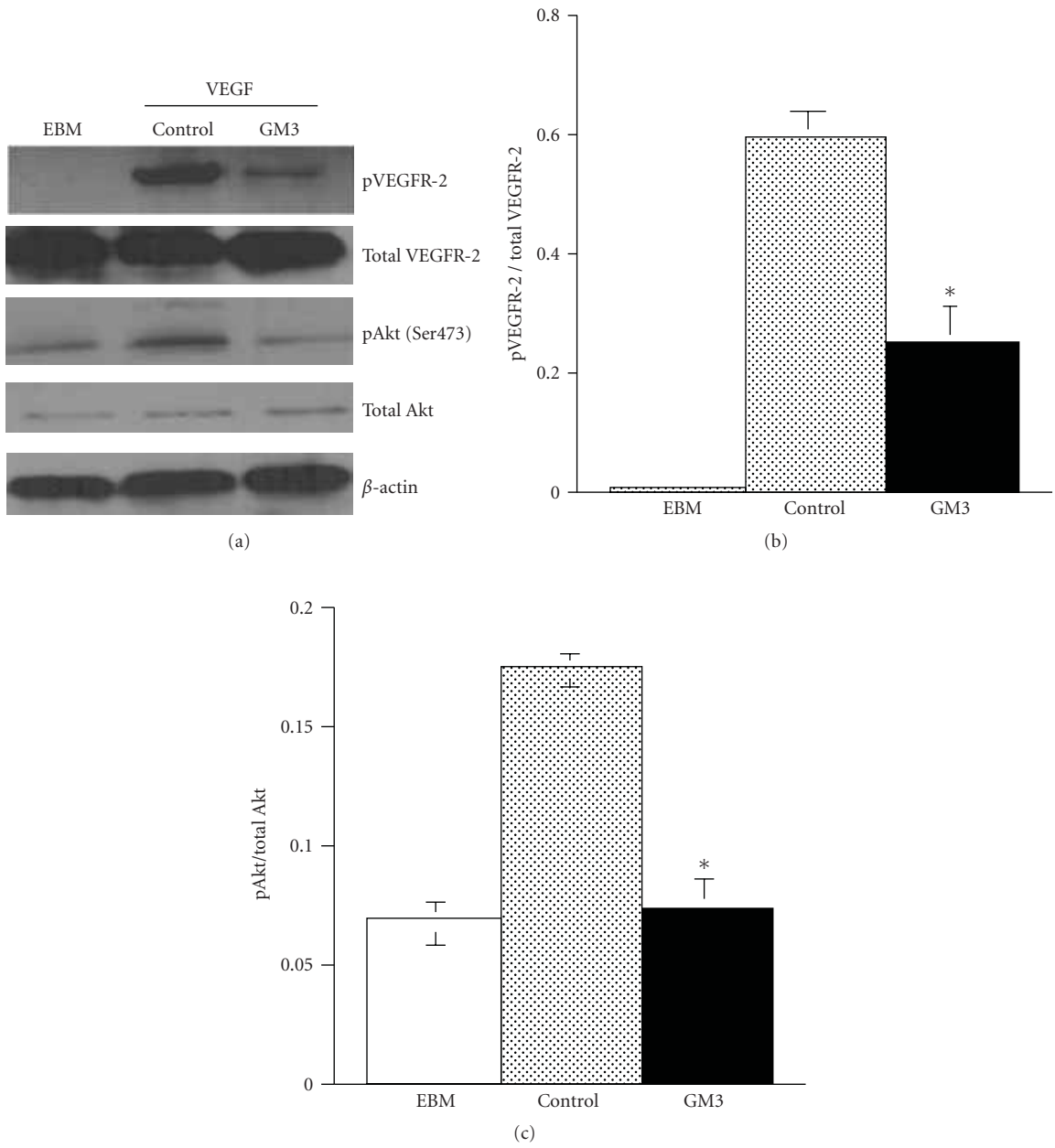


Figure 8: GM3 inhibits VEGFR-2 and Akt phosphorylation in HUVEC. HUVECs were incubated with GM3 (100 ng/ml) in endothelial basal medium (EBM) for 24 hours and were then stimulated with VEGF (100 ng/ml) for 5 minutes as we described [49]. (a) Cell lysates were prepared and measurement of VEGFR-2 and Akt phosphorylation over total was analyzed using Western blots [49]. (b) VEGFR-2 and (c) Akt phosphorylation were significantly lower in GM3-treated HUVEC than in control HUVEC ($P < .001$). Values are expressed as means ± SEM ($n = 3$ independent experiments).

In summary, our results show that GM3 inhibits brain tumor angiogenesis. GM3 targets both tumor cells and endothelial cells. Although GM3 is elevated in human malignant brain tumors, its concentration is less than that of complex gangliosides especially GD3. We suggest that increasing the ratio of GM3 to complex gangliosides may be effective in reducing angiogenesis and growth in human glioblastomas. Our findings suggest that pharmacological application of GM3 is warranted as a potential nontoxic anti-angiogenic therapy for malignant brain cancer.

References

[1] J. K. Lowry, J. J. Snyder, and P. W. Lowry, "Brain tumors in the elderly: recent trends in a Minnesota cohort study," *Archives of Neurology*, vol. 55, no. 7, pp. 922–928, 1998.

[2] J. Kaiser, "No meeting of minds on childhood cancer," *Science*, vol. 286, no. 5446, pp. 1832–1834, 1999.

[3] P. Kaatsch, C. H. Rickert, J. Kühl, J. Schüz, and J. Michaelis, "Population-based epidemiologic data on brain tumors in German children," *Cancer*, vol. 92, no. 12, pp. 3155–3164, 2001.

[4] P. J. Jukich, B. J. McCarthy, T. S. Surawicz, S. Freels, and F. G. Davis, "Trends in incidence of primary brain tumors in the United States, 1985–1994," *Neuro-Oncology*, vol. 3, no. 3, pp. 141–151, 2001.

[5] H. Sontheimer, "A role for glutamate in growth and invasion of primary brain tumors," *Journal of Neurochemistry*, vol. 105, no. 2, pp. 287–295, 2008.

[6] C.-L. Tso, P. Shintaku, J. Chen, et al., "Primary glioblastomas express mesenchymal stem-like properties," *Molecular Cancer Research*, vol. 4, no. 9, pp. 607–619, 2006.

[7] L. E. Abate, P. Mukherjee, and T. N. Seyfried, "Gene-linked shift in ganglioside distribution influences growth and vascularity in a mouse astrocytoma," *Journal of Neurochemistry*, vol. 98, no. 6, pp. 1973–1984, 2006.

[8] D. Zagzag, M. Esencay, O. Mendez, et al., "Hypoxia- and vascular endothelial growth factor-induced stromal cell-derived factor-1α/CXCR4 expression in glioblastomas: one plausible explanation of Scherer's structures," *American Journal of Pathology*, vol. 173, no. 2, pp. 545–560, 2008.

[9] M. Kirsch, G. Schackert, and P. M. Black, "Anti-angiogenic treatment strategies for malignant brain tumors," *Journal of Neuro-Oncology*, vol. 50, no. 1-2, pp. 149–163, 2000.

[10] H. Bai, J. Orlando, and T. N. Seyfried, "Altered ganglioside composition in virally transformed rat embryo fibroblast," *Biochimica et Biophysica Acta*, vol. 1136, no. 1, pp. 23–27, 1992.

[11] M. El-Abbadi, T. N. Seyfried, A. J. Yates, C. Orosz, and M. C. Lee, "Ganglioside composition and histology of a spontaneous metastatic brain tumour in the VM mouse," *British Journal of Cancer*, vol. 85, no. 2, pp. 285–292, 2001.

[12] S. Hakomori, "Glycosylation defining cancer malignancy: new wine in an old bottle," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 16, pp. 10231–10233, 2002.

[13] H.-J. Choi, T.-W. Chung, S.-K. Kang, et al., "Ganglioside GM3 modulates tumor suppressor PTEN-mediated cell cycle progression—transcriptional induction of $p21^{WAF1}$ and $p27^{kip1}$ by inhibition of PI-3K/AKT pathway," *Glycobiology*, vol. 16, no. 7, pp. 573–583, 2006.

[14] H. Bai and T. N. Seyfried, "Influence of ganglioside GM3 and high density lipoprotein on the cohesion of mouse brain tumor cells," *Journal of Lipid Research*, vol. 38, no. 1, pp. 160–172, 1997.

[15] A. J. Yates and A. Rampersaud, "Sphingolipids as receptor modulators: an overview," *Annals of the New York Academy of Sciences*, vol. 845, pp. 57–71, 1998.

[16] T. N. Seyfried, R. K. Yu, M. Saito, and M. Albert, "Ganglioside composition of an experimental mouse brain tumor," *Cancer Research*, vol. 47, no. 13, pp. 3538–3542, 1987.

[17] J. A. Ecsedy, K. A. Holthaus, H. C. Yohe, and T. N. Seyfried, "Expression of mouse sialic acid on gangliosides of a human glioma grown as a xenograft in SCID mice," *Journal of Neurochemistry*, vol. 73, no. 1, pp. 254–259, 1999.

[18] H.-H. Chou, H. Takematsu, S. Diaz, et al., "A mutation in human CMP-sialic acid hydroxylase occurred after the Homo-Pan divergence," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11751–11756, 1998.

[19] E. A. Muchmore, M. Milewski, A. Varki, and S. Diaz, "Biosynthesis of N-glycolyneuraminic acid. The primary site of hydroxylation of N-acetylneuraminic acid is the cytosolic sugar nucleotide pool," *Journal of Biological Chemistry*, vol. 264, no. 34, pp. 20216–20223, 1989.

[20] I. Kawashima, H. Ozawa, M. Kotani, et al., "Characterization of ganglioside expression in human melanoma cells: immunological and biochemical analysis," *Journal of Biochemistry*, vol. 114, no. 2, pp. 186–193, 1993.

[21] M. El-Abbadi and T. N. Seyfried, "Influence of growth environment on the ganglioside composition of an experimental mouse brain tumor," *Molecular and Chemical Neuropathology*, vol. 21, no. 2-3, pp. 273–285, 1994.

[22] M. Ziche, G. Alessandri, and P. M. Gullino, "Gangliosides promote the angiogenic response," *Laboratory Investigation*, vol. 61, no. 6, pp. 629–634, 1989.

[23] W. Song, M. F. Vacca, R. Welti, and D. A. Rintoul, "Effects of gangliosides G_{M3} and de-*N*-acetyl G_{M3} on epidermal growth factor receptor kinase activity and cell growth," *Journal of Biological Chemistry*, vol. 266, no. 16, pp. 10174–10181, 1991.

[24] A. Rebbaa, J. Hurh, H. Yamamoto, D. S. Kersey, and E. G. Bremer, "Ganglioside GM3 inhibition of EGF receptor mediated signal transduction," *Glycobiology*, vol. 6, no. 4, pp. 399–406, 1996.

[25] K. Kabayama, T. Sato, F. Kitamura, et al., "TNAα-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3," *Glycobiology*, vol. 15, no. 1, pp. 21–29, 2005.

[26] S. Tagami, J.-I. Inokuchi, K. Kabayama, et al., "Ganglioside GM3 participates in the pathological conditions of insulin resistance," *Journal of Biological Chemistry*, vol. 277, no. 5, pp. 3085–3092, 2002.

[27] E. G. Bremer, J. Schlessinger, and S. Hakomori, "Ganglioside-mediated modulation of cell growth. Specific effects of GM_3 on tyrosine phosphorylation of the epidermal growth factor receptor," *Journal of Biological Chemistry*, vol. 261, no. 5, pp. 2434–2440, 1986.

[28] M. S. Toledo, E. Suzuki, K. Handa, and S. Hakomori, "Effect of ganglioside and tetraspanins in microdomains on interaction of integrins with fibroblast growth factor receptor," *Journal of Biological Chemistry*, vol. 280, no. 16, pp. 16227–16234, 2005.

[29] Y. Nakatsuji and R. H. Miller, "Selective cell-cycle arrest and induction of apoptosis in proliferating neural cells by ganglioside GM3," *Experimental Neurology*, vol. 168, no. 2, pp. 290–299, 2001.

[30] G. Alessandri, K. S. Raju, and P. M. Gullino, "Interaction of gangliosides with fibronectin in the mobilization of capillary endothelium. Possible influence on the growth of metastasis," *Invasion and Metastasis*, vol. 6, no. 3, pp. 145–165, 1986.

[31] G. Alessandri, P. Cornaglia-Ferraris, and P. M. Gullino, "Angiogenic and angiostatic microenvironment in tumors: role of gangliosides," *Acta Oncologica*, vol. 36, no. 4, pp. 383–387, 1997.

[32] M. Ziche, L. Morbidelli, G. Alessandri, and P. M. Gullino, "Angiogenesis can be stimulated or repressed in vivo by a change in GM3:GD3 ganglioside ratio," *Laboratory Investigation*, vol. 67, no. 6, pp. 711–715, 1992.

[33] G. Alessandri, S. Filippeschi, P. Sinibaldi, et al., "Influence of gangliosides on primary and metastatic neoplastic growth in human and murine cells," *Cancer Research*, vol. 47, no. 16, pp. 4243–4247, 1987.

[34] M. G. Manfredi, S. Lim, K. P. Claffey, and T. N. Seyfried, "Gangliosides influence angiogenesis in an experimental mouse brain tumor," *Cancer Research*, vol. 59, no. 20, pp. 5392–5397, 1999.

[35] M. H. Ravindranath, T. Tsuchida, D. L. Morton, and R. F. Irie, "Ganglioside GM3:GD3 ratio as an index for the management of melanoma," *Cancer*, vol. 67, no. 12, pp. 3029–3035, 1991.

[36] R. Jennemann, H.-D. Mennel, B. L. Bauer, and H. Wiegandt, "Glycosphingolipid component profiles of human gliomas correlate with histological tumour types: analysis of inter-individual and tumour-regional distribution," *Acta Neurochirurgica*, vol. 126, no. 2-4, pp. 170–178, 1994.

[37] S. Koochekpour and G. J. Pilkington, "Vascular and perivascular GD3 expression in human glioma," *Cancer Letters*, vol. 104, no. 1, pp. 97–102, 1996.

[38] P. Fredman, H. von Holst, V. P. Collins, B. Dellheden, and L. Svennerholm, "Expression of gangliosides GD3 and 3′-isoLM1 in autopsy brains from patients with malignant tumors," *Journal of Neurochemistry*, vol. 60, no. 1, pp. 99–105, 1993.

[39] B. Berra, S. M. Gaini, and L. Riboni, "Correlation between ganglioside distribution and histological grading of human astrocytomas," *International Journal of Cancer*, vol. 36, no. 3, pp. 363–366, 1985.

[40] T. D. Traylor and E. L. Hogan, "Gangliosides of human cerebral astrocytomas," *Journal of Neurochemistry*, vol. 34, no. 1, pp. 126–131, 1980.

[41] G. Zeng, L. Gao, S. Birkle, and R. K. Yu, "Suppression of ganglioside GD3 expression in a rat F-11 tumor cell line reduces tumor growth, angiogenesis, and vascular endothelial growth factor production," *Cancer Research*, vol. 60, no. 23, pp. 6670–6676, 2000.

[42] L. Manuelidis, R. K. Yu, and E. E. Manuelidis, "Ganglioside content and pattern in human gliomas in culture. Correlation of morphological changes with altered gangliosides," *Acta Neuropathologica*, vol. 38, no. 2, pp. 129–135, 1977.

[43] L. C. Huysentruyt, P. Mukherjee, D. Banerjee, L. M. Shelton, and T. N. Seyfried, "Metastatic cancer cells with macrophage properties: evidence from a new murine tumor model," *International Journal of Cancer*, vol. 123, no. 1, pp. 73–84, 2008.

[44] T. N. Seyfried, M. El-Abbadi, and M. L. Roy, "Ganglioside distribution in murine neural tumors," *Molecular and Chemical Neuropathology*, vol. 17, no. 2, pp. 147–167, 1992.

