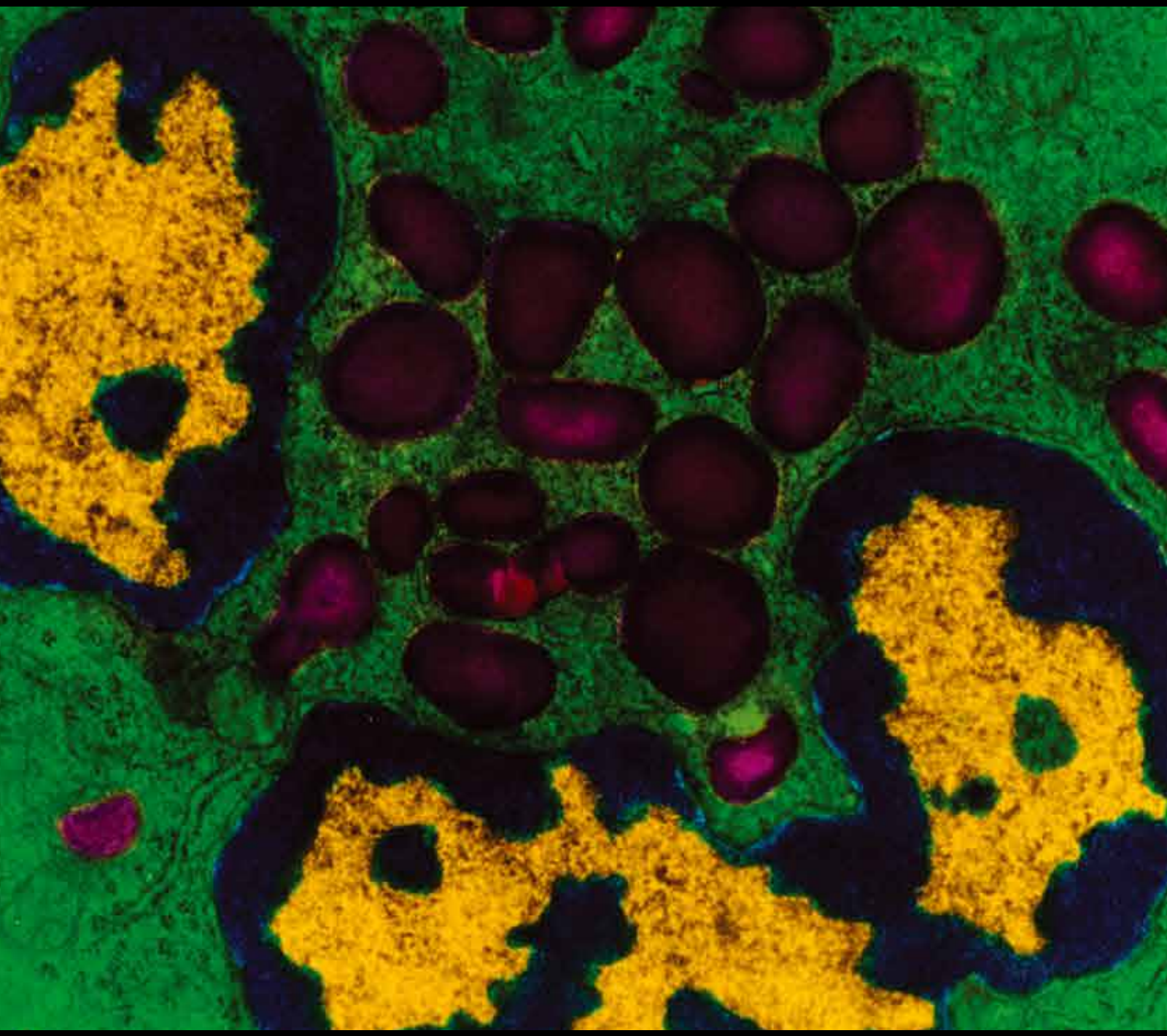
Mediators of Inflammation

Inflammatory Mediators of Angiogenesis

Guest Editors: Grzegorz Szewczyk, Janusz Rak, and Jeffrey H. Ruth



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Editorial Board

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Editorial

Inflammatory Mediators of Angiogenesis

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Angiogenesis gained a stable position in the field of pathogenesis. Vascular changes are a recognized hallmark of cancer growth, inflammatory disorders, and retina disorders, particularly in the context of diabetes. The inflammatory process, defined by the release of mediators from activated neutrophils, macrophages, and other myeloid cells, intersects with vascular changes, but the nature of this interrelationship frequently remains complex and puzzling, and the consequences remain difficult to predict. Clearly, a better understanding of molecular and cellular pathways that link angiogenesis and inflammation would be helpful in the development of treatment of conditions where both processes are involved. Although many of these mediators involved in angiogenesis are well known, there is still a need for further clarification of their activity.

This special issue contains eight independent articles, of which three are focused on the pathogenesis of inadequate angiogenesis in human disease, and two are experimental papers using rodent models. There are three review articles which describe both physiological aspects of angiogenesis and its possible keys for modification.

Articles included in this special issue also reflect the notion that angiogenesis is a fragment of the biological continuum involved in tissue development, remodeling, and repair, including haemostasis, inflammation, and vascular growth. These mechanisms are often subverted in pathological states, such as chronic injury, diabetes, or cancer, but remain intimately linked. Inflammation and angiogenesis have often been studied as separate entities; however, several articles in this issue document the existence of important biological linkages between them.

"*Fractalkine (CX3CL1) and its receptor CX3CR1 may contribute to increased angiogenesis in diabetic placenta*" by D. Szukiewicz et al. describes how an inflammatory chemokine known as fractalkine (CX3CL1) may act as direct modulator of angiogenesis by virtue of interacting with its receptors (CX3CR1) present on endothelial cells. This process is upregulated in placenta of patients with diabetes class C, leading to increased microvascular density. This is important as abnormal remodeling of placental vasculature may impact oxygen exchange and fetal health.

"*Antiangiogenic VEGF isoform in inflammatory myopathies*" by N. Volpi et al. shows that VEGF-A165b is significantly upregulated in idiopathic inflammatory myopathies, as well as TGF-β. VEGF-A is also shown to be diffusely expressed on unaffected myofibres, whereas regenerating/atrophic myofibres strongly react for both VEGF-A isoforms. Most inflammatory cells and endomysial vessels express both isoforms, but VEGF-A165b levels show a positive correlation to inflammatory scores, endomysial vascularization, and TGF-β. These findings indicate that skeletal muscle expression of antiangiogenic VEGF-A165b and its preferential upregulation in idiopathic inflammatory myopathies suggest that modulation of VEGF-A isoforms may occur in myositides.

"*Thrombospondin and VEGF-R: is there a correlation in inflammatory bowel disease?*" by J. Wejman et al. shows significantly higher vascular density and vascular area percentage in all layers of bowel wall (not only mucosal bioptates) in patients with Crohn's disease (CD), which was evaluated by immunohistochemistry analysis. This study found differences in vascular density distribution between ulcerative colitis (CU) and CD and between CU and controls, but no statistically significant correlation between those findings and VEGFR-1 or thrombospondin-1 (TSP-1) expression.

Overall, the results suggest the existence of different TSP-1 independent pathways of antiangiogenesis in inflammatory bowel disease.

"*Inhibitory effect of herbal remedy PERVIVO and anti-inflammatory drug sulindac on L-1 sarcoma tumor growth and tumor angiogenesis in Balb/c mice*" by P. Skopiński et al. reveals the interesting observation concerning antiangiogenic properties of the herbal mixture PERVIVO. The experimental model was developed in Balb/c mice after grafting of L-1 sarcoma cells. PERVIVO shows antiangiogenic properties by decreasing de novo vessel development thereby inhibiting tumor growth. Both effects are augmented after combining PERVIVO with sulindac—in which a synergistic effect was observed.

"*The effect of anti-inflammatory and antimicrobial herbal remedy PADMA 28 on immunological angiogenesis and granulocytes activity in mice*" by D. M. Radomska-Leśniewska et al. was also focused on traditional medicine rationalizing the use of herbal drugs. The in vivo stimulatory effect of PADMA 28 (which is a mixture of herbal extract) for angiogenic activity can be modulated by dose and is associated with deterioration of granulocytes after high-dose treatment.

"*Cytokines and angiogenesis in the corpus luteum*" by A. M. Galvão describes angiogenesis in the corpus luteum as an extremely rapid sequence of events that determines the dramatic changes on vascular and nonvascular structures. The main purpose of this review is to highlight the interaction between immune, endothelial, and luteal steroidogenic cells, regarding vascular dynamics/changes during the establishment and regression of the equine corpus luteum.

"*Matrix metalloproteinases: inflammatory regulators of cell behaviour in vascular formation and remodelling*" by Q. Chen et al. gives the systematic review of the role of matrix metalloproteinases (MMP) in the process of angiogenesis. The main purpose of this review is to show a complex interaction between MMP stimulators, their mechanism of activity and inflammatory cell recruitment.

"*Angiogenesis and its therapeutic opportunities*" by S. Y. Yo and S. M. Kwon offers a general review of angiogenesis with a particular focus on clinical applications.

There are general implications concerning the crosstalk between inflammatory and angiogenic circuitries. For instance, effective ways to lower pathological angiogenesis may require targeting not only "professional" regulators of endothelial cell function, but also elements of the inflammatory network, a notion that has already been linked to resistance of certain cancers to antiangiogenic agents. While articles published in this special issue were not intended to fully address these questions (the area is simply too vast), they do draw attention to specific scenarios in which the inflammatory-vascular continuum is particularly manifest or functionally significant. We hope that this integrated view will be of interest and of stimulating value to the readers.

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Review Article

Angiogenesis and Its Therapeutic Opportunities

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Angiogenesis plays critical roles in human physiology that range from reproduction and fetal growth to wound healing and tissue repair. The sophisticated multistep process is tightly regulated in a spatial and temporal manner by "on-off switch signals" between angiogenic factors, extracellular matrix components, and endothelial cells. Uncontrolled angiogenesis may lead to several angiogenic disorders, including vascular insufficiency (myocardial or critical limb ischemia) and vascular overgrowth (hemangiomas, vascularized tumors, and retinopathies). Thus, numerous therapeutic opportunities can be envisaged through the successful understanding and subsequent manipulation of angiogenesis. Here, we review the clinical implications of angiogenesis and discuss pro- and antiangiogenic agents that offer potential therapy for cancer and other angiogenic diseases.

1. Introduction

The growth of new capillaries from existing blood vessels, which is called angiogenesis, is mediated by a complex multistep process comprising a series of cellular events that lead to neovascularization [1, 2]. Angiogenesis plays a central role in various physiological processes within human body, not only during fetal development but also in tissue repair after surgery or trauma. Angiogenesis can be a hallmark of wound healing, the menstrual cycle, cancer, and various ischemic and inflammatory diseases [3–5]. The realization that tumor growth is associated with new blood vessels led us to investigate the chemical factors that mediate angiogenesis, broadened our knowledge of pathological processes, and thus opened new possibilities for the diagnosis and treatment of these diseases.

The pivotal process of angiogenesis can be simply described as multiple steps. First, angiogenic stimuli cause increased endothelial cell (EC) permeability and cellular proliferation, which continues as the new capillary sprout elongates [6]. Second, proteolysis of basement membrane matricellular components is a necessary process to promote the invasion of ECs into the stroma of the neighboring tissue [7], in which the cooperative activity of the plasminogen activator (PA) system and matrix metalloproteinases (MMPs) is required. Third, migrated ECs trigger lumen formation as the sprout forms a multicellular structure. Then, a new capillary channel is formed. Finally, the capillary is stabilized through the construction of basement membrane, adherent junctions, and ECs (Figure 1).

A number of molecules are involved in these complex angiogenic cascades. Their names and functions are described briefly in the next section and are listed in Table 1. These factors are commonly used as the targets in strategies to manipulate angiogenesis. The pathological disruption of angiogenesis can be caused by either vascular insufficiency (myocardial or critical limb ischemia) or vascular overgrowth (hemangiomas, tumors, and retinopathies) (Table 2). Thus, therapeutic benefits may be realized by manipulating angiogenesis.

2. Angiogenic Factors and Regulation of Angiogenesis

In recent decades, numerous studies focused on identifying angiogenesis stimulators, which led to the identification of several angiogenic factors. Angiogenic factors can be categorized as follows: (1) soluble growth factors such as acidic and basic fibroblast growth factor (aFGF and bFGF)

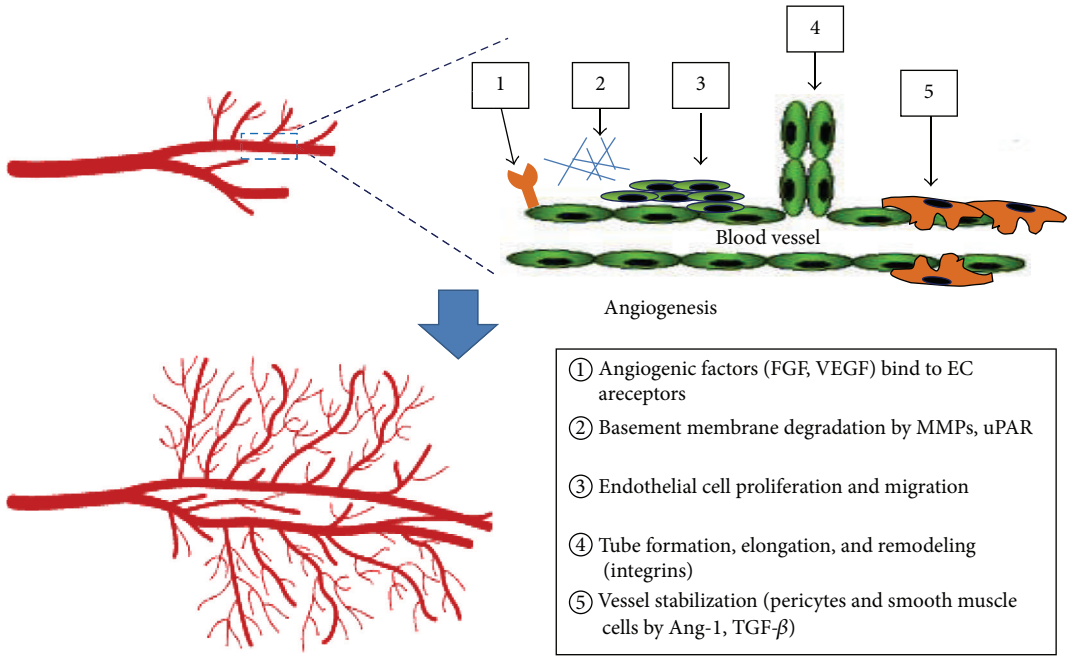

Figure 1: Processes in angiogenesis. (1) Angiogenic factors bind to their receptors on endothelial cells and activate the signal transduction pathways. (2) Matrix metalloproteinases are activated, and they degrade the extracellular matrix. (3) Endothelial cells migrate out of the preexisting capillary wall and proliferate. (4) Integrins are expressed by endothelial cells, facilitating their adhesion to the extracellular matrix and their migration for tube formation. (5) Angiopoietin 1 binds to Tie-2 receptors and stimulates pericyte recruitment and vessel stabilization.

and vascular endothelial growth factor (VEGF), which are associated with EC growth and differentiation [8–10]; (2) inhibiting factors that inhibit the proliferation and enhance the differentiation of ECs, such as transforming growth factor β (TGF-β), angiogenin, and several low molecular weight substances [11–13]; and (3) extracellular matrix-bound cytokines that are released by proteolysis, which may contribute to the regulation of angiogenesis and include angiostatin, thrombospondin, and endostatin [14–18]. In addition, a number of microphages secreting bFGF, tumor necrosis factor (TNF), and VEGF were shown to be associated with tumor angiogenesis. Angiogenesis is governed by a balance between inducers and inhibitors [19]. It also can be regulated by EC proliferation, which is regulated or restrained by pericytes through the sequestration of potent mitogens in the extracellular matrix, changes in EC shape that reduce the sensitivity of the cells to growth factors, and certain endothelial integrins.

3. Angiogenic Disorders

The hypervascularity of tumors was first thought to be the inflammatory vasodilation of preexisting vessels in response to tumor metabolites and necrotic tumor products. It was also thought that tumor growth and metastasis depended on angiogenesis and that the tumor secreted the chemicals that shifted resting ECs into rapidly growing ECs. These ideas are not widely accepted yet. The concept of the role of angiogenesis in cancer and other diseases has now become clear.

3.1. Angiogenesis in Cancer. Most tumors (up to 2-3 mm^3) persist *in situ* without neovascularization for months to years, but they become vascularized by "switches" in cells during the turnover to an angiogenic state when the tumor needs to keep growing. Cells in prevascular tumors may replicate as rapidly as those in expanding vascularized tumors; however, without the growth of new vessels, their growth is limited. A change in the local equilibrium between positive and negative angiogenic regulators of the growth of microvessels is involved in the switch to the angiogenic state [20, 21]. Of the angiogenic inducers (Table 1), those that are most commonly found in tumors appear to be VEGF and bFGF. Their angiogenic activities are synergistic [22]. VEGF plays a critical role in vasculogenesis and angiogenesis during fetal development. In a knockout mouse model, VEGF or VEGF receptor (VEGFR) inactivation resulted in defects in vasculogenesis in the early stages of development and was embryonic lethal [23].

VEGF is overexpressed in tumor stromal cells as well as tumor cells of renal cancer [24], lung cancer [25], breast cancer [26], and ovarian cancer [27]. VEGF expression is regulated positively by oncogenes such as Ras [28] and negatively by tumor suppressors such as von Hippel-Lindau (VHL) [29]. VEGF binds to VEGFR1 or VEGFR2 on the EC surface. Most of the angiogenic effects of VEGF result from VEGFR2 activation. Hypoxia is a major stimulator of VEGF expression that results from hypoxia-inducible factor 1 (HIF-1) binding to a hypoxia response element (HRE) within the VEGF promoter. It is also stimulated by inflammatory mediators (interleukin 1 (IL1), TGF-β, and

Table 1: Overview of the different angiogenic factors.

Category	Names	Major functions	References
Proteolytic enzymes	(i) Matrix metalloproteinases (MMPs): matrilysin (MMP-7), interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), metalloelastase (MMP-12), MMP-19, enamelysin (MMP-20), gelatinase A (MMP-2), gelatinase B (MMP-9), MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT3-MMP (MMP-16), MT4-MMP (MMP-17) (ii) Plasminogen activators (PAs)	MMPs; taking different substrates according to MMPs; substrates can be collagen, gelatin, laminin, fibronectin, proteoglycans, and proMMPs	[67–69]
Angiogenesis inducers	Vascular endothelial growth factor family (VEGF-A or VEGF, P1GF, VGGF-B, VEGF-C, VEGF-D, orf virus VEGF or VEGF-E), fibroblast growth factor family (aFGF, bFGF, etc.), angiopoietin 1 (Ang-1), transforming growth factor-alpha/beta (TGF α/β), platelet-derived growth factor (PDGF), hepatocyte growth factor/scatter factor (HGF/SF), tumor necrosis factor-alpha (TNFα), interleukin-1/8, angiogenin, ephrins, integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, cyclooxygenase-2 (COX-2)	(i) Induction of EC proliferation, migration, and differentiation (ii) TGF-β shows opposite effect in some contexts	[8–10, 70–79]
Angiogenesis inhibitors	Thrombospondin-1/2 (TSP-1/2), angiostatin (plasminogen fragment), endostatin (collagen XVIII fragment), vasostatin (calreticulin fragment), tumstatin, platelet factor-4 (PF4), antiangiogenic antithrombin III, kringle 5 (plasminogen fragment), prolactin 16-kD fragment, fragment of SPARC, 2-methoxyestradiol, metalloproteinase inhibitors (TIMPs), interferon-alpha/beta/gamma (IFN $\alpha/\beta/\gamma$), interleukin-12 (IL-12), IP-10, Ang-2	(i) Inhibit EC proliferation/migration (ii) Induce EC apoptosis (iii) TIMPs: inhibit MMP or uPA activity (iv) Ang-2: inhibit blood vessels maturation, antagonist of Ang-1	[11–15, 18, 80–85]

prostaglandin E2 (PGE2)) or mechanical forces (shear stress and cell stretch). Increased PGE2 by cyclooxygenase-2 (COX-2) induces VEGF expression and angiogenesis in tumor cells or tumor stromal cells [30]. FGF signaling may be through the recruitment of other growth factor signaling pathways. The simultaneous VEGF signaling through VEGFR1 was required in the bFGF-stimulated capillary organization. bFGF also initiates the transcription of hepatocyte growth factor (HGF) [31]. However, the upregulation of an angiogenic inducer is not sufficient for tumor angiogenesis. The downregulation of certain negative regulators or inhibitors (Table 1) for vessel growth may be required [21].

Tumor growth is augmented by the onset of neovascularization through a perfusion effect and a paracrine effect. The perfusion effect is more efficient at allowing nutrients and oxygen to enter and catabolites to exit in crowded tissues, and the paracrine effect results from the production of growth factors (e.g., bFGF, insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and granulocyte colony stimulating factor (GCSF)) by ECs or their release by macrophages and other host cells that are delivered to the tumor by blood vessels. Neovascularization gradually reduces a tumor's accessibility to chemotherapeutic drugs because tumors compress their blood supply. Antiangiogenic therapy in rodents showed increased delivery of chemotherapy agents to a tumor [32], which may be associated with lowered interstitial pressure and an unpacked mass of tumor cells.

3.2. Angiogenesis in Other Diseases. Either excessive or deficient angiogenesis can be classified as an angiogenic disease, which is an abnormal growth of microvessels (Table 2). Recent studies reported that the angiogenic protein VEGF is the chief mediator of ocular neovascularization, a major cause of blindness worldwide [33, 34]. The upregulation of VEGF is

Table 2: Clinical manipulation of angiogenesis.

Therapeutic goal	Diseases	Definitions/symptoms	Reference
Inhibition of angiogenesis	Hemangiomas	Benign and usually a self-involuting tumor (swelling or growth) of the endothelial cells that line blood vessels and is characterised by increased number of normal or abnormal vessels filled with blood	[86]
	Psoriasis	Immune-mediated disease that affects the skin. The immune system mistakes a normal skin cell for a pathogen and sends out faulty signals that cause overproduction of new skin cells	[37]
	Kaposi's sarcoma	Tumor caused by human herpesvirus 8 (HHV8)	[87]
	Ocular neovascularization	Abnormal or excessive formation of blood vessels in the eye	[88]
	Rheumatoid arthritis	Inflammatory response of the capsule around the joints (synovium), secondary to swelling (hyperplasia) of synovial cells, excess synovial fluid, and the development of fibrous tissue (pannus) in the synovium	[36]
	Endometriosis	A gynecological medical condition in which cells from the lining of the uterus (endometrium) appear and flourish outside the uterine cavity, most commonly on the membrane which lines the abdominal cavity	[89]
	Atherosclerosis	Artery wall thickens caused largely by the accumulation of macrophage white blood cells and promoted by low-density lipoproteins (LDL, plasma proteins that carry cholesterol and triglycerides)	[35]
	Tumor growth and metastasis	Tumor-associated neovascularization is involved in tumor growth, invasion, and metastasis	[4]
Stimulation of angiogenesis	Induction of collateral vessel formation: Myocardial ischemia, Peripheral ischemia, Cerebral ischemia	After blood vessels blockage (occulsion), collateral vessels can be developed to improve blood supply to the area.	[90]
	Wound healing	Intricate process in which the skin (or another organ-tissue) repairs itself after injury. Angiogenesis occurs concurrently with fibroblast proliferation when endothelial cells migrate to the area of the wound	[91]
	Reconstructive surgery	Surgery to restore the form and function of the body	

a response to the local hypoxia that is produced in tumors by vascular compression and ischemia. Thus, a similar process may cause the formation of collateral vessels in an ischemic heart or limb. Neovascularization in atherosclerotic plaques [35] may be mediated by the overexpression of VEGF and by local hypoxia, which contribute to the growth and rupture of plaques. The expression of both VEGF and bFGF is excessive in hemangiomas of infancy. In the case of rheumatoid arthritis, infiltrated macrophages, immune cells, or inflammatory cells produce excessive angiogenic factors that may mediate the ingrowth of a vascular pannus in a joint [36]. In psoriasis, hypervascular skin lesions over expressed the angiogenic polypeptide IL8 and under expressed the angiogenic inhibitor thrombospondin 1 (TSP-1) [37]. Peptic ulcers in animals appear to be deficient in microvessels in the ulcer bed. The oral administration of aFGF induced angiogenesis in the ulcer bed and accelerated the healing of ulcers in animals [38]. Bowel atresia, vascular malformations, hemangiomas, and unilateral facial atrophy are developmental disorders that are caused also by abnormal vascular development through defects in angiogenesis [39].

4. Angiogenesis in Clinical Applications

Abnormal angiogenesis is the major cause of numerous diseases; therefore, angiogenesis itself can be useful for diagnostic/prognostic applications and can be manipulated for further clinical applications. In the case of ischemic diseases, which develop because of deficient angiogenesis, an angiogenesis stimulator can be used to induce therapeutic angiogenesis. In the case of cancer, which has excessive angiogenesis, angiogenic inhibitors including antiangiogenic factors can be used to attenuate angiogenesis. The Food and Drug Administration (FDA) approved becaplermin (Regranex, recombinant human PDGF-BB) for diabetic foot ulcer disease in 1977, which was the first angiogenesis-stimulating drug that was used as a therapeutic angiogenesis approach. For cancer therapeutics, the FDA approved

bevacizumab (Avastin, humanized anti-VEGF monoclonal antibody), an angiogenesis inhibitor, to treat metastatic colorectal cancer in 2004 [40].

4.1. Diagnostic and Prognostic Applications. The quantitation of angiogenesis in a biopsy specimen may help predict the risk of metastasis or recurrence. The quantitation of microvessel density in histologic specimens of invasive breast cancer, for example, has provided an indication of the risk of metastasis [41]. A positive association between tumor angiogenesis and the risk of metastasis, tumor recurrence, or death has also been reported with regard to breast cancers and other types of tumors [42–44]. A high microvessel density may be a successful predictor of metastatic risk. An increased area of the vascular surface because of a high density may facilitate the escape of cancer cells into the circulation. An angiogenic cell that is shed from a primary tumor is more likely than a nonangiogenic cell to develop into a detectable metastasis.

The quantitation of angiogenic proteins in body fluids can be used to indirectly measure angiogenic activity. Higher concentrations of bFGF were found in the serum and urine of nearly 10% and more than 37% of cancer patients, respectively [45]. Concentrations of biologically active bFGF were abnormally high in the cerebrospinal fluid of children with brain tumors but not in children with hydrocephalus or malignant disease outside the central nervous system [46].

4.2. Therapeutic Angiogenesis. Therapeutic angiogenic drugs that accelerate the angiogenesis process are useful for treating diseases of deficient angiogenesis. In preclinical studies, orally administered bFGF was shown to stimulate angiogenesis and to accelerate the healing of duodenal ulcers in rats [38]. Phase I clinical trials were then prompted to evaluate this therapy in patients with gastric or duodenal ulcers that were refractory to conventional therapy [47]. Orally administrated bFGF was found to heal gastric ulcers that were caused by nonsteroidal anti-inflammatory drugs. Orally administrated aFGF induced angiogenesis in the ulcer bed and accelerated the healing of ulcers in animals [38].

A phase I clinical trial of therapeutic angiogenesis using bFGF protein in heparin-alginate slow-release microcapsules in 8 patients with symptomatic, severe coronary artery disease that was not amenable to complete revascularization by either percutaneous transluminal coronary angioplasty or coronary artery bypass grafting demonstrated the feasibility and safety of bFGF administration for coronary artery diseases [48]. These clinical studies suggest that angiogenic and other growth factors can heal gastrointestinal ulceration as well as coronary artery diseases. It is interesting to note that the antiulcer drug sucralfate (sucrose aluminum sulfate or Carafate) appears to act by protecting endogenous mucosal bFGF from degradation by acid.

Angiogenic agents can be used in ischemic heart disease as a therapeutic angiogenesis approach for cardiac tissue repair and regeneration [49, 50]. The therapeutic goal of this condition is to stimulate angiogenesis to improve perfusion, deliver survival factors to sites of tissue repair, mobilize regenerative stem cell populations, and ultimately restore form and function to the tissue. Although more than 2,000 patients with heart disease have received some form of experimental angiogenic therapy [51, 52], currently there are no FDA-approved angiogenic drugs to treat ischemic cardiovascular disease. The first FDA-approved device to stimulate new blood vessel growth in diseased hearts is a laser that is used in a technique called direct myocardial revascularization (DMR) or transmyocardial revascularization (TMR).

5. Antiangiogenic Therapies

Many studies using ECs that were isolated from either capillaries or large vessels led to considerable insights into the molecular and cellular biology of angiogenesis and the discovery and evaluation of potential antiangiogenic compounds (Table 3). They were identified using classical angiogenesis assays such as the chick chorioallantoic membrane (CAM), rabbit cornea assay, sponge implant models, and matrigel plugs [53–57]. Recent research has focused on the specific effects of antiangiogenic compounds on individual angiogenic processes. In this section, we discuss their clinical applications as antiangiogenic therapies.

5.1. Interferon Alpha-2a to Treat Hemangiomas. Hemangiomas occur in 1 out of 100 neonates and in 1 out of 5 premature infants [58]. Most do not need treatment because these tumors grow rapidly in the first year of life (the proliferating phase), slow down during the next 5 years (the involuting phase), and gradually regress by the age of 10–15 years (the involuted phase). However, about 10% of them may have serious tissue damage that includes interfering with a vital organ, obstructive airway, heart failure, or Kasabach-Merritt syndrome. Kasabach-Merritt syndrome, a platelet-trapping thrombocytopenic coagulopathy, and hepatic hemangiomas have a mortality rate of 30%–50%. Corticosteroid therapy worked for 30% of hemangiomas [59]. Radiation, cyclophosphamide treatment, and embolization were also tried and showed favorable outcomes; however, sometimes they showed toxicity. Interferon alpha-2a (IFNα-2a) is an angiogenic agent that could be useful for treating these hemangiomas. It was successfully used in a 7-year-old child with pulmonary hemangiomas [60]. It was also found that therapy with IFNα-2a accelerated the tumor regression in 18 of 20 hemangioma patients [61]. IFNα-2a suppresses the production of FGFs in human tumor cells, which could work for hemangiomas because bFGF is an angiogenic factor that is overexpressed in hemangiomas.

5.2. Ocular Neovascularization. Ophthalmology angiogenesis in the eye, an ocular neovascularization, includes age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), diabetic macular edema (DME), neovascular glaucoma, corneal neovascularization (trachoma), and pterygium. Inhibiting VEGF is presently an antiangiogenic therapy that is approved for ophthalmic conditions. Two currently approved antiangiogenic therapies for ophthalmic diseases are an anti-VEGF aptamer (pegaptanib, Macugen) and a Fab fragment of a monoclonal antibody directed against

Table 3: Antiangiogenic compounds and their mechanism of action (adapted from references [2, 92–102]).