[45] P. Mukherjee, L. E. Abate, and T. N. Seyfried, "Antiangiogenic and proapoptotic effects of dietary restriction on experimental mouse and human brain tumors," *Clinical Cancer Research*, vol. 10, no. 16, pp. 5622–5629, 2004.

[46] E. G. Bremer and S. I. Hakomori, "GM3 ganglioside induces hamster fibroblast growth inhibition in chemically-defined medium: ganglioside may regulate growth factor receptor function," *Biochemical and Biophysical Research Communications*, vol. 106, no. 3, pp. 711–718, 1982.

[47] T.-W. Chung, S.-J. Kim, H.-J. Choi, et al., "Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: direct interaction of GM3 with VEGFR-2," *Glycobiology*, vol. 19, no. 3, pp. 229–239, 2009.

[48] E. N. E. Noll, J. Lin, Y. Nakatsuji, R. H. Miller, and P. M. Black, "GM3 as a novel growth regulator for human gliomas," *Experimental Neurology*, vol. 168, no. 2, pp. 300–309, 2001.

[49] P. Mukherjee, A. C. Faber, L. M. Shelton, R. C. Baek, T. C. Chiles, and T. N. Seyfried, "Ganglioside GM3 suppresses the proangiogenic effects of vascular endothelial growth factor and ganglioside GD1A," *Journal of Lipid Research*, vol. 49, no. 5, pp. 929–938, 2008.

[50] H. M. Zimmerman and H. Arnold, "Experimental brain tumors: I. tumors produced with methylcholanthrene," *Cancer Research*, vol. 1, pp. 919–938, 1941.

[51] J. Marsh, P. Mukherjee, and T. N. Seyfried, "Akt-dependent proapoptotic effects of dietary restriction on late-stage management of a phosphatase and tensin homologue/tuberous sclerosis complex 2-deficient mouse astrocytoma," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7751–7762, 2008.

[52] R. Martínez-Murillo and A. Martínez, "Standardization of an orthotopic mouse brain tumor model following transplantation of CT-2A astrocytoma cells," *Histology and Histopathology*, vol. 22, no. 12, pp. 1309–1326, 2007.

[53] J. Jośko and M. Mazurek, "Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis," *Medical Science Monitor*, vol. 10, no. 4, pp. RA89–RA98, 2004.

[54] J. Fang, M. Ding, L. Yang, L.-Z. Liu, and B.-H. Jiang, "PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis," *Cellular Signalling*, vol. 19, no. 12, pp. 2487–2497, 2007.

[55] H. D. Skinner, J. Z. Zheng, J. Fang, F. Agani, and B.-H. Jiang, "Vascular endothelial growth factor transcriptional activation is mediated by hypoxia-inducible factor 1α, HDM2, and p70S6K1 in response to phosphatidylinositol 3-kinase/AKT signaling," *Journal of Biological Chemistry*, vol. 279, no. 44, pp. 45643–45651, 2004.

[56] O. Nakamura, M. Iwamori, M. Matsutani, and K. Takakura, "Ganglioside GD3 shedding by human gliomas," *Acta Neurochirurgica*, vol. 109, no. 1-2, pp. 34–36, 1991.

[57] K. Kaucic, Y. Liu, and S. Ladisch, "Modulation of growth factor signaling by gangliosides: positive or negative?" *Methods in Enzymology*, vol. 417, pp. 168–185, 2006.

[58] S. Ladisch, S. Kitada, and E. F. Hays, "Gangliosides shed by tumor cells enhance tumor formation in mice," *Journal of Clinical Investigation*, vol. 79, no. 6, pp. 1879–1882, 1987.

[59] R. Olshefski and S. Ladisch, "Intercellular transfer of shed tumor cell gangliosides," *FEBS Letters*, vol. 386, no. 1, pp. 11–14, 1996.

[60] Y. Liu, R. Li, and S. Ladisch, "Exogenous ganglioside GD1a enhances epidermal growth factor receptor binding and dimerization," *Journal of Biological Chemistry*, vol. 279, no. 35, pp. 36481–36489, 2004.

[61] Z. Lang, M. Guerrera, R. Li, and S. Ladisch, "Ganglioside GD1a enhances VEGF-induced endothelial cell proliferation and migration," *Biochemical and Biophysical Research Communications*, vol. 282, no. 4, pp. 1031–1037, 2001.

[62] H. van Cruijsen, M. Ruiz, P. van der Valk, T. D. de Gruijl, and G. Giaccone, "Tissue micro array analysis of ganglioside N-glycolyl GM3 expression and signal transducer and activator of transcription (STAT)-3 activation in relation to dendritic cell infiltration and microvessel density in non-small cell lung cancer," *BMC Cancer*, vol. 9, article 180, 2009.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 659231, 7 pages
doi:10.1155/2010/659231

Research Article

Role of Angiopoietin-2 in Regulating Growth and Vascularity of Astrocytomas

Gelareh Zadeh,[1] Keyvan Koushan,[1] Qian Baoping,[1] Patrick Shannon,[2] and Abhijit Guha[1,3]

[1] *Arthur & Sonia Labatts Brain Tumor Center, Hospital for Sick Children, University of Toronto, Toronto, Canada M5G 2G4*
[2] *Department of Pathology, Western Hospital, University of Toronto, Toronto, Canada M5G 2G4*
[3] *Division of Neurosurgery, Western Hospital, University of Toronto, Toronto, Canada M5G 2G4*

Correspondence should be addressed to Gelareh Zadeh, gelareh.zadeh@uhn.on.ca

Received 13 December 2009; Accepted 15 February 2010

Academic Editor: Arkadiusz Dudek

Angiopoietins and Tie2 are angiogenic-specific ligand and receptor complex that have been shown to play a critical role in tumor angiogenesis. Angiopoietin-2 (Ang2) is one of four ligands for receptor Tie2 and it is the naturally occurring antagonist to Tie2, inhibiting the action of Angiopoietin-1 (Ang1). Over the last decade, significant research has focused on elucidating the role of Ang2 in cancer biology and its exact role in tumor angiogenesis remains elusive. In this study we have focused on establishing the role of Ang2 in angiogenesis of malignant astrocytomas. We have demonstrated that Ang2 significantly enhances the vascular growth of malignant astrocytomas and constant upregulation of Ang2 throughout all phases of tumor growth generates abnormal vascular structures that are not typically seen in human astrocytomas, suggesting that Ang2 plays a tumor stage-dependent role and is not a consistently elevated throughout all growth stages of malignant astroctyomas.

1. Introduction

Based on the originally proposed paradigm by Holash et al., Angiopoietin-2 (Ang2) works in concert with VEGF to promote neoangiogenesis, and in the absence of VEGF, vessels that have been destabilized by Ang2 will undergo apoptosis and regress. Ang2 is the naturally occurring antagonist to Ang1 and inhibits Ang1-induced activation of Tie2/TEK. Though there has been numerous biochemical data to support this paradigm [1–7], there is sufficient data to suggest a more complex role for Ang2. For example, at high concentrations Ang2 acts as an agonist of Tie2/TEK, providing a prosurvival signal to endothelial cell (EC), which is a similar function as Ang1 [1]. Although all tumor models show an upregulation of Ang2, its role in tumor angiogenesis has proven to be quite complex and variable, depending on the tumor model investigated [8–14]. Ang2 upregulation is seen primarily in EC of small cell lung cancer, hepatocellular carcinoma, neuroblastoma, gastric cancer, colon cancer, and Kaposi sarcoma, with Ang2 being associated with poor prognosis in many of these tumors [8–16].

Upregulation of Ang2 along with VEGF upregulation suggests that vessel destabilization by Ang2 is a critical step required to allow for VEGF-induced neoangiogenesis. In astrocytomas, Ang2 has been found to be upregulated in GBMs compared to LGAs and NB [11, 17–19]. The source of Ang2 is mainly reported to be the EC; however, one study and our own unpublished data suggest that Ang2 may also be expressed by malignantly transformed astrocytoma cells [11]. A noteworthy observation made by Stratmann et al. is that expression of Ang2 appears to be vessel size- or vessel type-dependent [20]. Ang2 expression was confined to EC of smaller vessels and not seen in larger vessels suggesting that Ang2 promotes *in-situ* angiogenesis and is more intimately involved with capillary-like vascular structures in tumors [18, 20].

A more recent study identifies Ang2 as a marker of tumor cell invasion in high-grade astrocytomas, with little Ang2 expression seen in the center of human GBM compared to the invasive peripheral edge of the tumors where Ang2 is expressed by both the vascular and neural elements [13]. They also found that upregulation of Ang2 in U87 xenografts had a pronounced invasive phenotype compared

to the parental U87MG xenografts that had no Ang2 expression [13]. They propose that Ang2 confers a more invasive phenotype to the tumor cells via either activation of MMP-2, independent of Tie2/TEK receptor activation, or perhaps via activation of integrins [13].

In this study, we have focused on deciphering the distinct contribution of Ang2 to GBM angiogenesis and vessel development. We have found that Ang2 promotes vascular growth of GBMs. Additionally, Ang2 induces vascular architectural changes that are pathological and aberrant in comparison to control tumor vessels. This aberrancy in vasculature is not seen in human GBMs, which suggests that Ang2 is not constantly upregulated in human tumors and alludes to a stage-dependent upregulation of Ang2.

2. Materials and Methods

2.1. Cells and Reagents. Established human U87-MG GBM cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and U373-MG cell lines were a gift from B. Westermark (Uppsala, Sweden). These GBM lines were chosen as they provide variability in their degree of baseline Angiopoietin and VEGF-A expression [17], in addition to variable tumorigenicity potential and differences in genetic aberrations. They were maintained in Dulbecco's minimal essential medium (DMEM) (Cellgro, Herndon, VA) supplemented with 10% FBS and penicillin-streptomycin antibiotics.

2.2. Stable Clones

2.2.1. Constitutively Overexpressing Clones. Full-length human ANG2 cDNA (a gift from K. Alitalo, Helsinki, Finland) was subcloned into the pSec vector (Invitrogen) to allow generation of Myc-Histidine epitope-tagged constructs. The Ang-Myc/HIS sequence was subcloned into the BamH1 and EcoR1 sites of the pCAGG vector that contained a CMV promoter along with a chicken β-actin enhancer element. Stable cell lines expressing Ang2, were generated by transfection of the vector "pCAGG-Ang-Myc/HIS-Zeocin" into U87 and U373 GBM lines using Lipofectamine 2000 (Gibco/BRL) as per the manufacturer's instructions. Twenty stable clones, selected with 1mg/ml of Zeocin (Invitrogen), were examined for Ang2 expression by western blot analysis as described below. Two single clones with highest expression of Ang2 above baseline parental levels, as well as one pooled clone of Ang2 were selected for each of the three GBM lines (U87:Ang2, U373:Ang2). Corresponding control stable cell lines were generated using empty-vector transfectants.

2.2.2. Tetracycline Inducible Clones Overexpressing Angiopoietins. As described previously, stable Tet-Off U87-MG cells were established [21]. Briefly, U87-MG cell lines were transfected with pTet-Off (Clontech, Palo Alto) vector and stable clones selected and maintained in 1 mg/mL and 500 μg/mL of G418, respectively. Thirty of the Tet-Off clones were assayed by transfecting with the reporter construct pTRE-LUC and subsequent examination of luciferase activity with a luciferase assay. The highest tetracycline inducible clone was selected to generate double stable Tet-Off cell lines (data not shown). Double stable Tet-Off U87-MG cell lines overexpressing Ang2 were generated by cotransfecting U87-MG:Tet-Off stable cells with pTRE-Ang2 with the pTK-Puromycin vector. Stable clones were selected in 3 mg/mL of Puromycin, and twenty clones were tested for induction of Ang2 expression by immunoprecipitation followed by western blotting, as described below. For control U87-MGTet-off double stable cell lines, pTRE-Red vector expressing the ds-RED fluorescent protein was used. *In vitro* testing of tetracycline induction of Ang2 expression was determined using varying doses of Doxycycline, with the most tightly regulated clones expressing Ang2 selected for *in vivo* experiments.

2.3. In Vivo Tumor Models

2.3.1. Subcutaneous Models. Subcutaneous xenografts were generated by growing U87-MG stable clones overexpressing Ang2 in the flanks of NOD-SCID mice. For each stable clone, seven mice were injected with 10^7 cells suspended in 300 μL of PBS, with five mice injected with control empty vector transfectants. Tumor growth was measured biweekly, using calipers by two observers in a blinded fashion. Tumor volume was calculated using the formula: (diameter$^2\times$ length)/2. As per animal protocol, mice were sacrificed by cervical dislocation after 100 mg/kg BrDU injection (Sigma-Aldrich). Tumors were cut in cross sections, with two cross-sections kept in formaldehyde for paraffin blocks and immunohistochemical analysis and the remaining tumor stored in liquid nitrogen. All in vivo tumor models were repeated in duplicate.

2.3.2. Intracranial Models. For orthotopic xenograft models, Tet-Off regulated human U87-MG:Ang2 cells (10^6) were stereotactically injected 3 mm deep into the frontal cortex of NOD-SCID mice. Mice were treated with Dox in the drinking water with three doses of 0, 1, and 10 mg/mL. These doses were selected based on prior published studies demonstrating that Dox crosses the blood-brain barrier efficiently to regulate gene expression in the brain [21]. When animals exhibited symptoms consistent with failure to thrive or raised intracranial pressure, the mice were sacrificed by perfusion fixation after BrDU injection and tail vein injection of 2% Evans Blue solution (2 mL/kg) to determine intraluminal blood flow and vessel permeability. The time interval between the injection of Evans Blue and the perfusion and killing of the mice was approximately 30 minutes. All in vivo experiments were repeated in duplicate.

2.4. Tumor Vascularity. Four different tumor portions were each cut at 5 μm consecutive paraffin sections and stained with the EC specific marker anti-FactorVIII (DAKO; 1 : 2000), followed by detection with an avidin-biotin complex method-3,3′-diaminobenzidine (VectaStain Elite; Vector Laboratories) system. Microvessel density (MVD) was calculated by counting the number of hollow lumen vessels

in ten high-power fields (HPF:500x) and in five HPF at vascular "hot spots". Areas that included abnormal vascular structures, such as glomeruloid bodies, were not included in the MVD count as the functional status of these vascular units in both human and xenograft tumors is not known. All analyses were carried out using the MicroComputer Image Device (MCID-Imaging Research, Inc.) linked to a color CCD camera (Sony DXC 970 MD) mounted on a transmitted-light microscope (Zeiss Axioskop). IHC for EC and SMC staining was performed using FactorVIII antibody and smooth muscle antigen (SMA) staining.

2.5. Immunohistochemistry. Standard hematoxylin and eosin (H&E) staining and immunohistochemical analysis was performed on 5 μm tissue sections from paraffin embedded tissue blocks. Primary antibodies used include: FactorVIII (rabbit polyclonal antibody #A0082; DAKO; used at 1 : 2500) and a polyclonal goat anti-Ang2 antibody (1 : 200 and 1 : 400, Santa Cruz). Secondary antibody was a goat antimouse antibody (Zymed) used at 1 : 200, and antigens were detected using the avidin-biotin complex method (Vector Laboratories) and diaminobenzidine substrate.

2.6. Statistical Analysis. All analyses were completed using StatView 4.1 for the Macintosh (Abacus Concepts, Berkeley, CA). All errors were calculated as the standard error of the mean (S.E.M.). One-tailed Student's t-test was used to compare means (two sample, unequal variance) with $P < .05$ considered statistically significant.

3. Results

3.1. Overexpression of Ang2 in GBM Cell Lines. Parental U87MG and U373MG cells have no detectable Ang2 (Figures 1(a) and 1(b)) [17]. Overexpression of Ang2 did not alter the *in vitro* proliferation rate, morphology, or the VEGF expression of the cells compared to parental controls (data not shown). Stable transfectants overexpressing the highest levels of Ang2 (A2-1) and one pooled (A2-p) clone were selected for subsequent experiments (Figures 1(a) and 1(b)). Tet-Off regulated Ang2 stable clones were also established in U87MG cells, with the most tightly regulated clones selected for *in vivo* studies (Figure 1(c)). In the U87MG:Ang2 Tet-Off clone, Dox at 5000 ng/mL was sufficient to decrease Ang2 expression to undetectable levels, as seen in control cell lines (Figure 1(c)).

3.2. Effect of Ang2 on Tumor Growth and Proliferation. We assessed the impact of Ang2 on the growth of GBM xenografts in both subcutaneous (s.c). and intracranial (i.c.) tumor models using stable cell lines of U87MG and U373MG overexpressing Ang2 (Figure 1(b)). In s.c. xenografts, Ang2 overexpression resulted in a significantly faster tumor growth and larger final tumor size compared to controls (Figure 1(b) and Table 1). In i.c. xenografts, Ang2 conferred a growth advantage as suggested by a significant decrease in survival and tumor proliferation (Figure 1(c) and Table 2). The response to Ang2 was dose-dependent with respect to survival, tumor proliferation, and vascularity (Figure 1(c) and Table 2). Mice treated with 0 mg/mL of Dox in the drinking water, hence those with xenografts expressing high levels of Ang2, had a significantly shorter survival time, and tumor proliferation was increased by 2.2-fold compared to the mice receiving either 1 or 10 mg/mL of Dox, which had comparable survival to controls (Figure 1(c) and Table 2). Ang2 is not expressed endogenously by U87MG cells (Figure 1(a)), therefore, addition of Dox can completely turn-off exogenous Ang2 and result in similar tumor growth and survival of mice as that of controls.

Ang2 upregulation resulted in increased MVD and altered vessel size and EC distribution in both s.c. and i.c. xenografts (Figure 2, Tables 1 and 2). Additionally, in both s.c. and i.c. U87MG:Ang2 xenografts, there was an abnormal vascular architecture, characterized by preponderance of small vessels, "cord"-like distribution of EC and whirling of EC present throughout the tumors, in addition to increased numbers of dilated vessels (Figure 2(a)*i* and *ii*). The alterations in the microvasculature were dependent on levels of Ang2 expression that were regulated by Dox in the i.c. U87MG:Ang2 models (Figure 1(b) and Table 1). At high levels of Ang2 (0 mg/mL Dox), a large number of dilated vessels were present, along with abnormal EC distribution throughout the tumor (Figure 2(b)). With Dox suppression (10 mg/mL Dox) of Ang2, EC distribution, vessel size, and the overall microvasculature architecture returned to similar structural patterns as is seen in control U87MG tumors (Figure 2(b) and Table 2). These vascular alterations have not been reported previously; though the recent publication by Hu et al. [13] and Lee et al. [19] makes the observation of impaired angiogenesis by Ang2, they do not report similar structural changes as ours on tumor vascularity.

4. Discussion

Astrocytomas angiogenesis is postulated to be highly tumor stage-dependant. At their initial growth phase, they coopt and parasitize existing host vessels in an attempt to support their growth, thus the first phase being independent of the tumor angiogenic process [18, 22–24]. The second growth phase begins when the host mounts a defensive response and the parasitized vascular supply regresses resulting in tumor hypoxia and necrosis, which in turn triggers upregulation of Ang2 and VEGF [23, 24]. Therefore, Ang2 appears to play a highly phase-dependent role in the progression of malignant astrocytomas. It plays a pivotal role in the cooption of host vessels in the initial phase; and in supporting *in-situ* tumor angiogenesis, while in the second phase it allows destabilization of mature vessels, by antagonizing Ang1-mediated Tie2/TEK activation, in order to facilitate mitogenic stimulation of ECs by VEGF and promoting tumor neovascularization [22, 24]. However, the role of Ang2 in tumor angiogenesis remains controversial.

We have found that Ang2 upregulation, in both s.c. and i.c. xenografts, led to an increase in growth rate, final volume and proliferation of GBMs along with an increase in tumor angiogenesis. Ang2 upregulation resulted in an alteration of vascular structures, marked by abnormal EC distribution,

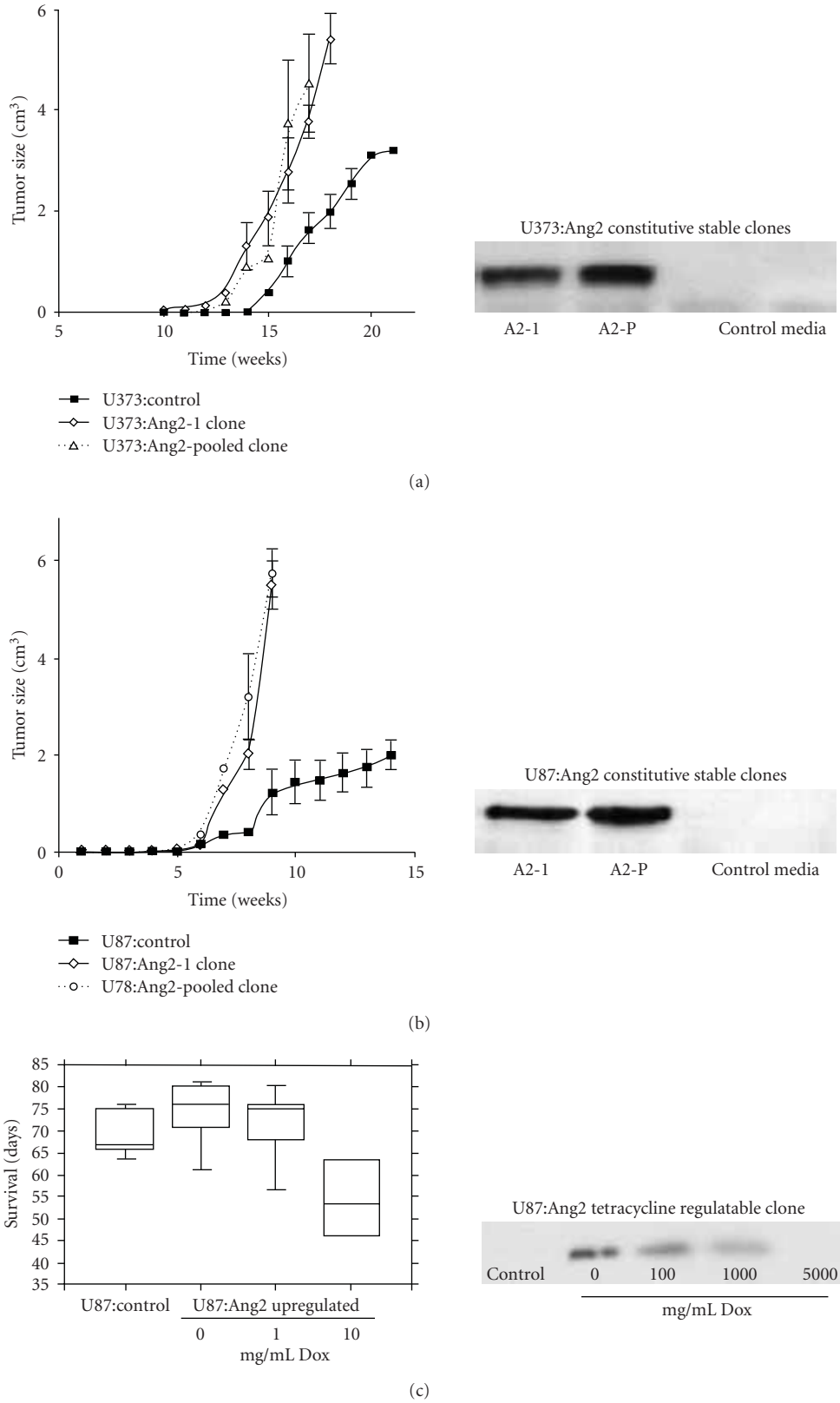


Figure 1: Effect of Ang2 over-expression on growth of GBM xenografts. Stable clones of U87 and U373 were generated to over-express Ang2 constitutively. Neither of the cell lines expresses Ang2 at baseline. One highest expressing clone and one pooled clone of each cell line was grown as subcutaneous models. Ang2 restricted tumor growth in U373 (a) tumors while it conferred a growth advantage in U87 tumors (b). Similarly, a growth advantage was maintained in U87 intracranial xenografts as evidenced by a significantly lowered survival time of mice with these grafts compared to mice with control tumors, and this increased tumor growth was dose dependent on Ang2 (c).

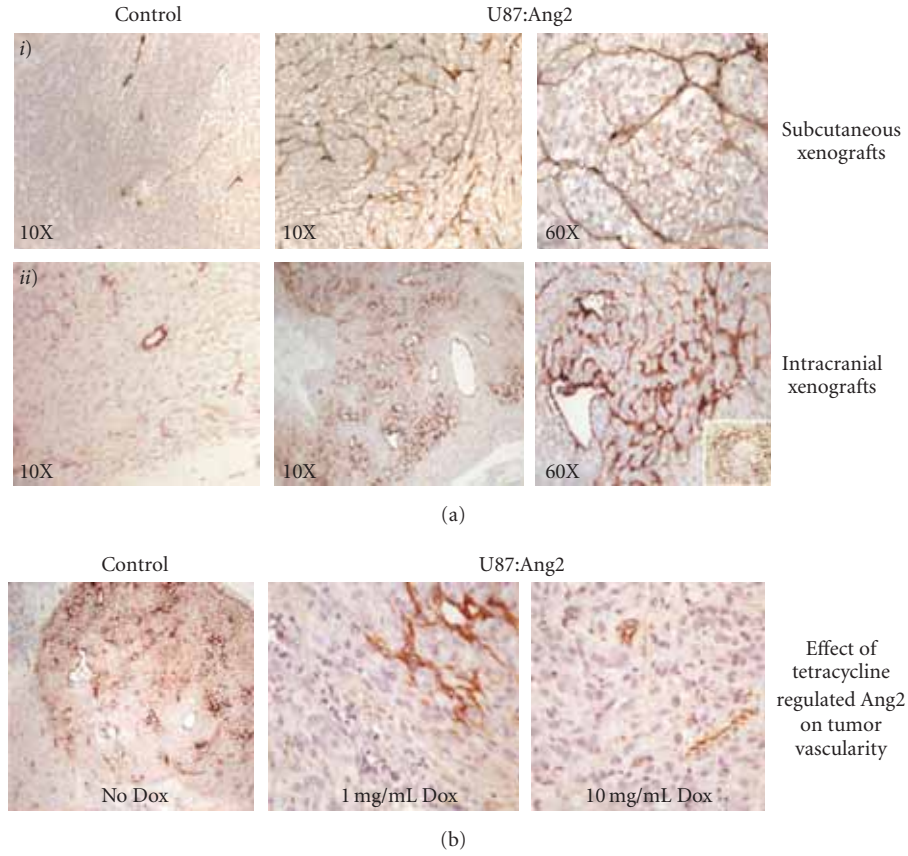


Figure 2: Effect of Ang2 on tumor vascularity. Immunohistochemical analysis of tumor vessels as determined by Factor VIII stains was performed on Ang2 upregulated xenografts. (a) *i*) subcutaneous and *ii*) intracranial tumors of U87MG:Ang2 demonstrated in addition to an increase in MVD, abnormal EC distribution with fine "cord" like-structures dispersed throughout the tumor, areas of EC whirling (inset *ii*) and dilated vessels. This process was seen in both the intracranial and subcutaneous xenografts and was dependent on the level of Ang2 expression. (b) Tumors with high levels of Ang2 expression (No Dox in the drinking water) had a small, highly infiltrative EC pattern together with dilated vessels, whereas these structural changes are lost with turning off of Ang2 using 1 mg/mL, and to a greater extent at 10 mg/mL, of Dox in the drinking water.

Table 1: Effect of Ang2 on subcutaneous xenograft models of GBM.

	U87MG:Ctl	U87MG:Ang2	U373:Ctl	U373MG:Ang2
	$n = 8$	$n = 15$	$n = 15$	$n = 15$
Final Tumor Size (cm^3)	2.01 (SEM 0.3)	5.7 * (SEM 0.4) $P = 4 \times 10^{-4}$	3.18 (SEM 0.3)	5.96 (SEM 0.5) $P = 6 \times 10^{-5}$
Proliferation Index	0.23	0.75* $P = .0042$	0.042	0.28 $P = .001$
MVD (vessels/HPF) mean of 10 counts	2.12 (SEM 0.1)	9.5* (SEM 0.1) $P = .001$	3 (SEM 0.1)	3.9* (SEM 0.2) $P = .001$

SEM = Standard Error of Mean
*indicates statistical significance.

with EC forming "cord" or capillary-like structures and areas of EC whorling present throughout the tumor, in addition to a high number of dilated vessels. In the model used in this study, there is constant upregulation of Ang2 throughout all stages and phase of GBM tumor growth, potentially providing a continual trigger for host vessel cooption and promoting *in-situ* angiogenesis, thereby increased tumor growth.

On the other hand, Lee et al. have demonstrated a complex temporal and stage-dependent role for Ang2 [19].

Table 2: Effect of Ang2 on intracranial U87MG xenografts.

	U87MG:Ctl	U87MG:Ang2		
		no. Dox.	1 mg/mL Dox	10 mg/mL Dox
	$n = 10$	$n = 10$	$n = 10$	$n = 10$
Overall survival (days)	63.7 (SEM = 2.3)	54.7* (SEM = 3.3) $P = .015$	71.7* (SEM = 2.3) $P = .021$	74.4 (SEM = 3.5) $P = .0149$
Proliferation index	0.043 (SEM = 0.01)	0.094* (SEM = 0.01) $P = .0039$	0.038* (SEM = 0.00) $P = .3989$	0.035* (SEM = 0.00) $P = .4262$
MVD (vessels/HPF) mean of 10 counts	5.8 (SEM = 0.478)	8.8* (SEM = 0.859) $P = .0132$	4.6* (SEM = 0.616) $P = .038$	4.0* (SEM = 0.785) $P = .0189$

They observe a bimodal expression pattern of Ang2 in astrocytomas and support the postulate that Ang2 is a vessel destabilize, seen at sites of tumor cell growth, tumor periphery, and around sites of necrosis, presumably to promote neoangiogenesis and support tumor cell growth [19]. However, quite contrary to what one would predict based on this observation, Lee et al. also found that Ang2 treatment of U87MG xenografts did not promote but rather restricted astrocytoma growth. Moreover, at first glance these results appear to be in opposition with our observations; however, on closer analysis, both findings can be seen as corroborative and together explain the complex tumor phase-dependent role of Ang2. Lee et al. treated the U87MG i.c. xenografts on day 4 after tumor implantation followed by biweekly injections of Ang2, whereas in our model Ang2 is upregulated constantly throughout all stages of tumor growth. The difference between the level and stages of Ang2 upregulation in the two models supports the postulate that Ang2 plays a highly tumor stage-dependent role. Another evidentiary data that Ang2 plays a stage dependent role in glioma angiogenesis is the fact that tumor vascular structures observed in our xenograft models are not evident in human GBM specimens, indicating that Ang2 is not upregulated throughout all stages of human GBMs, and most likely plays a very precise role at specific stages of GBM growth.

The mechanism by which Ang2 causes abnormal vascular structures is not established. The abundant "cord" or capillary-like vessels in the U87MG:Ang2 xenografts may be a result of Ang2-mediated modulation of EC motility, migration, and invasion. Hu et al. demonstrate regions of Ang2-expressing tumors actively invading the brain, high levels of MMP-2 expression, and increased angiogenesis. The direct impact of Ang2 on EC invasion *in vivo* remains unknown.

Additionally, Ang2 is known to influence the fate of new tumor vessels, differentiating them into capillaries versus arteries or venous structures. We observe dilated vessels throughout the GBM xenografts. The most likely explanation is that Ang2 presents an inhibitory signal, preventing Ang1-mediated maturation by of the newly formed tumor vessels. Taken together our data indicates that increased Ang2 promotes angiogenic growth of GBMs. Constant upregulation of Ang2 throughout all phases of tumor growth results in the abnormal vascular structures seen in our xenografts that are not present in human GBMs, suggesting that Ang2 upregulation in GBMs is very much a tumor stage dependent process and not constant throughout all stages of GBM growth. Future studies are required to decipher the precise temporal role of Ang2 and whether the combinatorial impact of other angiogenic cytokines with Ang2 can be used for therapeutic targets in treatment of GBMs.