Inhibiting angiogenic process	Antiangiogenic compounds	Mechanism of action
Inhibitors of ECM remodeling	Batimastat, Marimastat, AG3340, Neovastat, PEX, TIMP-1,2,3,4	MMP inhibitors, block endothelial and tumor cell invasion
	PAI-1,2, uPA Ab, uPAR Ab, Amiloride	uPA inhibitors, block ECM breakdown
	Minocycline, tetracyclines, cartilage-derived TIMP	Collagenase inhibitors, disrupt collagen synthesis and deposition
Inhibitors of adhesion molecules	$\alpha_v\beta_3$ Ab: LM609 and Vitaxin, RGD containing peptides, $\alpha_v\beta_5$ Ab	Block EC adhesion, induce EC apoptosis
	Benzodiazepine derivatives	Antagonist of $\alpha_v\beta_3$
Inhibitors of activated ECs	Endogenous inhibitors: endostatin, angiostatin, aaAT	Block EC proliferation, induce EC apoptosis, inhibit angiogenic switch
	IFN-α, IFN-γ, IL-12, nitric oxide synthase inhibitors, TSP-1	Block EC migration and/or proliferation
	TNP-470, Combretastatin A-4	Block EC proliferation
	Thalidomide	Inhibits angiogenesis in vivo
	Linomide	Inhibits EC migration
Inhibitors of angiogenic inducers or their receptors	IFN-α, PF-4, prolactin fragment	Inhibit bFGF, Inhibit bFGF-induced EC proliferation
	Suramin and analogues	Bind to various growth factors including bFGF, VEGF, PDGF, inhibit EC migration and proliferation
	PPS, distamycin A analogues, bFGF Ab, antisense-bFGF	Inhibit bFGF activity
	Protamine	Binds heparin, inhibits EC migration and proliferation
	SU5416, soluble Flt-1, dominant-negative Flk-1, VEGF receptor, ribozymes, VEGF Ab	Block VEGF activity
	Aspirin, NS-398	COX inhibitors
	6AT, 6A5BU, 7-DX	TP antagonists
Inhibitors of EC intracellular signaling	Genistein	Tyrosine kinase inhibitor, blocks uPA, EC migration and proliferation
	Lavendustin A	Selective inhibitor of protein tyrosine kinase
	Ang-2	Inhibits Tie-2

VEGF (ranibizumab, Lucentis). A photodynamic therapy called Visudyne (QLT Therapeutics/CIBA Vision) has shown effectiveness for treating macular degeneration and was the first FDA-approved blood vessel therapy for eye disease in 2004 [62, 63].

5.3. Rheumatoid Arthritis. Clinical trials of angiogenic inhibitors have not been performed yet in patients with arthritis; however, minocycline and TNP-470 (also known as AGM-470) have shown efficacy as potent inhibitors of the vascular pannus in experimental arthritis [36, 64].

5.4. Cancer. Angiogenesis plays a critical role in the growth and spread of cancer because a blood supply is necessary for tumor growth and metastases. Tumors secrete chemical signals that stimulate angiogenesis and thus stimulate nearby normal cells. Therefore, many natural or synthetic angiogenesis inhibitors, also called antiangiogenic agents, have been studied to prevent or slow the growth of cancer. These inhibitors can interfere with angiogenesis in various ways. Bevacizumab (Avastin) is a monoclonal antibody that specifically recognizes and binds to VEGF, which prevents VEGF from activating VEGFR [40]. In contrast, other angiogenesis inhibitors, including sorafenib and sunitinib, bind to receptors on the ECs or to other proteins in the downstream signaling pathways to block their activities [65].

Bevacizumab was FDA-approved to be used alone to treat glioblastoma and to be used in combination with other drugs to treat metastatic colorectal cancer, some nonsmall cell lung cancers, and metastatic renal cell cancer. Bevacizumab is the first approved angiogenesis inhibitor that was shown to slow tumor growth and, more importantly, to extend the lives of patients with some cancers. The other FDA-approved antiangiogenic drugs are sorafenib (Nexavar) for hepatocellular carcinoma and kidney cancer, sunitinib (Sutent) for kidney cancer and neuroendocrine tumors, pazopanib (Votrient) for kidney cancer and neuroendocrine tumors, and everolimus

Table 4: Selected angiogenesis inhibitors in clinical trials.

Inhibiting target	Drug	Sponsor	Clinical trials/mechanism	References
Epidermal growth factor receptor (EGFR)	Gefitinib (Iressa)	AstraZeneca and Teva	FDA-approved in 2003 for NSCLC/effective in cancers with mutated and overactive EGFR	[103, 104]
	Lapatinib (Tykerb)	GSK	FDA-approved in 2007 for breast cancer/dual tyrosine kinase inhibitor which interrupts the HER2/neu and epidermal growth factor receptor (EGFR) pathways	[105]
	Erlotinib (Tarceva)	Genentech/OSI pharmaceuticals/Roche	FDA-approved in 2005/used to treat nonsmall cell lung cancer, pancreatic cancer, and several other types of cancer	[106]
	Canertinib (CI-1033)	Selleck Chemicals	Phase II/irreversible tyrosine-kinase inhibitor with activity against EGFR, HER-2, and ErbB-4	[107]
VEGFR	Vatalanib (PTK787 or PTK/ZK)	Bayer Schering and Novartis	Phase III/it inhibits all known VEGF receptors, as well as platelet-derived growth factor receptor-beta and c-kit, but is most selective for VEGFR-2	[108]
VEGFR-2	IMC-1C11	ImClone Systems	Phase I/chimerized monoclonal antibody	[109]
VEGFR-3	mF4-31C1	ImClone Systems	Phase I/rat monoclonal antibody to murine VEGFR-3, which potently antagonizes the binding of VEGF-C to VEGFR-3	[110]
Multiple growth factor receptors	Imatinib (Glivec)	Novartis	FDA-approved in 2001/competitive tyrosine-kinase inhibitor used in the treatment of multiple cancers, most notably Philadelphia chromosome-positive (Ph^+) chronic myelogenous leukemia (CML)	[111]
	Sunitinib (Sutent)	Pfizer	FDA-approved in 2006 for renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST)/the simultaneous inhibition of receptors for platelet-derived growth factor (PDGF-Rs) and vascular endothelial growth factor receptors (VEGFRs)	[112]
	Sorafenib (Nexavar)	Bayer and Onyx pharmaceuticals	FDA-approved in 2005/a small molecular inhibitor of several tyrosine protein kinases (VEGFR and PDGFR) and Raf kinases (more avidly C-Raf than B-Raf)	[113]
	Pazopanib (Votrient)	GlaxoSmithKline	FDA-approved in 2009 for advanced renal cancer/multitargeted receptor tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, VEGFR-3, PDGFR-a/β, and c-kit	[114]
VEGF	Bevacizumab (Avastin)	Genentech/Roche	FDA-approved in 2004 for metastatic colorectal cancer/humanized anti-VEGF mAb, licensed to treat various cancers including colorectal, lung, breast (outside the USA), glioblastoma (USA only), kidney, and ovarian	[115, 116]
Integrin $\alpha_V\beta_3$	Vitaxin	Applied molecular evolution	Phase II as a treatment for colorectal cancer/humanized monoclonal antibody against the vascular integrin $\alpha_V\beta_3$	[117]

(Afinitor) for kidney cancer. Researchers are exploring the use of angiogenesis inhibitors in some clinical trials (Table 4).

5.5. Conventional Anticancer Therapy versus Antiangiogenic Therapy. Conventional anticancer therapy generally faces the problems of drug resistance and impaired drug delivery because of genomic instability and the tumor mass of cancers [66]. Tumor mass has an interstitium composed of a collagen-rich matrix between cancer cells and vascular cells. Anticancer drugs need to pass through the interstitium from blood vessels to reach the cancer cells. However, tumor mass has an interstitium with an abnormally high interstitial fluid pressure, which acts as a barrier to drug delivery. However, angiogenesis inhibitors inhibit the growth of blood vessels rather than tumor cells. In some cancers, angiogenesis inhibitors are most effective when they are combined with additional therapies, especially chemotherapy. It has been

hypothesized that these drugs help normalize the blood vessels that supply the tumor, facilitating the delivery of other anticancer agents [32]. Angiogenesis inhibitor therapy may prevent tumor growth instead of killing tumors. Therefore, antiangiogenic therapy may require a long period for treatment.

6. Concluding Remarks and Prospects

Currently, chemotherapeutic drugs are being used to treat cancer as well as other diseases. Unfortunately, many compounds showed limited efficacy with impaired delivery, penetration, and selectivity for the tumor cells, thereby causing serious side effects and bystander effects. The activity of these compounds is mainly restricted by the drug resistance of tumor cells. Preclinical studies and clinical trials suggest that angiogenesis-based therapy may be useful in the future care of patients. In particular, antiangiogenic therapy is a unique approach to kill tumor cells because it does not directly target cancer cells; instead, it inhibits the growth of blood vessels. So far, antiangiogenic agents are not likely to result in bone marrow suppression, gastrointestinal symptoms, or hair loss. The approach to slow the growth of blood vessels may require several months to a year; thus, the administration of the agents at lower doses and longer uninterrupted periods than the usual doses and periods of conventional cytotoxic agents should be considered in the design of clinical trials. The development of resistance to angiogenic inhibitors has not been a big problem so far. Furthermore, a combination of antiangiogenic therapy and conventional therapy may be more effective than either therapy alone. Angiogenesis-based therapy may provide a novel, selective, safe, and reasonable treatment in future medicine.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Thrombospondin and VEGF-R: Is There a Correlation in Inflammatory Bowel Disease?

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Up to date several authors discussed interactions between cells forming inflammatory infiltrates in the course of inflammatory bowel disease (IBD), mainly dealing with endoscopic biopsy specimens. These usually contain only mucosa. We have evaluated full bowel wall sections, which seems to be especially important in patients with Crohn's disease (CD). The purpose of our study was to evaluate the relationship between vascular density and expression of thrombospondin-1 (TSP-1) and vascular endothelial growth factor receptor 1 (VEGFR-1) in full-thickness tissue fragments of intestinal wall taken from patients after colectomy, comparing those with IBD to non-IBD control group. Histological sections were immunostained with antibodies against CD-31, TSP-1, and VEGFR-1 and analyzed by pathologists with the use of computer-assisted morphometrics. Our research showed significantly higher vascular density and vascular area percentage in all layers of bowel wall in patients with CD when compared to control. We have also demonstrated differences in vascular density distribution between ulcerative colitis (CU) and CD and between CU and control. However we have not found statistically significant correlation between those findings and VEGFR-1 or TSP-1 expression. Our results might suggest existence of different, TSP-1 independent pathways of antiangiogenesis in IBD.

1. Introduction

Ulcerative colitis (CU) and Crohn's disease (CD) are known as chronic inflammatory bowel diseases (IBD). There is still some controversy about IBD etiology. Among factors associated with inflammatory bowel disease, angiogenesis plays important role in development of clinical symptoms. It has been shown that exacerbation of IBD increases angiogenesis especially in ulcerative colitis [1, 2]. This finding produced a concept of antiangiogenic therapy in IBD, as presented by Danese et al. [3].

It was reported that anti-angiogenesis in the development of IBD can be measured by detection of thrombospondin [4, 5]. Since anti-angiogenesis is almost always associated with angiogenesis, numerous angiogenic factors have been indicated as possible counterparts to thrombospondin. Among them, vascular endothelial growth factor (VEGF) is thought to play a crucial role by stimulating migration and proliferation of endothelial cells (ECs) and the expression of angiogenesis-related genes. There are 7 types of VEGF family and VEGF-A with two receptors VEGFR-1 and VEGFR-2 that are most important in angiogenesis [6, 7]. Thrombospondin (TSP) is a 450 kD adhesive glycoprotein that was initially discovered in platelet α-granule [8]. Thrombospondin is one of the extracellular matrix adhesive molecules, including also laminin, fibronectin, fibrinogen, and von Willebrand

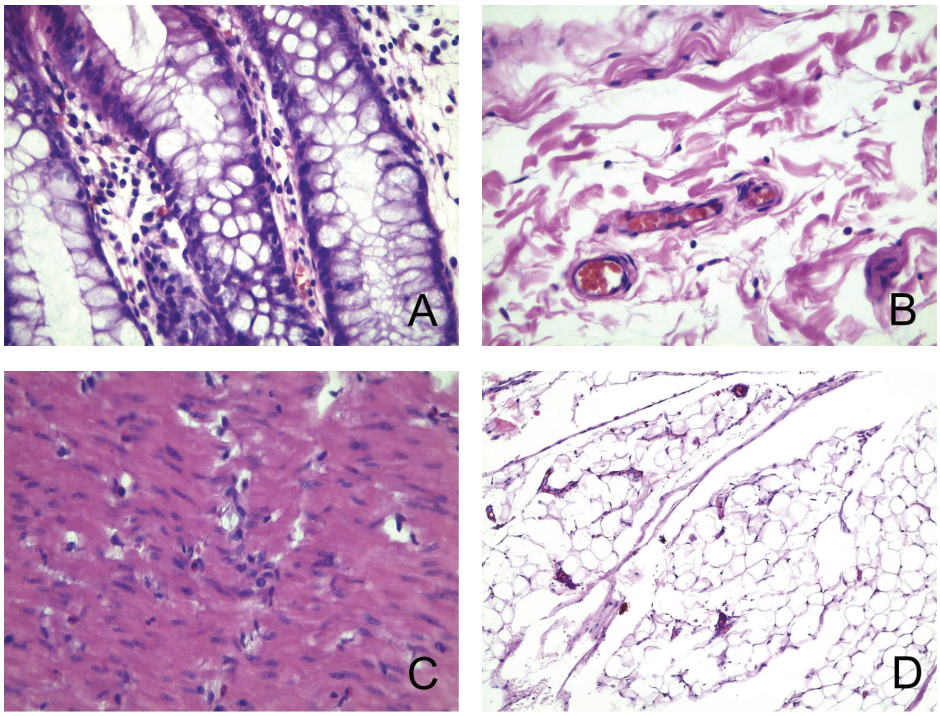

Figure 1: Microphotographs from hematoxylin & eosin stained sections of normal large bowel wall. A: mucosa (400x); B: submucosa (400x); C: muscularis (400x); D: subserosa (100x).

factor. Thrombospondins are secreted glycoproteins that modulate cell-matrix interactions, influence platelet aggregation, and support neutrophil chemotaxis and adhesion [9]. Thrombospondin-1 (TSP-1) is known as an antiangiogenic factor. There are several articles about the role of TSP-1 in IBD, most of these based upon animal models [4, 5].

The purpose of our study was to assess angiogenesis in correlation with expression of VEGFR-1 and thrombospondin-1 to see if they mediate in maintaining balance between angiogenesis and anti-angiogenesis in the course of IBD. We have decided to use computer-assisted morphometrics to facilitate the analysis of vascular density and immunohistochemically highlighted expression of angiogenic factors.

2. Materials and Methods

We have analyzed full-thickness intestinal wall histological sections routinely prepared out of formalin fixed, paraffin embedded tissue samples. All samples were collected after colectomy in the course of IBD. All our IBD-affected intestines were resected because of an active phase of inflammation. CU bowels were presented with grade 5 of the disease, according to grading scale for histological assessment of inflammation in ulcerative colitis by Geboes et al. [10]. Intestines with CD have showed a severe phase of the disease according to Geboes [11]. Patients in the control group underwent bowel removal due to noninflammatory, nonvascular conditions. All tissue specimens were collected during standard grossing procedures. The number of patients in each group was shown in Table 1.

Table 1: Groups of patients in the study.

Group	Number of patients (N)
Control	32
Crohn's disease	38
Ulcerative colitis	36

Only one tissue fragment containing full thickness of intestinal wall was collected from each patient. In IBD groups tissue fragments were collected from sites involved by the disease. The control group was formed with the use of fragments that were collected from macroscopically unchanged bowel wall. All tissue samples were fixed in formalin, embedded in paraffin, and sectioned into 4 micrometer sections, according to standard histopathology protocol.

One section in each case was stained routinely with hematoxylin & eosin to evaluate the extent of the disease or to confirm the lack of pathology (in the control group) (see Figures 1, 2, and 3).

Other sections were immunostained with antibodies against CD-31 (to highlight vessel walls), thrombospondin-1 and VEGFR-1 (to show the expression of both). Antibodies and their respective dilutions are shown in Table 2. Visualization was performed with En-Vision Flex visualization system (Dako). All antibodies were earlier validated for routine use with immunohistochemistry in our facility.

Immunostained sections were subsequently photographed and analyzed.

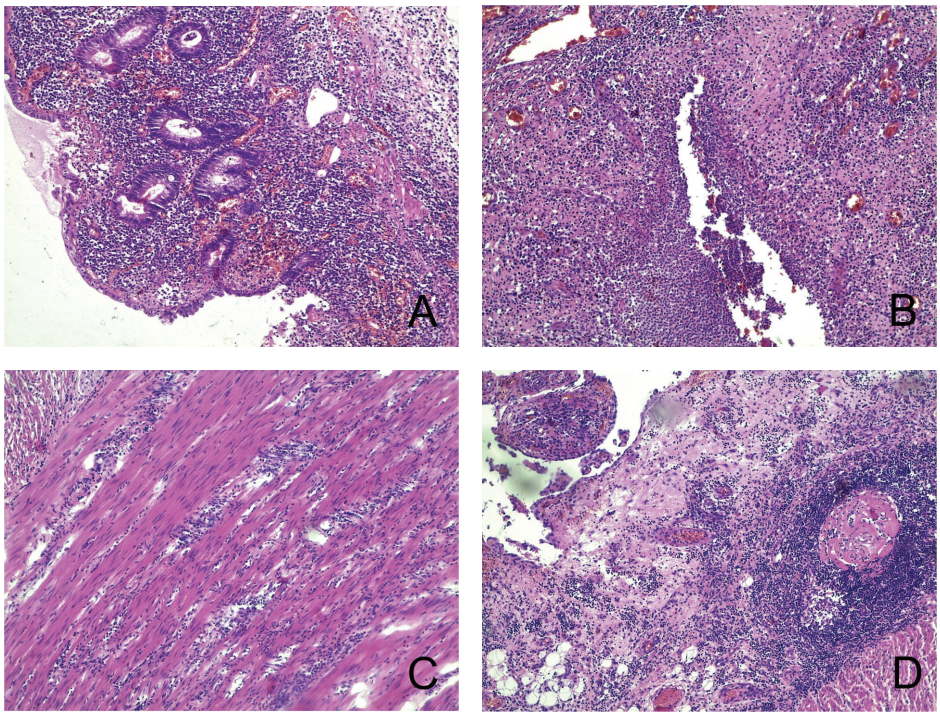

Figure 2: Microphotographs from hematoxylin & eosin stained sections of bowel wall from a patient with Crohn's disease. A: partially ulcerated mucosa (100x); B: fissure-like ulcer penetrating from mucosa into submucosa (100x); C: inflammatory changes in muscularis propria (100x); D: Inflammatory changes in subserosa (100x).

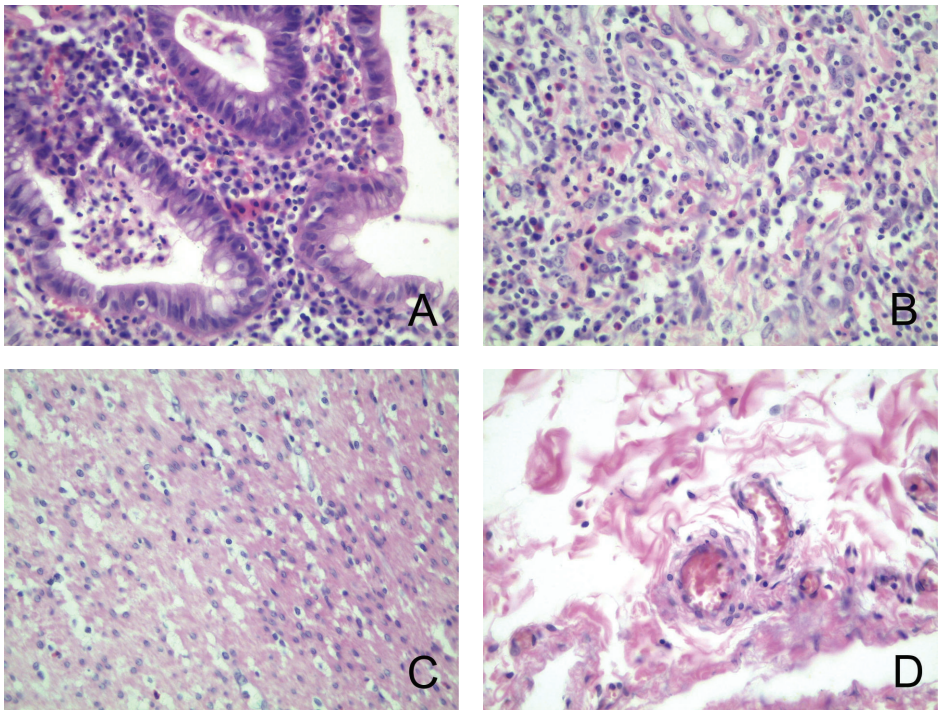

Figure 3: Microphotographs from hematoxylin & eosin stained sections of bowel wall from a patient with ulcerative colitis. A: inflammatory changes within mucosa (400x); B: inflammatory changes in submucosa (400x); C: muscularis (400x); D: subserosa (400x).

Table 2: Antibodies used for immunostaining.

Antibody	Clone	Manufacturer	Dilution	Epitope retrieval
Monoclonal mouse anti-human CD31	Endothelial cell, Clone JC70A	Dako	Ready-to-use (manufacturer prediluted)	HIER, pH = 9.0
Monoclonal mouse anti-human thrombospondin	Clone TSP-B7	Sigma-Aldrich	1 : 100	HIER, pH = 6.0
Rabbit anti-VEGFR-1 antibody	Polyclonal	Sigma-Aldrich	1 : 20	HIER, pH = 6.0

HIER: heat induced epitope retrieval.

Table 3: Mean vessel count differences [vessel count/mm^2 (SD)]. Comparison of Crohn's disease versus control, ulcerative colitis versus control, and ulcerative colitis versus Crohn's disease.

Layer	Control n/mm^2	CD n/mm^2	CU n/mm^2
Mucosa	171.10 (99.36)	346.43 (113.50)$^{*\#}$	215.98 (125.26)$^{\#}$
Submucosa	87.58 (38.42)	318.06 (112.34)$^{*\#}$	138.82 (114.96)$^{\#}$
Muscularis	38.35 (15.83)	168.29 (104.71)$^{*\#}$	44.55 (46.51)$^{\#}$
Subserosa	74.00 (57.66)	174.11 (96.84)$^{*\#}$	41.53 (52.11)$^{*\#}$

Mean number of vessels/mm^2. Numbers in parentheses show standard deviation.
*Significant differences between CD or CU and Control; $^{\#}$Significant differences between CD and CU.
CD: Crohn's disease, CU: ulcerative colitis.

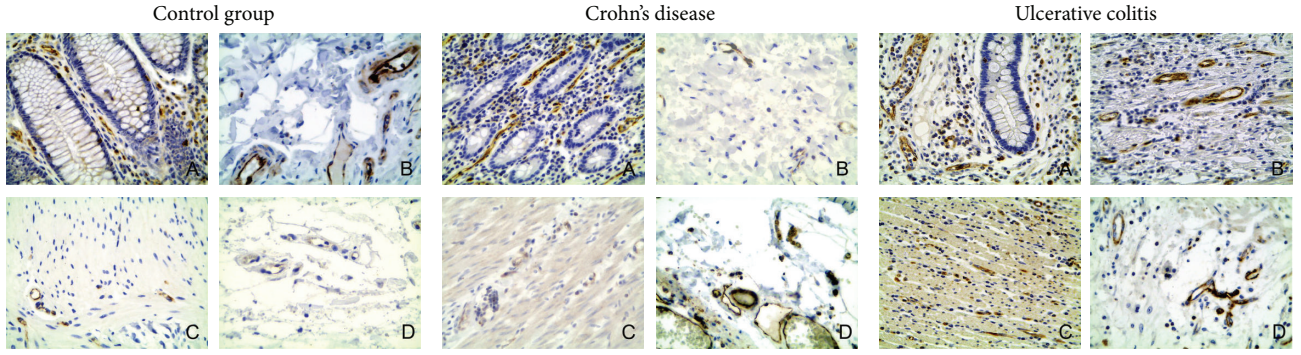

Figure 4: Immunohistochemical expression of VEGFR-1 (magnification 400x). A: mucosa; B: submucosa; C: muscularis; D: subserosa.

For morphometric analysis we have used Leica Quantimet workstation along with ImageJ custom macros [12–14].

Each layer of vessel wall was analyzed separately. Vascular density was evaluated based upon two parameters—immunostaining against CD31 per square mm and percentage of section area covered by vessels. Thrombospondin expression was independently evaluated by two pathologists on a semiquantitative manner. VEGFR-1 expression was evaluated by counting the vessels that showed immunohistochemical reaction in endothelial cells. Immunohistochemical expression of VEGFR-1 has been shown in Figure 4.

Statistical analysis of the results (Mann-Whitney U test, Kruskall-Wallis test, correlation analysis) was performed with the use of R programming language [15].

3. Results

Figures 5–7 show vascular density values in tissue layers of bowel wall in our study.

We found statistically significant differences in both mean vessel count and mean vessel area fraction in Crohn's disease when compared to normal bowel and ulcerative colitis in comparison to normal bowel (see Table 3, Figures 6 and 8).

Differences in vascular area percentage between ulcerative colitis and control were insignificant for submucosa and muscularis propria. It is interesting, that our results showed significantly higher vascular density in subserosa of control group patients when compared to ulcerative colitis as shown in Figures 6 and 8.

We have also shown significant differences in both mean vessel count and mean vessel area fraction throughout the all layers between Crohn's disease and ulcerative colitis ($P < 0.002$).

Our study showed no statistically significant correlation between IBD type and VEGFR-1 (Flt1) expression and between vascular density and VEGFR-1 expression (see Table 4 and Figure 7).

TSP-1 was sporadically found (4 cases, expression within intestinal epithelium, see Figure 9) only in samples from patients with IBD (3 in CU and 1 in CD), and it seems to be rather artifactual and insufficient to make any statistical correlation between TSP-1 expression and vascular density

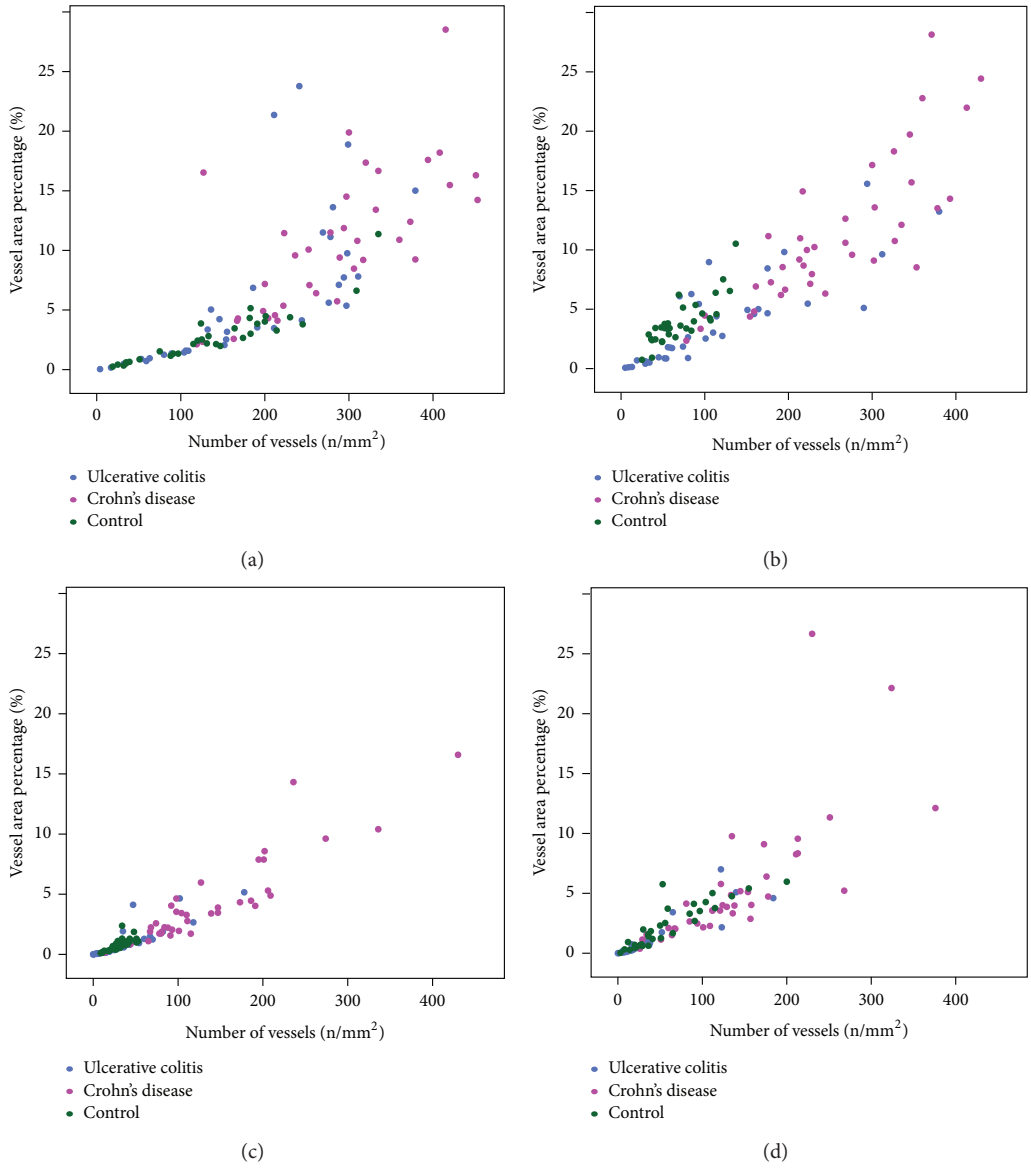

Figure 5: Number of vessels versus vessel area percentage in intestinal wall layers. (a) Mucosa, (b) submucosa, (c) muscularis propria, and (d) subserosa.

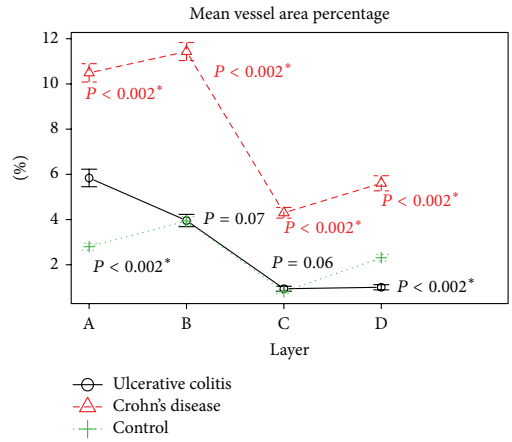

Figure 6: Mean vessel area percentage in bowel wall layers by group. A: mucosa; B: submucosa; C: muscularis; D: subserosa statistically significant values were marked with (∗).