References

[1] I. Kim, J.-H. Kim, S.-O. Moon, H. J. Kwak, N.-G. Kim, and G. Y. Koh, "Angiopoietin-2 at high concentration can enhance enthelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway," *Oncogene*, vol. 19, no. 39, pp. 4549–4552, 2000.

[2] P. C. Maisonpierre, C. Suri, P. F. Jones, et al., "Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis," *Science*, vol. 277, no. 5322, pp. 55–60, 1997.

[3] W. S. Moon, K. H. Rhyu, M. J. Kang, et al., "Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma?" *Modern Pathology*, vol. 16, no. 6, pp. 552–557, 2003.

[4] K. Teichert-Kuliszewska, P. C. Maisonpierre, N. Jones, et al., "Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2," *Cardiovascular Research*, vol. 49, no. 3, pp. 659–670, 2001.

[5] R. P. Visconti, C. D. Richardson, and T. N. Sato, "Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 12, pp. 8219–8224, 2002.

[6] N. L. Ward and D. J. Dumont, "The angiopoietins and Tie2/Tek: adding to the complexity of cardiovascular development," *Seminars in Cell and Developmental Biology*, vol. 13, no. 1, pp. 19–27, 2002.

[7] G. Zadeh and A. Guha, "Molecular regulators of angiogenesis in the developing nervous system and adult brain tumors (review)," *International Journal of Oncology*, vol. 23, no. 3, pp. 557–565, 2003.

[8] Q. Yu and I. Stamenkovic, "Angiopoietin-2 is implicated in the regulation of tumor angiogenesis," *American Journal of Pathology*, vol. 158, no. 2, pp. 563–570, 2001.

[9] T. Etoh, H. Inoue, S. Tanaka, G. F. Barnard, S. Kitano, and M. Mori, "Angiopoietin-2 is related to tumor angiogenesis in gastric carcinoma: possible in vivo regulation via induction of proteases," *Cancer Research*, vol. 61, no. 5, pp. 2145–2153, 2001.

[10] A. Stratmann, T. Acker, A. M. Burger, K. Amann, W. Risau, and K. H. Plate, "Differential inhibition of tumor angiogenesis by tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants," *International Journal of Cancer*, vol. 91, no. 3, pp. 273–282, 2001.

[11] K. Koga, T. Todaka, M. Morioka, et al., "Expression of angiopoietin-2 in human glioma cells and its role for angiogenesis," *Cancer Research*, vol. 61, no. 16, pp. 6248–6254, 2001.

[12] G. Zadeh and A. Guha, "Neoangiogenesis in human astrocytomas: expression and functional role of angiopoietins and their cognate receptors," *Frontiers in Bioscience*, vol. 8, pp. e128–e137, 2003.

[13] B. Hu, P. Guo, Q. Fang, et al., "Angiopoietin-2 induces human glioma invasion through the activation of matrix metalloprotease-2," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 15, pp. 8904–8909, 2003.

[14] L. Chen, Z. Yang, G. Wang, and C. Wang, "Expression of angiopoietin-2 gene and its receptor Tie2 in hepatocellular carcinoma," *Journal of Tongji Medical University*, vol. 21, no. 3, pp. 228–230, 2001.

[15] L. F. Brown, B. J. Dezube, K. Tognazzi, H. F. Dvorak, and G. D. Yancopoulos, "Expression of Tie1, Tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma," *American Journal of Pathology*, vol. 156, no. 6, pp. 2179–2183, 2000.

[16] L. M. Ellis, S. Ahmad, F. Fan, et al., "Angiopoietins and their role in colon cancer angiogenesis," *Oncology (Huntingt)*, vol. 16, no. 4, supplement 3, pp. 31–35, 2002.

[17] H. Ding, L. Roncari, X. Wu, et al., "Expression and hypoxic regulation of angiopoietins in human astrocytomas," *Neuro-Oncology*, vol. 3, no. 1, pp. 1–10, 2001.

[18] I. Fischer, J.-P. Gagner, M. Law, E. W. Newcomb, and D. Zagzag, "Angiogenesis in gliomas: biology and molecular pathophysiology," *Brain Pathology*, vol. 15, no. 4, pp. 297–310, 2005.

[19] O. H. Lee, J. Fueyo, J. Xu, et al., "Sustained angiopoietin-2 expression disrupts vessel formation and inhibits glioma growth," *Neoplasia*, vol. 8, no. 5, pp. 419–428, 2006.

[20] A. Stratmann, W. Risau, and K. H. Plate, "Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis," *American Journal of Pathology*, vol. 153, no. 5, pp. 1459–1466, 1998.

[21] S. Wang, A. Khan, F. F. Lang, and T. S. Schaefer, "Conditional gene expression in human intracranial xenograft tumors," *BioTechniques*, vol. 31, no. 1, pp. 196–202, 2001.

[22] D. Zagzag, A. Hooper, D. R. Friedlander, et al., "In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis," *Experimental Neurology*, vol. 159, no. 2, pp. 391–400, 1999.

[23] J. Holash, P. C. Maisonpierre, D. Compton, et al., "Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF," *Science*, vol. 284, no. 5422, pp. 1994–1998, 1999.

[24] D. Zagzag, R. Amirnovin, M. A. Greco, et al., "Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis," *Laboratory Investigation*, vol. 80, no. 6, pp. 837–849, 2000.

Hindawi Publishing Corporation
Journal of Oncology
Volume 2010, Article ID 729725, 13 pages
doi:10.1155/2010/729725

Review Article

Vascular Endothelial Growth Factor-Related Pathways in Hemato-Lymphoid Malignancies

Michael Medinger,[1] Natalie Fischer,[2] and Alexandar Tzankov[3]

[1] *Department of Hematology, University Hospital Basel, Petersgraben 4, CH-4031 Basel, Switzerland*
[2] *Clinic for Medical Oncology, University Hospital Basel, Petersgraben 4, CH-4031 Basel, Switzerland*
[3] *Institute of Pathology, University Hospital Basel, Schoenbeinstrasse 40, CH-4031 Basel, Switzerland*

Correspondence should be addressed to Alexandar Tzankov, atzankov@uhbs.ch

Received 6 November 2009; Revised 4 February 2010; Accepted 3 March 2010

Academic Editor: Arkadiusz Dudek

Angiogenesis is essential for malignant tumor growth. This has been documented for solid tumors, and there is emerging evidence suggesting that tumor progression of hemato-lymphoid malignancies also depends on the induction of new blood vessel formation. The most important pro-angiogenic agent is vascular endothelial growth factor (VEGF), activating VEGF receptors 1 and 2. The available data on angiogenesis in hemato-lymphoid malignancies such as acute leukemias, myelodysplastic syndromes, myeloproliferative neoplasms, multiple myeloma and lymphomas, point towards the significance of autocrine and paracrine VEGF-mediated effects for proliferation and survival of leukemia/lymphoma cells in addition to tumor vascularization. Anti-angiogenic strategies have become an important therapeutic modality for solid tumors. Several anti-angiogenic agents targeting VEGF-related pathways have are also being utilized in clinical trials for the treatment of hemato-lymphoid malignancies, and in some instances these pathways have emerged as promising therapeutic targets. This review summarizes recent advances in the basic understanding of the role of angiogenesis in hemato-lymphoid malignancies and the translation of such basic findings into clinical studies.

1. Introduction

New blood vessel formation (angiogenesis) is fundamental to tumor growth and spread. In adults, physiological angiogenesis is limited to a small number of specific processes, such as wound healing, tissue repair, and the female reproductive cycle [1]. The pioneering work of Judah Folkman led to the recognition that angiogenesis plays an important role in tumor development, progression, and metastasis [2]. Tumors require nutrients and oxygen to grow, and new blood vessels, formed by angiogenesis, provide these substrates. Tumor blood vessels are generated by various mechanisms, such as cooption of the existing vascular network, expansion of the host vascular network by budding of endothelial sprouts (sprouting angiogenesis), remodeling and expansion of vessels by the insertion of interstitial tissue columns into the lumen of preexisting vessels (intussusceptive angiogenesis), and homing of endothelial cell precursors (EPC; CEP) from the bone marrow or peripheral blood into the endothelial lining of neovessels (vasculogenesis) [3]. Bone marrow-derived progenitor cells contribute significantly to neovascularization in a variety of tumors [4, 5].

The key mediator of angiogenesis is the vascular endothelial growth factor (VEGF). Its expression is regulated by a plethora of intrinsic and extrinsic factors, with hypoxia and hypoglycemia being the major stimuli [6]. Hypoxia-induced transcription of *VEGF* mRNA is mediated by binding of hypoxia-inducible factor 1 (HIF-1) [7]. Cytokines may also modulate angiogenesis by regulating *VEGF* expression, for example, tumor necrosis factor (TNF)-α increases *VEGF* mRNA in glioma cells [8], and transforming growth factor (TGF)-β results in the induction of *VEGF* mRNA and protein in human lung adenocarcinoma cells [9]. In solid tumors, intratumoral hypoxia and HIF-1 mediation have been found to be a key angiogenesis triggering event [10]. Less is known about the exact trigger mechanisms of VEGF expression

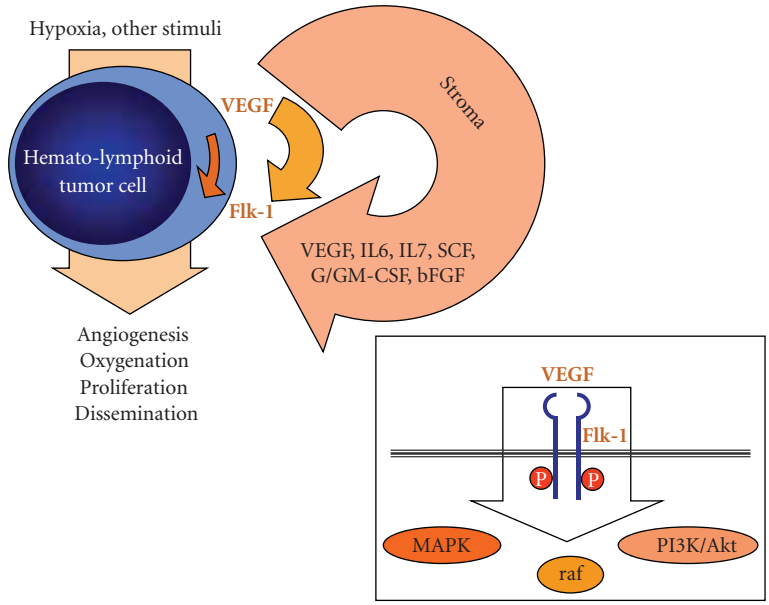


Figure 1: Possible vascular endothelial growth factor- (VEGF) and VEGF receptor-related (e.g., Flk-1, i.e., VEGFR-2) autocrine and paracrine loops in hemato-lymphoid neoplasms; insert: receptor tyrosine kinase activity and signaling cascades through VEGFR-2.

in hemato-lymphoid tumors, but mechanisms analogous to those observed in solid tumors are anticipated [11, 12]. Tight control of angiogenesis is maintained by a balance of endogenous antiangiogenic and proangiogenic factors. VEGF has a key, rate-limiting role in promoting tumor angiogenesis and exerts its effects by binding to one of three tyrosine kinase receptors (Figure 1): VEGF receptor-1 (VEGFR-1; fms-like tyrosine kinase-1, Flt-1), VEGFR-2 (human kinase domain region, KDR/murine fetal liver kinase-1, Flk-1), and VEGFR-3 (Flt-4). VEGFR-1 (ligands include VEGF-A, -B, and placental growth factor [PIGF]) and VEGFR-2 (ligands include VEGF-A, -C, and -D) are predominantly expressed on vascular endothelial cells, and activation of VEGFR-2 appears to be both necessary and sufficient to mediate VEGF-dependent angiogenesis and induction of vascular permeability [13, 14]. Both receptor tyrosine kinases are expressed in all adult endothelial cells except the brain. VEGFR-1 is also expressed on hematopoietic stem cells (HSC), vascular smooth muscle cells, monocytes, and leukemic cells [15, 16], while VEGFR-2 is expressed on endothelial progenitor cells and megakaryocytes [17, 18]. Although the exact contribution of VEGFR-1 signaling to angiogenesis is unclear, it has been shown that VEGFR-1 directly cooperates with VEGFR-2 via heterodimerization, as well as binding two additional VEGF homologues, VEGF-B and PIGF [19]. VEGFR-3, largely restricted to lymphatic endothelial cells, binds the VEGF homologues VEGF-C and VEGF-D and may play an important role in the regulation of lymphangiogenesis.

VEGF and VEGFR represent significant anticancer therapy targets that elegantly bypass potential tumor-related treatment barriers [13]. VEGF signaling inhibition has been shown to result in significant tumor growth delay in a wide range of animal models [20]. In the case of VEGF, even a single *VEGF* allele knock-out led to embryonic lethality in mice [21]. The clinical benefit of this approach has also been confirmed, and concerted efforts in recent years have resulted in a number of novel antiangiogenic agents [22]. The first antiangiogenic agent to be approved was bevacizumab (Avastin, Genentech), a humanized anti-VEGF monoclonal antibody. Administration of bevacizumab, in combination with cytotoxic chemotherapy, conferred benefits to patients with metastatic colorectal cancer, nonsquamous, nonsmall cell lung cancer, and metastatic breast cancer [23–25], and it is now under investigation for patients with relapsed and refractory acute leukemia in combination with standard chemotherapy [26]. Additionally, two small-molecule inhibitors targeting VEGFR and other kinases, sorafenib (Nexavar, Bayer and Onyx pharmaceuticals) and sunitinib (Sutent, Pfizer), have been approved based on their efficacy in treating renal cell- and hepatocellular carcinoma [27, 28]. A growing number of antiangiogenics are now either in various stages of clinical development or in clinical use as components of standard regimens (Table 1). The major classes of antiangiogenic therapy include (1) direct anti-VEGF acting molecules (anti-VEGF antibodies, *VEGF*-antisense nucleotides), (2) immunomodulatory drugs (IMIDs) with antiangiogenic properties, (3) receptor tyrosine kinase inhibitors that target VEGFR signaling as well as receptors of other (proangiogenic) factors, (4) the antiendothelial approach of metronomic therapy, and (5) other new compounds targeting signaling downstream to proangiogenic growth factors, such as mammalian target of rapamycin (mTOR) inhibitors, histone deacetylases' (HDAC) inhibitors, and proteasome inhibitors.

Several studies suggested that angiogenesis plays an important role, as might autocrine and paracrine VEGF/VEGFR-related loops in hemato-lymphoid malignancies

Table 1: Summary of clinical trials and approved antiangiogenic therapies in hemato-lymphoid malignancies.

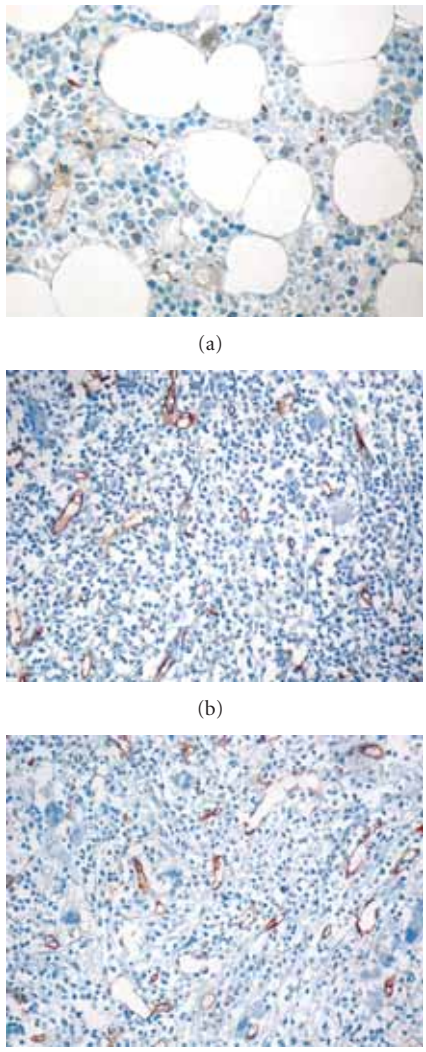
Drug class	Target	Study entities	Approved for
Anti-VEGF strategies			
Bevacizumab (Avastin)	VEGF-A	AML, MDS, CLL, CML, NHL, MM	Metastatic colorectal cancer, NSCLC, breast cancer
RTK inhibitors			
Vatalanib	VEGFR1-3, PDGFRβ, CD117	AML, PMF, MDS, CML, DLBCL, MM	
Cediranib (Recentin)	VEGFR1-3, PDGFRβ, CD117	AML, MDS, CLL	
Immunomodulators			
Thalidomide		AML, MDS, MPN, CLL, NHL, MM	MM
Lenalidomide (Revlimid)		AML, MDS, CLL, NHL	MM, 5q-MDS

such as acute and chronic leukemias, myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), lymphomas, and multiple myeloma (MM) [29–39] (Figure 1). Moreover, angiogenesis appears to be targeted even by conventional chemotherapy in hemato-lymphoid malignancies; for example, patients with acute myelogenous leukemia (AML) show increased microvessel density (MVD) in the bone marrow with subsequent MVD reduction under chemotherapy and return to normal levels in cases of complete remission (CR) [31]. This review will focus on the current knowledge of angiogenesis and antiangiogenic therapies (related to classes 1 to 3 of antiangiogenic treatment approaches) in hemato-lymphoid malignancies.

2. Acute Leukemias

Leukemias have been ever since associated with angiogenesis since the AML cell line HL-60 was first used to clone the *VEGF* gene [40]. The first demonstration that leukemia progression might be accompanied by an increase of bone marrow vascularization was provided by Judah Folkman's group [41], who demonstrated that the bone marrow of acute lymphoblastic leukemia (ALL) patients had increased blood vessel content compared to normal counterparts. Detailed analysis of bone marrow sections from ALL patients led to the development of a model to illustrate their irregular, albeit abundant, bone marrow vasculature. Moreover, it was also shown that urine and peripheral blood samples from ALL patients contained elevated levels of proangiogenic growth factors, namely, basic fibroblast growth factor (bFGF) and VEGF, which correlated with the increase of bone marrow angiogenesis [41, 42]. These studies raised the question of whether the growth of other types of hemato-lymphoid malignancies is also accompanied by increased angiogenesis, while proving that the basic molecular/cellular mechanisms occurring during leukemia expansion might be similar to those seen in solid tumors. The existence of an "angiogenesis switch", first proposed for solid tumors [43], was therefore suggested to apply to hemato-lymphoid malignancies as well. "Angiogenesis switch" in leukemia is documented by increased bone marrow MVD (Figures 2(a) and 2(b)), increased expression of HIF-1, multiple proangiogenic factors (VEGF, bFGF, angiopoietin-2), soluble VEGFR, and decreased expression of endogenous angiogenesis inhibitors, such as thrombospondin-1 [11, 12].

In a recent study by Norén-Nyström et al. [44], MVD, analyzed on 185 bone marrow biopsies, was higher in T-ALL compared to B-ALL. In the B-ALL group, cases with t(12;21) were characterized by a low MVD, while patients with hyperdiploid leukemia showed a high MVD. There was a correlation between MVD and white blood cell count in high-risk B-ALL patients. In addition, patients with a high marrow reticulin fiber density and high MVD had an unfavorable outcome. Similarly, in previously untreated AML, increased levels of plasma VEGF correlated with reduced survival and lower remission rates [45]. Moreover, the level of plasma/serum VEGF correlated with the number of circulating blasts [46], indicating the probable cellular origin of this proangiogenic factor. Such in vivo clinical studies are further supported by in vitro demonstrations of the capacity of leukemia cells to produce proangiogenic growth factors such as VEGF and bFGF [47–49]. Importantly, leukemia cells release increased amounts of proangiogenic factors in response to proinflammatory molecules, suggesting interactions with other cell types (e.g., the bone marrow stroma). In contrast to the abundant literature demonstrating that acute leukemia cells secrete significant amounts of angiogenesis activators such as VEGF, fewer studies have addressed the possibility that reduced production of angiogenesis inhibitors by these cells might also trigger the neovascularization process by shifting the local (bone marrow) angiogenesis balance [50, 51]. In addition to the modulation of bone marrow angiogenesis by leukemia cells, it was demonstrated that subsets of cases express endothelial-specific tyrosine kinase receptors, such as VEGFR-1, -2, and -3, or members of the FGF receptor family [49, 52, 53]. Recent studies showed that even hematopoietic stem cells (HSC) express VEGFR and are capable of generating functional autocrine loops that support their proliferation and survival [54], raising the question of whether this might be of relevance for leukemic stem cells.



(a)

(b)

(c)

Figure 2: (a) Microvascular architecture in normal bone marrow highlighted by CD34; note single capillaries. (b) Increased microvessel density in acute myelogenous leukemia and in (c) myeloproliferative neoplasm; note dilated sinus and atypical megakaryocytes in the latter.

2.1. Antiangiogenic Therapy in Acute Leukemias. Bevacizumab is a humanized murine antihuman VEGF monoclonal IgG_1 antibody that blocks the binding of human VEGF to its receptors, thereby also disrupting autocrine and paracrine survival mechanisms mediated by VEGFR-1 and VEGFR-2 [55]. In a Phase II clinical trial by Karp et al., bevacizumab was administered after chemotherapy to adults with refractory or relapsed AML [26]. Bevacizumab 10 mg/kg was administered on day 8 after cytarabine beginning day 1 and mitoxantrone beginning day 4. Forty-eight adults received induction therapy and the overall response was 23 of 48 (48%), with CR in 16 (33%). Eighteen patients (14 CR and 4 partial responses) underwent one consolidation cycle and 5 (3 CR and 2 partial responses) underwent allogeneic transplant. Median overall and disease-free survivals for CR patients were 16.2 months (64%, 1 year) and 7 months (35%, 1 year), respectively. Bone marrow samples demonstrated marked MVD decrease after bevacizumab administration. VEGF was detected in pretreatment serum in 67% of patients tested, increased by day 8 to 52%, and decreased to 93% (67% undetectable) 2 hours after bevacizumab administration. Currently, bevacizumab is being evaluated as treatment option for newly diagnosed AML in combination with cytarabine and idarubicin in a phase II study.

Thalidomide, originally marketed as a sedative and antiemetic drug, was withdrawn from use subsequent to reports of teratogenicity in the 1960s. The initial use of thalidomide was the treatment of erythema nodosum leprosum. It has been shown that thalidomide has important immunomodulatory effects in that it decreases TNF-α synthesis and selectively modulates T-cell subsets, shifting the T-cell population towards T helpers [56]. The interest in thalidomide as an antineoplastic agent rose after demonstration of its antiangiogenic activity in a rabbit model of corneal neovascularization that was induced in response to bFGF [57]. This report led to thalidomide application in MM, where it demonstrated a clinical benefit. Thalidomide and the newer IMIDs (e.g., lenalidomide) have been shown to significantly decrease the expression of the proangiogenic factors VEGF and interleukin-6 (IL-6) in MM [58]. The newer IMIDs were found to have 2-3 times more potent antiangiogenic activity than thalidomide in various in vivo assays [59]. The antiangiogenic activity of IMIDs has been shown to be independent of their immunomodulatory effects [60]. In AML patients, thalidomide therapy was assessed alone and in combination with other compounds. In a phase II study by Thomas et al. [61], thalidomide was analyzed in patients with relapsed or refractory AML previously treated with cytarabine-containing regimens. A total of 16 patients were treated with 200–800 mg/d oral thalidomide. Overall, one patient (6%) achieved CR lasting for 36 months, and two patients had a transient reduction in marrow blasts from 8% and 7% to less than 5%. There was no correlation between reduction of angiogenesis marker levels and response. In a phase I/II trial by Steins et al. [62], a dose-escalating trial was performed to study the safety and efficacy of thalidomide in 20 AML patients. Thirteen patients were assessable for both toxicity and response, tolerating a maximum dose of 200–400 mg/d for at least 1 month. Overall, adverse events were fatigue, constipation, rash, and neuropathy (grades 1 to 2 in most patients). In 4 patients, a partial response, defined as reduction of at least 50% of the blast cell infiltration in the bone marrow accompanied by increases of platelet counts and hemoglobin values, was observed. In parallel, MVD significantly decreased in these 5 patients during treatment with thalidomide. In a study by Barr et al. [63], thalidomide was examined in combination with fludarabine, carboplatin, and topotecan in 42 patients with poor prognosis AML, and 10 of 42 (24%) patients achieved a CR. Serious thrombotic adverse events were observed in 5 patients, suggesting that the combination of cytotoxic chemotherapy and thalidomide may be thrombogenic despite significant thrombocytopenia.

VEGF levels did not correlate with response to therapy, while a trend towards decreased MVD was noted in patients who achieved CR.

Small tyrosine kinase inhibitors that target VEGFR are a further important class of antiangiogenic drugs with application to AML, although their efficacy in hematolymphoid neoplasias, especially AML, might be attributable to inhibition of a variety of pathways, particularly those related to c-kit and flt3.

Vatalanib (formerly PTK787/ZK 222584) is an oral angiogenesis inhibitor that is active against VEGFR and PDGFR tyrosine kinases, thereby offering a novel approach to inhibiting tumor growth [64] by interfering with the ATP binding sites of VEGFR. In our phase I study, vatalanib was well tolerated and showed clinical activity in a variety of solid tumors [65]. In MM, its action primarily reduces the number of tumor microvessels and dilates the remaining vessels [66, 67]. Ongoing studies are now evaluating the efficacy of vatalanib in combination with imatinib in a phase I/II trial for patients with AML, PMF, and blast phase of chronic myelogenous leukemia. Vatalanib was studied in a phase I clinical trial alone or in combination with cytosine-arabinoside and daunorubicin in patients with MDS and AML [68]. Sixty-three patients received vatalanib at doses of 500–1000 mg/bid orally. At 1000 mg/bid, dose-limiting toxicities resulting in lethargy, hypertension, nausea, emesis, and anorexia were observed. CR was observed in 5 of 17 evaluable AML patients treated with vatalanib combined with chemotherapy. The authors concluded that vatalanib is generally well tolerated and can be given in combination with chemotherapy in patients with MDS and AML. In a recent study by Barbarroja et al. [69], vatalanib was examined in combination with idarubicin in 4 AML cell lines and 7 AML patient samples. Vatalanib decreased VEGF levels and VEGFR phosphorylation in AML cells, which showed *FLT3* internal tandem reduplications/mutations (ITD), raising questions about the actual targeted tyrosine kinase (VEGFR of flt3).

Cediranib (AZD2171, Recentin) is a potent inhibitor of both VEGFR-1 and VEGFR-2; it also has activity against c-kit, PDGFR-β, and VEGFR-3 at nanomolar concentrations [70]. In our study, cediranib was well tolerated up to 45 mg/d in patients with a broad range of solid tumors [71], with the most common adverse side-effects being diarrhea, dysphonia, and hypertension. In a phase I study with cediranib in 35 AML patients, the most common adverse events were diarrhea, hypertension, and fatigue. Six patients experienced an objective response (3 each at 20 and 30 mg). Dose- and time-dependent reductions of soluble VEGFR-2 were observed, and there was a correlation between cediranib exposure and plasma VEGF levels [72].

3. Myelodysplastic Syndromes

In MDS, VEGF is overexpressed by immature myeloid cells in the bone marrow and associated with increased bone marrow vascularity [52]. MVD parallels disease progression from refractory anemia to secondary AML [32]. MDS patients also have increased proangiogenic factors in peripheral blood [73]. Higher levels of VEGF were found by immunohistochemistry and corroborated by reverse transcriptase-polymerase chain reaction in patients with refractory anemia with excess blasts (RAEB) and RAEB in transformation (RAEB-T), compared to patients with refractory anemia (RA) and with ringed sideroblasts (RARS) or normal bone marrow controls [74]. These differences were thought to result from expression of VEGF in immature myeloid cells in RAEB and RAEB-T. To evaluate the interplay between angiogenesis and cytokines, we conducted a study of 89 MDS cases and showed [34] that TNF-α expression and bone marrow MVD correlated with each other as well. Importantly, thalidomide, a drug that modulates T-cell function and inhibits TNF-α activity as well as angiogenesis, is under investigation in clinical trials for the treatment of MDS [29, 75].

3.1. Antiangiogenic Therapy in Myelodysplastic Syndromes. A combination therapy of thalidomide and 5-azacytidine, a hypomethylating drug, was assessed in 40 patients with MDS and AML [76]. Hematological improvement was observed in 15 of 36 patients (42%), stable disease was observed in 5 of 36 patients (14%), 10 of 36 patients (28%) had disease progression, and six had CR. Lenalidomide, a synthetic compound derived by modifying the chemical structure of thalidomide, has also shown immunomodulatory and antiangiogenic properties and lower adverse effects rates [77]. Lenalidomide was investigated in a study of 148 MDS patients with 5q deletion [78]; 112 patients (76%) had a reduced need for transfusions, and 99 (67%) eliminated the need entirely regardless of karyotype complexity. Among 85 evaluable patients, 62 showed cytogenetic improvement, and 38 of that 62 showed complete cytogenetic remission. Therefore, lenalidomide was approved as a monotherapy for the treatment of transfusion-dependent MDS patients with 5q deletion with or without additional cytogenetic abnormalities.

4. Multiple Myeloma

MM is characterized by proliferation of malignant plasma cells that accumulate in the bone marrow and often produce a monoclonal immunoglobulin. MM was the first hematolymphoid malignancy in which increased angiogenesis was detected [36, 79]. New vessel formation in the bone marrow seems to play an important role in the pathogenesis of MM [80, 81]. Increased bone marrow MVD in patients with MM also appears to be an important prognostic factor [82]. Malignant plasma cells can secrete various cytokines, including VEGF, bFGF, and hepatocyte growth factor (HGF), all known for their proangiogenic activity [83]. It has been shown that MM cells are capable of secreting VEGF in response to IL-6 stimulation, and in response to this VEGF stimulation, microvascular endothelial cells and bone marrow stromal cells in turn secrete IL-6, a potent growth factor for malignant plasma cells, thus closing a paracrine loop [84, 85]. Rajkumar et al. showed a gradual increase of bone marrow angiogenesis along the disease

spectrum from monoclonal gammopathy of undetermined significance (MGUS) to smoldering MM, newly diagnosed MM, and relapsed MM [86], though the expression levels of VEGF, bFGF, and their receptors were similar among MGUS, smoldering MM, and newly diagnosed MM [87], suggesting that MVD increase in plasma cell neoplasias could be a function of chronology. In a recent study, Pour et al. examined 96 MM patients at diagnosis and after high-dose chemotherapy with regard to angiogenesis factor/inhibitor concentrations in the peripheral blood and bone marrow plasma [88]. Based on a significant decrease of VEGF and hepatocyte growth factor levels, and a significant increase in TSP-1 thrombospondin-1 concentrations in the bone marrow plasma of patients who achieved complete or very good partial response versus those who had partial or no response, they concluded that a reduction in the rate of angiogenesis had occurred.

4.1. Antiangiogenic Therapy in Multiple Myeloma. Thalidomide monotherapy in a phase II trial of 84 patients with relapsed and refractory MM who had received doses ranging from 200 to 800 mg/d resulted in an overall response rate of 32%. The 2-year event-free and overall survival were 20 and 48%, respectively [89, 90]. In combination with dexamethasone, the response rate was 63% compared to 41% with dexamethasone alone in patients with newly diagnosed MM [91]. Subsequent to these studies, thalidomide was approved for the treatment of newly diagnosed MM. In 2 phase III trials, lenalidomide in combination with dexamethasone resulted in remarkable response rates and significantly less toxicity than thalidomide [92, 93], and increased the response rate from 22.5% to 59.2% compared to dexamethasone alone in patients with previously-treated relapsed/refractory MM. Lenalidomide was approved in combination with dexamethasone for second-line treatment of MM.