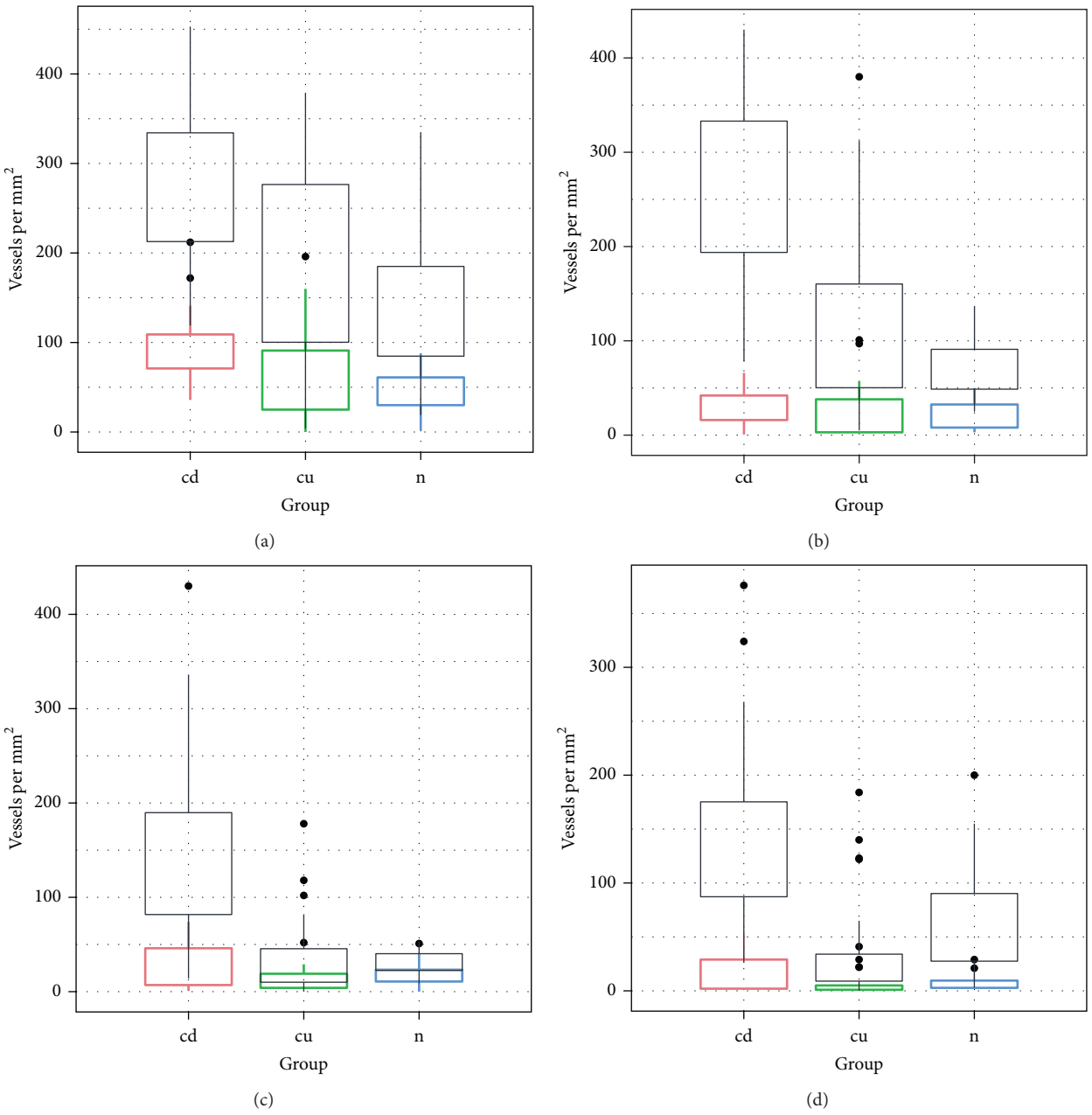

Figure 7: Comparison of total number of vessels (black) and number of VEGFR-1 positive vessels (color). (a) Mucosa, (b) submucosa, (c) muscularis propria, and (d) subserosa. cd: Crohn disease, cu: ulcerative colitis, and n: control.

Table 4: Vessel count and VEGFR-1 positive vessel count correlation analysis (Spearman's rho).

	Control	Crohn's disease	Ulcerative colitis
Mucosa	R Spearman = −0.01399 P = 0.9590	R Spearman = 0.1156 P = 0.1156	R Spearman = 0.5004 P = 0.0484
Submucosa	R Spearman = 0.1864 P = 0.4893	R Spearman = −0.03824 P = 0.8802	R Spearman = 0.02432 P = 0.9288
Muscularis	R Spearman = 0.06038 P = 0.8242	R Spearman = −0.05168 P = 0.8386	R Spearman = 0.2426 P = 0.3652
Subserosa	R Spearman = 0.1167 P = 0.6669	R Spearman = −0.04359 P = 0.8636	R Spearman = 0.1828 P = 0.4981

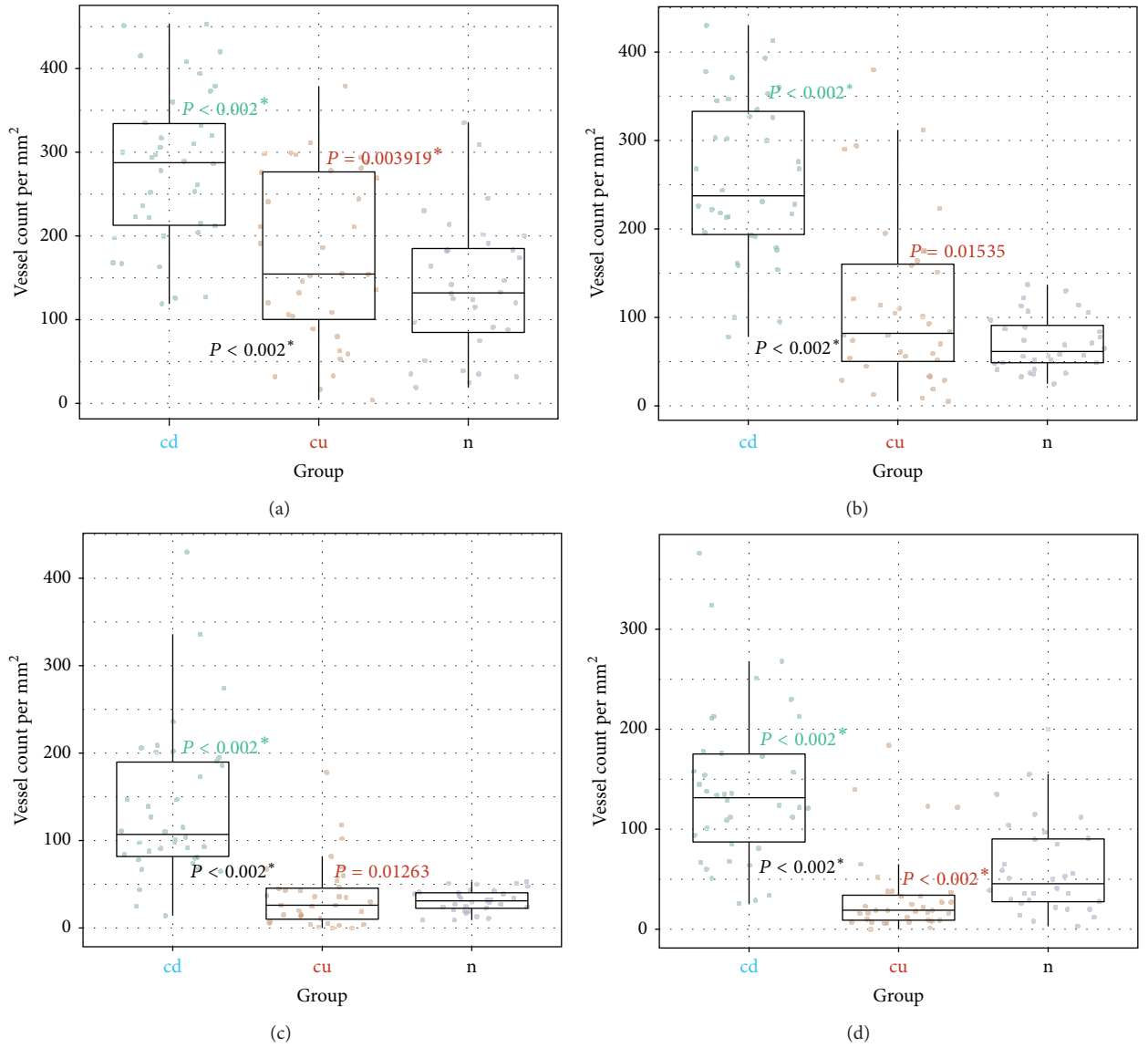

Figure 8: Vessel count differences between groups by bowel wall layer. cd: Crohn's disease; cu: ulcerative colitis; n: control. (a) Mucosa; (b) submucosa; (c) muscularis; (d) subserosastatistically significant differences were marked with (∗). *P* values: color: cd/cu versus control; grey: cd versus cu.

or VEGFR-1 expression. We have observed strong internal immunohistochemical control reaction in thrombocytes within blood vessels. Answering the title question, we could not find a strict correlation between thrombospondin and vascular endothelial growth factor receptor expression.

4. Discussion

The results of our study support macro- and microscopic pattern in cases with active phase of inflammatory bowel disease—intensive blood vessels congestion. We have also revealed significantly increased number of blood vessels in almost all of the four layers of bowel wall while comparing Crohn's disease and ulcerative colitis with normal bowel wall. Staining with CD31 was shown to be of great importance not only allowing the assessment of the number of vessels but also showing blood vessels essentially dilated. Increased vessel count could support the fact of angiogenesis accompanied by VEGF. However, in our opinion, Flt1 (VEGFR1) is not a marker of angiogenesis—this study not revealed statistically significant reaction in normal and IBD bowels. This is contrary to Konno et al. who stated that expression of Flt-1 (among others) was increased in active CU [16]. We believe that a better marker of angiogenesis could be the expression of another VEGF receptor-VEGFR2 (KDR/Flk-1), which was also studied by the same authors. Importance of VEGFR2 in angiogenesis was also described by Ferrara et al. [6] and Usui et al. [17]. We share the opinion of Scaldaferri et al. who stated that, after the induction of colitis, the expression of both VEGF-A and VEGFR-2 was markedly enhanced, whereas no increase in the expression of VEGFR-1 was observed [18]. Only four positive thrombospondin

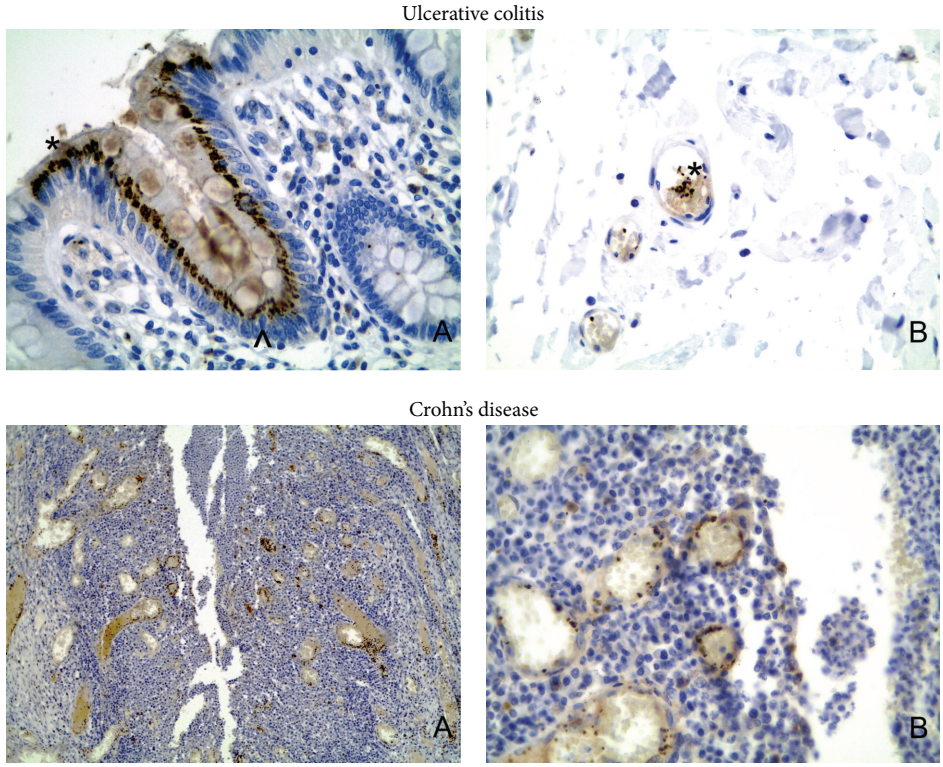

Figure 9: Immunohistochemical expression of TSP-1. Ulcerative colitis: A: TSP-1 expression within surface (∗) and crypt (arrowhead) epithelium (400x); B: expression of TSP-1 in thrombocyteswithin subserosal blood vessel (∗). Crohn's disease: expression of thrombospondin in thrombocytes within vessels surrounding fissure-like ulcer. A: low magnification (100x), B: detailed view (400x).

expression findings in our research could be explained by several mechanisms. All our cases with IBD were collected from patients with acute and severe phases of the diseases. This could explain inflammation-induced changes in TSP-1 expression. Also, increased stimulation of angiogenic pathways could completely suppress anti-angiogenesis (inhibition of thrombospondin expression). It is also possible that the pathways of anti-angiogenesis in IBD rely on mediators other than TSP-1. We also cannot exclude that our antibody reacted with different epitopes (nonspecific for that of TSP-1) leading to artifactual staining. Our findings did not confirm previous reports on the possible role of TSP-1. Zak et al. and Punekar et al. [4, 5] have studied TSP-1-deficient mice with experimental colitis and suggested that TSP-1 might decrease angiogenesis. Alkim et al. studied only mucosal samples and found that the expression of TSP-1 was higher in IBD groups relative to healthy control group [19]. We could not find such a correlation because TSP-1 tissue reaction was sporadic and weak. We would like to emphasize that we studied the full thickness of bowel wall in contrast to most reports dealing with samples from the intestinal mucosa only. According to our findings, the number of blood vessels increased, and they became dilated in all layers of bowel wall (especially in the course of Crohn's disease) showing that angiogenesis is one of the main pathological processes occurring not only within mucosa but also within other layers of bowel wall.

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Research Article

Fractalkine (CX3CL1) and Its Receptor CX3CR1 May Contribute to Increased Angiogenesis in Diabetic Placenta

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Chemokine CX3CL1 is unique, possessing the ability to act as a dual agent: chemoattractant and adhesive compound. Acting via its sole receptor CX3CR1, CX3CL1 participates in many processes in human placental tissue, including inflammation and angiogenesis. Strongly upregulated by hypoxia and/or inflammation-induced inflammatory cytokines secretion, CX3CL1 may act locally as a key angiogenic factor. Both clinical observations and histopathological studies of the diabetic placenta have confirmed an increased incidence of hypoxia and inflammatory reactions with defective angiogenesis. In this study we examined comparatively (diabetes class C complicated versus normal pregnancy) the correlation between CX3CL1 content in placental tissue, the mean CX3CR1 expression, and density of the network of placental microvessels. A sandwich enzyme immunoassay was applied for CX3CL1 measurement in placental tissue homogenates, whereas quantitative immunohistochemical techniques were used for the assessment of CX3CR1 expression and the microvascular density. Significant differences have been observed for all analyzed parameters between the groups. The mean concentration of CX3CL1 in diabetes was increased and accompanied by augmented placental microvessel density as well as a higher expression of CX3CR1. In conclusion, we suggest involvement of CX3CL1/CX3CR1 signaling pathway in the pathomechanism of placental microvasculature remodeling in diabetes class C.

1. Introduction

Augmented immune tolerance during pregnancy prevents onset of inflammatory immune responses that may cause fetal rejection [1]. Specific roles of the cytokine network in the human placenta include local modulation of the balance between pro and anti-inflammatory factors [2]. Thus, together with pregnancy-specific hormones, adequate participation of placental cytokines in immune control is crucial for normal intrauterine growth of the fetus [3]. Since many mediators of inflammation are angiogenic, any disorder associated with the shift in the precise quantitative balance between proinflammatory cytokines and their inhibitors may influence the development of placental vessels [4]. Pathomechanisms of diabetes mellitus during pregnancy include changed oxygen and metabolic pathways, resulting in abnormal placental villous growth and function [5]. It was reported that dysregulation of angiogenic response within diabetic placental tissue may significantly affect fetal well-being by an increase in susceptibility to hypoxia and hypoxia-associated apoptotic triggers [6].

Clinical observations and histopathological studies of the placenta have confirmed an increased incidence of inflammatory reactions in diabetes [7, 8]. The class C of diabetes in pregnancy (after White) is the last stage without recognized vascular morphological changes in light microscopy [9] (Table 1). We previously showed that increased density of the villous vascular network in class C human diabetic

Table 1: Clinical characteristics of the two studied groups: diabetes class C after White (group I) versus normal-course pregnancies (group II).

Parameter	Group I (diabetes class C)	Group II (normal controls)
Number of patients/placentae/newborns (*n*)	11/11/11	11/11/11
Age of the patients in full years (range; mean; median)	26–33; 29; 28	22–33; 27; 26
Parity	0	0
Gestational age in days (range; mean; median)	242–258; 250; 252	249–257; 253; 255
Method of delivery	Cesarean section	Cesarean section
1st minute Apgar's score (range; mean)	8–10; 9.3	9-10; 9.7
Blood pressure during pregnancy	All records within normal range[a]	All records within normal range[a]
Proteinuria during pregnancy	Not present[b]	Not present
Liver blood tests (aminotransferases enzymes AST and ALT levels)	Within normal range[c]	Within normal range[c]
Smoking during pregnancy	1 declared >5 cigarettes per day	2 declared >5 cigarettes per day
Body Mass Index <21 or >35	None	None
Other identified risk factors	None	None
Birth weight in grams (range; mean; median)	2995–3590; 3386; 3277	3056–3440; 3307; 3320
Newborns gender (M—male; F—female)	4M + 7F	6M + 5F
Weight of placenta in grams (range; mean, median)	425–616; 571; 563	482–626; 554; 539

[a]The normal range of the blood pressure was defined as systolic pressure between 100 and 140 mm Hg and diastolic pressure between 60 and 90 mm Hg.
[b]Microalbuminuria (urinary albumin excretion in the range of 30–300 mg/24 h) not present.
[c]The normal range of values for AST is from 5 to 40 units per liter of serum, and the normal range of values for ALT is from 7 to 56 units per liter of serum.

placenta was correlated with higher histamine and vascular endothelial growth factor (VEGF) concentrations and increased number of placental mast cells [10, 11]. Since several mastocyte-derived mediators, including histamine, are angiogenic and regulate endothelial cell proliferation and function, degranulation of mast cells may augment local angiogenesis [12]. One explanation for the increase in mast cell number is migration of mast cells in diabetic placental tissue. Considering this, the role of chemotactic cytokines—chemokines in the pathogenesis of diabetes-induced neovascularization—should be suspected [13].

Chemokines form a superfamily of cytokines with the major roles involved in the modulation of immune response and the guidance of migrating leukocytes towards or away from the chemotactic substance (chemoattractant or chemorepellent activities, resp.) [14]. First description of chemokine CX3CL1 (known also as fractalkine or neurotactin) was given in 1997 by Bazan et al. and Pan et al. [15, 16]. To date, encoded on human chromosome 16 and possessing three amino-acid residues located between the first two cysteine residues in the molecule, CX3CL1 is the lone member of the CX3C (delta) subfamily of chemokines [17]. Unlike other chemokines, CX3CL1 is of nonhaemopoietic origin and exists in a soluble form as chemotactic protein and in a membrane-anchored form mainly on endothelial cells as a cell-adhesion molecule. The main roles of the soluble CX3CL1 domain include chemotactic activity for natural killer (NK) cells, T cells, monocytes, and mast cells, but not neutrophils, whereas membrane-anchored form of the chemokine is involved in promotion of leukocyte binding and adhesion. This dual function (chemoattractant and adhesive compound) makes CX3CL1 unique among the known chemokine subfamilies [18]. Together with some other chemokines (CCL4, CCL7, CCL14) CX3CL1 participates in the processes of implantation, trophoblast invasion into the spiral uterine arteries, placental angiogenesis, response to inflammatory and immunologic factors within the uteroplacental unit, and induction of labor [19–21]. Endothelial cells of the placental vasculature, vascular smooth muscle cells, and amniotic epithelial cells are the main sources of CX3CL1 in the human placenta including the membranes [22–24].

Actions of CX3CL1 are mediated by its sole receptor CX3CR1 (previously known as V28), Gαi protein-linked seven-transmembrane receptor [25]. Expression of CX3CR1 was confirmed in endothelial cells, as well as on mast cells and other cell types, including monocytes, NK cells, microglial cells, neurons, and subpopulations of T-lymphocytes [26]. CX3CR1 receptor stimulation leads to the activation of both CX3CL1-dependent and integrin-dependent migrations of cells with augmented adhesion in result of synergistic reactions [27].

Strongly upregulated by hypoxia and/or inflammation-induced inflammatory cytokines secretion, especially tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin-1 beta (IL-1β), CX3CL1 may act locally as a key angiogenic factor [28]. It was reported that activation of CX3CL1/CX3CR1 signaling pathway induces angiogenesis through two sequential steps: the induction of hypoxia inducible factor 1 alpha (HIF-1α) and vascular endothelial growth factor-(VEGF-) A gene expression and the subsequent VEGF-A/vascular endothelial growth factor receptor type 2 (VEGFR2 or KDR) induced angiogenesis [29, 30].

The aim of this study was to examine comparatively (diabetes class C—complicated versus normal pregnancy) the

correlation between CX3CL1 content in placental tissue, the mean CX3CR1 expression, and density of the network of placental microvessels.

2. Materials and Methods

The study was conducted in compliance with international and local laws of human experimentation and was officially approved by local ethics committee, and written consent from the women was obtained for use of their placentae. Strictly speaking, the work has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and the uniform requirements for manuscripts submitted to biomedical journals have been fulfilled.

2.1. Placental Tissue Collection. Eleven placentae obtained from nulliparas after single pregnancies complicated by diabetes class C (group I; the mean gestational age 250 ± 8 days), were compared with 11 placentae obtained from gestationally matched near-term controls (group II; 253 ± 4 days). All pregnancies were terminated by cesarean sections due to the fetal interest (group I), including breech presentation (pelvic longitudinal lie of the fetus) in group II. The control of glycemia in all cases was satisfactory; the levels of fraction of glycosylated hemoglobin in all trimesters of diabetic pregnancy were kept within the normal range (5%–7.5%). The courses of pregnancies in group II were normal, except for a near-term initiation of the contractile activity of the uterus. More detailed clinical characteristics of the two studied groups are given in Table 1.

Five specimens were excised in a standardized manner from each placenta: three from the region contiguous to the maternal surface (the first one—from the central part, the next two—from peripheral regions of the placental maternal surface) and two specimens from the region contiguous to the fetal surface (the first—from place of umbilical cord insertion, the next—from the peripheral region) (Figure 1). The tissue material obtained by this procedure was subjected either to freezing in carbon dioxide snow for CX3CL1 concentration measurement or fixed in paraffin wax and cut at 5 μm, before staining procedures with hematoxylin/eosin and immunohistochemistry.

2.2. Measurement of CX3CL1 Content. Concentration of CX3CL1 was estimated in the frozen placental excisions. During handling, the material was kept on ice. Before generating a lysate, the tissue was initially cut into about 1 mm^3 cubes by using a razor blade on a glass plate held on ice. In order to perform a gentle cell disruption, the cubes were then transferred into a hand-held Potter S homogenizer. The liquefied tissue was placed in the 1.5 mL tubes and centrifuged at 13000 rpm for 3 min at 4°C. The clear supernatant was used for in vitro quantitative measurement of CX3CL1 in the placental tissue homogenates by a sandwich enzyme immunoassay. Chemokine C-X3-C-Motif Ligand 1 (CX3CL1) BioAssay ELISA Kit (Human; Cat. no. 024096) was applied with detection range 0.156–10 ng/mL and sensitivity of 0.053 ng/mL. The mean value was calculated for each examined placenta and expressed in pg/g of wet tissue weight.

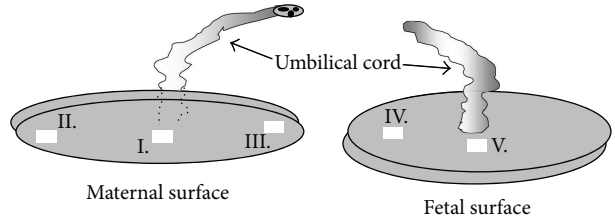


Figure 1: Location of samples collected in standardized manner from the maternal surface of the placenta (I–III) and from the fetal surface (IV-V). The mean weight of the sample: 10.49 ± 0.89 g.

2.3. Immunohistochemistry of CX3CR1. Human placenta paraffin 5 μm sections were subjected to the standard immunohistochemical procedures that led to visualization of CX3CR1. Rabbit polyclonal antibody IgG to CX3CR1 (ab8020; Abcam Inc., USA; concentration of 10 μg/mL) was used as primary and goat anti-rabbit IgG as biotinylated secondary antibody (ab64256; Abcam; 0.5% v/v). In order to visualize the primary anti-CX3CR1 antibodies, the StreptAB-Complex/HRP Duet (Dako Cytomation, Glostrup, Denmark) was used, following the protocol recommended by the manufacturer, with 3,3′-diaminobenzidine that served as a chromogen. The respective negative controls for immunostainings were prepared simultaneously by replacement of the polyclonal primary antibody by normal rabbit preimmune IgG diluted with phosphate buffered saline, containing 3% bovine serum albumin at the same protein concentration as that used for the primary antibody.

2.4. Mean Density of Placental Microvessels. Identification of the vasculature elements in placental sections was performed using endothelial cell marker, rabbit polyclonal antibody anti-CD31 (dilution 1 : 50, ab28364; Abcam Inc., Cambridge, MA, USA). The tissue was incubated with the primary antibody for 30 minutes. Next, a biotinylated goat anti-rabbit antibody was used as the secondary (Abcam).

Using light microscopy with computed morphometry for quantitative analysis (Quantimet 500C+ image analysis workstation provided by Leica, UK), the vascular/extravascular tissular index (V/EVTI) was estimated in calibrated areas of the placental sections. Each preparation (paraffin section) underwent three area analyses repeated by two experienced, independent observers. The single area measured with the picture analyzer amounted to 721320 μm^2 and the total number of preparations 55 per group. The picture analysis procedure consisted in a measurement of the total vascular area. Consequently, the total lumen area of all types of identified vessels was summed up in both groups. With the purpose of a minimizing disruption caused by technical errors, especially unaxial section of the vessel, the lowest value of Ferret's diameter was accepted as the diameter of single lumen. Thus, V/EVTI represents the ratio, which reflects intensity of vascularization and is most closely correlated with the mean density of placental microvessels [31].

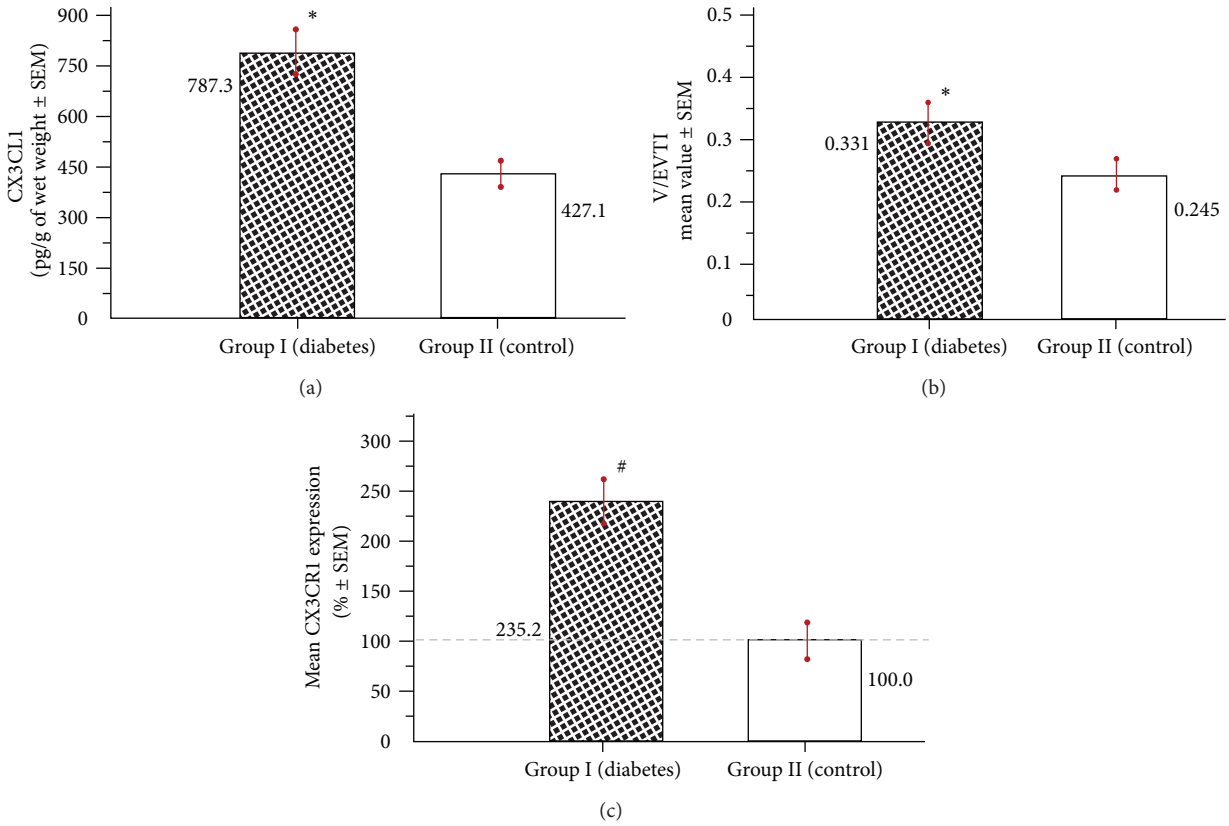


Figure 2: Placental samples collected after diabetes class C (group I) versus specimens obtained after normal pregnancy (group II): (a) the mean concentration of CX3CL1; (b) density of the microvessels estimated using the mean vascular/extravascular tissular index (V/EVTI); (c) the mean percentage expression of CX3CR1. The values obtained for normal pregnancy (0.016 ± 0.0014; abstract numbers ± SEM) were taken as 100% ($^{*}P < 0.05$; $^{\#}P < 0.01$).