5. Myeloproliferative Neoplasms

The available data on angiogenesis and expression of VEGF and VEGFR in the bone marrow of patients with *BCR-ABL1*-negative MPN suggest a significant increase of MVD (Figure 2(c)), especially in primary myelofibrosis (PMF), which might inversely correlate with survival [30, 33, 94–97]. The identification of an acquired somatic mutation in the *JAK2* gene, resulting in a valine to phenylalanine substitution at position 617 (*JAK2-V617F*), has provided new insights into the pathogenesis of *BCR-ABL1*-negative MPN, which is found in most patients with polycythemia vera (PV) and in about 50% of patients with essential thrombocythemia (ET) and PMF [98, 99]. The correlations between angiogenesis and *JAK2* status in MPN have been addressed in two studies with contradictory results [100, 101]. In a recent study [30], we found a significantly increased MVD and VEGF expression in MPN compared to controls, particularly in cases with high *JAK2-V617F* mutant allele burdens. Our results imply that higher activity of Jak2-related pathways, as observed in cases with higher *JAK2-V617F* mutant allele burdens, may influence angiogenesis in MPN. This assumption is further supported by our observation that the number of VEGF expressing cells did not rise concurrent with the increasing *JAK2-V617F* mutant allele burden regardless of rising MVD. Further support is provided by the study of Zhu et al. showing that H-2g, a glucose analog of the blood group H antigen, mediates endothelial cell chemotaxis and induces expression of the proangiogenic factors VEGF and bFGF through pathways involving Jak2 and phosphoinositide-3 kinase that could be abolished by treatment with the respective inhibitors AG490 and LY294002 [102]. The importance of VEGF/Jak2/STAT5 pathways in angiogenesis is substantiated by evidence from another study as well suggesting tight interactions between VEGF and Jak2 [103]. Thus, it could be speculated that in at least some hemato-lymphoid neoplasms, such as *BCR-ABL1*-negative MPN, key tumor-related gate-keeping genetic mechanisms might also directly influence angiogenesis. A very recent study identified the *JAK2-V617F* mutation in microdissected endothelial cells form the liver veins of Budd-Chiari syndrome patients [104], raising the hypothesis that endothelial cells in PV are direct players in the neoplastic process.

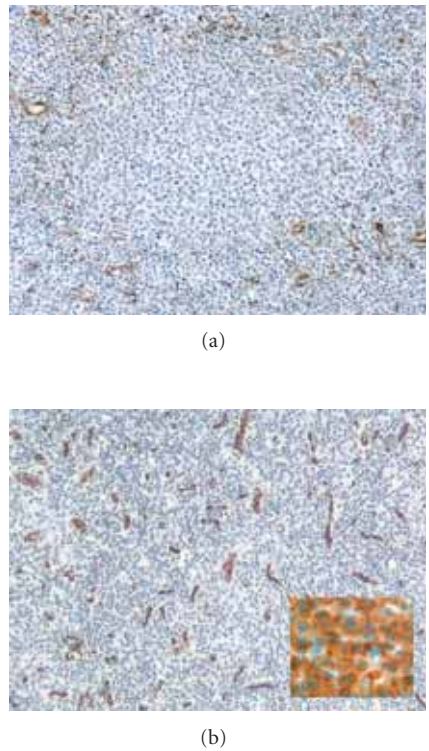
5.1. Antiangiogenic Therapy in Myeloproliferative Neoplasms. In a phase II study of 44 PMF patients, the efficacy of thalidomide monotherapy was assessed [105]. Seventeen of 41 evaluable patients (41%) receiving treatment for at least 15 days showed a response. CR (without reversal of bone marrow fibrosis) was achieved in 4 patients (10%), partial response in 4 patients (10%), and hematological improvements of anemia, thrombopenia, and/or splenomegaly were observed in 9 patients (21%). A further phase II thalidomide and placebo study assessed the efficacy of thalidomide in the treatment of anemia in PMF [106]. The primary outcome was a 2 g/l increase of hemoglobin levels resulting in a 20% reduction of transfusion needs. At 180 days, in an intention-to-treat analysis, no difference was observed between the thalidomide and placebo groups with regard to hemoglobin levels. In phase II studies with lenalidomide monotherapy in patients with symptomatic PMF, the overall response rates were 22% for anemia, 33% for splenomegaly, and 50% for thrombocytopenia [107]. In a combination study of lenalidomide with prednisone in 40 PMF patients [108], responses were recorded in 12 patients (30%) and are ongoing in 10 (25%), with a median time to response of 12 weeks. Three patients (7.5%) had partial response and nine (22.5%) had clinical improvement lasting a median of 18 months. Overall response rates were 30% for anemia and 42% for splenomegaly. Interestingly, all eight *JAK2-V617F*-positive responders also experienced a reduction of the baseline mutant allele burden.

In another study, vatalanib was administered to 29 PMF patients at doses of 500 or 750 mg/bid. One patient (3%) achieved CR and 5 (17%) achieved clinical improvement. Cumulatively, these studies indicated that vatalanib had modest activity in PMF patients [109].

6. Lymphomas

MVD is significantly higher in nodal lymphomas, particularly in those that are highly proliferative, than in reactive lymph nodes (Figures 3(a) and 3(b)) [35, 110–113]. In extranodal lymphomas, for example, cutaneous T-cell and B-cell lymphomas, MVD are higher than in normal skin or benign cutaneous lymphoproliferative disorders [114–116]. The interplay between lymphoma cells and tumor vessels is complex [117]; lymphoma-specific chromosomal aberrations such as t(8;14), t(11;14), and t(14;18) were discovered in tumor endothelial cells, calling into question the histogenesis of B-cell lymphoma vasculature [118]. Lymphoma growth and progression appear to be promoted by at least two distinct angiogenic mechanisms: autocrine stimulation of tumor cells via expression of VEGF and VEGFR by lymphoma cells, and paracrine influences of the proangiogenic tumor microenvironment on both local neovascular transformation and recruitment of circulating bone marrow-derived EPC [119]. The lymphoma microenvironment has been increasingly recognized as influencing neoplastic progression, in part by modulating angiogenic responses to distinct proangiogenic growth factors and cytokine milieu. In follicular lymphoma and diffuse large B-cell lymphoma (DLBCL), large-scale gene expression profiling studies demonstrated that genetic signatures expressed by stromal and infiltrating immune cells defined distinct prognostic groups [120, 121]. In the study of Lenz et al. [121], DLBCL gene-expression signatures correlated with survival. A multivariate model, created from three gene-expression signatures termed "germinal-center B-cell", "stromal-1", and "stromal-2", predicted survival and was influenced by differences in immune cells, fibrosis, and particularly angiogenesis in the tumor microenvironment.

The prognostic and predictive value (as possible treatment target) of MVD and angiogenic factors in lymphomas is still controversial due to the heterogeneity of diseases, different classifications, and methods for analysis (immunohistochemistry, serum levels of angiogenic markers, mRNA extraction, etc.). In B-cell lymphomas, VEGF protein and mRNA have been identified in DLBCL, mantle cell lymphoma (MCL), central nervous system DLBCL, and viral-related lymphomas [122]. A large study of 200 patients showed that high pretreatment levels of both serum VEGF and bFGF were independent prognostic factors for survival in multivariate analysis [123]. In a study with *de novo* DLBCL treated with anthracycline-based therapy, increased tumor vascularity was associated with poor overall survival independent of the international prognostic index [124]. Our results on the in situ expression of VEGF in B-cell lymphomas did not suggest a prognostic value, but showed distinct expression patterns among the different entities with higher prevalence in DLBCL [35]. Since VEGF expression in lymphomas parallels their proliferative activity [110–112], our findings of strong VEGF expression (20 out of 109 cases, 18%) and highest MVD in DLBCL were not surprising (Figure 2(b)) [35]. We found no direct correlation between increased MVD and VEGF expression in DLBCL.



(a)

(b)

Figure 3: (a) Microvascular organization of the cortical (B-cell-) zone of a normal lymph node highlighted by CD34; note a perifollicular condensation of microvessels and paucity inside the follicle. (b) Increased microvessel density in a mantle cell lymphoma case; insert: vascular endothelial growth factor expression in a diffuse large B-cell lymphoma case highlighted by immunohistochemistry.

Regarding MVD, follicular lymphoma is of special interest. Several studies have recognized increased vascularization in the perifollicular compartment of affected lymph nodes [35, 111, 113, 125]. The MVD inside the neoplastic follicles seems similar to that in reactive lymph nodes and somewhat lower than in DLBCL and MCL. Koster and Raemaekers [113] showed that in follicular lymphoma, increased vascularization is associated with improved clinical outcome. Furthermore, VEGF expression seemed not to be involved in follicular lymphoma angiogenesis.

In T-cell lymphomas, the *VEGF* gene is overexpressed in both microdissected lymphoma- and endothelial cells of angioimmunoblastic T-cell lymphoma (AITL) [126]. Accordingly, VEGF protein expression was also found in both types of cells in lymph nodes and bone marrow samples with AITL involvement.

In Hodgkin lymphoma (HL), VEGF is expressed along with HIF-1, VEGFR-2, and platelet-derived endothelial growth factor (PDGF) and its receptor (PDGFRα) at both the protein and RNA levels [127, 128]. MVD seems also to be increased in HL, especially in progressive disease [128].

6.1. Antiangiogenic Therapy in Lymphomas. Given the low proportion of DLBCL cases strongly expressing VEGF and

the lack of correlation between VEGF and MVD in DLBCL, it could be anticipated that bevacizumab application in DLBCL would be not very successful, explaining the low observed response rates. In a phase II study of the Southwest oncology group, single agent bevacizumab was examined in 52 DLBCL or MCL patients in first or second relapses of aggressive lymphomas [129]. Patients were treated with bevacizumab at 10 mg/kg every 2 weeks, resulting in a six-month progression-free survival of 16% with a response rate of 2%, and median duration of response or stable disease of 5.2 months (range 3.5–72.7). Treatment was generally well tolerated, with grade 3 hypertension being the most significant adverse side effect in two patients. Clinical trials combining active chemotherapy regimens with VEGF-targeted agents are currently in progress. Thalidomide was evaluated in a study of 19 patients with recurrent/refractory lymphomas until disease progression or prohibitive toxicity was observed [130]. One patient (5%) with evidence of recurrent gastric mucosa-associated lymphoid tissue B-cell lymphoma achieved CR, and 3 patients (16%) achieved stable disease. There is more promising data with lenalidomide treatment of indolent lymphomas, including follicular lymphoma [131, 132], with reported response rates about 30% in pretreated patients, but the significance of lenalidomide effects other than modulation of angiogenesis, for example, immunomodulation, should be considered in this instance.

Given this strong VEGF production in AITL, it is not surprising that the results of anti-angiogenesis therapy in relapsed AITL are promising. There are published case reports [133, 134] with successful bevacizumab treatment in AITL leading to remissions lasting for several months. One patient received 4th line bevacizumab leading to CR lasting for 10 months; another received 5th line bevacizumab leading to CR with such excellent tolerability that an allogeneic transplant could be planned. Furthermore, there is additional small, but promising, body of evidence in favor of thalidomide [135–137] and lenalidomide [http://www.org/ASCOv2/Meetings/Abstracts?&vmview=abst_detail_view&ID=65&abstractID=30959]. In peripheral T-cell lymphoma, antiangiogenic substances are not yet integrated in primary, curative-intended therapies. In the relapsed, palliative setting, however, there are some promising data, especially with lenalidomide (25 mg/d during 3 weeks in a 4-week cycle) [138], including unpublished data on 24 T-cell lymphomas, including 7 patients with AITL [http://www.asco.org/ASCOv2/Meetings/Abstracts?&vmview=abst_detail_view&confID=65&abstractID=30959]. In these heavily pretreated patients, the response rate was 30%, and long-lasting remissions were also observed in the responding patients.

After a successful tumor growth delay by bevacizumab administration in a xenograft HL model, Reiners et al. also showed some promising effects of combined gemcitabine/bevacizumab regimen in heavily pretreated HL patients with multiple relapses [139]. In a combination study, thalidomide was examined with vinblastine in patients with refractory HL [140]; of the 11 patients, 4 showed a partial response to treatment (response rate 36%) and two patients had stable disease.

7. Conclusion

Angiogenesis is essential to the development of hematolymphoid malignancies, including acute and chronic leuk emias, MPN, and lymphomas. In such instances, VEGF/VEGFR-related pathways are the most relevant regulators of neoangiogenesis, vasculogenesis, and recruitment of endothelial progenitor cells. Furthermore, VEGF/VEGFR interactions can stimulate proliferation, migration, and survival of leukemia/lymphoma cells by autocrine and paracrine loops. Finally, in some hemato-lymphoid neoplasms, VEGF/VEGFR-related pathways represent a promising therapeutic target.

Abbreviations

AML: Acute myelogenous leukemia
DLBCL: Diffuse large B-cell lymphoma
CLL: Chronic lymphocytic leukemia
NHL: Non-Hodgkin lymphoma
NSCLC: Nonsmall cell lung cancer
MDS: Myelodysplastic syndrome
MM: Multiple myeloma
MPN: Myeloproliferative neoplasm
PDGFR: Platelet derived growth factor receptor
PMF: Primary myelofibrosis
RTK: Receptor tyrosine kinase
VEGF: Vascular endothelial growth factor (receptor).

References

[1] J. Folkman, "Angiogenesis in cancer, vascular, rheumatoid and other disease," *Nature Medicine*, vol. 1, no. 1, pp. 27–31, 1995.

[2] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[3] D. Lyden, K. Hattori, S. Dias, et al., "Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth," *Nature Medicine*, vol. 7, no. 11, pp. 1194–1201, 2001.

[4] S. Rafii, D. Lyden, R. Benezra, K. Hattori, and B. Heissig, "Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy?" *Nature Reviews Cancer*, vol. 2, no. 11, pp. 826–835, 2002.

[5] B. A. Peters, L. A. Diaz Jr., K. Polyak, et al., "Contribution of bone marrow-derived endothelial cells to human tumor vasculature," *Nature Medicine*, vol. 11, no. 3, pp. 261–262, 2005.

[6] D. Shweiki, A. Itin, D. Soffer, and E. Keshet, "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis," *Nature*, vol. 359, no. 6398, pp. 843–845, 1992.

[7] Y. Dor, R. Porat, and E. Keshet, "Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis," *American Journal of Physiology*, vol. 280, no. 6, pp. C1367–C1374, 2001.

[8] M. Ryuto, M. Ono, H. Izumi, et al., "Induction of vascular endothelial growth factor by tumor necrosis factor α in human glioma cells: possible roles of SP-1," *Journal of Biological Chemistry*, vol. 271, no. 45, pp. 28220–28228, 1996.

[9] L. Pertovaara, A. Kaipainen, T. Mustonen, et al., "Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells," *Journal of Biological Chemistry*, vol. 269, no. 9, pp. 6271–6274, 1994.

[10] W. G. Kaelin Jr., "The von Hippel-Lindau tumor suppressor protein and clear cell renal carcinoma," *Clinical Cancer Research*, vol. 13, no. 2, pp. 680s–682s, 2007.

[11] X. Dong, Z. C. Han, and R. Yang, "Angiogenesis and antiangiogenic therapy in hematologic malignancies," *Critical Reviews in Oncology/Hematology*, vol. 62, no. 2, pp. 105–118, 2007.

[12] J. L. Frater, N. E. Kay, C. L. Goolsby, S. E. Crawford, G. W. Dewald, and L. C. Peterson, "Dysregulated angiogenesis in B-chronic lymphocytic leukemia: morphologic, immunohistochemical, and flow cytometric evidence," *Diagnostic Pathology*, vol. 3, no. 1, article 16, 2008.

[13] N. Ferrara, H.-P. Gerber, and J. LeCouter, "The biology of VEGF and its receptors," *Nature Medicine*, vol. 9, no. 6, pp. 669–676, 2003.

[14] H. Gille, J. Kowalski, B. Li, et al., "Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2): a reassessment using novel receptor-specific vascular endothelial growth factor mutants," *Journal of Biological Chemistry*, vol. 276, no. 5, pp. 3222–3230, 2001.

[15] K. Hattori, S. Dias, B. Heissig, et al., "Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells," *Journal of Experimental Medicine*, vol. 193, no. 9, pp. 1005–1014, 2001.

[16] W. T. Bellamy, L. Richter, Y. Frutiger, and T. M. Grogan, "Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies," *Cancer Research*, vol. 59, no. 3, pp. 728–733, 1999.

[17] M. Gill, S. Dias, K. Hattori, et al., "Vascular trauma induces rapid but transient mobilization of $VEGFR2^+AC133^+$ endothelial precursor cells," *Circulation Research*, vol. 88, no. 2, pp. 167–174, 2001.

[18] I. Casella, T. Feccia, C. Chelucci, et al., "Autocrine-paracrine VEGF loops potentiate the maturation of megakaryocytic precursors through Flt1 receptor," *Blood*, vol. 101, no. 4, pp. 1316–1323, 2003.

[19] M. Autiero, A. Luttun, M. Tjwa, and P. Carmeliet, "Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders," *Journal of Thrombosis and Haemostasis*, vol. 1, no. 7, pp. 1356–1370, 2003.

[20] K. J. Kim, B. Li, J. Winer, et al., "Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo," *Nature*, vol. 362, no. 6423, pp. 841–844, 1993.

[21] W. Risau, "Mechanisms of angiogenesis," *Nature*, vol. 386, no. 6626, pp. 671–674, 1997.

[22] M. Benouchan and B. M. Colombo, "Anti-angiogenic strategies for cancer therapy," *International Journal of Oncology*, vol. 27, no. 2, pp. 563–571, 2005.

[23] H. Hurwitz, L. Fehrenbacher, W. Novotny, et al., "Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer," *The New England Journal of Medicine*, vol. 350, no. 23, pp. 2335–2342, 2004.

[24] A. Sandler, R. Gray, M. C. Perry, et al., "Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer," *The New England Journal of Medicine*, vol. 355, no. 24, pp. 2542–2550, 2006.

[25] K. Miller, M. Wang, J. Gralow, et al., "Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer," *The New England Journal of Medicine*, vol. 357, no. 26, pp. 2666–2676, 2007.

[26] J. E. Karp, I. Gojo, R. Pili, et al., "Targeting vascular endothelial growth factor for relapsed and refractory adult acute myelogenous leukemias: therapy with sequential 1-β-D-arabinofuranosylcytosine, mitoxantrone, and bevacizumab," *Clinical Cancer Research*, vol. 10, no. 11, pp. 3577–3585, 2004.

[27] B. Escudier, T. Eisen, W. M. Stadler, et al., "Sorafenib in advanced clear-cell renal-cell carcinoma," *The New England Journal of Medicine*, vol. 356, no. 2, pp. 125–134, 2007.

[28] R. J. Motzer, T. E. Hutson, P. Tomczak, et al., "Sunitinib versus interferon alfa in metastatic renal-cell carcinoma," *The New England Journal of Medicine*, vol. 356, no. 2, pp. 115–124, 2007.

[29] A. Aguayo, "The role of angiogenesis in the biology and therapy of myelodysplastic syndromes," *Current Hematology Reports*, vol. 3, no. 3, pp. 184–191, 2004.

[30] M. Medinger, R. Skoda, A. Gratwohl, et al., "Angiogenesis and vascular endothelial growth factor-/receptor expression in myeloproliferative neoplasms: correlation with clinical parameters and JAK2-V617F mutational status," *British Journal of Haematology*, vol. 146, no. 2, pp. 150–157, 2009.

[31] T. Padró, S. Ruiz, R. Bieker, et al., "Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia," *Blood*, vol. 95, no. 8, pp. 2637–2644, 2000.

[32] G. Pruneri, F. Bertolini, D. Soligo, et al., "Angiogenesis in myelodysplastic syndromes," *British Journal of Cancer*, vol. 81, no. 8, pp. 1398–1401, 1999.

[33] M. Steurer, H. Zoller, F. Augustin, et al., "Increased angiogenesis in chronic idiopathic myelofibrosis: vascular endothelial growth factor as a prominent angiogenic factor," *Human Pathology*, vol. 38, no. 7, pp. 1057–1064, 2007.

[34] G. Stifter, S. Heiss, G. Gastl, A. Tzankov, and R. Stauder, "Over-expression of tumor necrosis factor-alpha in bone marrow biopsies from patients with myelodysplastic syndromes: relationship to anemia and prognosis," *European Journal of Haematology*, vol. 75, no. 6, pp. 485–491, 2005.

[35] A. Tzankov, S. Heiss, S. Ebner, et al., "Angiogenesis in nodal B cell lymphomas: a high throughput study," *Journal of Clinical Pathology*, vol. 60, no. 5, pp. 476–482, 2007.

[36] A. Vacca, D. Ribatti, L. Roncali, et al., "Bone marrow angiogenesis and progression in multiple myeloma," *British Journal of Haematology*, vol. 87, no. 3, pp. 503–508, 1994.

[37] M. Majka, A. Janowska-Wieczorek, J. Ratajczak, et al., "Numerous growth factors, cytokines, and chemokines are secreted by human $CD34^+$ cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner," *Blood*, vol. 97, no. 10, pp. 3075–3085, 2001.

[38] S. Dias, K. Hattori, B. Heissig, et al., "Inhibition of both paracrine and autocrine VEGF/VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 19, pp. 10857–10862, 2001.

[39] S. Loges, G. Heil, M. Bruweleit, et al., "Analysis of concerted expression of angiogenic growth factors in acute myeloid leukemia: expression of angiopoietin-2 represents an independent prognostic factor for overall survival," *Journal of Clinical Oncology*, vol. 23, no. 6, pp. 1109–1117, 2005.

[40] D. W. Leung, G. Cachianes, W.-J. Kuang, D. V. Goeddel, and N. Ferrara, "Vascular endothelial growth factor is a secreted angiogenic mitogen," *Science*, vol. 246, no. 4935, pp. 1306–1309, 1989.

[41] A. R. Perez-Atayde, S. E. Sallan, U. Tedrow, S. Connors, E. Allred, and J. Folkman, "Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia," *American Journal of Pathology*, vol. 150, no. 3, pp. 815–821, 1997.

[42] S. Yetgin, I. Yenicesu, M. Çetin, and M. Tuncer, "Clinical importance of serum vascular endothelial and basic fibroblast growth factors in children with acute lymphoblastic leukemia," *Leukemia & Lymphoma*, vol. 42, no. 1-2, pp. 83–88, 2001.

[43] D. Hanahan and J. Folkman, "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis," *Cell*, vol. 86, no. 3, pp. 353–364, 1996.

[44] U. Norén-Nyström, M. Heyman, P. Frisk, et al., "Vascular density in childhood acute lymphoblastic leukaemia correlates to biological factors and outcome," *British Journal of Haematology*, vol. 146, no. 5, pp. 521–530, 2009.

[45] A. Aguayo, H. M. Kantarjian, E. H. Estey, et al., "Plasma vascular endothelial growth factor levels have prognostic significance in patients with acute myeloid leukemia but not in patients with myelodysplastic syndromes," *Cancer*, vol. 95, no. 9, pp. 1923–1930, 2002.

[46] A. Aguayo, E. Estey, H. Kantarjian, et al., "Cellular vascular endothelial growth factor is a predictor of outcome in patients with acute myeloid leukemia," *Blood*, vol. 94, no. 11, pp. 3717–3721, 1999.

[47] W. Fiedler, U. Graeven, S. Ergün, et al., "Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia," *Blood*, vol. 89, no. 6, pp. 1870–1875, 1997.

[48] H. Chen, A. T. Treweeke, D. C. West, et al., "In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells," *Blood*, vol. 96, no. 9, pp. 3181–3187, 2000.

[49] S. Dias, K. Hattori, Z. Zhu, et al., "Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration," *Journal of Clinical Investigation*, vol. 106, no. 4, pp. 511–521, 2000.

[50] Q. Hu, A. L. Dey, Y. Yang, et al., "Soluble vascular endothelial growth factor receptor 1, and not receptor 2, is an independent prognostic factor in acute myeloid leukemia and myelodysplastic syndromes," *Cancer*, vol. 100, no. 9, pp. 1884–1891, 2004.

[51] R. Lai, E. Estey, Y. Shen, et al., "Clinical significance of plasma endostatin in acute myeloid leukemia/myelodysplastic syndrome," *Cancer*, vol. 94, no. 1, pp. 14–17, 2002.

[52] W. T. Bellamy, "Expression of vascular endothelial growth factor and its receptors in multiple myeloma and other hematopoietic malignancies," *Seminars in Oncology*, vol. 28, no. 6, pp. 551–559, 2001.

[53] M. Allouche, F. Bayard, S. Clamens, G. Fillola, P. Sie, and F. Amalric, "Expression of basic fibroblast growth factor (bFGF) and FCF-receptors in human leukemic cells," *Leukemia*, vol. 9, no. 1, pp. 77–86, 1995.

[54] H.-P. Gerber, A. K. Malik, G. P. Solar, et al., "VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism," *Nature*, vol. 417, no. 6892, pp. 954–958, 2002.

[55] L. G. Presta, H. Chen, S. J. O'Connor, et al., "Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders," *Cancer Research*, vol. 57, no. 20, pp. 4593–4599, 1997.

[56] B. Moncada, M. L. Baranda, R. González-Amaro, R. Urbina, and C. E. Loredo, "Thalidomide—effect on T cell subsets as a possible mechanism of action," *International Journal of Leprosy*, vol. 53, no. 2, pp. 201–205, 1985.

[57] R. J. D'Amato, M. S. Loughnan, E. Flynn, and J. Folkman, "Thalidomide is an inhibitor of angiogenesis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 9, pp. 4082–4085, 1994.

[58] D. Gupta, S. P. Treon, Y. Shima, et al., "Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications," *Leukemia*, vol. 15, no. 12, pp. 1950–1961, 2001.

[59] S. K. Teo, "Properties of thalidomide and its analogues: implications for anticancer therapy," *AAPS Journal*, vol. 7, no. 1, pp. E14–E19, 2005.

[60] K. Dredge, J. B. Marriott, C. D. Macdonald, et al., "Novel thalidomide analogues display anti-angiogenic activity independently of immunomodulatory effects," *British Journal of Cancer*, vol. 87, no. 10, pp. 1166–1172, 2002.

[61] D. A. Thomas, E. Estey, F. J. Giles, et al., "Single agent thalidomide in patients with relapsed or refractory acute myeloid leukaemia," *British Journal of Haematology*, vol. 123, no. 3, pp. 436–441, 2003.

[62] M. B. Steins, T. Padró, R. Bieker, et al., "Efficacy and safety of thalidomide in patients with acute myeloid leukemia," *Blood*, vol. 99, no. 3, pp. 834–839, 2002.

[63] P. Barr, P. Fu, H. Lazarus, et al., "Antiangiogenic activity of thalidomide in combination with fludarabine, carboplatin, and topotecan for high-risk acute myelogenous leukemia," *Leukemia & Lymphoma*, vol. 48, no. 10, pp. 1940–1949, 2007.

[64] J. Drevs, R. Müller-Driver, C. Wittig, et al., "PTK787/ZK 222584, a specific vascular endothelial growth factor-receptor tyrosine kinase inhibitor, affects the anatomy of the tumor vascular bed and the functional vascular properties as detected by dynamic enhanced magnetic resonance imaging," *Cancer Research*, vol. 62, no. 14, pp. 4015–4022, 2002.

[65] K. Mross, J. Drevs, M. Müller, et al., "Phase I clinical and pharmacokinetic study of PTK/ZK, a multiple VEGF receptor inhibitor, in patients with liver metastases from solid tumours," *European Journal of Cancer*, vol. 41, no. 9, pp. 1291–1299, 2005.

[66] J. M. Wood, G. Bold, E. Buchdunger, et al., "PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration," *Cancer Research*, vol. 60, no. 8, pp. 2178–2189, 2000.

[67] B. Lin, K. Podar, D. Gupta, et al., "The vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584 inhibits growth and migration of multiple myeloma cells in the bone marrow microenvironment," *Cancer Research*, vol. 62, no. 17, pp. 5019–5026, 2002.

[68] G. J. Roboz, F. J. Giles, A. F. List, et al., "Phase 1 study of PTK787/ZK 222584, a small molecule tyrosine kinase receptor inhibitor, for the treatment of acute myeloid leukemia and myelodysplastic syndrome," *Leukemia*, vol. 20, no. 6, pp. 952–957, 2006.

[69] N. Barbarroja, L. A. Torres, M. J. Luque, et al., "Additive effect of PTK787/ZK 222584, a potent inhibitor of VEGFR phosphorylation, with Idarubicin in the treatment of acute

myeloid leukemia," *Experimental Hematology*, vol. 37, no. 6, pp. 679–691, 2009.

[70] S. R. Wedge, J. Kendrew, L. F. Hennequin, et al., "AZD2171: a highly potent, orally bioavailable, vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor for the treatment of cancer," *Cancer Research*, vol. 65, no. 10, pp. 4389–4400, 2005.

[71] J. Drevs, P. Siegert, M. Medinger, et al., "Phase I clinical study of AZD2171, an oral vascular endothelial growth factor signaling inhibitor, in patients with advanced solid tumors," *Journal of Clinical Oncology*, vol. 25, no. 21, pp. 3045–3054, 2007.

[72] W. Fiedler, R. Mesters, M. Heuser, et al., "An open-label, phase I study of cediranib (RECENTIN^{TM}) in patients with acute myeloid leukemia," *Leukemia Research*, vol. 34, no. 2, pp. 196–202, 2010.

[73] B. Brunner, E. Gunsilius, P. Schumacher, H. Zwierzina, G. Gastl, and R. Stauder, "Blood levels of angiogenin and vascular endothelial growth factor are elevated in myelodysplastic syndromes and in acute myeloid leukemia," *Journal of Hematotherapy and Stem Cell Research*, vol. 11, no. 1, pp. 119–125, 2002.

[74] F. Wimazal, M.-T. Krauth, A. Vales, et al., "Immunohistochemical detection of vascular endothelial growth factor (VEGF) in the bone marrow in patients with myelodysplastic syndromes: correlation between VEGF expression and the FAB category," *Leukemia & Lymphoma*, vol. 47, no. 3, pp. 451–460, 2006.

[75] P. Musto, "Thalidomide therapy for myelodysplastic syndromes: current status and future perspectives," *Leukemia Research*, vol. 28, no. 4, pp. 325–332, 2004.

[76] A. Raza, M. Mehdi, M. Mumtaz, F. Ali, S. Lascher, and N. Galili, "Combination of 5-azacytidine and thalidomide for the treatment of myelodysplastic syndromes and acute myeloid leukemia," *Cancer*, vol. 113, no. 7, pp. 1596–1604, 2008.

[77] V. Kotla, S. Goel, S. Nischal, et al., "Mechanism of action of lenalidomide in hematological malignancies," *Journal of Hematology and Oncology*, vol. 2, article 36, 2009.

[78] A. List, G. Dewald, J. Bennett, et al., "Lenalidomide in the myelodysplastic syndrome with chromosome 5q deletion," *The New England Journal of Medicine*, vol. 355, no. 14, pp. 1456–1465, 2006.

[79] A. M. Roccaro, T. Hideshima, N. Raje, et al., "Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells," *Cancer Research*, vol. 66, no. 1, pp. 184–191, 2006.

[80] O. Sezer, K. Niemöller, J. Eucker, et al., "Bone marrow microvessel density is a prognostic factor for survival in patients with multiple myeloma," *Annals of Hematology*, vol. 79, no. 10, pp. 574–577, 2000.