2.5. Expression of CX3CR1. After immunostaining, a quantitative immunohistochemistry based on morphometric software (Quantimet 500C+) was applied for CX3CR1 receptors identification in paraffin 5 μm sections of the placental specimens under light microscopy. All morphometric procedures were carried out twice by two independent researchers and the average values uploaded in the result recording tab. Intensity of immunostaining was evaluated using mean colour saturation parameter and thresholding in grey-level histograms. Thus, expression of CX3CR1 corresponded to the total immunostained calibrated area of examined sections, where colour saturation comprises segmentation-separation criteria for objects. Single analysed image area amounted to 138692 μm^2 (magnification ×200). In total, 165 visual fields have been analysed (15 visual fields per placenta) in each studied group. To achieve maximum accuracy of measurements, the following factors have been controlled or monitored: averaging of image intake, hue, illumination, luminance, power supply, relation of illumination to quantification of area percentage of positively staining structures, shading correction, and warming up. More detailed description of these morphometric procedures was given previously elsewhere [31, 32]. Assuming that the accuracy of CX3CR1 expression measurement may be significantly affected by the local differences in density of placental microvessels, in both groups we examined comparatively vascular density-matched samples with the tolerated range of discrepancy ±5% [33]. Morphometric results comprising 90% confidence intervals were reported as mean percentage values ± SEM.

2.6. Statistical Analysis. Statistical analyses were performed using Statistica 8.0 software (Stat-Soft, Poland). Mann-Whitney's U test was applied. The results were expressed as means ± SEM or mean percentage values ± SEM. Differences between group I (diabetes class C) and group II (normal-course pregnancy) were deemed statistically significant if $P < 0.05$.

3. Results

The results pertaining to CX3CL1 content in placental tissue, the mean density of microvessels, and CX3CR1 expression are summarized in Figures 2(a), 2(b), and 2(c), respectively. Significant differences have been observed for all analyzed parameters between the groups.

The mean concentration of CX3CL1 in placental cuts from the diabetic group (group I) was increased compared to controls (group II) and amounted to 787.3 ± 70.2 pg/g of wet weight ± SEM versus 427.1 ± 37.4 (Figure 2(a)). There were no significant differences in CX3CL1 levels between the

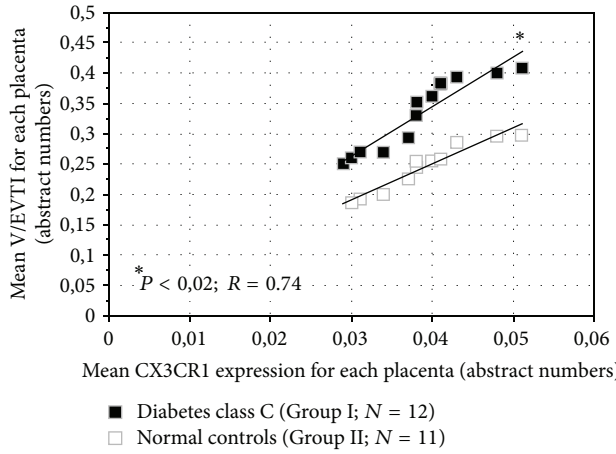


Figure 3: The mean values of V/EVTI versus the mean CX3CR1 expressions: diabetic and normal placentae, comparatively.

specimens collected from maternal and fetal surface of the placenta within the group.

The mean V/EVTI value (absolute number ± SEM) in placental tissue obtained after diabetes class C-complicated pregnancies was increased ($P < 0.05$), amounting to 0.331 ± 0.037 versus 0.245 ± 0.025 in normal controls (Figure 2(b)). These mean values of V/EVTI were evaluated totally for each group, using the partial data gained from all collected placental samples. However, we observed in both groups that in the samples obtained from the maternal surface, the mean density of microvessels expressed as V/EVTI was significantly higher ($P < 0.05$) compared to the mean V/EVTI calculated for the specimens of placental tissue obtained from the fetal surface (the data not shown on the chart).

Evaluation of the relationship between the mean CX3CR1 expression and the mean V/EVTI revealed strong positive correlation and significant differences between the groups (Figure 3). The higher expression of CX3CR1 in diabetes corresponded to the augmentation of the placental vascularization, as assessed by V/EVTI.

Immunohistochemical technique used for identification of CX3CR1 revealed that this receptor is predominantly located in placental endothelial cells (Figure 4). The mean percentage value of CX3CR1 expression estimated in the vascular density-matched samples was remarkably higher ($P < 0.01$) in diabetes and reached 235.2 ± 24.4 (%, ±SEM) of the reference value established in group II (Figure 2(c)).

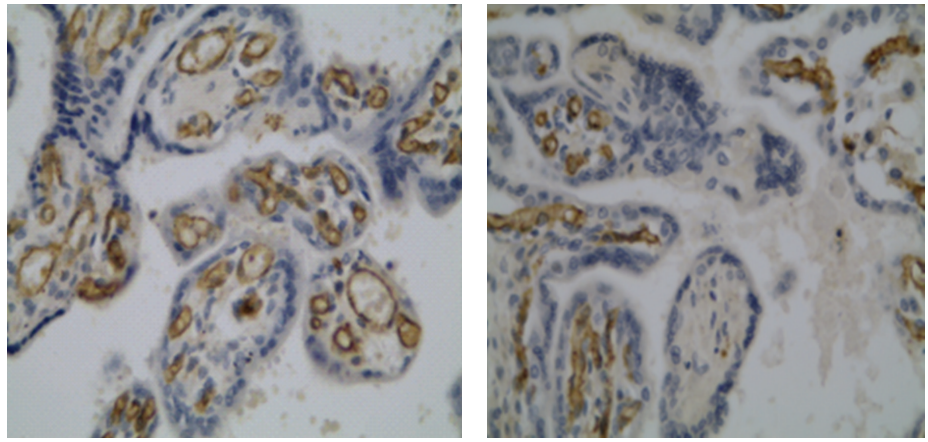
4. Discussion

The growth, maintenance and functioning of placental microvessels may be affected by the majority of the pathological changes in maternal/fetal hemodynamics and by alterations in blood composition or content [34]. Different authors working independently have reported that the induction of the pro-inflammatory milieu is inherently involved in the pathophysiology of diabetes mellitus, including diabetic placenta [7, 35]. In such an environment, frequent episodes of local hypoxia and transient hyperglycemia (even in well-controlled cases) are observed and are associated with elevated levels of free oxygen radicals, advanced glycation end products (AGEs), and some proinflammatory cytokines, especially those with angiogenic properties [6, 35]. However, it has also been reported that placental expression of the inflammatory cytokines in response to oxidative stress is significantly reduced in gestational diabetes mellitus [36]. This study did not involve women with diabetes class C, and CX3CL1 concentrations were not examined.

Secondary to hypoxia, increases in the local levels of TNFα, IFNγ, and IL-1β potentiate the angiogenic action of CX3CL1 that is simply correlated with an increase in the concentration of this chemokine [28, 29, 37]. CX3CL1 stimulates ex vivo and in vivo angiogenesis in a dose- and time-dependent manner and acts on endothelial cells even more strongly than the well-known angiogenic factor VEGF [29]. It has been reported that CX3CL1 (100 nM) induced endothelial cells to form capillary tubes in synthetic matrix with at least the same efficiency as the angiogenic mediators bFGF (60 nM) and VEGF (100 nM) [38]. Based on the data that are available to date, there is no clear standpoint on the roles of VEGF and its receptor VEGFR-2 (KDR/Flt-1) in CX3CL1-mediated angiogenesis. Some authors have reported that the activation of CX3CL1/CX3CR1 by vascular endothelial cells induces angiogenesis through VEGF-A/KDR. Increased VEGF production can be achieved through the pathways involving hypoxia-inducible factor-1 alpha (HIF-1α) and p42/44 mitogen-activated protein (MAP) kinase [29]. In our previous study of the diabetic placenta, we found that higher VEGF expression levels were positively correlated with increased microvascular density [11]. However, the results of another study indicate that the mechanism of CX3CL1 expression and its angiogenic effects differ from those of VEGF. Whereas hypoxia strongly induces VEGF expression, an ischemic environment actually inhibits the expression of CX3CL1. This finding may suggest that hypoxia promotes VEGF-mediated angiogenesis, while inflammation involves CX3CL1-related new vessel formation [39]. Considering this, our findings are rather correlative and not mechanistic in nature. The results provide no formal evidence for the causal involvement of the CX3CL1/CX3CR1 system in altered placental vascularity, especially because we have previously reported changes in the placental VEGF expression [11].

Studies of the molecular mechanism by which CX3CL1 regulates angiogenesis in human umbilical vein endothelial cells (HUVEC) showed that CX3CL1 did not increase the levels of VEGF mRNA. These results indicate that CX3CL1 may act as a direct angiogenic modulator in endothelial cells without inducing VEGF expression. It is likely that the angiogenic activities of CX3CL1 and VEGF are independent of one another but utilize the Raf1/MEK/ERK kinase cascade and P13 K/Akt/eNOS activation as common mediators in their angiogenic signaling pathways. In other words, it was proposed recently that CX3CL1, acting directly on angiogenesis via CX3CR1, may activate the two previously mentioned distinct signaling pathways [40].

All of the characteristic steps in the complex process of new vessel formation are augmented after CX3CL1 treatment,



(a) (b)

Figure 4: Immunohistochemical localization of the receptor CX3CR1 in the placental tissue at 400x magnification: (a) diabetes class C (group I); (b) normal controls (group II).

including endothelial cell proliferation, migration, and tube-like structure formation [30, 41].

The statistically significant upregulation of CX3CR1 expression documented in diabetic endothelial cells should also be considered, particularly because of the existence of an autoregulatory mechanism between CX3CL1 and CX3CR1 [42]. A form of autoregulation between CX3CR1 and CX3CL1 via the autocrine loop (CX3CR1/CX3CL1 axis) was proposed by independent authors with respect to many cell types, including endothelial cells [43–45]. So far, however, there is no convincing scientific evidence that the same human endothelial cells possess the ability to produce CX3CL1 and simultaneously express CX3CR1. However, because CX3CR1 is the only known receptor for CX3CL1, it is possible that CX3CL1-induced endothelial cell migration and capillary tube formation was mediated through the interaction between CX3CL1 and its endothelial receptor CX3CR1 in an autocrine manner [38].

It is worth noting that the CX3CR1 signal documented in Figure 4 and considered to be associated with endothelial cells may also come from pericytes. A double-staining technique or high-resolution, high-power imaging should be implemented in future studies to elucidate this issue.

An excess of CX3CL1 and other inflammatory mediators may be responsible for augmented angiogenesis within the placental unit. According to the results of other studies, the regulation of CX3CL1 function may be maintained on both protein and gene expression levels. Increased expression of matrix metalloproteinases (MMPs) and disintegrin metalloproteinases (ADAMs, including ADAM17/TACE) or sheddases have been reported in the diabetic placenta [46, 47]. These increases may lead to an increase in the concentration of the soluble form of CX3CL1, which is derived from its membrane form.

In contrast, the proinflammatory background of diabetes, which includes increased placental levels of TNFα, IFNγ, and IL-1β, promotes enhanced CX3CL1 gene expression [48].

Further studies are needed to determine which mechanism of CX3CL1 regulation predominates in diabetes class C.

The classic example of a diabetic complication with increased defective angiogenesis mediated by CX3CL1 with the inflammatory background is proliferative retinopathy [38, 49].

Both in this study and in previous reports, we have provided evidence of altered vascularization in the diabetic placenta. The results of the present investigation strongly support the hypothesis that CX3CL1-related pathways is involved in the pathomechanism of placental angiogenesis in diabetes. Increased V/EVTI values are consistent with our previous approach to evaluating the density of placental microvessels in diabetes class C with respect to histamine concentration and mast cell number [10, 11].

Chemotaxis assays performed in vitro by other researchers have demonstrated that increased CX3CL1 levels initiate and augment the migration of specific CX3CR1-positive subpopulations of inflammatory cells [50]. For example, we previously observed both an increase in the number of mast cells in the diabetic placenta and changes in their heterogeneity, namely, a shift of the quantitative balance between tryptase-positive and tryptase/chymase-positive cells [51]. This shift may influence the placental cytokine network profile in diabetes, possibly including an increase in the local CX3CL1 concentration. Considering the dual role of CX3CL1, mechanisms that influence placental angiogenesis and are related to the direct action of CX3CL1 and the chemoattractant properties of CX3CL1 should be considered. Interestingly, despite the significant expression of CX3CR1 by human mast cells, CX3CL1 does not directly produce mast cell degranulation [52]. Thus, CX3CL1 should not be simply linked with mast cell-mediated angiogenesis [12].

It should be clearly stated that altered vascular patterns are not necessarily a result of angiogenesis and could represent a form of vascular remodeling rather than overt capillary growth. This phenomenon is illustrated by the

larger, rather than more numerous vessels depicted in Figure 4. The algorithm used to enumerate vascular changes does not permit the capturing of microvessels [53].

In conclusion, it is very likely that increased CX3CL1 concentration in diabetes class C, together with the upregulation of its specific and sole receptor, CX3CR1, are involved in the pathomechanism of placental microvasculature remodeling. Further studies are needed to elucidate the rationale for anti-CX3CL1 or anti-CX3CR1 therapies during diabetic pregnancy.

Conflict of Interests

The authors report no conflict of interests related to this study or the findings specified in this paper.

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Research Article

Inhibitory Effect of Herbal Remedy PERVIVO and Anti-Inflammatory Drug Sulindac on L-1 Sarcoma Tumor Growth and Tumor Angiogenesis in Balb/c Mice

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Anticancer activity of many herbs was observed for hundreds of years. They act as modifiers of biologic response, and their effectiveness may be increased by combining multiple herbal extracts . PERVIVO, traditional digestive herbal remedy, contains some of them, and we previously described its antiangiogenic activity. Numerous studies documented anticancer effects of nonsteroidal anti-inflammatory drugs. We were the first to show that sulindac and its metabolites inhibit angiogenesis. In the present paper the combined *in vivo* effect of multicomponent herbal remedy PERVIVO and nonsteroidal anti-inflammatory drug sulindac on tumor growth, tumor angiogenesis, and tumor volume in Balb/c mice was studied. These effects were checked after grafting cells collected from syngeneic sarcoma L-1 tumors into mice skin. The strongest inhibitory effect was observed in experimental groups treated with PERVIVO and sulindac together. The results of our investigation showed that combined effect of examined drugs may be the best way to get the strongest antiangiogenic and antitumor effect.

1. Introduction

Tumor angiogenesis, the development of new blood vessels within the primary tumor or in metastasis hotspots, is an essential process for the growth and progression of metastases. Both innate and adaptive immune systems are involved in positive and negative regulations of this process. All over the world various research teams are conducting studies to find agents, which could potentially be able to inhibit the process. Despite the discovery of many such agents, the need for further research exists, because the angiogenesis inhibitors obtained so far are either very costly to synthesize, or they exhibit a number of side effects. Angiogenesis is a complex process with many contributing factors and one regulated by endogenic stimulators and inhibitors. Among the scientists involved in such studies a common conviction exists; that is the single angioinhibitors are not able to suppress the process effectively. Most antiangiogenic natural health products block new vessel formation at multiple molecular levels. Thus the need to recourse to low-toxic vegetal compounds and studies of combined effects of a few low-dosage compounds with different modes of action on the processes of angiogenesis in

tumor growth. Many phytochemicals and diet derivatives are able to exert chemopreventive and antitumor activity targeting the tumor environment and inflammatory angiogenesis [1–3].

PERVIVO is a digestive herbal remedy, mixture of 27 herbs alcoholic extracts dissolved in 32% ethyl alcohol, some of them traditionally used as anti-inflammatory agents also possessing antimicrobial or anti-tumor properties. We were the first to show angioinhibitory effect of this remedy in local cutaneous model of tumor angiogenesis [4]. The most important anti-tumor substance in PERVIVO is *Radix zingiberis* (ginger). Ginger has a long history of medicinal use dating back 2500 years. The anticancer properties of ginger are connected mainly with gingerols, shogaols, and zingerone [5]. 6-gingerol, a natural component of ginger, was shown to inhibit growth of colon cancer cells via induction of G2/M arrest [6]. The second ginger component, 6-shogaol, induced apoptosis in human hepatocellular carcinoma cells and exhibited anti-tumor activity *in vivo* through endoplasmic reticulum stress [7]. Ginger treatment suppressed the proliferation and colony formation in breast cancer cell lines [8]. Chakraborty et al. observed the *in vitro* effect of 6-gingerol on HeLa cells. Their results suggest that 6-gingerol has potential to bind with DNA and induce cell death by autophagy and caspase-3-mediated apoptosis [9]. Silva et al. demonstrated specific antiproliferative activities of gingerols against MDA-MB-231 tumor cell line [5]. Shogaols are dehydration products of corresponding gingerols during storage or thermal processing. Shogaols have stronger inhibitory effect than gingerols on growth of cancer cells, arachidonic acid release, and nitric oxide (NO) synthesis [10]. In experiments of Weng et al. both 6-shogaol and 6-gingerol effectively inhibited invasion and metastasis of hepatocellular carcinoma but through diverse molecular mechanisms. Both of them regulate MMP-2/-9 transcription. 6-Gingerol directly decreased expression of urokinase plasminogen activator and 6-shogaol indirectly by upregulation plasminogen activator inhibitor [11] and via blockade of nuclear factor-κB activation [12].

It was also documented that ginger and its compounds inhibit angiogenesis *in vitro* and *in vivo* [13–15].

The next PERVIVO ingredient, *Artemisia absinthium,* inhibited TNF alpha production and accelerated healing of patients with Crohn's disease [16]. Artemisinin, active antimalarial compound isolated from herbs belonging to *Artemisia* species, dihydroartemisinin (DHA), a semisynthetic derivative of artemisinin, and hispidulin, small flavonoid from *Artemisia vestita*, inhibited growth of pancreatic, prostate, and ovarian cancer cells and were shown to be cytotoxic to cancer cells through induction of apoptosis. The antiangiogenic effect of artemisinin *in vitro* and *in vivo* was also described [17–25].

The claims concerning anti-tumor activity of some other PERVIVO compounds are not largely supported by scientific evidence as yet. Radix *Angelicae sinensis* is a medicinal herb and health food supplement that has been widely used in Asia for centuries. Cytotoxicity against tumor cell lines of its extracts, epigenetic modifications of cancer oncogenes, and tumor suppressor genes were described. It was also reported that Radix *Angelicae sinensis* may be a potential source of glutathione S-transferase inhibitors and counteract multidrug resistance [26–30]. One report described antiproliferative activity of *Gentiana triflora* root extract on cultured and implanted tumor cells [29]. Fruit oils of *Litsea cubeba* from Taiwan exhibited cytotoxic activity against human lung, liver, and oral cancer cells *in vitro* [30, 31]. Galangin, flavonol present in *Alpinia galanga* rhizome, induced apoptosis of cancer cells [32, 33]. Extract of Radix Liquiritiae (licoricidin from *Glycyrrhiza uralensis*) inhibited the metastatic potential of human prostate cancer cells [34].

Most components of PERVIVO possess antimicrobial activity. Essential oil and decoction of Carlinae Radix (*Carlina acanthifolia* L.) showed significant antimicrobial effect against *Staphylococcus aureus* [35]. Compounds from *Artemisia* are antiplasmodial and antitrypanosomal drugs, and could be an alternative drug against trichinellosis [36–38]. Compounds from *Alpinia galanga* are potent inhibitors for the influenza virus replication [39] and exhibit significant activity *in vitro* against promastigotes of *Leishmania donovani* [40]. Phenylpropanoids of *Alpinia galanga* was efflux pump inhibitors in *Mycobacterium smegmatis* mc^2 155 cells [41]. Essential oils from *Litsea cubeba* contain fungicidal and antibacterial terpenoids [42, 43]. Sesquiterpene lactones from *Inula helenium* root essential oil exhibited antistaphylococcal activity [44].

Epidemiological studies have suggested that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk of cancer. Numerous studies have been devoted to the action of such anti-inflammatory agents as aspirin, indomethacin, piroxicam, and sulindac. The anticancer effect of NSAIDs was mainly found to result from their proapoptotic and antiproliferative effects which, therefore, restrict tumorigenesis [45–49].

We were the first to show that sulindac and its metabolites (sulindac sulfone and sulindac sulphide), described previously by other authors as pro-apoptotic anticancer drugs [50, 51], inhibit tumor growth and angiogenesis induced in mice skin by cells isolated from murine L-1 sarcoma as well as angiogenesis induced by cells collected from human kidney and pulmonary cancers [52–55]. The aim of the present work was to study the combined *in vivo* effect of multicomponent herbal remedy PERVIVO and non-steroidal anti-inflammatory drug sulindac on tumor growth, tumor angiogenesis, and tumor volume in Balb/c mice. These effects were checked after grafting of cells collected from syngeneic sarcoma L-1 tumors into mice skin.

2. Material and Methods

2.1. Drugs. PERVIVO (Richard Bittner GmbH, Weitensfeld, Austria) is herbal remedy composed of 27 herbal extracts dissolved in 32% ethyl alcohol (Table 1). Sulindac (Sudaklin, Polpharma SA, Starogard Gdański, Poland) is nonsteroidal anti-inflammatory drug.

2.2. Mice. The study was performed on female, 8–10-week old inbred Balb/c mice, about 20 g of body mass, delivered

Table 1: Composition of PERVIVO preparation.

(a) Active components

Radix *Angelicae*	1.360 g
Radix *Gentianae*	0.500 g
Menyanthis folium	0.120 g
Herba Absinthii	0.035 g
Radix *Zingiberis*	0.015 g
Camphora racemica	0.950 g
Theriak	0.970 g

(b) Additional

Fructus Anisi stellati	0.046 g
Myrrha	0.700 g
Herba Cardui benedicti	0.015 g
Herba Centaurii	0.013 g
Flos Caryophylli	0.030 g
Radix Galangae	0.014 g
Radix Liquiritiae	0.170 g
Radix Calami	0.047 g
Radix Helenii	0.020 g
Radix Zedoariae	1.380 g
Manna	1.360 g
Flos Verbasci	0.014 g
Radix Carlinae	0.680 g
Semen Myristicae	0.280 g
Herba Ivae moschatae	0.006 g
Radix Iridis	0.005 g
Pericarpium Aurantii amari	0.031 g
Cortex Curacao	0.038 g
Fructus Cubebae	0.017 g
Cortex Aurantii dulcis	0.011 g

from the Polish Academy of Sciences Breeding Colony. For all performed experiments animals were handled according to the Polish law on the protection of animals and NIH (National Institutes of Health) standards. All experiments were accepted and conducted according to ethical guidance of Local Bioethical Committee. Mice were housed 4-5 per cage and maintained under conventional conditions (room temperature 22.5–23.0°C, relative humidity 50–70%, and 12 h day/night cycle) with free access to standard rodent diet and water.

2.3. Sarcoma L-1 Tumor Cells. L-1 sarcoma cells from *in vitro* culture stock were delivered from Warsaw's Oncology Center collection, passaged *in vivo,* and grafted subcutaneously (for evaluation of tumor growth) or intradermally (for evaluation of angiogenic activity) to syngeneic Balb/c mice.

2.4. Treatment of Mice with PERVIVO and/or Sulindac. Mice received orally by Eppendorff pipette 20 μL of PERVIVO with 20 μL of water, or sulindac 0.6 mg, both drugs, for 3 days in cutaneous tumor-induced angiogenesis (TIA) test or for 14 days in evaluation of the effect of drugs on tumor growth. These doses correspond to 10 mL of PERVIVO and 300 mg of sulindac given to 70 kg person (applying the counter 7 for differences between mouse and human in relation of the surface to body mass). Control mice were fed with 40 μL of 16% ethyl alcohol.

2.5. Preparation of Tumor Cells after In Vivo Passage. Briefly, sarcoma L-1 cells from *in vitro* stock were grafted (10^6/0.1 mL) subcutaneously into subscapular region of Balb/c mice. After 14 days, the tumors were excised, cut to smaller pieces, rubbed through sieve, and suspended in 5 mL of PBS. The suspension was left for 10 min at room temperature.

After sedimentation, the supernatant was collected and centrifuged for 10 min at 1500 rpm. Obtained sarcoma cells were washed once with PBS for 10 min, then centrifuged at 1500 rpm, and resuspended in Parker medium in concentration of 4×10^6 cells/mL (for tumor-induced angiogenesis) or 10^7 cells/mL (for experiments with tumor growth). Viability of cells was about 95% of living cells as estimated by trypan-blue method.

2.6. Cutaneous Angiogenesis Assay (Tumor-Induced Angiogenesis (TIA) Test). Multiple 0.05 mL samples of 200 thousands of cells were injected intradermally into partly shaved, narcotised Balb/c mice (at least 3-4 mice per group). In order to facilitate the localization of cell injection sites later on, the suspension was colored with 0.1% of trypan blue. Mice were fed with drugs for 3 days. After 72 hours, mice were sacrificed with lethal dose of Morbital (Biowet, Puławy). All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6x, in 1/3 central area of microscopic field. Identification was based on the fact that the new blood vessels are thin, directed to the point of cells injection and (or) differ from the background vasculature in their tortuosity and divarications (Figure 1).

All experiments were performed in anaesthesia (3.6% chloral hydrate, Sigma-Aldrich), 0.1 mL per 10 g of body mass.

2.7. Subcutaneous Tumor Growth Assay. Suspensions of sarcoma cells were grafted (2 millions of cells) subcutaneously into mice. On the day of cells grafting and on the following 13 days mice were fed PERVIVO, sulindac, PERVIVO and sulindac, or diluted ethyl alcohol as a control. Mice were observed during these days, with number of appearing tumors noted at 7, 9, and 11 days after grafting. At days 9 and 14 the tumors volume was measured with electronic caliper (The Fowler Ultra-Cal Mark III caliper). After 14 days mice were sacrificed.

2.8. Morphological Examination. Morphological examination was done using light-microscopic analysis. Immediately after resection, tumor specimens were fixed in 10% formaldehyde solution. After fixation the specimens were dehydrated in increased concentration of alcohol and embedded in paraffin. Paraffin tissue block was sectioned on 4 μm thin sections. The specimens were stained by hematoxyline and eosine.

Table 2: Statistical analysis of the results presented in Figure 2.

(a)

One-way analysis of variance	
P value*	<0.0001
P value summary	***
Are means signif. different? ($P < 0.05$)	Yes
Number of groups	4
F	88.90
R square	0.8114

(b)

Bonferroni test	Mean diff.	t	Significant? $P < 0.05$?	Summary
Control versus PERVIVO	7.900	8.926	Yes	***
Control versus sulindac	10.60	11.98	Yes	***
Control versus PERVIVO + sulindac	13.20	15.19	Yes	***
PERVIVO versus sulindac	2.700	2.854	Yes	*
PERVIVO versus PERVIVO + sulindac	5.300	5.691	Yes	***

*$P < 0.05$, ***$P < 0.001$.

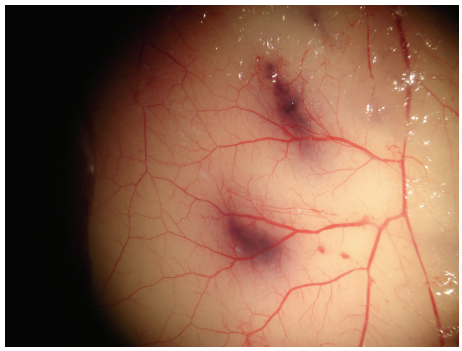

Figure 1: Typical picture of newly-formed blood vessels.

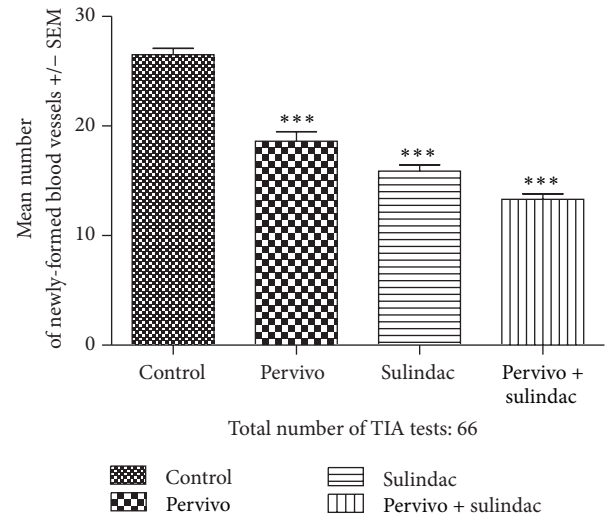

Figure 2: Inhibitory effect of PERVIVO and sulindac on neovascular reaction induced in mice skin after grafting L-1 sarcoma cells. ***$P < 0.001$.

2.8.1. Statistical Evaluation of the Results. Evaluation of the results was performed by chi-square test and one-way ANOVA with Bartlett's test for equal variances, and the significance of differences between the groups was verified with a Bonferroni Multiple Posttest (GraphPad Prism).

3. Results

Histological examination revealed no major differences between tumors collected from control and experimental groups of mice. The dominant picture was mass of poorly differentiated atypical cells with features of sarcoma. As shown in Figure 2 and Table 2 the number of newly formed vessels that were induced by the tumor presence was decreased both by PERVIVO and sulindac, with the effect of sulindac comparatively stronger. Joint application of both drugs resulted in even stronger effect than treatment with separate compounds. The influence of the study drugs on measurable tumor emergence in respective days after sarcoma load inoculation is shown in Figure 3 and Table 3. Similar to the antiangiogenic effect, the influence of sulindac alone was greater than this by PERVIVO, and the common appliance of both drugs resulted in even greater delay in measurable tumor appearance. The time of the strongest impediment of tumor growth was similar in case of all study settings (day 7). The effect was intermittent, and only after application of both drugs together, the measurable tumors have not developed in all animals.

The influence of the study drugs on the mean tumor volume at the respective study time points is shown in Figures 4 and 5. The mean volume was statistically significantly lower at day 9 after administration of sulindac and, to the lesser extent, by both drugs (Table 4). The effect was not clearly defined in the case of PERVIVO. The mean tumor volume was lower than in placebo group but the difference was

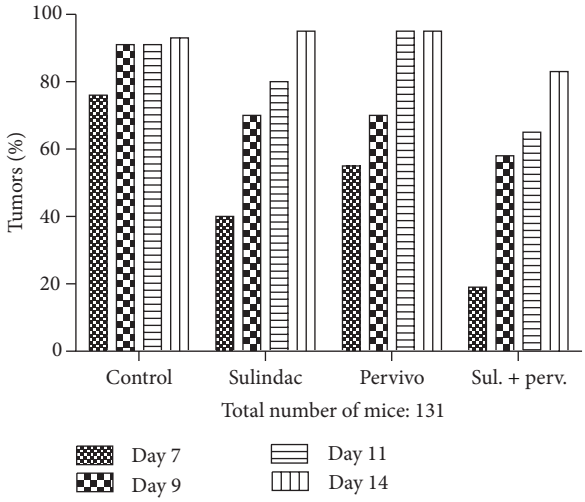

Figure 3: % of mice with measurable tumors in various days after L-1 sarcoma cells grafting.

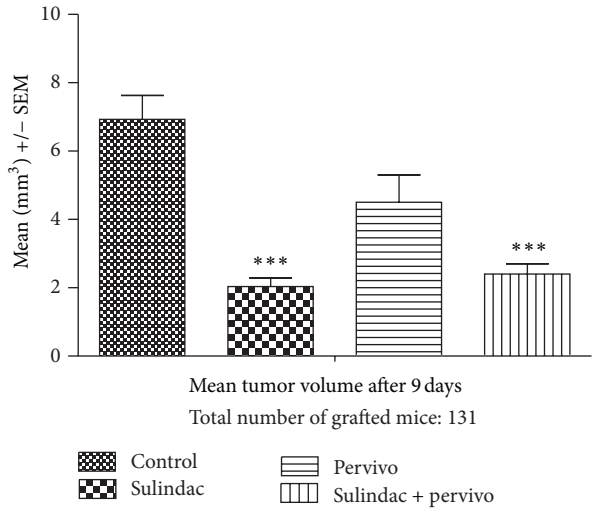

Figure 4: Mean tumor volume 9 days after Sarcoma L-1 cells grafting.

Table 3: Statistical analysis of the results presented in Figure 3.

Chi-square	
Chi-square, df.	23.55, 9
P value	0.0051
P value summary	**
One- or two-sided	NA
Statistically significant? (alpha < 0.05)	Yes
Data analyzed	
Number of rows	4
Number of columns	4

** $P < 0.01$.

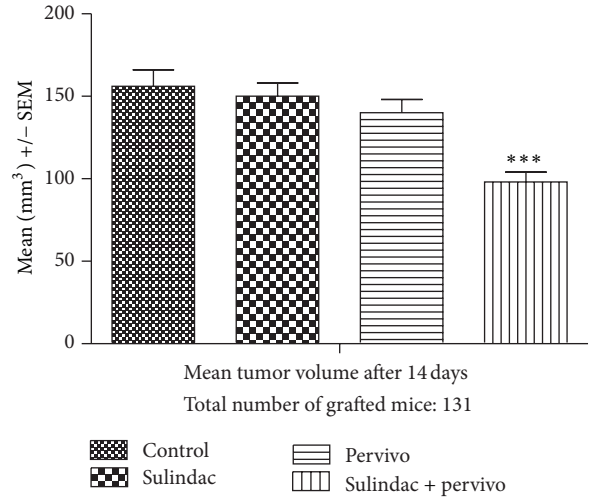

Figure 5: Mean tumor volume 14 days after L-1 sarcoma cells grafting *** $P < 0.001$.

not significant. At day 14 only in the group that received both drugs the mean tumor volume was significantly lower compared to the placebo (Figure 5 and Table 5).

4. Discussion

L-1 sarcoma tumor arose spontaneously in the lung of Balb/c mouse and was described by Przemysław Janik from Warsaw Oncology Center (58). This tumor has been maintained since then by subcutaneous serial passages in Balb/c mice and frozen and stored in Warsaw Oncology Center Tissue Collection. Isolated L-1 cells from tumors were adapted to grow *in vitro*.

L-1 sarcoma cells from culture, after grafting to animals, form tumors in *in vivo* conditions.

L-1 sarcoma is a perfect experimental model for assessing the impact of various substances upon the tumor growth and activity of its cells.

Previously, we used L-1 sarcoma cells for evaluation of pro- and antiangiogenic activity of various substances of synthetic and natural origin. We were the first to report the anti-angiogenic activity of sulindac and its metabolites, as well as that of theobromine, catechins in the cacao tree seeds, salidroside and rosavin isolated from the *Rhodiola rosea* and the *Rhodiola quadrifida*, convallamaroside isolated from the *Convallaria majalis* rhizome, alkylglycerols found in the shark liver oil, and other substances of natural origin [56–62].

We also demonstrated inhibitory effect of these substances in the angiogenic reaction induced in the mouse skin by cells or tissue homogenates obtained from surgically removed human cancers of the lung, kidney, ovary, and urinary bladder. We also proved the synergic anti-angiogenic activity of particular combinations of synthetic and natural inhibitors in relation to the reaction induced in mice skin with the human serum. We demonstrated the synergic activity of small doses of sulindac, convallamaroside, ursolic acid and epigallocatechin gallate (EGCG) in inhibition of cutaneous angiogenesis induced in mice by intracutaneous

Table 4: Statistical analysis of the results presented in Figure 4.

(a)

One-way analysis of variance	
P value	<0.0001
P value summary	* * * *
Are means. signif. different? ($P < 0.05$)	Yes
Number of groups	4
F	14.42
R square	0.3223

(b)

Bonferroni test	Mean diff.	t	Significant?	$P < 0.05$?
Control versus sulindac	4.890	6.973	Yes	* * *
Control versus PERVIVO	2.430	3.465	No	ns.
Control versus sulindac + PERVIVO	4.530	8.125	Yes	* * *
Sulindac versus PERVIVO	−2.460	2.892	No	ns.
Sulindac versus sulindac + PERVIVO	−0.3600	0.4886	No	ns.
PERVIVO versus sulindac + PERVIVO	2.100	2.850	No	ns.
Sulindac versus sulindac + PERVIVO	−0.3600	0.4886	No	ns.
PERVIVO versus sulindac + PERVIVO	2.100	2.850	No	ns.

$^{***}P < 0.001$, $^{****}P < 0.0001$.

Table 5: Statistical analysis of the results presented in Figure 5.

(a)

One-way ANOVA				
Source of variation	% of total variation	P value	P value summary	Significant?
Interaction	1.53	0.0101	*	Yes
Drug	4.25	<0.0001	* * *	Yes

(b)

Bonferroni test	Mean difference	t	P value	Summary
Control versus sulindac	−6.000	0.5758	$P > 0.05$	ns
Control versus PERVIVO	−16.00	1.535	$P > 0.05$	ns
Control versus sulindac + PERVIVO	−58.00	6.935	$P < 0.001$	* * *
Sulindac versus sulindac + PERVIVO	−52.00	4.990	$P < 0.001$	* * *
PERVIVO versus sulindac + PERVIVO	−42.00	4.030	$P < 0.001$	* * *
Control versus sulindac	−6.000	0.5758	$P > 0.05$	ns
Control versus PERVIVO	−16.00	1.535	$P > 0.05$	ns
Control versus sulindac + PERVIVO	−58.00	6.935	$P < 0.001$	* * *
Sulindac versus sulindac + PERVIVO	−52.00	4.990	$P < 0.001$	* * *
PERVIVO versus sulindac + PERVIVO	−42.00	4.030	$P < 0.001$	* * *

$^{*}P < 0.05$, $^{***}P < 0.001$.

administration of serum obtained from patients with diabetic retinopathy. Additionally, we demonstrated that human recombined cytokines (VEGF, bFGF, IL-8, and IL-18) induced the cutaneous angiogenesis inhibited by natural and synthetic inhibitors. Sulindac and its metabolites inhibited angiogenesis induced by bFGF and IL-18 and did not affect the angiogenic VEGF and IL-8 activity [63, 64].

Our experience, dealing with the question of angiogenic inhibitors, indicates that it is crucial to use simultaneously multiple anti-angiogenic factors of different handle points in the treatment. Many inhibitors display a synergic activity; therefore, their appropriate combinations may significantly reduce drugs doses and their possible side effects [65]. One of the examples of remarkable synergistic effect is inhibition of both the neovascularization and growth of tumor in head and neck squamous cell carcinoma by retinoic acid and interferon alfa by different mechanisms of action. Tumor cells treated by interferon alfa stopped secretion of interleukin 8, the major angiogenic factor, while retinoic acid caused them to secrete an inhibitor of angiogenesis [66]. There is also a proof for

synergism between cyclooxygenase inhibitors and cytotoxic chemotherapy drugs in inhibition of angiogenesis. Both celecoxib and 5-fluorouracil impaired angiogenesis by inhibiting vascular endothelial growth factor (VEGF). Additionally celecoxib influenced interferon gamma that has a pivotal role in tumor suppression [67]. Thalidomide has shown synergistic effect with low, nontoxic dose of cisplatin. It was shown that this effect is related to antiangiogenic influence of thalidomide on different tumor-related mediators: VEGF, basic fibroblast growth factor, hepatocyte growth factor, and IL-8 [68]. All evidence, of which examples are noted above, may lead to the concept of integrative approach of managing a patient with cancer. Cytotoxic drugs that are used as "golden standard" in cancer chemotherapy currently have high toxicity in therapeutic doses. The research is ongoing on combining them with compounds that target multiple biochemical pathways in processes that promote different aspects of cancer development, angiogenesis being one of the most important. These are i.a. natural health products that may be used as biological response modifiers and adaptogens, providing quality assurance of extracts is assured, and the effectiveness of combinations is proved in clinical trials.

We described inhibitory *in vivo* effect of two combinations of natural substances on angiogenesis and L-1 sarcoma growth in Balb/c mice. First of them was a composition of two Scandinavian folk medicine products, Greenland shark liver oil (rich in alkoxyglycerols) and arctic birch ashes, supplemented with squalene. All these substances alone and in combination significantly diminished cutaneous angiogenesis induced by tumor cells and tumor growth [69]. The second remedy composed of *Echinacea purpurea* extract, *Allium sativum* extract, and cocoa [70]. Again, a significant inhibitory effect on tumor angiogenesis and L-1 sarcoma growth was observed.

Other authors reported enhancing effect of theanine, a component of green tea leaves, on the antitumor activity of adriamycin [71], inhibition of liver metastasis of human pancreatic carcinoma by angiogenesis inhibitor TNP-470 (analog of fumagillin derived from *Aspergillus fumigatus*) in combination with cisplatin in nude mice [72], and augmented antitumor effects of combination therapy cisplatin/TNP-470/IL-12 in melanoma (B6D2F1 mice) and colon carcinoma (Balb/c mice) [73]. Recently, anticancer activity of noscapine, an opioid alkaloid in combination with cisplatin in human nonsmall cell lung cancer, *in vitro* and *in vivo*, in murine xenograft model was reported [74].

Selvendiran et al. [75] reported the results of *in vitro* and *in vivo* experiments with HO-3867, a curcumin analog, combined with cisplatin. This compound sensitized cisplatin-resistant ovarian carcinoma, leading to therapeutic synergy through STAT3 inhibition. Similar effect was obtained for dihydroartemisinin (derivative of *Artemisia* compound artemisinin, one of the PERVIVO components) which induced apoptosis and sensitized human ovarian cancer cells to carboplatin therapy [76] and improved the efficiency of chemotherapeutics (cisplatin, cyclophosphamide) in lung carcinomas *in vivo* and *in vitro* [77]. Lu et al. reported synergistic action of the C-Jun N-terminal Kinase (JNK) inhibitor and dihydroartemisinin in apoptosis induction through accelerating Bax translocation into mitochondria in human lung adenocarcinoma cells [78].

Suganuma et al. described synergistic proapoptotic effects of epigallocatechin gallate and epicatechin on human lung cancer cell line PC-9 *in vitro*. This effect was increased by sulindac or tamoxifen [79]. Recently, beneficial effect of sulindac, in combination with dimethylamino parthenolide (nuclear factor-κB inhibitor) and gemcitabine, in genetically engineered mouse model of pancreatic cancer was described [80]. It was revealed that sulindac sulfide can inhibit tumor cell invasion *in vitro* at concentrations less than those required to inhibit tumor cells growth, by suppressing NF-κB-mediated transcription of microRNAs [81]. Novel sulindac derivatives that do not inhibit COX-1 and COX 2 suppressed colon tumor cell growth *in vitro* by inhibiting cGMP phosphodiesterase and β-catenin transcriptional activity [82] and inhibited *in vivo* malignant pleural adenocarcinoma dissemination in mice [83].

Conflict of Interests

The authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

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Research Article

The Effect of Anti-Inflammatory and Antimicrobial Herbal Remedy PADMA 28 on Immunological Angiogenesis and Granulocytes Activity in Mice

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PADMA 28 is a herbal multicompound remedy that originates from traditional Tibetan medicine and possesses anti-inflammatory, antioxidant, antimicrobial, angioprotecting, and wound healing properties. The aim of the present study was to evaluate the influence of this remedy on immunological angiogenesis and granulocytes metabolic activity in Balb/c mice. Mice were fed daily, for seven days, with 5.8 mg of PADMA (calculated from recommended human daily dose) or 0.085 mg (dose in the range of active doses of other herbal extracts studied by us previously). *Results*. Highly significant increase of newly formed blood vessels number in *ex vivo* cutaneous lymphocyte-induced angiogenesis test (LIA) after grafting of Balb/c splenocytes from both dosage groups to F1 hybrids (Balb/c × C3H); increase of blood lymphocytes and granulocytes number only in mice fed with lower dose of remedy; and significant suppression of metabolic activity (chemiluminescence test) of blood granulocytes in mice fed with higher dose of PADMA. *Conclusion*. PADMA 28 behaves as a good stimulator of physiological angiogenesis, but for this purpose it should be used in substantially lower doses than recommended by producers for avoiding the deterioration of granulocyte function.

1. Introduction

PADMA 28 is a multicomponent, traditional Tibetan herbal plant remedy comprised of 20 specific herbs and 2 non-herbal ingredients. The main PADMA's active substances are bioflavonoids, tannins, phenolic acids, phenolic alcohols, and terpenoids [1, 2]. Weseler et al. presented evidence of antimicrobial activity of this remedy. Both aqueous and alcohol-based PADMA 28 preparations exhibited evident antibacterial effects against Gram-positive bacteria and *Klebsiella pneumonia in vitro* [3]. From 20 herbs present in PADMA, 13 have well-documented antimicrobial activity. *Azadirachta indica* exhibited activity against Gram-positive bacteria. *Aegle marmelos* fruit expresses antifungal activity. *Elettaria cardamomum* (cardamom) essential oil has activity against *Bacillus subtilis* spores. This herb also presents anti-inflammatory and immunotropic activity, enhancing Th2 and suppressing Th1 cytokine release by lymphocytes. Recently,

chemopreventive effects of cardamom on chemically induced skin carcinogenesis in mice were described. *Sida cordifolia*, known for its regenerating properties, is active against *Corynebacterium diphtheria* and in combination with nystatin and clotrimazole exhibited antimicrobial effects against five *Candida* strains. Terpenoid eugenol present in *Syzygium aromaticum* expresses general antimicrobial effect; phenolic alcohol from *Glycyrrhiza glabra* was active against *Mycobacterium tuberculosis*, *Staphylococcus aureus,* and *Plasmodium* [2, 4–9]. Recently, anti-influenza viral effects of nuclear export inhibitors from *Valerianae radix* were described [10].

Another PADMA 28 component, *Saussurea lappa* root, and its active principle dehydrocostus lactone inhibit prostate cancer cell migration *in vitro* and have been shown to have anticancer activity. Santamarin, a sesquiterpene lactone isolated from this herb, represses LPS-induced inflammatory response in murine macrophages and potently inhibits the growth of *Trypanosoma brucei rhodesiense* [11–15]. Another PADMA 28 component, *Terminalia chebula* Retz., is called the "King of Medicine" in Tibet. The plant possesses multiple activities, among them, antioxidant, antimicrobial, anti-inflammatory, and wound healing activities [16].

Some immunotropic activities of PADMA 28 and beneficial effect of this remedy in experimental models of inflammation and wound healing were reported [17–24].

In humans, PADMA 28 has been used as a beneficial tonic for heart and blood vessels and as an antioxidant. PADMA 28 has been registered in Switzerland since 1977 by Intercantonal Office for the Control of Medicines as a remedy to alleviate symptoms of claudication, impaired peripheral circulation, pain on walking, leg cramps, and paresthesia. A profitable influence of PADMA 28 was also observed in patients with atherosclerosis and in patients with multiple sclerosis [25, 26]. In 1992, PADMA 28 was registered in Poland. Its efficacy was further proved in prophylactics and treatment of some disorders with inflammatory, sclerotic, and degenerative origins. Treatment of chronic infective pulmonary diseases studied in Poland in a big group of children with PADMA has brought positive results [27, 28]. However, as PADMA is being used for a variety of diseases and usually for a long time (e.g., couple of weeks) and because it possesses strong antioxidative properties, it would affect various parameters of immune system, among them, oxidative burst of granulocytes. In fact, some authors reported that this remedy inhibited the respiratory burst of human neutrophils *in vitro* [29, 30]. That is why we decided to evaluate in the present study, on the experimental model in mice, the *in vivo* effect of PADMA 28 (in high dose, comparable to that recommended for humans, and in low dose, comparable to these which we used previously in studies of various other herbal extracts) on immunological angiogenesis and, simultaneously, on granulocytes metabolic activity evaluated by chemiluminescence. This test is widely accepted as a method of measuring granulocytes oxygen-dependent killing potential. It is important to know if selected doses of this remedy do not disturb granulocytes activity and stimulate immunological angiogenesis and, accordingly, could be used as a safe drug for therapeutic angiogenesis in vascular and immune system disturbances.

2. Material and Methods

PADMA 28 tablets (batch 28/6311, PADMA AG, Suisse), herbal mixture consisting of 22 ingredients: *Aegle marmelos* fruit (20 mg), *Pimenta dioica* fruit (25 mg), *Aquilegia vulgaris* aerial part (15 mg), *Calendula officinalis* flower (5 mg), *Elettaria cardamomum* fruit (30 mg), *Syzygium aromaticum* flower bud (12 mg), *Saussurea lappa* root (40 mg), *Hedychium spicatum* rhizome (10 mg), *Lactuca sativa* leaf (6 mg), *Cetraria islandica* thallus (40 mg), *Glycyrrhiza glabra* root (15 mg), *Azadirachta indica* fruit (35 mg), *Terminalia chebula* fruit (30 mg), *Plantago lanceolata* aerial part (15 mg), *Polygonum aviculare* aerial part (15 mg), *Potentilla aurea* aerial part (15 mg), *Pterocarpus santalinus* wood (30 mg), *Sida cordifolia* aerial part (10 mg), *Aconitum napellus* tuber (1 mg), *Valeriana officinalis* root (10 mg), camphor (4 mg), and calcium sulfate (20 mg).

2.1. Animals. The study was performed on 48 female inbred Balb/c mice 6–8 weeks old, weighing about 20 g, and on 24 female F1 hybrids (Balb/c × C3H), 6 weeks old, delivered from the Polish Academy of Sciences breeding colony. PADMA 28 was administered to mice *per os* in daily doses 5.8 mg or 0,085 mg. Higher dose was calculated according to the highest daily dose (6 tablets), recommended for humans (applying the factor 7 for differences between mouse and human in relation to the surface to body mass). Lower dose conforms to the range of active doses of other herbal extracts and their polyphenolic compounds used in our previous experiments [31–35].

2.2. Lymphocyte-Induced Angiogenesis Test (LIA). Balb/c mice were fed with PADMA (5.8 mg or 0.085 mg) by Eppendorf pipette, in 40 μL of water or 40 μL of water (controls), for 7 days, then bled in anaesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, BIOWET, Pulawy, Poland) and sacrificed by cervical dislocation. Splenocytes were isolated from spleens of Balb/c donors under sterile conditions by straining through stainless sieve and cotton gauze and centrifugation on Histopaque 1077 (Sigma-Aldrich, USA) for 8 min at 400 g in order to remove erythrocytes. Isolated splenocytes were resuspended in Parker culture medium (TC199, BIOMED, Lublin) and pooled within the groups. A local GVH reaction (lymphocyte-induced angiogenesis, LIA test) was performed according to [36] with some modifications [33]. Shortly, spleen cells suspensions were grafted intradermally (1 million cells in 0.05 mL of Parker medium per graft) into F1 (Balb/c × C3H) recipients. Before performing injections, mice were anaesthetized intraperitoneally with 3.6% chloral hydrate (Sigma-Aldrich, USA; 0.1 mL per 10 g of body mass). Both flanks of each mouse were finely shaved with a razor blade; each flank was injected with cells 2-3 times. Cell suspensions were supplemented with 0.05 mL/mL of 0.01% trypan blue in order to facilitate recognition of injection sites later on. Grafted Balb/c splenic lymphocytes recognized C3H antigens and produced many immunological mediators including proangiogenic factors (immunological angiogenesis). In this test, the number of newly formed blood vessels was the

measure of T-cell reactivity. After 72 hours the mice were treated with a lethal dose of Morbital (Biowet, Puławy, Poland). All newly formed blood vessels were identified and counted in dissection microscope on the inner skin surface, using criteria suggested by the authors of the method, at magnification of 6x, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cells injection, are thin and differ from the background vasculature in their tortuosity and divarications.

Experiment was performed twice (24 Balb/c mice and 24 F1 hybrids as a total).

2.3. Estimation of Leukocytes Number and Their Metabolic Activity (Luminol-Dependent Chemiluminescence Test, CL). Balb/c mice were fed with PADMA (5.8 mg or 0.085 mg) by Eppendorf pipette, in 40 μL of water or 40 μL of water (controls), for 7 days, then bled in anaesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg, BIOWET Pulawy, Poland) from retroorbital plexus and sacrificed by cervical dislocation. CL was measured using the method of Easmon et al. [37] with some modifications [38–40] at room temperature, in scintillation counter (RackBeta 1218, LKB, Sweden). Briefly, samples of 0.05 mL of heparinised blood were diluted 1 : 4 with phosphate buffered saline (PBS, Biomed Lublin, Poland) and supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, USA) and 0.1% glucose (Polfa, Poland). Next, 0.05 mL of this diluted blood was mixed with 0.2 mL of luminol (Sigma-Aldrich, USA) solution (10^{-5} M) in PBS and placed in a scintillation counter in the "out of coincidence" mode for background chemiluminescence measurement. Then, the cells were activated by the addition of 0.02 mL solution of opsonized zymosan (10 mg/mL, Serva, USA), and chemiluminescence activity was measured for the next 15 min. Counting of leukocytes and blood smears examination were performed by routine methods, and the results were shown as the maximum value of chemiluminescence (cpm) obtained for 10^3 granulocytes. Experiment was performed twice (24 mice as a total).

For all experiments, animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the Local Ethical Committee.

2.4. Statistical Analysis. Statistical evaluation of the results was performed by one-way ANOVA, and the significance of differences between the groups was verified with a Bonferroni multiple comparison post test (Graph Pad Prism software package).

3. Results

The effect of PADMA 28 (0.085 or 5.8 mg) supplementation of Balb/c donors on the angiogenic ability of their splenic lymphocytes to induce newly formed blood vessels in the skin of F1 (Balb/c × C3H) recipient mice is presented in Figure 1. According to one-way analysis of variance the P value <0.0001 is considered extremely significant. Variation among column means is significantly greater than expected by chance. Bonferroni multiple comparison test revealed that PADMA 28 in both doses highly significantly stimulated lymphocytes angiogenic activity as compared to the control and that this stimulation was better after higher dose of remedy (for comparison of lower and higher doses, $P < 0.01$).

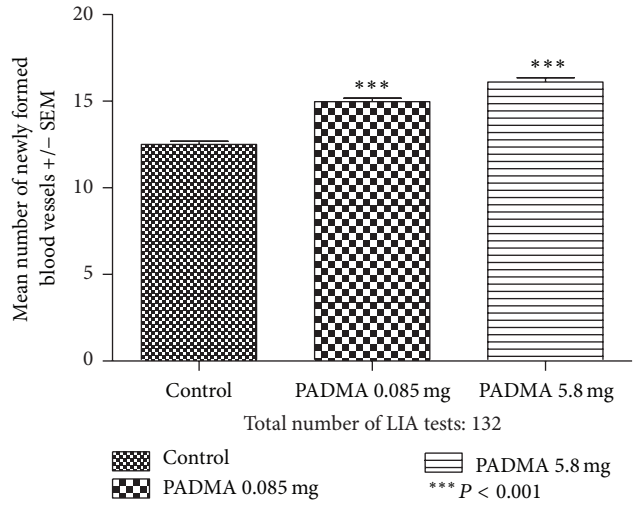

Figure 1: Effect of PADMA 28 (0.085 or 5.8 mg) supplementation of Balb/c donors on the number of newly formed blood vessels measured by lymphocyte-induced angiogenesis test (LIA) in F1 (Balb/c × C3H) recipient mice. Balb/c mice were fed daily *per os* with 0.085 mg or 5.8 mg of PADMA 28. After 7 days, mice were bled and sacrificed, and splenocytes were isolated. Splenocytes were pooled within the groups, resuspended in Parker culture medium (1 million of cells in 0.05 mL), and injected intradermally (5-6 injections per mouse) to 24 F1 (Balb/c × C3H) hybrids. After 72 hours the mice were euthanized, and newly formed blood vessels were counted in dissection microscope (6x magnification). Results are shown as mean ± SEM. $^{***}P < 0.001$.

The results of granulocytes chemiluminescence are presented in Figure 2. According to one-way analysis of variance, the P value 0.0019 is considered as significant. Bonferroni multiple comparison test revealed significantly lower chemiluminescence of blood leukocytes collected from mice fed with higher PADMA 28 dose, as compared to the control.

The results of PADMA 28 feeding on the blood granulocytes number are presented in Figure 3. According to one-way analysis of variance the P value 0.0006 is considered as highly significant. Bonferroni test revealed highly significantly statistical increase of blood granulocytes number in the blood collected from mice fed with lower PADMA 28 dose in comparison to the control ($P < 0.001$) and significant increase ($P < 0.05$) in comparison to the group fed with high dose of remedy. No difference was observed between this last group and control mice.

Number of blood lymphocytes is presented in Figure 4. According to ANOVA, the P value 0.0011 is considered as significant. Bonferroni test revealed highly significant increase of blood lymphocytes number in the blood collected from mice fed with lower PADMA 28 dose ($P < 0.001$) in comparison to the control and significant increase ($P < 0.05$)

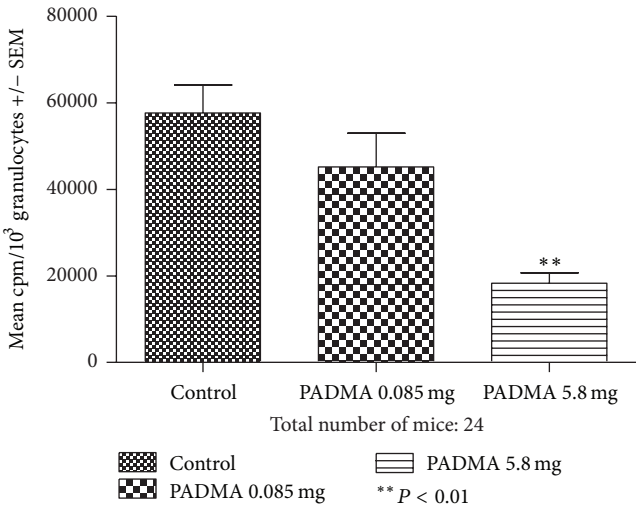

Figure 2: Effect of PADMA 28 (0.085 or 5.8 mg) supplementation on chemiluminescent activity of mouse granulocytes. Balb/c mice were fed daily *per os* with 0.085 mg or 5.8 mg of PADMA 28. After 7 days mice were bled and sacrificed. The chemiluminescence was measured with luminol after activating with blood with zymosan, in scintillation counter (RackBeta 1218, LKB, Sweden). Results are presented as the mean cpm +/− standard error (SEM) per 1000 granulocytes. $^{**}P < 0.01$.

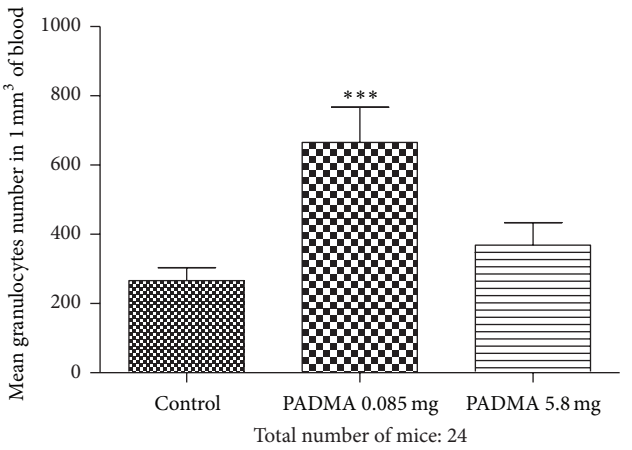

Figure 3: Effect of PADMA 28 (0.085 or 5.8 mg) supplementation on the number of blood granulocytes in mice. Balb/c mice were fed daily with 0.085 mg or 5.8 mg PADMA 28. After 7 days mice were bled and sacrificed. Counting of leukocytes and blood smears examination were performed by routine methods. $^{***}P < 0.001$.

in comparison to the group fed with high dose of remedy. No difference was observed between this last group and control mice.

4. Discussion

In this paper, we report for the first time stimulatory effect of multiherbal remedy PADMA 28 on immunological angiogenesis observed in the skin of recipient mice during the local cutaneous graft-versus-host reaction. In our previous

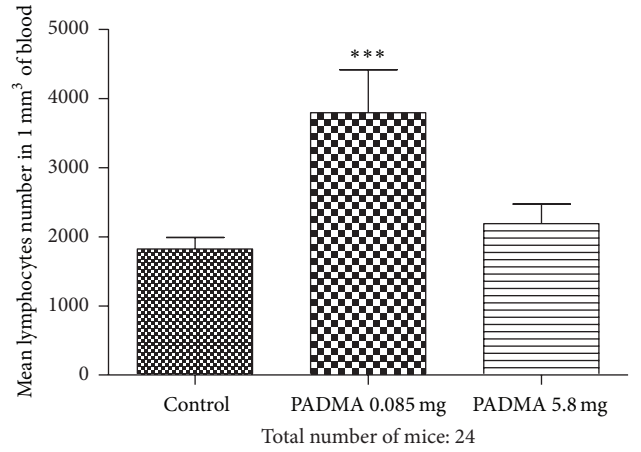

Figure 4: Effect of PADMA 28 (0.085 or 5.8 mg) supplementation on the number of blood lymphocytes in mice. Balb/c mice were fed daily with 0.085 mg or 5.8 mg of PADMA 28. After 7 days mice were bled and sacrificed. Counting of leukocytes and blood smears examination were performed by routine methods. $^{***}P < 0.001$.

studies we obtained similar effects, when we administered to donor mice some other herbal extracts (from plants *Echinacea purpurea*, *pallida*, and *angustifolia* and *Rhodiola rosea*, *quadrifida*, and *kirilowii*) and remedies Echinasal and Bioaron C. [31–35]. However, no stimulatory effect was obtained after feeding donor mice with extract from *Centella asiatica* or multicomponent herbal remedy PERVIVO [31, 41]. On the other hand, inhibitory effect was observed when donor mice were fed with FIBS—an aqueous solution of biogenic stimulators (coastal salt lake mud distillate mixed with cinnamic acid and coumarin) elaborated in 1948 year by Professor V. P. Filatov team [42].