[81] S. V. Rajkumar, T. Leong, P. C. Roche, et al., "Prognostic value of bone marrow angiogenesis in multiple myeloma," *Clinical Cancer Research*, vol. 6, no. 8, pp. 3111–3116, 2000.

[82] N. C. Munshi and C. Wilson, "Increased bone marrow microvessel density in newly diagnosed multiple myeloma carries a poor prognosis," *Seminars in Oncology*, vol. 28, no. 6, pp. 565–569, 2001.

[83] S. Kumar, T. E. Witzig, M. Timm, et al., "Expression of VEGF and its receptors by myeloma cells," *Leukemia*, vol. 17, no. 10, pp. 2025–2031, 2003.

[84] K. Podar, Y.-T. Tai, F. E. Davies, et al., "Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration," *Blood*, vol. 98, no. 2, pp. 428–435, 2001.

[85] B. Dankbar, T. Padró, R. Leo, et al., "Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma," *Blood*, vol. 95, no. 8, pp. 2630–2636, 2000.

[86] S. V. Rajkumar, R. A. Mesa, and A. Tefferi, "A review of angiogenesis and anti-angiogenic therapy in hematologic malignancies," *Journal of Hematotherapy and Stem Cell Research*, vol. 11, no. 1, pp. 33–47, 2002.

[87] S. Kumar, T. E. Witzig, M. Timm, et al., "Bone marrow angiogenic ability and expression of angiogenic cytokines in myeloma: evidence favoring loss of marrow angiogenesis inhibitory activity with disease progression," *Blood*, vol. 104, no. 4, pp. 1159–1165, 2004.

[88] L. Pour, H. Svachova, Z. Adam, et al., "Levels of angiogenic factors in patients with multiple myeloma correlate with treatment response," *Annals of Hematology*, vol. 89, no. 4, pp. 385–389, 2010.

[89] S. Singhal, J. Mehta, R. Desikan, et al., "Antitumor activity of thalidomide in refractory multiple myeloma," *The New England Journal of Medicine*, vol. 341, no. 21, pp. 1565–1571, 1999.

[90] B. Barlogie, R. Desikan, P. Eddlemon, et al., "Extended survival in advanced and refractory multiple myeloma after single-agent thalidomide: identification of prognostic factors in a phase 2 study of 169 patients," *Blood*, vol. 98, no. 2, pp. 492–494, 2001.

[91] S. V. Rajkumar, E. Blood, D. Vesole, R. Fonseca, and P. R. Greipp, "Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group," *Journal of Clinical Oncology*, vol. 24, no. 3, pp. 431–436, 2006.

[92] M. Dimopoulos, A. Spencer, M. Attal, et al., "Lenalidomide plus dexamethasone for relapsed or refractory multiple myeloma," *The New England Journal of Medicine*, vol. 357, no. 21, pp. 2123–2132, 2007.

[93] D. M. Weber, C. Chen, R. Niesvizky, et al., "Lenalidomide plus dexamethasone for relapsed multiple myeloma in North America," *The New England Journal of Medicine*, vol. 357, no. 21, pp. 2133–2142, 2007.

[94] L. G. Lundberg, R. Lerner, P. Sundelin, R. Rogers, J. Folkman, and J. Palmblad, "Bone marrow in polycythemia vera, chronic myelocytic leukemia, and myelofibrosis has an increased vascularity," *American Journal of Pathology*, vol. 157, no. 1, pp. 15–19, 2000.

[95] E. Zetterberg, L. G. Lundberg, and J. Palmblad, "Characterization of blood vessels in bone marrow from patients with chronic myeloid leukemia and polycythemia vera," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 64, no. 7, pp. 641–647, 2004.

[96] T. Wróbel, G. Mazur, P. Surowiak, D. Wołowiec, M. Jeleń, and K. Kuliczkowski, "Increased expression of vascular endothelial growth factor (VEGF) in bone marrow of patients with myeloproliferative disorders (MPD)," *Pathology and Oncology Research*, vol. 9, no. 3, pp. 170–173, 2003.

[97] M. Ponzoni, D. G. Savage, A. J. M. Ferreri, et al., "Chronic idiopathic myelofibrosis: independent prognostic importance of bone marrow microvascular density evaluated by CD105 (endoglin) immunostaining," *Modern Pathology*, vol. 17, no. 12, pp. 1513–1520, 2004.

[98] R. Kralovics, F. Passamonti, A. S. Buser, et al., "A gain-of-function mutation of JAK2 in myeloproliferative disorders," *The New England Journal of Medicine*, vol. 352, no. 17, pp. 1779–1790, 2005.

[99] R. Kralovics, S.-S. Teo, S. Li, et al., "Acquisition of the V617F mutation of JAK2 is a late genetic event in a subset of patients with myeloproliferative disorders," *Blood*, vol. 108, no. 4, pp. 1377–1380, 2006.

[100] E. Boveri, F. Passamonti, E. Rumi, et al., "Bone marrow microvessel density in chronic myeloproliferative disorders: a study of 115 patients with clinicopathological and molecular correlations," *British Journal of Haematology*, vol. 140, no. 2, pp. 162–168, 2008.

[101] U. Gianelli, C. Vener, P. R. Raviele, et al., "VEGF expression correlates with microvessel density in Philadelphia chromosome-negative chronic myeloproliferative disorders," *American Journal of Clinical Pathology*, vol. 128, no. 6, pp. 966–973, 2007.

[102] K. Zhu, M. A. Amin, Y. Zha, L. A. Harlow, and A. E. Koch, "Mechanism by which H-2g, a glucose analog of blood group H antigen, mediates angiogenesis," *Blood*, vol. 105, no. 6, pp. 2343–2349, 2005.

[103] A. C. Dudley, D. Thomas, J. Best, and A. Jenkins, "A VEGF/JAK2/STAT5 axis may partially mediate endothelial cell tolerance to hypoxia," *Biochemical Journal*, vol. 390, no. 2, pp. 427–436, 2005.

[104] S. Sozer, M. I. Fiel, T. Schiano, M. Xu, J. Mascarenhas, and R. Hoffman, "The presence of JAK2V617F mutation in the liver endothelial cells of patients with Budd-Chiari syndrome," *Blood*, vol. 113, no. 21, pp. 5246–5249, 2009.

[105] D. A. Thomas, F. J. Giles, M. Albitar, et al., "Thalidomide therapy for myelofibrosis with myeloid metaplasia," *Cancer*, vol. 106, no. 9, pp. 1974–1984, 2006.

[106] J.-F. Abgrall, I. Guibaud, J.-N. Bastie, et al., "Thalidomide versus placebo in myeloid metaplasia with myelofibrosis: a prospective, randomized, double-blind, multicenter study," *Haematologica*, vol. 91, no. 8, pp. 1027–1032, 2006.

[107] A. Tefferi, J. Cortes, S. Verstovsek, et al., "Lenalidomide therapy in myelofibrosis with myeloid metaplasia," *Blood*, vol. 108, no. 4, pp. 1158–1164, 2006.

[108] A. Quintás-Cardama, H. M. Kantarjian, T. Manshouri, et al., "Lenalidomide plus prednisone results in durable clinical, histopathologic, and molecular responses in patients with myelofibrosis," *Journal of Clinical Oncology*, vol. 27, no. 28, pp. 4760–4766, 2009.

[109] F. J. Giles, A. F. List, M. Carroll, et al., "PTK787/ZK 222584, a small molecule tyrosine kinase receptor inhibitor of vascular endothelial growth factor (VEGF), has modest activity in myelofibrosis with myeloid metaplasia," *Leukemia Research*, vol. 31, no. 7, pp. 891–897, 2007.

[110] D. Ribatti, A. Vacca, B. Nico, M. Fanelli, L. Roncali, and F. Dammacco, "Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphomas. An immunohistochemical and ultrastructural study," *European Journal of Haematology*, vol. 56, no. 1-2, pp. 45–53, 1996.

[111] A. Vacca, D. Ribatti, L. Ruco, et al., "Angiogenesis extent and macrophage density increase simultaneously with pathological progression in B-cell non-Hodgkin's lymphomas," *British Journal of Cancer*, vol. 79, no. 5-6, pp. 965–970, 1999.

[112] V. Arias and F. A. Soares, "Vascular density (tumor angiogenesis) in non-Hodgkin's lymphomas and florid follicular hyperplasia: a morphometric study," *Leukemia & Lymphoma*, vol. 40, no. 1-2, pp. 157–166, 2001.

[113] A. Koster and J. M. M. Raemaekers, "Angiogenesis in malignant lymphoma," *Current Opinion in Oncology*, vol. 17, no. 6, pp. 611–616, 2005.

[114] A. Vacca, S. Moretti, D. Ribatti, et al., "Progression of mycosis fungoides is associated with changes in angiogenesis and expression of the matrix metalloproteinases 2 and 9," *European Journal of Cancer*, vol. 33, no. 10, pp. 1685–1692, 1997.

[115] L. Schaerer, M. H. Schmid, B. Mueller, R. G. Dummer, G. Burg, and W. Kempf, "Angiogenesis in cutaneous lymphoproliferative disorders. Microvessel density discriminates between cutaneous B-cell lymphomas and B-cell pseudolymphomas," *American Journal of Dermatopathology*, vol. 22, no. 2, pp. 140–143, 2000.

[116] G. Mazur, Z. Woźniak, T. Wróbel, J. Maj, and K. Kuliczkowski, "Increased angiogenesis in cutaneous T-cell lymphomas," *Pathology and Oncology Research*, vol. 10, no. 1, pp. 34–36, 2004.

[117] E. Passalidou, M. Stewart, M. Trivella, et al., "Vascular patterns in reactive lymphoid tissue and in non-Hodgkin's lymphoma," *British Journal of Cancer*, vol. 88, no. 4, pp. 553–559, 2003.

[118] B. Streubel, A. Chott, D. Huber, et al., "Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas," *The New England Journal of Medicine*, vol. 351, no. 3, pp. 250–259, 2004.

[119] J. Ruan, K. Hajjar, S. Rafii, and J. P. Leonard, "Angiogenesis and antiangiogenic therapy in non-Hodgkin's lymphoma," *Annals of Oncology*, vol. 20, no. 3, pp. 413–424, 2009.

[120] S. S. Dave, G. Wright, B. Tan, et al., "Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells," *The New England Journal of Medicine*, vol. 351, no. 21, pp. 2159–2169, 2004.

[121] G. Lenz, G. Wright, S. S. Dave, et al., "Stromal gene signatures in large-B-cell lymphomas," *The New England Journal of Medicine*, vol. 359, no. 22, pp. 2313–2323, 2008.

[122] C.-L. Ho, L.-F. Sheu, and C.-Y. Li, "Immunohistochemical expression of basic fibroblast growth factor, vascular endothelial growth factor, and their receptors in stage IV non-Hodgkin lymphoma," *Applied Immunohistochemistry and Molecular Morphology*, vol. 10, no. 4, pp. 316–321, 2002.

[123] P. Salven, A. Orpana, L. Teerenhovi, and H. Joensuu, "Simultaneous elevation in the serum concentrations of the angiogenic growth factors VEGF and bFGF is an independent predictor of poor prognosis in non-Hodgkin lymphoma: a single-institution study of 200 patients," *Blood*, vol. 96, no. 12, pp. 3712–3718, 2000.

[124] D. Gratzinger, S. Zhao, R. J. Tibshirani, et al., "Prognostic significance of VEGF, VEGF receptors, and microvessel density in diffuse large B cell lymphoma treated with anthracycline-based chemotherapy," *Laboratory Investigation*, vol. 88, no. 1, pp. 38–47, 2008.

[125] J. M. Jørgensen, F. B. Sørensen, K. Bendix, et al., "Angiogenesis in non-Hodgkin's lymphoma: clinico-pathological correlations and prognostic significance in specific subtypes," *Leukemia & Lymphoma*, vol. 48, no. 3, pp. 584–595, 2007.

[126] W.-L. Zhao, S. Mourah, N. Mounier, et al., "Vascular endothelial growth factor-A is expressed both on lymphoma cells and endothelial cells in angioimmunoblastic T-cell lymphoma and related to lymphoma progression," *Laboratory Investigation*, vol. 84, no. 11, pp. 1512–1519, 2004.

[127] I. A. Doussis-Anagnostopoulou, K. L. Talks, H. Turley, et al., "Vascular endothelial growth factor (VEGF) is expressed

by neoplastic Hodgkin-Reed-Sternberg cells in Hodgkin's disease," *Journal of Pathology*, vol. 197, no. 5, pp. 677–683, 2002.

[128] T. Mainou-Fowler, B. Angus, S. Miller, S. J. Proctor, P. R. A. Taylor, and K. M. Wood, "Micro-vessel density and the expression of vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PdEGF) in classical Hodgkin lymphoma (HL)," *Leukemia & Lymphoma*, vol. 47, no. 2, pp. 223–230, 2006.

[129] A. T. Stopeck, J. M. Unger, L. M. Rimsza, et al., "A phase II trial of single agent bevacizumab in patients with relapsed, aggressive non-Hodgkin lymphoma: southwest oncology group study S0108," *Leukemia & Lymphoma*, vol. 50, no. 5, pp. 728–735, 2009.

[130] B. Pro, A. Younes, M. Albitar, et al., "Thalidomide for patients with recurrent lymphoma," *Cancer*, vol. 100, no. 6, pp. 1186–1189, 2004.

[131] P. H. Wiernik, I. S. Lossos, J. M. Tuscano, et al., "Lenalidomide monotherapy in relapsed or refractory aggressive non-Hodgkin's lymphoma," *Journal of Clinical Oncology*, vol. 26, no. 30, pp. 4952–4957, 2008.

[132] T. E. Witzig, P. H. Wiernik, T. Moore, et al., "Lenalidomide oral monotherapy produces durable responses in relapsed or refractory indolent non-Hodgkin's lymphoma," *Journal of Clinical Oncology*, vol. 27, no. 32, pp. 5404–5409, 2009.

[133] I. Bruns, F. Fox, P. Reinecke, et al., "Complete remission in a patient with relapsed angioimmunoblastic T-cell lymphoma following treatment with bevacizumab," *Leukemia*, vol. 19, no. 11, pp. 1993–1995, 2005.

[134] D. Aguiar Bujanda, "Complete response of relapsed angioimmunoblastic T-cell lymphoma following therapy with bevacizumab," *Annals of Oncology*, vol. 19, no. 2, pp. 396–397, 2008.

[135] K. Ramasamy, Z. Lim, A. Pagliuca, J. R. Salisbury, G. J. Mufti, and S. Devereux, "Successful treatment of refractory angioimmunoblastic T-cell lymphoma with thalidomide and dexamethasone," *Haematologica*, vol. 91, supplement 8, article ECR44, pp. 117–118, 2006.

[136] A. Dogan, L. S. P. Ngu, S. H. Ng, and P. L. Cervi, "Pathology and clinical features of angioimmunoblastic T-cell lymphoma after successful treatment with thalidomide," *Leukemia*, vol. 19, no. 5, pp. 873–875, 2005.

[137] C. Strupp, M. Aivado, U. Germing, N. Gattermann, and R. Haas, "Angioimmunoblastic lymphadenopathy (AILD) may respond to thalidomide treatment: two case reports," *Leukemia & Lymphoma*, vol. 43, no. 1, pp. 133–137, 2002.

[138] S. M. Horwitz, "Novel therapies for cutaneous T-cell lymphomas," *Clinical Lymphoma & Myeloma*, vol. 8, supplement 5, pp. S187–S192, 2008.

[139] K. S. Reiners, A. Gossmann, E. P. von Strandmann, B. Böll, A. Engert, and P. Borchmann, "Effects of the anti-VEGF Monoclonal antibody bevacizumab in a preclinical model and in patients with refractory and multiple relapsed hodgkin lymphoma," *Journal of Immunotherapy*, vol. 32, no. 5, pp. 508–512, 2009.

[140] J. Kuruvilla, K. Song, P. Mollee, et al., "A phase II study of thalidomide and vinblastine for palliative patients with Hodgkin's lymphoma," *Hematology*, vol. 11, no. 1, pp. 25–29, 2006.