In our present experiments PADMA 28 has behaved as a strong stimulator of proangiogenic factors production by splenic lymphocytes in mice. It remains to elucidate which factors are involved, and this will be the matter of our further studies. It is important that PADMA exerted this angiostimulatory effect also in a substantially lower dose than that recommended by producer, because higher recommended dose significantly inhibited granulocyte respiratory burst measured by chemiluminescence. It should be expected as PADMA 28 contains many compounds demonstrating antioxidant effects—gallic acid, eugenol, ellagitannins, bioflavonoids: quercetin, luteolin, and apigenin, and others [29].

5. Conclusion

The ability of PADMA 28 to increase angiogenic activity of lymphocytes may partly explain its beneficial effect on regenerative/repair processes, but for this purpose this remedy should be used in evidently lower doses than these recommended by producers, for avoiding deterioration of granulocyte function.

Conflict of Interests

The authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

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Review Article

Matrix Metalloproteinases: Inflammatory Regulators of Cell Behaviors in Vascular Formation and Remodeling

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Abnormal angiogenesis and vascular remodeling contribute to pathogenesis of a number of disorders such as tumor, arthritis, atherosclerosis, restenosis, hypertension, and neurodegeneration. During angiogenesis and vascular remodeling, behaviors of stem/progenitor cells, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs) and its interaction with extracellular matrix (ECM) play a critical role in the processes. Matrix metalloproteinases (MMPs), well-known inflammatory mediators are a family of zinc-dependent proteolytic enzymes that degrade various components of ECM and non-ECM molecules mediating tissue remodeling in both physiological and pathological processes. MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MT1-MMP, are stimulated and activated by various stimuli in vascular tissues. Once activated, MMPs degrade ECM proteins or other related signal molecules to promote recruitment of stem/progenitor cells and facilitate migration and invasion of ECs and VSMCs. Moreover, vascular cell proliferation and apoptosis can also be regulated by MMPs via proteolytically cleaving and modulating bioactive molecules and relevant signaling pathways. Regarding the importance of vascular cells in abnormal angiogenesis and vascular remodeling, regulation of vascular cell behaviors through modulating expression and activation of MMPs shows therapeutic potential.

1. Introduction

As blood vessels nourish all the tissues and organs in the body, it is unsurprising that abnormal formation and maintenance of blood vessels contribute to pathogenesis of numerous disorders. Indeed, excessive vascular formation causes cancer, arthritis, psoriasis and infectious disease, and so forth, whereas atherosclerosis, restenosis, hypertension, stroke, neurodegeneration, preeclampsia, respiratory distress, and osteoporosis are characterized by insufficient vessel growth or abnormal vascular remolding, and the list is still growing [1].

Vascular formation is coordinated in several modes comprising vasculogenesis—primitive vascular labyrinth assembly, angiogenesis—vascular sprouting and branching, and arteriogenesis—endothelial cell tubules covered by vascular mural cells [2–4]. After vessel maturation, enduring changes in the vascular composition and structure, known as vascular remodeling, occur in response to a number of stimuli. Vascular remodeling not only associates with vessel repair and adaptation, but also eventually affects luminal diameter and the thickness of vessel wall in every direction [5].

Both vascular formation and remodeling are complicated processes including recruitment, migration, proliferation, and apoptosis of vascular cells consisting of stem/progenitor cells, endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and other mural cells. It is common that extracellular matrix (ECM) molecules play important roles in vessel development and morphogenesis by providing supportive matrix scaffold for cells, interacting with certain matrix

receptors on cells and supplying growth factors that impact cellular function. Reciprocal interaction between vascular cells and their ECM is critical in blood vessel formation and remodeling.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes that degrade various components of ECM and mediate ECM remodeling in both physiological and pathological processes. A major function of MMPs is degradation of ECM to facilitate the progression of cell migration and invasion. However, a variety of research works reveal that proteolytic activity of MMPs controls availability of active molecules such as growth factors [6, 7]. Increasing evidence suggests that MMPs play a critical role in vascular formation and remodeling through degrading vascular basement membrane and ECM proteins and modifying angiogenic growth factors and cytokines. In normal physiological conditions, MMPs activities are regulated at multiple levels, including gene transcription, activation of zymogens and interaction with specific inhibitors in order to limit MMPs activity. Striking the balance enlarges MMPs activity and probably leads to pathological alterations in blood vessels [8].

The purpose of this review is to discuss the biological activities of MMPs in vascular formation and remodeling, especially to focus on increasing evidence elucidating the effects of MMPs on stem/progenitor cells, ECs, and VSMCs and their pathogenic role in related diseases.

2. MMPs: Regulators in Vascular Tissue

2.1. MMP Classification. MMPs are expanding family of endopeptidases first discovered in 1962 as a collagenase in resorption of the tadpole tail [9]. To date, the MMP family has at least 24 members, although some of them have not been well understood. Typical MMPs consist of a propeptide, a catalytic domain, a linker peptide (hinge region) and a hemopexin domain. The Zn^{2+} binding motif HEXXHXXGXXH in catalytic domain is the signature to assign proteinases to MMP family [6, 10].

MMPs are categorized by their structure and substrates into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs, and others. Collagenases including MMP-1, MMP-8, MMP-13, and MMP-18 cleave interstitial collagen type I, II, and III and digest some of other ECM and non-ECM proteins. Gelatinases degrade both collagens and gelatins and include MMP-2 (gelatinase A) and MMP-9 (gelatinase B) which are the most widely studied MMPs in blood vessel. Stromelysins comprise MMP-3, MMP-10 and MMP-11. MMP-3 and MMP-10 have similar substrate specificities, and both digest wide range of ECM molecules and participate in proMMPs proteolysis, while the structure and function of MMP-11, also called stromelysin-3, diverge from other two stromelysins. Matrilysins which lack hemopexin domain include MMP-7 (matrilysin-1) and MMP-26 (matrilysin-2). MMP-7 processes many cell surface molecules, while MMP-26 degrades a number of ECM components. Membrane-type (MT)-MMPs consist of four transmembrane MMPs, MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), and MMP-24 (MT5-MMP) and two glycosyl-phosphatidylinositol-anchored MMPs, MMP-17 (MT4-MMP) and MMP-25 (MT6-MMP). MT-MMPs are expressed on the cell surface and activate proMMPs. Other MMPs that are not classified in previous categories include MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27, and MMP-28 [6, 10, 11].

Many studies of *in vitro* cultured cells and normal or diseased blood vessels reveal that both vascular cells and inflammatory cells in vessel wall can produce MMPs which participate in diverse vascular physiology and pathogenesis. To date, major MMPs expressed in vascular tissue and cell types include MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, MMP-13, MT1-MMP, and MT3-MMP [8, 12].

2.2. MMP Activation and Function. Most MMPs are synthesized and secreted as inactive zymogens called proMMPs which have a cysteine switch motif PRCGXPD coordinating with Zn^{2+} in catalytic domain to maintain latency [13]. ProMMPs can be activated *in vitro* by chemical agents, such as thiol-modifying agents, oxidized glutathione, sodium dodecyl sulfate, chaotropic agents, and reactive oxygen species through the disturbance of cysteine-Zn^{2+} interaction of the cysteine switch [14]. However, stepwise activation of proMMPs *in vivo* is more complicated with removal of propeptide, disruption of cysteine-zinc binding, and detachment of the hemopexin domain, which is more likely conducted by other MMPs or other classes of proteinases such as plasmin and neutrophil elastase [7]. For example, proMMP-2 is activated by most MT-MMPs but not MT4-MMP [15].

Once activated, MMP catalytic domains contact with protein substrates and cleave at specific sites so as to breakdown the extracellular scaffold or modify biologically active molecules residing in ECM. In blood vessels, matrix scaffold degradation is mediated by activation of MMPs to facilitate endothelial and mural cell migration and invasion. In addition, various MMPs are necessary for releasing and processing of non-matrix molecules including growth factors such as fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α) and their receptors, as well as integrins, plasminogen and adhesion molecules, and so forth, which impact vascular cell recruitment, proliferation, migration, and apoptosis in angiogenesis and vascular remodeling.

2.3. Vascular MMPs as Inflammatory Mediators. Vascular inflammation is a complex process encompassing multiple types of cells and various biological factors, which is initiated by tissue damage. Coordination of pro- and anti-inflammatory cytokines and chemokines regulates cell proliferation, adhesion, chemotaxis, migration, and apoptosis in inflammatory sites [16, 17]. In diseases related to angiogenesis and vascular remodeling, inflammation is identified and is always imbalanced, exacerbated, and chronic [18, 19].

There is complicated interplay between angiogenesis and inflammation. Angiogenesis sustains inflammation by providing metabolic demands to inflammatory cells, while inflammation promotes angiogenesis via releasing several

cytokines and chemokines which impact vascular cell function and behavior [18]. Similarly, vascular inflammatory process is prominent in hypertension, atherosclerosis and restenosis, and so forth, in which abnormal vascular remodeling plays a predominant role [20]. During vascular remodeling, various cytokines are secreted from inflammatory cells such as monocytes, macrophages, and neutrophils, contributing to further recruitment of inflammatory cells and proliferation, migration, and apoptosis of ECs and VSMCs [17, 20].

MMPs function as such inflammatory cytokines during vascular formation or remodeling. In matured and quiescent vessels, active MMPs are absent or expressed at low levels. But in tissues undergoing abnormal angiogenesis and vascular remodeling, MMPs are markedly expressed, secreted, and activated [8]. Inflammatory cells such as macrophages and neutrophils are an important resource of MMPs in vascular tissue. Inflammatory factors, including tumor necrosis factor-alpha (TNF-α) and interleukins (ILs), stimulate MMP expression [21]. In turn, MMPs degrade ECM to facilitate migration and recruitment of cells including inflammatory cells and cleave cell surface receptors and other non-ECM molecules to mediate adhesion, proliferation, and apoptosis of cells in vessel wall which are involved in inflammatory process [22]. Therefore, MMPs are inflammatory mediators linking inflammation with angiogenesis and vascular remodeling.

2.4. Major Stimuli of Vascular MMPs and Their Contributions to Vascular Formation and Remodeling. Many studies investigating the relationship between MMPs and vascular biology demonstrate that a number of factors leading to angiogenesis and vascular remodeling-related diseases regulate MMP expression and activation, such as hemodynamics, oxidative stress, inflammation, hormonal factors, and hypoxia, (Figure 1).

Hemodynamic forces such as shear stress and arterial pressure regulate MMP expression and participate in vascular remodeling. Elevated transmural pressure activates MMP-2 and MMP-9 in *ex vivo* porcine carotid arteries [23]. In a murine model of blood flow cessation induced arterial remodeling, an early and significant increase in MMP-9 expression precedes the formation of intimal hyperplasia [24]. Low fluid shear stress induced MMP-9 expression involves integrins-p38 MAPK or ERK1/2-NF-kappaB signaling pathways [25]. Remodeling due to hemodynamic adaptation of the vein to the arterial condition leads to saphenous vein graft bypass failure. *Ex vivo* vein support system and *in vivo* animal model of vein graft reflect that high shear stress and increased venous pressure and wall tension induce overexpression of MMP-2 and MMP-9, which in turn drives vein graft wall remodeling [26–29].

Oxidative stress induced by imbalance between elimination and production of reactive oxygen species (ROS) has deleterious effect on vascular biology via excessive activation of MMPs. ROS formation via the NADPH oxidase (Nox) induced by mechanical stretch enhances MMP-2 mRNA expression and pro-MMP-2 release [30]. Oxidative stress induces the loss of retinal capillary cells by regulating the proapoptotic role of MMP-2 [31]. MMP-9 secretion and activity in monocytes are enhanced by increased Nox-dependent superoxide radical ($^{\bullet}O_2^-$) production in the atherosclerotic process [32]. In hindlimb ischemia, Nox2 derived ROS production increases MT1-MMP expression and MMP-9 activity, leading to neovascularization and tissue repair [33]. Moreover, ROS-dependent activation of MMPs is necessary for arteriolar inward remodeling [34]. Recent evidence also revealed the role of Nox in MMP transcription and activation. NOX4 induces MMP2 transcription via stimulating FoxO activity [35], while Nox1 in neointimal VSMCs initiates redox-dependent phosphorylation of ERK1/2 and subsequent MMP-9 activation following vascular injury [36]. In addition, a variety of other vascular MMP stimuli modify MMP functions via ROS-dependent ways. Homocysteine contributing to arterial remodeling induces increased activation of latent MMP-2 through an oxidative/nitrative dependent mechanism [37, 38]. High glucose stimulating MMP-1, MMP-2, and MMP-9 expression in cultured endothelial cells and macrophages is associated with ROS production [39–41]. Smoking-induced MMP activation promotes vascular remodeling through vascular inflammation and oxidative stress [42]. Regarding to the effects of oxidative stress in vascular remodeling, antioxidant approaches are used to reduce the upregulation of MMPs and attenuate the tissue remodeling during vascular diseases [43, 44].

Inflammatory process involving MMP activities is essential for the vascular remodeling, entailing reorganization of the ECM scaffold of the vascular wall, and particularly mediating atherosclerotic plaque progression and rupture [12, 21]. Inflammatory cells such as macrophages, mast cells, and neutrophils are the main sources of MMPs, and inflammatory cytokines like TNF-α and ILs stimulate MMP expression and activation [21, 22]. Taking TNF-α, IL-17 and IL-18 as examples, TNF-α activates MMP-9 gene expression at transcription level [45], while IL-17 and IL-18 also stimulate MMP-9 expression via nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1) signaling activation [46, 47].

Hormonal stimuli including angiotensin II (Ang II), estrogen, and progesterone take part in vascular remodeling through activating vascular MMPs as well. Ang II stimulates MMP-8 and MMP-13 activity in atherosclerotic lesions, inducing intraplaque hemorrhages and plaque rupture via MMPs mediated degradation of interstitial collagen I [48]. Ang II also increases MMP-2 expression and activity in vascular remodeling during atherosclerosis, intimal and medial thickening, and hypertension [49–51]. In addition, an early increase in MT1-MMP expression with a subsequent increase in MMP-2 and MMP-9 activity has been observed in Ang II induced aneurysm formation [52], and MMP-9 production in aortic aneurysms relies on Ang II/ERK pathway [53]. Postmenopausal women receiving hormone replacement therapy are more likely to suffer from intimal hyperplasia after vascular intervention and bypass graft failure, which implies the significant role of estrogen and progesterone in vascular remodeling. Estrogen deficiency induced by

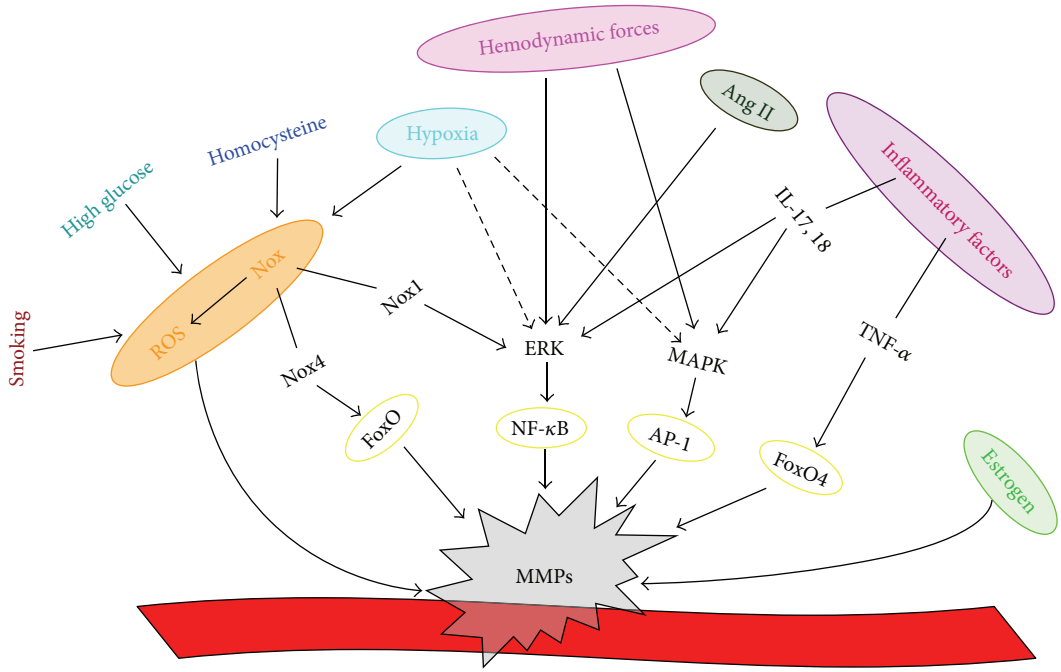

Figure 1: Major stimuli activating vascular MMPs.

ovariectomy gives rise to a reduction of active MMP-2 in the initial phase and a concurrent elevation of MMP-2 and MT1-MMP expression in latter period [54]. VSMCs incubated with estrogen and progesterone show upregulation of MMP pathway by increasing MMP-2 activity via enhancement of MT1-MMP expression [55], and inhibition of MT1-MMP prevents estrogen-stimulated increases in MMP-2 activity [56].

Hypoxia increasing expression levels of hypoxia inducible factor 1 (HIF-1) and vascular growth factors has a significant role in vasculogenesis, angiogenesis, and vascular remodeling [57, 58]. Hypoxia results in an overall upward tendency of vascular MMP-2 and MMP-9 expression. *In vitro* studies reveal that exposure of ECs to prolonged hypoxia enhances MMP-2 expression and activity [59, 60]. Consistently, rat exposed to hypoxia shows increased MMP-2 protein level and activity in aorta [61]. Chronic hypoxia also accelerates the development of atherosclerosis along with activated MMP-9 in apoE(−/−) mice [62]. The mechanisms of MMP upregulation by chronic hypoxia are not fully understood. However, hypoxia might regulate MMP expression transcriptionally due to its effect on activation of some transcriptional factors such as AP-1 and NF-κB which are primarily involved in MMP gene expression [63, 64].

3. MMPs, Stem/Progenitor Cell Mobilization, Recruitment, and Angiogenesis

Multipotent adult stem/progenitor cells implicated in angiogenesis and vascular remodeling contribute to physiological and pathological processes through recruitment and migration into blood vessels and secretion of growth factors and cytokines. Bone marrow serves as a reservoir for several stem/progenitor cell populations, including mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and hematopoietic stem cells (HSCs) [65]. Another source of stem/progenitor cells involved in vascular diseases is blood vessel wall harboring MSCs, EPCs, and other stem/progenitor cells [66, 67]. Recruitment of stem/progenitor cells undergoes escaping from their niche, invading ECM and engrafting into target site where they proliferate and differentiate. To facilitate this mobilization, degradation of ECM via MMPs is an imperative requirement. MT1-MMP, MMP-2, MMP-9, and MMP-8 have been reported to function in stem/progenitor cell mobilization and recruitment in blood vessel formation and vascular remodeling (Table 1).

3.1. MT1-MMP in Bone Marrow-Derived Stem/Progenitor Cell Migration. Bone marrow-derived stem cells (BMSCs) induce an angiogenic effect and elicit vessel morphogenesis both *in vitro* and *in vivo* depending on proteolytic ability of MT1-MMP [68]. Lumican inhibits *in vitro* tube-like structure formation by bone marrow-derived MSCs via downregulation of MT1-MMP expression and activity in MSCs, and overexpression of MT1-MMP enhances MSC migration and invasion [69].

3.2. MMP-9 in Bone Marrow-Derived Stem/Progenitor Cell Mobilization. MMP-9 induced in bone marrow cells releases soluble Kit-ligand, allowing translocation of bone marrow EPCs and HSCs from quiescent bone marrow niche to proliferative vascular niche [70]. Deficiency of MMP-9 attenuates

Table 1: MMPs, stem/progenitor cell mobilization and recruitment, and angiogenesis.

MMPs	Stem/progenitor cells	Functions/effects	Substrates	Outcomes	References
MT1-MMP	BMSCs	Migration/invasion↑	ECM (fibrin)	Angiogenesis	[68, 69]
MMP-9	BM-EPCs/BM-HSCs (c-kit+)	Adhesion/migration↑	c-kit ligand	Neovascularization	[70, 71]
	BM-myelomonocytic cells (CD11b+)	Recruitment/invasion↑	ECM	Tumor vasculogenesis	[72]
	BMSCs (CD34+)	Migration/invasion↑	ECM	Restenosis	[73, 74]
MMP-2	BM-EPCs (c-kit+)	Invasion/proliferation↑	ECM VEGF signaling	Neovascularization	[75]
MMP-8	SPC (CD34+/c-kit+/Sca-1+/Flk-1+)	Recruitment/migration↑	ECM ADAM10	Atherosclerosis	[76]

ischemia-induced neovascularization through an impairment of bone marrow-derived EPC adhesion, migration, and proangiogenic functions [71]. In tumor angiogenesis studies using MMP-9 knockout mice, transplanting tumors are unable to grow after irradiation in MMP-9 knockout mice, but tumor growth could be restored by transplantation of wild-type bone marrow. CD11b+ myelomonocytic cells rather than EPCs in transplanted bone marrow are responsible for immature vessels formation and tumor growth [72]. Moreover, it has been reported that elevated MMP-9 expression is associated with postinterventional restenosis [126]. Studies on coronary stent implantation patients reveal that active MMP-9 produced by neutrophils related to vascular injury possibly leads to mobilization of BMSCs (CD34+ stem cells), which may contribute to reendothelialization as well as restenosis [73, 74].

3.3. MMP-2 in Bone Marrow-Derived Stem/Progenitor Cell Mobilization. MMP-2, as another major vascular MMP, shows a significant role in normal and tumor angiogenesis and development of atherosclerotic and neointimal lesions [127–129]. These biological effects may attribute to a novel role of MMP-2 in stem/progenitor cells function. Bone marrow-derived EPCs (c-Kit+ stem cells) from MMP-2(−/−) mice display marked reduction in invasive and proliferative abilities and angiogenic responses. Ischemia-induced neovascularization in MMP-2(−/−) mice is impaired and can be restored by transplantation of bone marrow-derived mononuclear cells from MMP-2(+/+) mice [75], that suggests MMP-2 as a critical modulator of EPC mobilization and vascular formation.

3.4. MMP-8 in Vascular Stem/Progenitor Cell Migration. The pathogenic role of MMP-8 in progression and instability of atherosclerotic plaque has been discovered [130, 131]. We generated ApoE(−/−) MMP-8(−/−) double knockout mice and found substantially reduced extent of atherosclerotic plaque in these mice [131]. Furthermore, recent study from our group clearly shows that MMP-8 plays an important role in stem/progenitor cell migration and their recruitment from arterial lumen and adventitia into atherosclerotic plaque. ADAM10 cleavage and maturation by MMP-8 and subsequent E-cadherin shedding from cell surface could be the underlying molecular mechanism by which MMP-8 mediated stem/progenitor cell mobilization [76].

Previous studies elucidating the function of MMPs on stem/progenitor cells in blood vessels formation and vascular remodeling mainly emphasize on degradation of ECM promoting stem/progenitor cell mobilization and recruitment. However, MMPs are able to improve cell differentiation in other tissues [132–134]. Therefore, whether MMPs regulate proliferation and differentiation of stem/progenitor cells in vascular tissue via cutting and activating non-ECM molecules such as transcription factors needs further investigations.

4. MMPs and Vascular EC Function in Angiogenesis

Vascular ECs comprising a single layer of cells on the inferior surface of the blood vessels have unique and distinct function in vascular biology, especially in new blood vessel formation and angiogenesis. During vascular formation or angiogenesis, ECs are activated in response to environmental cues and express MMPs. Active MMPs degrade vascular basement membrane and other ECM resulting in detachment of ECs which is imperative in proliferation, migration, invasion, and even apoptosis of ECs and is an initial step of angiogenesis or vascular formation. In addition, MMPs can modify non-ECM molecules such as vascular endothelial growth factor receptors (VEGFR) and urokinase-type plasminogen activator receptor (uPAR) and their signaling pathways to affect EC vitality and behavior. Many MMPs have been shown to exert multiple impacts on ECs functions and angiogenesis (Table 2).

4.1. MMP-1/MMP-8 in Vascular EC Function and Angiogenesis. MMP-1 and MMP-8, two important collagenases, are highly active in vascular tissue and may sensitize EC function. Stimulation of ECs with active MMP-1 promotes expression of VEGFR2, a main binding receptor for VEGF-A and subsequent elevated endothelial proliferation through activation of protease activated receptor-1 (PAR-1) and NF-κB pathway [77, 78]. Our most recent study provides strong evidence to suggest that MMP-8 plays an important role in EC angiogenesis *in vitro*, *ex vivo*, and *in vivo* by regulating the conversion of Ang I to Ang II, PECAM-1 expression, β-catenin nuclear accumulation, and cell proliferation and migration related gene expression [79].

Table 2: MMPs and vascular EC function in angiogenesis.

MMPs	Functions/effects	Substrates/signaling pathway	Outcomes	References
MMP-1	Proliferation↑	PAR-1/NF-κB → VEGFR2	Angiogenesis Vascular remodeling	[77, 78]
MMP-8	Proliferation/migration↑	Ang I → PECAM-1 → β-catenin	Angiogenesis	[79]
MT1-MMP	Migration/invasion↑	ECM (collagen)	Vascular formation	[80–83]
MMP-9	Migration/invasion↑	ECM	Angiogenesis	[84]
MMP-2	Survival/apoptosis↑	Caspases and p38 MAPK	Angiogenesis Angioregression	[59, 85]
MMP-12	Proliferation/migration↑	uPAR	Systemic sclerosis	[86–88]
MMP-7	Proliferation/migration↑	Soluble VEGFR-1	Angiogenesis	[89, 90]

4.2. MT1-MMP in Vascular EC Migration and Function. Evidence suggests that MT1-MMP affects EC migration and function. *In vitro* wound healing migration model reveals that expression of MT1-MMP is upregulated during EC migration and its activity can modulate endothelial migration, invasion, and formation of capillary tubes [80]. MT1-MMP contributes significantly to EC migration in both 2D collagen-coated surfaces and 3D collagen matrices, while secretory-type MMP-1, MMP-2, MMP-9, and MMP-13 are not critical for EC movement in 3D collagen gels [81]. In 3D collagen matrices, MT1-MMP-dependent collagen proteolysis is required in EC lumen formation and generation of vascular guidance tunnels that allow subsequent EC migration and tube network formation which is vital for blood vessel assembly [82]. MT1-MMP also acts as a key effector of nitric oxide (NO) in NO-induced EC migration and angiogenic processes via its collagenolytic function [83]. Furthermore, MT1-MMP performs not only as a matrix-degrading enzyme, but also a signaling molecule on ECs. MT1-MMP mediates small GTPases RhoA and Rac1 activation and subsequent Ca^{2+} and Nox-dependent signaling pathway, ultimately promoting tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) protein expression, in thrombin-stimulated endothelial cells [135].

4.3. MMP-9/MMP-2 in Vascular EC Migration and Survival. MMP-9 and MMP-2, two gelatinases, have been shown as EC modulators. MMP-9 reduction is required for inhibition of invasion and angiogenesis in human microvascular ECs [84]. MMP-2 appears to be an essential molecule determining EC fate, paradoxical effects on both survival (angiogenesis) and cell death. Two major apoptotic pathways in ECs, caspases and p38 MAPK, enhance MMP-2 synthesis and affect EC behavior via different activation form of MMP-2. The partially active form supports survival and migration, while the fully active form leads to apoptosis, and eventually the ratio between these two MMP-2 activation forms in environment determines EC functions [59, 85].

4.4. MMP-12 in Vascular EC Proliferation and Migration. Studies in systemic sclerosis suggest that MMP-12 influences EC function and angiogenesis. Overexpression of MMP-12 cleaves urokinase-type plasminogen activator receptor (uPAR) and impairs uPA-dependent human microvascular EC proliferation, migration, and capillary morphogenesis in systemic sclerosis [86, 87]. Loss of function of MMP-12 in systemic sclerosis ECs restores the ability to induce vascularization [88].

4.5. MMP-7 in Vascular EC Proliferation and Migration. MMP-7, a matrilysin, accelerates the proliferation of human umbilical vein ECs *in vitro* and directly induces angiogenesis [89]. Moreover, MMP-7 impacts EC function through modulating VEGF pathway. Soluble VEGF receptor-1, an endogenous VEGF inhibitor via blocking VEGF access to membrane receptors, can be degraded by MMP-7, which liberates VEGF and enhances VEGF-induced VEGFR2 phosphorylation. The presence of MMP-7 finally promotes endothelial migration and tube formation via VEGF-VEGFR2 downstream activation in ECs [90].

5. MMPs and VSMC Behaviors

VSMCs are the major components of blood vessels. Abnormal VSMC proliferation, migration, and apoptosis are the main causes of vascular remodeling implicated in multiple vascular disorders, including hypertension, restenosis, and atherosclerotic plaque progression and rupture. Alterations of VSMC functions/behaviors also play important roles in physiological and pathological angiogenesis since VSMC and other mural cell recruitment is required for vessel formation and maturation. ECM degradation and remodeling indispensable to vascular structure alterations highlight MMP functions in VSMC behaviors. MMP-2, MMP-9, MT1-MMP, MMP-3, MMP-1, and MMP-7 have been recognized in vascular tissue and play pathogenic roles in vascular remodeling via regulating VSMC behaviors (Table 3).

5.1. MMP-2 in VSMC Proliferation and Migration. In cultured VSMCs, stimulation of MMP-2 production is related to mitogenesis and migration of VSMCs [91]. Pulmonary

Table 3: MMPs and VSMC behaviors in vascular remodeling.

MMPs	Functions/effects	Substrates/signaling pathways	Outcomes	References
MMP-2	Proliferation↑	ET-1, sphingolipid signaling	Hypertension Atherosclerosis	[91–94]
	Migration/invasion↑	ECM	Atherosclerosis Neointimal hyperplasia	[95–98]
MMP-9	Proliferation↑	N-cadherin	Atherosclerosis Neointimal hyperplasia	[99, 100]
	Migration/invasion↑	ECM VSMC-ECM attachment	Atherosclerosis neointimal hyperplasia	[46, 47, 95, 101, 102]
MT1-MMP	Proliferation/migration↑	pro-MMP-2	Neointimal hyperplasia	[103, 104]
	Migration/invasion↑	ECM (collagen)	Neointimal hyperplasia	[105]
	Proliferation/migration↑	PDGF-PDGFRβ Signaling (LRP1, ApoE)	Atherosclerosis Neointimal hyperplasia	[106–115]
MMP-1	Migration/invasion↑	ECM (collagen)	Neointimal hyperplasia	[116, 117]
MMP-1	Proliferation↓	Unclear	Preventive in pulmonary hypertension	[118]
MMP-3	Migration/invasion	ECM, pro-MMP-9	Neointimal hyperplasia	[119–123]
MMP-7	Apoptosis	N-cadherin	Atherosclerotic plaque instability	[124, 125]

arterial hypertension (PAH) characterized by medial hypertrophy and ECM remodeling of pulmonary arteries is associated with MMP-2. Elevated MMP-2 is found in PAH arteries and its overexpression and activation stimulate VSMC proliferation leading to medial wall thickness [92, 93]. Oxidized LDL (ox-LDL), a risk factor promoting atherogenesis, induces VSMC proliferation through activating multiple pathways. MMP-2 triggers ox-LDL induced activation of sphingomyelin/ceramide pathway and subsequent ERK1/2 activation and DNA synthesis that finally leads to VSMC proliferation [94].

The effects of MMP-2 on VSMC migration have also been widely described. MMP-2 deficiency resulted in deceased arterial SMC migration and invasion ability *in vitro* and attenuated intimal hyperplasia after carotid ligation *in vivo* [95]. SMCs from saphenous vein (SV) have similar responses. Transfection SV-SMCs with MMP-2 siRNA resulting in MMP-2 silencing inhibits invasive capacity of cultured human SV-SMCs [96]. Young and aged human aortic SMCs possessing different migratory ability at least partially attribute to MMP-2 expression and activation. Young SMCs show higher migratory capacity due to producing more active MMP-2, while aged SMCs only produce inactive zymogen form of MMP-2 [97]. Moreover, many factors stimulate migration of VSMCs in MMP-2-dependent manner. For example, Interleukin-lβ (IL-lβ), an inflammatory cytokine which is related to VSMC migration during neointimal formation, enhances active MMP-2 synthesis and activation of pro-MMP-2, stimulating VSMC migration [98].

5.2. MMP-9 in VSMC Proliferation and Migration. Rat VSMCs overexpressing MMP-9 show enhanced migration and invasion in the collagen invasion assay as well as Boyden chamber *in vitro* and increased invasion into medial and intimal layers when seeded on the outside of the artery *in vivo* [101]. Genetic MMP-9 knockout impairs migratory activity of isolated VSMCs and decreases intimal hyperplasia [101, 102]. In addition, lack of MMP-9 reorganizes collagenous matrix and reduces VSMC attachment to gelatin [95, 102]. It indicates that MMP-9 not only degrades ECM, but also conducts a connection between cell surface and matrix.

Various cytokines induced in vascular injury and immunoinflammatory responses contribute to atherosclerosis and restenosis through MMP-9 mediated VSMC migration. TNF-α mediates VSMC migration and neointimal formation through upregulation of MMP-9. TNF-α upregulates nuclear FoxO4, which in turn activates transcription of MMP-9 gene, through the N-terminal, Sp1-interactive domain, and the C-terminal transactivation domain of FoxO4 [45]. Both IL-18 and IL-17 stimulate VSMC migration in an MMP-9-dependent manner. MMP-9 expression induced by IL-18 and IL-17 is via NF-κB and AP-1 signaling activation [46, 47].

Studies also find that VSMC replication is significantly decreased in MMP-9(−/−) arteries [99]. However, there are limited data explaining how MMP-9 regulates cell cycle. Recent evidence shows that MMP-9 regulates VSMC proliferation by modulating cell adhesion as well as cadherin and β-catenin association. MMP-9 cleaves N-cadherin and releases β-catenin which translocates to the nucleus and regulates cyclin D1 expression in VSMCs [100].

5.3. MT1-MMP in VSMC Proliferation and Migration. MT1-MMP is initially described as an activator of MMP-2 in vascular remodeling. In neointimal development subjected to balloon catheter injury, increased MT1-MMP level is of importance to MMP-2 activation and neointimal formation

[103, 104]. However, since MT1-MMP possesses a broad spectrum of substrates, an increasing number of studies demonstrate that MT1-MMP participates in vascular remodeling not only via MMP-2 activating.

MT1-MMP functions as a collagenase as well as a signaling molecule. Proteinases such as plasmin, cysteine proteinases, MMP-2, and MMP-9 previously linked to VSMC migration in 2D substrata do not play a vital role in 3D matrix environment, whereas MT1-MMP, a key pericellular collagenolysin of type I and III collagens, enhances VSMC invasion into 3D collagenous barriers *in vitro*. Furthermore, MMT1-MMP deficiency attenuates neointimal hyperplasia and arterial lumen narrowing *in vivo* via a MMP-2, and MMP-9-independent role [105]. On the other hand, MT1-MMP cleaves or modulates cell surface molecules to influence VSMC behavior. Platelet-derived growth factor (PDGF)/PDGFRβ signaling pathway is critical in VSMC migration and proliferation during vasculature development and VSMC phenotypic switch [106, 107]. In VSMC investment of the vasculature, MT1-MMP associates with PDGFRβ as a necessary cofactor. Active MT1-MMP functions as a PDGF-B selective regulator of efficient induction of PDGF-B/PDGFRβ downstream signal transduction that eventually leads to proliferation and chemotaxis of VSMCs. MT1-MMP deficient mice display abnormal vessel wall morphology with notably reduced density of VSMCs [108]. Additionally, in VSMC dedifferentiation, MT1-MMP proteolytically processes LDL receptor-related protein 1 (LRP1) and promotes endocytosing of PDGFRβ-β3-integrin-MT1-MMP-LRP1 complex, which impairs the negative regulation of PDGFRβ depending on ligands binding to LRP1 [109, 110]. Moreover, ApoE is also shown to inhibit PDGF-induced VSMC migration and proliferation [111, 112]. This process is mediated via ApoE binding to LRP1 and subsequent activation of cAMP-dependent protein kinase A [113, 114]. Interestingly, apoE is identified as a MT1-MMP substrate, so that MT1-MMP can cleave apoE to suppress apoE-LRP1 binding and downstream signaling [115, 136]. To sum up, MT1-MMP-dependent LRP1 and apoE cleavage activate PDGF/PDGFRβ signaling pathway and then enhance VSMC proliferation and migration.

5.4. MMP-1 in VSMC Proliferation and Migration. The effects of MMP-1 on VSMC migration and proliferation have been studied. Interstitial flow is elevated in vascular injury and hypertension and is believed to participate in VSMC migration and vascular remolding. In an *in vitro* 3D collagen I system, upregulation of MMP-1 mediates interstitial flow enhanced VSMC migration, while MMPs inhibitor GM6001 attenuates flow enhanced migration [116]. ERK1/2 phosphorylation and increased expression of AP-1 transcription factor c-Jun are implicated in interstitial flow induced MMP-1 expression and VSMC motility [117]. Additionally, studies of MMP-1 in PAH depict the preventive effect of MMP-1 in medial hypertrophy and enhanced remodeling of pulmonary arteries. MMP-1 transgenic mice show decreased medial hyperplasia via impaired cell proliferation of VSMCs and reduced excessive collagen deposition [118]. However, the mechanism underlying MMP-1 mediated impaired VSMC proliferation is still unclear.

5.5. MMP-3 in VSMC Proliferation and Migration. MMP-3, also known as stromelysin-1, influences saphenous vein SMC migration. The main cause of vein graft failure is intimal hyperplasia, a process dominated by proliferation and migration of VSMCs. MMP-3 overexpression significantly reduces SMC migration and inhibits neointimal formation in arterialized vein grafts [119]. Recently, a common insertion/deletion polymorphism in MMP-3 gene promoter region, known as 5A/6A polymorphism, is reported. There is evidence suggesting that 5A/6A polymorphism is related to MMP-3 expression and activation and individuals' susceptibility to many cardiovascular diseases [120]. However, MMP-3 5A/6A polymorphism does not affect invasion of saphenous vein SMCs isolated from patients with different genotypes [121].

It has been reported that MMP-3 regulates VSMC migration via MMP-9 activation. MMP-3 is already known as an activator of pro-MMP-9 [122]. MMP-3 knockout significantly reduces VSMC migration *in vitro* and neointima formation after carotid ligation *in vivo*. Combination of MMP-3 and MMP-9 knockout or knockdown reveals that MMP-3 mediated activation of MMP-9 is required and efficient in neointimal hyperplasia [123].

Preventive effects of MMP-3 in venous neointima via gene transfer and deteriorative role of MMP-3 found in carotid neointimal hyperplasia using genetic knockout seem paradoxical. The possible explanation could be the diverse properties of vein and artery, the distinct procedures inducing neointimal formation, or even the different methods generating genetic modifications. The fact that MMP-3 possesses the broadest substrate specificity among all MMPs makes it more complicated to fully understand the exact roles of MMP-3 in VSMC functions and vessel formation.

5.6. MMP-7 in VSMC Apoptosis. Effects of MMPs on VSMC proliferation and migration are widely discussed in various studies. However, there are only a few studies to investigate the functional involvements of MMPs in VSMC apoptosis. MMP-7, detectable in unstable atherosclerotic plaques, cleaves N-cadherin which is vital in cell adhesion and survival and then promotes VSMC apoptosis [124]. VSMC apoptosis leads to instability of plaque through thinning of fibrous cap [125]. Therefore, MMP-7-dependent N-cadherin cleavage and cell apoptosis may promote plaque development and rupture.

It has been shown that several cell survival and VSMC viability maintaining factors including N-cadherin, platelet-derived growth factor (PDGF), heparin binding endothelial growth factor (HB-EGF), and insulin-like growth factor 1 (IGF-1) can be cleaved and modulated by MMPs [137]. However, direct evidence presenting MMPs mediated VSMC apoptosis still needs to be achieved in the future work.

6. Conclusions and Perspectives

In conclusion, MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MT1-MMP play a crucial role in blood vessel formation, remodeling, or angiogenesis through regulating the functions or behaviors of stem/progenitor and vascular cells. Numerous stimuli which are risk factors of blood vessel-related disorders such as oxidative stress, inflammatory factors, hemodynamic forces, hormones, and hypoxia provoke MMP expression and activation. Once activated, MMPs modulate various behaviors of stem/progenitor cells, vascular ECs and VSMCs, which in turn contribute to physiopathological processes in vascular formation, remodeling as well as angiogenesis. The major and predominant role of MMPs in angiogenesis is still the degradation of ECM components to promote recruitment of stem/progenitor cells and facilitate migration and invasion of ECs and VSMCs. In addition, effects of MMPs on cell proliferation and apoptosis are discovered. It has been well known that other important molecular mechanisms by which MMPs regulate vascular cell proliferation and apoptosis are via proteolytically cleaving and modulating non-ECM molecules in VEGF-VEGFR signaling, uPA-uPAR signaling, and cell-cell adhesion.

As vascular cells are fundamental elements participating in vascular formation, remodeling, or angiogenesis, regulation of vascular cell behaviors via modulating the expression and activation of MMPs seems to provide a reasonable way for therapeutic purpose. A great number of MMP inhibitors have been tested experimentally. Tissue inhibitors of MMPs, inhibitory antibodies, and chemically-synthesized MMP inhibitors show certain effects on amelioration of pathological changes in animal models of vascular diseases [11, 138]. However, only doxycycline has been approved by the Food and Drug Administration for clinical application until now. This is partially due to the broad substrate spectrum, overlapped proteolytic effects, and wide distribution of MMPs and nonspecificity of MMP inhibitors. Several approaches such as site specific delivery and generating MMP inhibitors with increased selectivity are thought to be helpful for MMPs-targeted therapy. However, further understanding of MMPs governing vascular cell behaviors and their specific underlying mechanisms is still essential to develop novel therapies. Except for MMP inhibitors, factors promoting MMP gene transcription and signaling pathways mediating MMPs-induced vascular cell alterations could be potential therapeutic targets.

Authors' Contribution

Q. Chen and M. Jin contributed equally to this work. Both Qingzhong Xiao and Li Zhang are the correspondent authors for this paper.

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Research Article

Antiangiogenic VEGF Isoform in Inflammatory Myopathies

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Objective. To investigate expression of vascular endothelial growth factor (VEGF) antiangiogenic isoform A-$_{165b}$ on human muscle in idiopathic inflammatory myopathies (IIM) and to compare distribution of angiogenic/antiangiogenic VEGFs, as isoforms shifts are described in other autoimmune disorders. *Subjects and Methods*. We analyzed VEGF-A$_{165b}$ and VEGF-A by western blot and immunohistochemistry on skeletal muscle biopsies from 21 patients affected with IIM (polymyositis, dermatomyositis, and inclusion body myositis) and 6 control muscle samples. TGF-β, a prominent VEGF inductor, was analogously evaluated. Intergroup differences of western blot bands density were statistically examined. Endomysial vascularization, inflammatory score, and muscle regeneration, as pathological parameters of IIM, were quantitatively determined and their levels were confronted with VEGF expression. *Results*. VEGF-A$_{165b}$ was significantly upregulated in IIM, as well as TGF-β. VEGF-A was diffusely expressed on unaffected myofibers, whereas regenerating/atrophic myofibres strongly reacted for both VEGF-A isoforms. Most inflammatory cells and endomysial vessels expressed both isoforms. VEGF-A$_{165b}$ levels were in positive correlation to inflammatory score, endomysial vascularization, and TGF-β. *Conclusions*. Our findings indicate skeletal muscle expression of antiangiogenic VEGF-A$_{165b}$ and preferential upregulation in IIM, suggesting that modulation of VEGF-A isoforms may occur in myositides.

1. Introduction

Idiopathic inflammatory myopathies (IIM) encompass three subsets: dermatomyositis (DM), polymyositis (PM), and sporadic inclusion body myositis (IBM) with distinct immunopathological patterns. In DM, complement deposition on endomysial capillaries, loss of microvessels, and mainly perivascular inflammation are observed, whereas PM and IBM show a T-cell invasion of muscle cells associated with degenerative features in IBM. Recently, necrotizing autoimmune myopathy (NAM), with scarce or no inflammation, has been recognized as a fourth IIM entity [1]. The expression of inflammatory effectors in IIM is extensively investigated, since pharmacological targeting of immunoregulatory factors is increasingly recognized as an effective treatment tool [2–4].

Vascular endothelial growth factor (VEGF) family comprises a group of potent endothelial cell mitogens. VEGF-A, an angiogenic growth factor, proinflammatory mediator, and promoter of vascular permeability, is produced by various cell types, among which are myocytes and inflammatory cells. Its expression is transcriptionally regulated by multiple molecules: growth factors, hormones, and oncogenes [5]. Alternative splicing from eight exons within the VEGF-A gene generates a family of proteins, named according to their amino acid number, VEGF$_{xxx}$. VEGF family encompasses proangiogenic$_{xxx}$ and antiangiogenic$_{xxxb}$ isoforms, originating by alternate splice site selection in the terminal exon. Molecules have identical length but differing C-terminal amino acid sequences and opposing biological effect on angiogenesis: proangiogenic VEGF$_{xxx}$ isoforms originate by

Table 1: Demographic data.

	PM	DM	IBM	Controls
No. of subjects	6	8	7	6
Gender: M, F	1, 5	3, 5	6, 1	4, 3
Biopsy site	Vastus lateralis: 4 Deltoid: 2	Vastus lateralis: 6 Deltoid: 2	Vastus lateralis: 6 Brachial biceps: 1	Vastus lateralis: 7
Age at biopsy, years (range)	61.33 ± 7.87 (53–63)	58.25 ± 12.07 (42–72)	72.43 ± 7.91 (61–85)	45.42 ± 23.22 (18–73)
Symptoms before biopsy, months	12.5 ± 5.6	14.6 ± 9	40 ± 11.8	—
Immunotherapy before biopsy (1–4 months) prednisolone: 5–10 mg/day	3/6	4/8	7/7	—

proximal splice site selection and antiangiogenic $VEGF_{xxxb}$ isoforms by distal splice site selection [6]. VEGF-A_{165b} is the first identified antiangiogenic VEGF molecule [6]. Inhibition of angiogenesis by $VEGF_{xxxb}$ isoforms is due to competitive receptor binding, inhibition of receptor phosphorylation, and downstream intracellular signalling [7].

In the present work, we investigated tissue localization of VEGF-A_{165b} and VEGF-A in IIM as well as transforming growth factor-β (TGF-β), involved in the angiogenic and proliferative processes and a prominent VEGF-A_{165b} inductor, along with pathological parameters of myositides.

2. Subjects and Methods

2.1. Patients and Diagnostic Procedures. Muscle samples were archival diagnostic biopsy specimens from twenty-one patients affected with IIM, diagnosed by current clinicopathological criteria [1], as DM ($n = 8$), PM ($n = 6$), or IBM ($n = 7$). Demographic data are presented in Table 1. As control samples, we processed biopsy muscle specimens ($n = 6$) from subjects complaining of muscle pain, or cramps, with normal or mildly increased levels of serum creatine kinase and normal or mild myopathic electromyographic findings. We selected as controls only subjects in which muscle histology, histochemistry, immunohistology, and ultrastructural morphology resulted as normal, ruling out inflammation, degeneration-regeneration, denervation, or changes suggestive of metabolic storage. All subjects signed an informed consent with allowance to scientific utilization of muscle samples for research purposes. Specimens were frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use. Cryostat sections, 10 μm thick, were submitted to diagnostic routine histological and histochemical stains.

2.2. Western Blot Analysis. WB analysis in muscle samples from control subjects and IIM patients was performed following standard procedures. Muscle samples were lysed in radioimmunoprecipitation assay (RIPA) buffer; proteins (40 μg load) were separated by SDS-PAGE 4–20% Tris-Glycine Pre-Cast Gel (Invitrogen S.R.L., Milan, Italy) and transferred to a nitrocellulose membrane. After blocking, samples were incubated with the primary antibodies for VEGF-A (cod. MAB3734; Millipore Corporation, Billerica, MA, USA), VEGF-A_{165b} (Abcam Plc, Cambridge, UK), TGF-β (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA) and then with the appropriate secondary antibodies. Bound antibodies were visualized by enhanced chemiluminescence (ECL Detection Kit, GE Healthcare, Milan, Italy), and the band densities were quantified using NIH image software by using β-actin band for normalization.

2.3. Immunohistochemistry. Immunohistology for diagnostic analysis of inflammation (HLA-ABC, C5-b9, CD4, CD8, CD11b, CD20, CD45Ro—Dako, Glostrup, Denmark; Carpinteria, CA, USA) was carried out on 7 μm thick cryostat sections on silane-coated slides (StarFrost; Knittel Gläser, Braunschweig, Germany). Immunolocalization of VEGF-A and VEGF-A_{165b} was performed on consecutive sections. Immunohistochemistry for TGF-β and endothelial marker CD31 (Dako), for vessel detection, were also carried out. Regenerating fibres were identified by antifoetal myosin heavy chain antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Analysis was performed by immunoperoxidase technique, by HRP-labeled polymer (Dako), and 3,3′-diaminobenzidine (Sigma-Aldrich, Milan, Italy) for visualization. Negative controls by omission of the primary antibody were performed.

2.4. Quantitative Analysis on Immunohistochemical Slides. Morphometry was carried out by Zeiss AxioPlan2 microscope equipped with AxioVision 4.6 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). The endomysial vessel density, the degree of inflammation, and the entity of muscle regeneration were evaluated, as histopathological parameters of myositis.

Density of endomysial vessels was expressed as number/mm^2 of muscle area by counting the whole sections immunostained for CD31. Inflammatory score was assessed on sections stained for marker of activated leukocytes CD45Ro. For each sample, three randomly selected fields at 100x magnification were analyzed by automatized colorimetric pixel evaluation, detecting the peroxidase reaction product. CD45Ro peroxidase labeled area and the area of the whole field were measured: inflammatory score was expressed as the CD45Ro+ percentage of the total area.

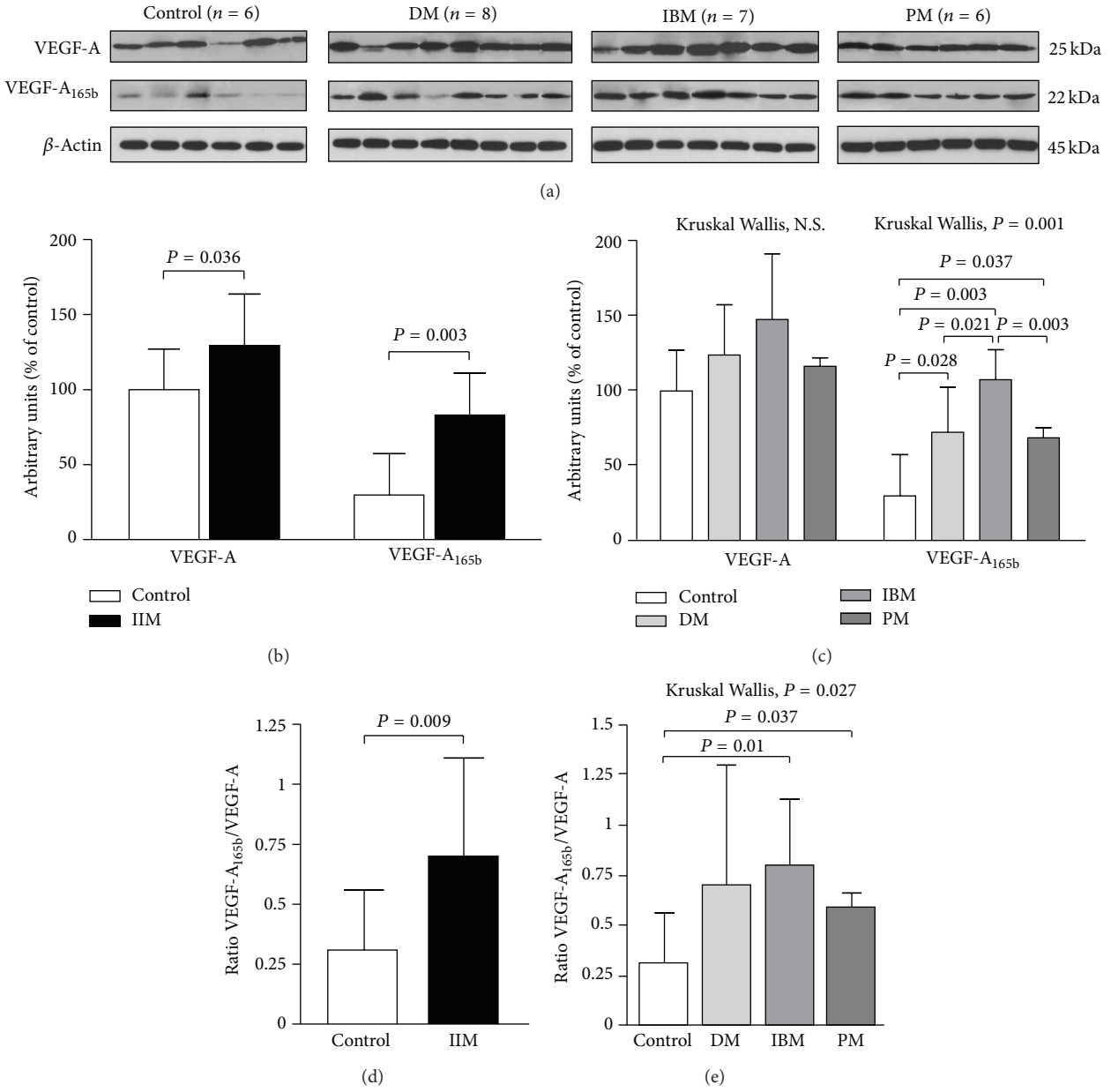


Figure 1: Increase of VEGF-A and VEGF-A$_{165b}$ protein expressions in inflammatory myopathies. Representative VEGF-A and VEGF-A$_{165b}$ western blots (a). Densitometric quantification of the VEGF-A and VEGF-A$_{165b}$ bands is shown as ratio with the loading control β-actin in IIM (b) and in DM, IBM, and PM subsets (c). VEGF-A$_{165b}$/VEGF-A ratio was obtained for all samples ((d), (e)). Data are expressed as arbitrary units from five independent experiments. Histograms represent mean values ± SD. All the statistical significant differences were reported.

Regeneration index was expressed as the percentage of foetal myosin heavy chain reactive fibres by examination of at least 700 fibres.

2.5. Statistics. Data were evaluated by SPSS statistics software. IIM were examined as a whole versus controls and as single subgroups. Intergroup variabilities were analyzed by Mann-Whitney test for paired analysis, and analysis of variance was performed by Kruskal-Wallis test for multiple groups. Spearman rank test was used to analyze correlations between WB band densities and the other investigated pathological parameters. Significance was set at $P < 0.05$. Data were expressed as means ± SD.

3. Results

3.1. Western Blot

3.1.1. VEGF-A and VEGF-A$_{165b}$. The protein levels of both VEGF isoforms were higher in IIM subjects (Figure 1). An increase of circa 30% for VEGF-A and up to 2.5-fold for VEGF-A$_{165b}$ versus controls (Figure 1(b)) was observed.

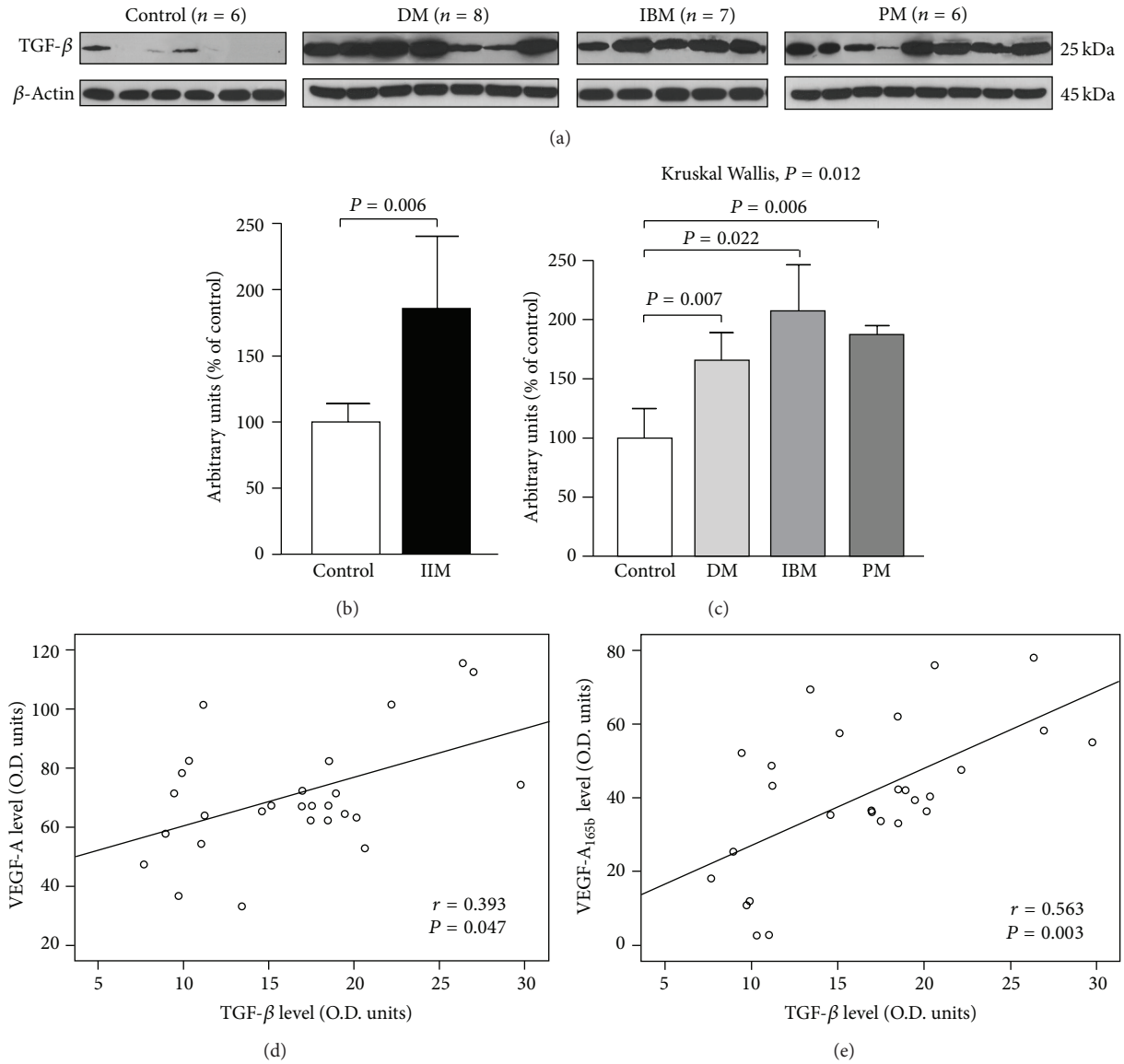


Figure 2: Increase of TGF-β protein expression in inflammatory myopathies. Representative TGF-β Western blots (a). Densitometric quantification of the TGF-β bands was obtained by β-actin normalization ((b), (c)). Histograms represent mean values ± SD from five independent experiments. All the statistical significant differences were reported. TGF-β levels were in positive correlation with VEGF-A (d) and VEGF-A_{165b} (e).

VEGF-A_{165b} was significantly upregulated in the IIM subgroups DM, IBM, and PM versus controls; analysis of intergroup variance evidentiated significantly higher levels of VEGF-A_{165b} in IBM versus PM and DM (Figure 1(c)). The ratio VEFG-A_{165b}/VEGF-A was significantly increased in IIM (Figure 1(d)), indicating a preferential upregulation of the antiangiogenic subunit: IIM 0.70 ± 0.40 (min. 0.15, max 2.09); ctrls: 0.31±0.25 (min 0.03, max 0.68). PM and IBM subgroups were significantly different from controls (Figure 1(e)).

3.1.2. TGF-β. IIM samples displayed an almost 2-fold increase in TGF-β levels versus controls (Figure 2(b)), with statistical significance in all the IIM subsets (Figure 2(c)). TGF-β protein levels in IIM were positively related with both VEGF isoforms content (Figures 2(d) and 2(e)).

3.2. Immunohistology

3.2.1. VEGF-A, VEGF-A_{165b}, and TGF-β Localization. Muscle fibres showed a constitutive diffuse cytoplasmic expression of VEGF-A, either in controls (Figure 3(a)) and in IIM (Figures 4(1b); 4(2b); 4(3b); 4(4a)). Cytoplasmic localization of VEGF-A_{165b} was very faint in control muscle, where endomysial vessels appear the main source of the protein (Figure 3(b)) and in morphologically normal fibres of IIM (Figures 4(1c); 4(2c); 4(3c); 4(4b)). In myositis, atrophic and regenerating fibres (Figure 4(1a)), identified by foetal myosin (Figure 4(1d)), were strongly reactive for either isoforms. A milder VEGF-A_{165b} upregulation was observed in most nonregenerating, morphologically normal fibres (Figure 4(1c)), with neolocalization of MHC I molecule

(a) VEGF-A (b) VEGF-A_{165b} (c) Negative control

Figure 3: Immunohistological distribution of VEGF isoforms in control muscle. (a)–(c) Consecutive sections. Diffuse VEGF-A cytoplasmic stain of myofibers is observed (a); very mild cytoplasmic VEGF-A_{165b} reactivity, with stronger stain of endomysial sparse mononuclear cells and endomysial small vessels (b). No stain on negative control slide by omission of primary antibody (c).

HLA-ABC (Figure 4(1e)). Necrotic fibres, identified by deposits of the terminal complex of complement (Figure 4(2a)), displayed no VEGF or VEGF-A_{165b} expression (Figures 4(2b)-4(2c)). Vacuolated fibres of IBM (Figures 4(3a)–4(3c)) strongly expressed VEGF-A_{165b} in adjacency of rimmed vacuoles, a pathological hallmark of IBM. Perifascicular atrophic fibres of DM also showed an increased expression of both VEGF isoforms (Figures 4(4a)-4(4b)). Areas of VEGF upregulation displayed a substantial VEGF-A/VEGF-A_{165b} co-localization but a stronger diffuse immunostain and a high occurrence of focal reactive deposits resulted distinctive VEGF-A_{165b} features (Figures 4(1b)-4(1c), 4(3b)-4(3c)). Both VEGFs were also expressed by endothelium of most capillaries (Figures 4(1b)-4(1c)) or larger endomysial vessels (Figures 4(3e)-4(3f)). Occurrence of mononuclear infiltrates with scarce VEGF-A stain was observed in subjects submitted to steroid treatment (Figure 4(3b)), whereas VEGF-A_{165b} expression was maintained (Figure 4(3c)). In biopsies performed prior to therapy, inflammatory cells strongly reacted for both VEGF isoforms (Figures 4(4a)-4(4b)). TGF-β was detected in inflammatory cells and muscle fibres in adjacency of infiltrates, as well as in vessel walls (Figure 4(3d)), in substantial colocalization with VEGFs (Figures 4(3e)-4(3f)).

3.3. Quantitative Analysis

3.3.1. Density of Endomysial Vessels. Variations of endomysial vascularization were detected, accordingly to literature data. IBM cases showed a significantly higher vessel density than controls (Figure 5(a)). Analysis of variance among IIM subsets evidentiated a significantly higher endomysial vascularization in IBM versus PM and DM.

Endomysial vessel density in IIM was in positive correlation with VEGF-A ($r = 0.585$, $P = 0.017$,) and VEGF-A_{165b} levels ($r = 0.503$, $P = 0.047$), as well as with VEGF-A_{165b}/VEGF-A ratio ($r = 0.812$, $P < 0.001$).

3.3.2. Inflammatory Score. IBM cases showed the highest degree of inflammation compared to the other groups (Figure 5(b)). Inflammatory score in IIM was in positive correlation with VEGF-A_{165b} levels ($r = 0.630$, $P = 0.009$), as well as with vessel density ($r = 0.499$, $P = 0.049$).

3.3.3. Regeneration Index. The occurrence of regenerating fibres was highly variable among and within the subgroups (Figure 5(c)): IBM and PM showed higher regeneration percentage as compared to DM.

The regeneration index was positively related to TGF-β protein level in the whole group of IIM ($r = 0.473$, $P = 0.03$) and to VEGF-A_{165b}/VEGF-A ratio ($r = 0.523$, $P = 0.012$).

4. Discussion

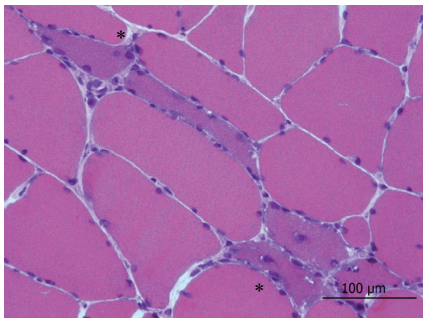
The present report demonstrates that VEGF-A_{165b} is expressed in human skeletal muscle and its level robustly increases in IIM.

Constitutive expression of VEGF by myocytes is found to be essential for regulation of capillarity [8] and, in aged humans, the lower density of endomysial capillaries is associated with VEGF local decrease [9]. Therefore, the VEGF increase that we report in IIM is not biased, but rather stressed, by the younger median age of our noninflammatory controls.

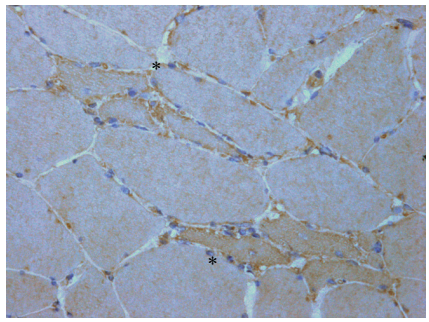
Increase of VEGF *in toto* in skeletal muscle following injury, in association with fibres regeneration, is documented [10].

A recent fundamental study highlighted increased muscle expression of VEGF-A in IIM, in correlation with clinicopathological stages of disease, and it also specifically addressed the issue of therapy influence [11]. Glucocorticoid treatment (3–6 months) in PM and DM lowered VEGF-A expression, which stayed anyway significantly higher than in controls, by decreasing the number of VEGF-A reactive capillaries and mononuclear inflammatory cells, as we also observed in our treated patients. Therefore a role of steroids is likely also in the modulation of muscle VEGF-A_{165b}; however, the highest levels of both VEGF isoforms were detected in our group of IBM patients, typically unresponsive to steroids [4], all submitted to 1–4 months glucocorticoid treatment, prior to biopsy and histological diagnosis.

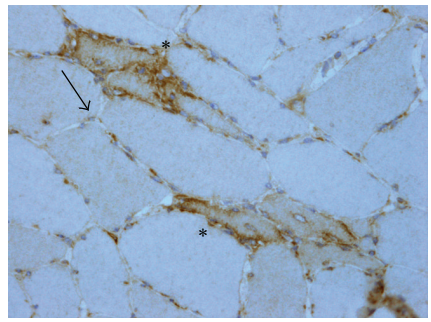
The constitutive expression of antiangiogenic VEGF isoforms is highly variable (reviewed by [12]). They are downregulated in highly angiogenic tissues, such as placenta [13], but they may reach 90% of the total VEGF protein in colon [14].



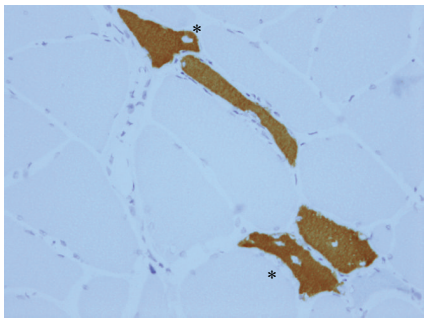
(1a) IBM HE



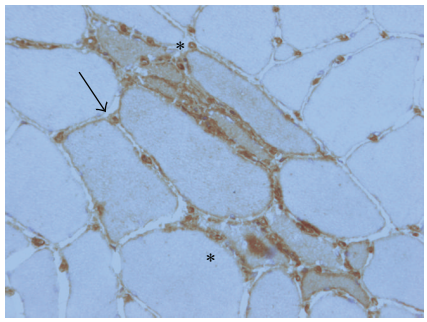
(1b) VEGF-A



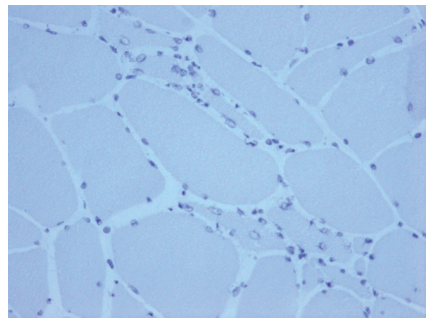
(1c) VEGF-A_{165b}



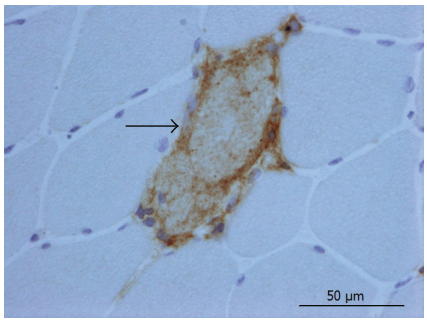
(1d) Foetal MyHC



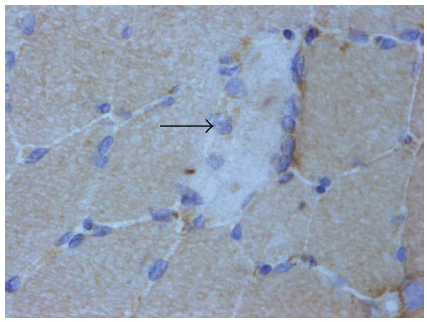
(1e) HLA-ABC



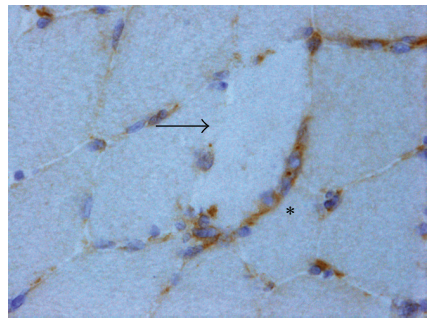
(1f) Negative control



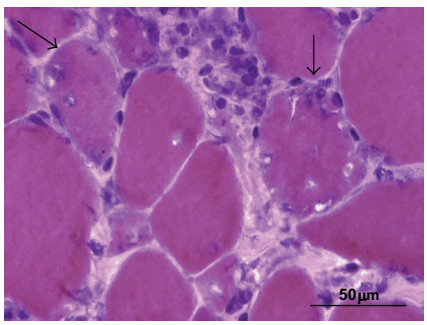
(2a) PM MAC



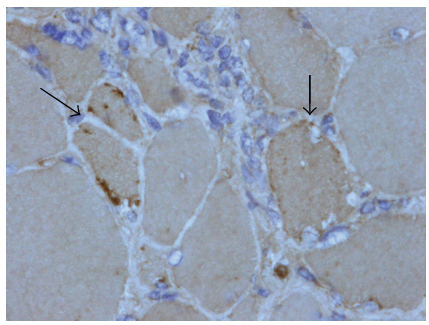
(2b) VEGF-A



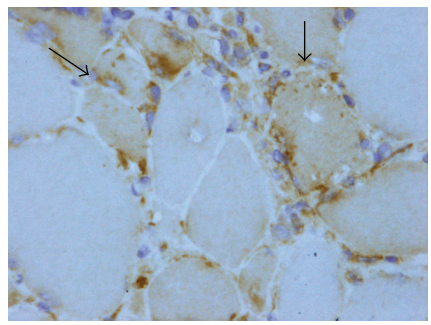
(2c) VEGF-A_{165b}



(3a) IBM HE

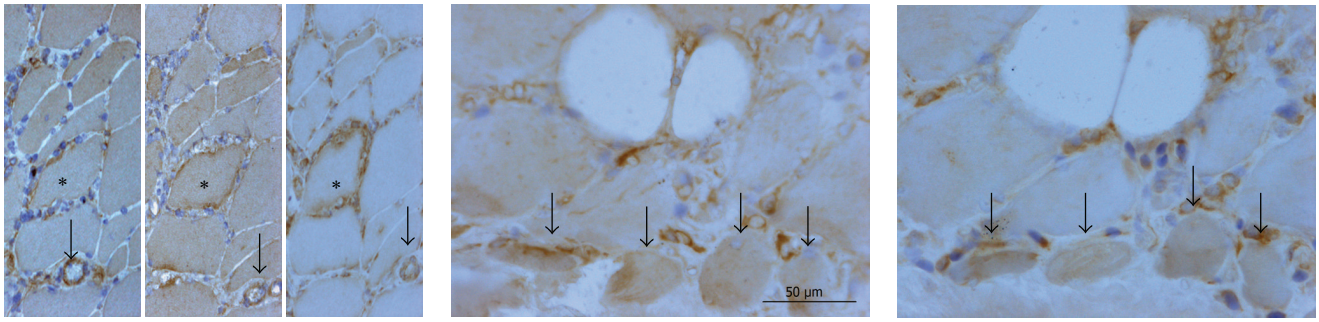


(3b) VEGF-A



(3c) VEGF-A_{165b}

Figure 4: Continued.



(3d) TGFβ (3e) VEGF-A (3f) VEGF-A_{165b} (4a) DM VEGF-A (4b) VEGF-A_{165b}

Figure 4: Representative images of immunohistological distribution of VEGF-A, VEGF-A_{165b}, and TGF-β in inflammatory myopathies. (1a)–(1f) Consecutive sections, IBM. Regenerating fibres (asterisk), basophilic (1a) and reactive for foetal myosin heavy chain (1d), show VEGF-A (1b) upregulation, against basal expression of adjacent fibres. These fibres are strongly VEGF-A_{165b} reactive (1c), in contrast with low or null reactivity of adjacent fibres, and show highly reactive foci. Small endomysial vessels are diffusely VEGF-A and VEGF-A_{165b} reactive. A mild VEGF-A_{165b} upregulation (1c: arrow) is observed in nonregenerating fibres displaying sarcolemmal and cytoplasmic HLA-ABC neoexpression (1e: arrow). (1f) Negative control slide. (2a)–(2c) Consecutive sections, PM. Necrotic fibres, identified by deposits of the terminal complex of complement, or membranolytic attack complex (MAC) (2a: arrow), do not express neither VEGF-A (2b: arrow) nor VEGF-A_{165b} (2c: arrow), appearing as unreactive pale elements. Mononuclear cells surrounding and partially invading the necrotic fibre (2c: asterisk) are strongly reactive for VEGF-A_{165b}. (3a)–(3c) Consecutive sections, IBM. Fibres with rimmed vacuoles (3a, arrows) show VEGF-A upregulation (3b, arrows), with occasional highly reactive foci, and VEGF-A_{165b} upregulation with multiple spots of intense reactivity (3c, arrows). Mononuclear infiltrates show a scarce reactivity for VEGF-A (3b), whereas they strongly express VEGF-A_{165b}. (3d)–(3f) IBM. TGF-β (3d) is expressed on a large endomysial vessel (arrow) and inflammatory cells. Muscle fibres of smaller diameter show higher reactivity. On nonadjacent serial sections, localization of both VEGF isoforms is observed ((3e)-(3f)) in the vessel. The asterisk identifies a fibre surrounded by inflammatory cells. (4a)-(4b) Consecutive sections, DM. Perifascicular atrophic fibres (arrows) show increased reactivity for angiogenic (3a) and antiangiogenic (3b) VEGF isoforms. Mononuclear cells also react for both isoforms.

A previous study investigating antiangiogenic isoforms of VEGF-A in human muscle did not detect VEGF-A_{165b}, basally or after submaximal exercise, in healthy subjects, by PCR [15], whereas previous PCR [6], and ELISA [16] identifications of $VEGF_{165b}$ in skeletal muscle are reported. As skeletal muscle is richly vascularized, either Western blot or ELISA, at the protein level, and PCR, at the mRNA level, do not rule out detection of endothelial molecules. As we agree that, within normal muscle, the antiangiogenic VEGF subunit is mainly expressed by endothelial cells, the events of inflammation and coexisting atrophy/regeneration in IIM seem to trigger a substantial $VEGF_{165b}$ upregulation in muscle. A basal constitutive VEGF-A_{165b} synthesis by myocytes, against a diffusion from adjacent inflammatory cells, is also suggested by the absent stain, for either VEGF isoforms, in necrotic fibres. The specificity of the utilized VEGF-A_{165b} antibody, directed against the antiangiogenic COOH-terminal sequence, was assessed by complete lack of recognition of angiogenic VEGF-A_{165} protein isoform by WB, as documented by manufacturer (http://www.abcam.com/VEGF-165B-antibody-MRVL56-1-ab14994.html). The differences in the staining pattern of the two isoforms further support antibody specificity.

Studies addressing the alternate distal/proximal site splicing in human disease describe a splicing switch to pro-angiogenic isoform in angiogenesis-associated disorders, such as solid tumors and diabetic retinopathy [17, 18]. Opposingly, a selective upregulation of VEGF-A_{165b} is reported in retinal detachment associated with proliferative vitreoretinopathy, glaucoma [19], and fibrosing autoimmune disorder systemic sclerosis [20, 21], where decrease of angiogenesis occurs.

In our IIM samples, a local VEGF-A_{165b} preferential upregulation is suggested by the increased VEGF-A_{165b}/VEGF-A ratio.

Research data on VEGF-A_{165b} offer clues to elucidate its increase in IIM: growth factors and related signal pathways act on $VEGF_{xxx}/VEGF_{xxxb}$ alternative splicing [5, 17], and pleiotropic fibrogenic TGF-β is a key factor in switch to distal site splice selection for synthesis of VEGF-A_{165b} [5, 20]. In our IIM samples, as previously described [2, 22], TGF-β was upregulated, and we observed a positive correlation TGF-β/VEGF-A_{165b} by western blot. Therefore, the peculiar VEGF-A_{165b} staining of degenerating/regenerating fibres may be linked to molecular events of muscle regeneration, where upregulation of TGF-β occurs [23], as supported by correlation of extent of regeneration and VEGF-A_{165b}/VEGF-A ratio. Nevertheless, the lack of a direct correlation VEGF-A/regeneration index and a milder VEGF-A_{165b} localization in nonregenerating fibres neoexpressing major histocompatibility complex-I, a change consistent with myofiber activation (reviewed in [24]), suggest that other factors contribute significantly to VEGF-A_{165b} upregulation in IIM. Immunohistology shows that TGF-β reactive endothelium and inflammatory cells are also a prominent source of VEGF-A_{165b}. The finding is associated to inflammatory score/VEGF-A_{165b} and both VEGF isoforms/vessel density positive correlations.

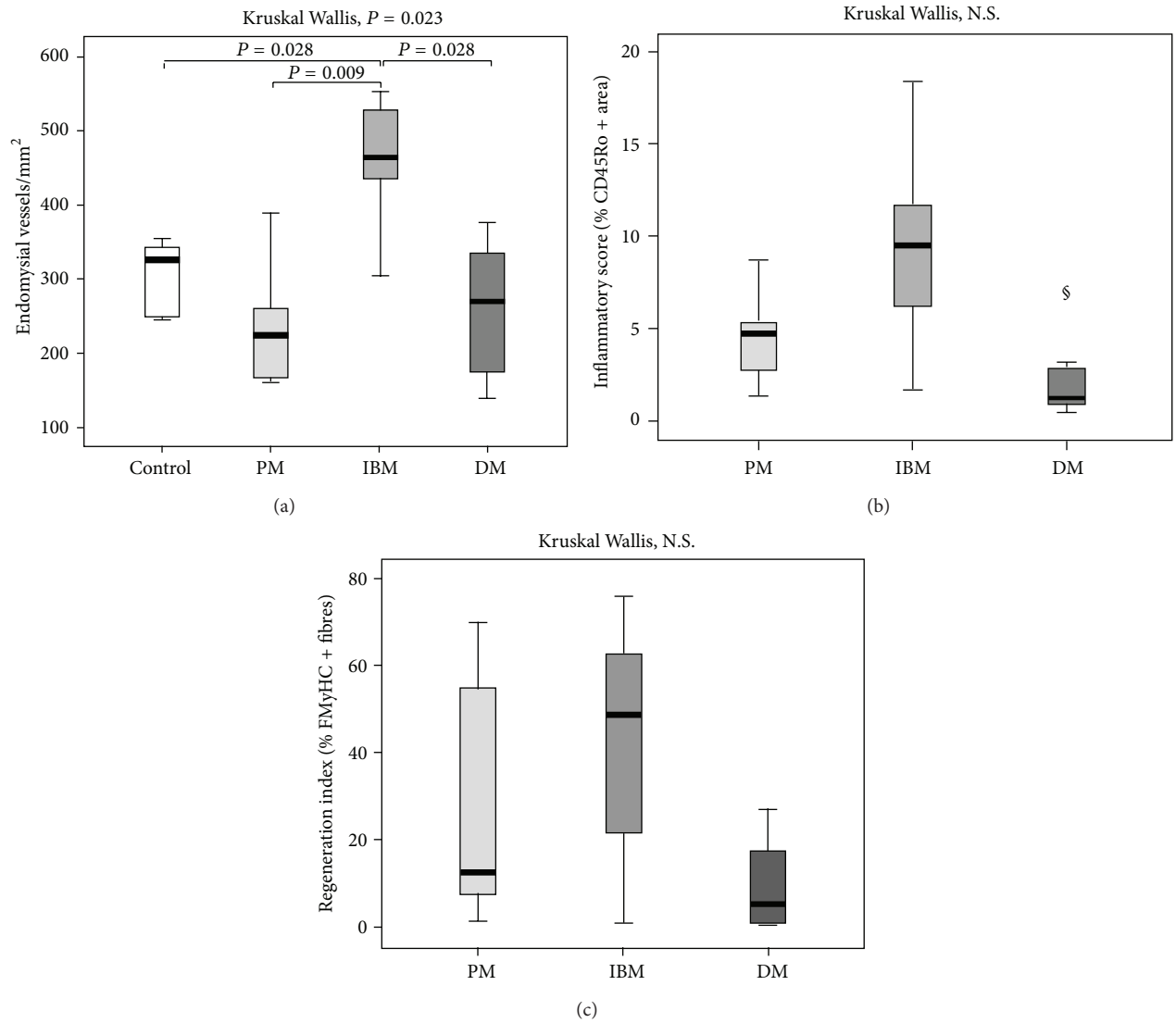


Figure 5: Quantitative analysis of pathological parameters in inflammatory myopathies. (a) A significant increase of the vessels density in IBM cases is detected. PM and DM cases showed a mild decrease of vessel density. (b) IBM cases showed the highest inflammatory score. § The DM outlier, with an unusually high inflammatory score, is a case of severe paraneoplastic DM, with high degree of myofiber necrosis and inflammation. (c) Regeneration index: the occurrence of regeneration showed a high variability among subjects, within the IIM subsets. The values are reported as means ± SD. All the statistical significant differences were reported.

Though our patients' subgroups are numerically too small to allow general conclusions, IBM higher inflammatory score and density of endomysial vessels, an acknowledged pathological feature of IBM [3], which resulted in our study strictly related to VEGF-A_{165b}/VEGF-A ratio, appear to concur to higher VEGF-A_{165b} expression. This apparently controversial issue might depend on a negative feedback triggered by VEGF-A_{165b}. In fact, as it has been proposed [6, 25], VEGF-A_{165b} can inhibit VEGF-A-mediated proliferation and migration of endothelial cells, via a counterregulatory mechanism, limiting the rate of *de novo* muscle capillarization.

Moreover, hypoxia, a strong VEGF modulating agent [14, 21, 26, 27], due to microvascular involvement, has a role in all the major IIM subsets by activating circuitries of cytokines, adhesion molecules, and leukocyte recruitment [3]. Skeletal muscle reacts with increased VEGF-A gene expression to hypoxia caused by acute exercise [27]. As an inductor of TGF-β [28], hypoxia might result in an additional causative factor of local VEGF-A_{165b} increase in IIM.

Interestingly, besides antiangiogenic effect, a cytoprotective activity of VEGF-A_{165b} against ischaemic damage has been recently documented on colon epithelium [14] and on retinal epithelium and endothelium [29], so that its compound biological effects still need to be elucidated.

5. Conclusions

Local VEGF antiangiogenic switch in inflammation and necrosis/regeneration of skeletal muscle is likely to depend on a cohort of humoral and cellular effectors, possibly involved in tissue protection against inflammatory process.

As therapeutic modulation of VEGF isoforms is currently investigated in cancer [12, 14] and in angiogenic eye disorders [17], and considering a potential development in autoimmune disorder systemic sclerosis [20, 21], further studies are needed for a complete understanding of the balance between antiangiogenic and proangiogenic VEGF isoforms in inflammatory myopathies.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Nila Volpi and Giuseppe Valacchi equally contributed to the study.

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Review Article

Cytokines and Angiogenesis in the Corpus Luteum

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In adults, physiological angiogenesis is a rare event, with few exceptions as the vasculogenesis needed for tissue growth and function in female reproductive organs. Particularly in the corpus luteum (CL), regulation of angiogenic process seems to be tightly controlled by opposite actions resultant from the balance between pro- and antiangiogenic factors. It is the extremely rapid sequence of events that determines the dramatic changes on vascular and nonvascular structures, qualifying the CL as a great model for angiogenesis studies. Using the mare CL as a model, reports on locally produced cytokines, such as tumor necrosis factor α (TNF), interferon gamma (IFNG), or Fas ligand (FASL), pointed out their role on angiogenic activity modulation throughout the luteal phase. Thus, the main purpose of this review is to highlight the interaction between immune, endothelial, and luteal steroidogenic cells, regarding vascular dynamics/changes during establishment and regression of the equine CL.

1. Introduction

The angiogenic process plays an essential role during organogenesis and embryo development [1]. Angiogenesis itself can be classified as the process of new blood vessels formation from the preexisting vasculature. In adult tissues angiogenesis is very limited, and blood vessels remain quiescent until there is an angiogenic stimulus, such as hypoxia or wounding [2]. Besides the essential role of angiogenesis on wound healing, it also plays a major function in various diseases and tumorigenesis [3]. In contrast, after puberty, tissue growth and function of female reproductive organs (placenta, ovary, and corpus luteum) under physiologic conditions are extremely dependent on new blood vessels formation [4, 5].

Angiogenesis is a highly regulated process involving the balance between pro- and antiangiogenic factors. Locally, endothelial surrounding cells produce growth factors, cytokines, enzymes, receptors, adhesion molecules, and metabolic factors that regulate the angiogenic process. In this way, interaction between immune and endothelial cells should not be neglected [6]. It is well known that inflammatory cells, namely, macrophages, T lymphocytes, and monocytes, fully participate in the angiogenic process by secreting pro- and/or anti-inflammatory cytokines, which may control endothelial cells migration and activation, proliferation, survival, and apoptosis [7]. With particular regard to the ovary, it is well established that immune cells contribute for ovarian function regulation [8]. Moreover, immune cells present in the corpus luteum (CL) can be considered as a large pool of mobile cells that putatively modulate luteal establishment, maintenance, and regression. Overall, little is known about the complex cross-talk between immune and vascular systems in the CL. The plethora of intervenient cytokines and their often pleiotropic range of actions demand our attention to better understand luteal angiogenesis regulation.

This review focuses on the modulation of angiogenesis by cytokines in the CL, addressing, in a chronological fashion, the events occurring from the follicle to the regressing CL. Special emphasis will be put on angiogenesis and cytokines action in the mare CL, since this research team has been gathering both descriptive and functional knowledge on the expression and role of cytokines like tumor necrosis factor alpha (TNF), interferon gamma (IFNG), Fas ligand (FASL), vascular endothelial growth factor A (VEGF), and nitric oxide (NO), among others, on equine luteal function. Due to the particular similarities between woman and mare

(monovulatory species) on many aspects of ovarian function, mare CL is a valuable study model for understanding the regulatory pathways involved on the control of ovarian physiology [9]. Besides, most physiologic studies on woman ovarian function are based on the knowledge generated from abnormal tissue or granulosa cells collected from *in vitro* fertilization [10]. Therefore, their physiologic relevance is questionable [10], being the mare ovary a better study model.

2. Angiogenesis in the Corpus Luteum: A Chronological Sequence of Events from the Follicle to the Regressed Corpus Luteum

Different studies indicate that the CL is one of the most vascularized organs in the body [11, 12]. The CL undergoes extremely rapid cellular and vascular changes, only comparable with tumors [13]. The coordination of those biological processes is the outcome of a complex cross-talk between several factors. In the CL, as in other organs, angiogenesis seems to be tightly controlled by stimulating and inhibiting factors [14] that might regulate its vascularization and function [14, 15]. Development of the microvasculature during luteal establishment and formation is required for the delivery of adequate levels of hormones and lipoprotein bound cholesterol [16]. Quantitative reports in ruminants showed that in early luteal phase CL (early CL) more than 85% of proliferating cells are endothelial cells, while in mature CL more than 50% [12].

2.1. Starting from the Follicular Vasculature. During follicular growth, angiogenesis is determinant for preantral follicle development, follicle dominance, and preovulatory development [17]. Around 40% of proliferating cells in the theca are of endothelial origin [18]. Moreover, the blood clot formed during ovulation might stimulate cell migration. Indeed, platelets are a better stimulant for endothelial cells migration than granulosa cells themselves [19]. Examples of proangiogenic cytokines acting on this stage of the cycle include the cytokines fibroblast growth factor 2 (FGF2), VEGF, platelet-derived growth factor (PDGF) family, and angiopoietin (Ang). The VEGF has been described as the main proangiogenic factor, which is also produced by luteal cells [13, 14]. In fact, treatments with antiangiogenic compounds (VEGF trap) impaired follicular development [20]. Definitely, VEGF plays a central role, since its blockade abolished endothelial cell proliferation, luteal vascularization, and progesterone (P4) production in rat [21] and mouse CL [22].

In mares, dominant follicles show an increase in blood flow prior to deviation, when compared with subordinated follicles [23]. This follicular vascular bed provides the basis on which luteal vasculature will be formed [24]. It has been noticed that in the developing follicle granulosa and theca cells produce proangiogenic factors [25]. At the time of ovulation, the LH surge induces several important cellular and biochemical changes [26]. Specifically, breakdown of the basement membrane and immune-like responses are determinant for angiogenesis promotion [27]. Breakdown and reorganization of the blood vessel basement membrane involve a plethora of proteases, including matrix metalloproteinase (MMP) family, such as collagenases, gelatinases, and membrane-type MMP. Several MMPs (MMP9, MMP13, and MT-MMP1), which are primarily secreted by macrophages in the ovary of many species (reviewed by Wu et al. [28]), are upregulated by the LH surge [29]. It should be noticed that some of these MMPs also participate on the ovulatory process [30]. Thus, the physical block to vascularization of the granulosa layer is removed, and breaking down and spreading extracellular matrix (ECM) components take place, creating a more spacious environment facilitating motility and migration of endothelial cells and others. Another important consequence is the release of angiogenic factors sequestered in the basement membrane. Disintegrin and metalloproteinase with a thrombospondin (TSP) type 1 motif (ADAMTS) are proteases that appear to be critical for angiogenesis following ovulation [31]. The ADAMTS1 cleaves the matrix proteoglycans, expressed in the periovulatory follicle. Besides, ADAMTS1 is increased by gonadotropin stimulation [32], possibly mediated by hypoxia-induced factor 1α (HIF1A) pathway [33]. This might be important for endothelial cell invasion, since it is upregulated when these cells invade the collagen matrix, following VEGF and FGF2 stimulation [34].

Another important trigger for the increased blood flow is the HIF1A, whose expression is upregulated in the collapsed follicle of pigs [35], suggesting that this tissue is hypoxic. The relationship between LH, VEGF, FGF2, and HIF1A is still not clear for the period of follicular-luteal transition, but it is possible that VEGF raise following LH surge is mediated by HIF1A [36].

The cytokine TNF also presents an increased expression at the ovulation time [37], suggesting its participation on the ovulation process and incoming steps of luteal growth. Tumor necrosis factor α and its receptors presence was shown in the early CL of cow [38], pig [39], human [40], and horse (further discussed in detail) [41]. Regarding the TNF cellular action, the type of receptor involved should be considered. Indeed, TNF can induce both cell proliferation and death, depending on which receptor it binds to (TNFRI, the proapoptotic receptor; or TNFRII, the prosurvival receptor) [42, 43]. Reports in bovine CL demonstrated the TNF mediates endothelial cells proliferation [44–46]. Moreover, TNF participation in early CL vascularization should be considered alongside with the generation of NO. Studies by Okuda and coworkers evidenced that endothelial cells of bovine CL treated with TNF exhibited an increase in NO secretion [47], confirming the relevance of TNF/NO interaction on luteal angiogenesis in cows. The NO is considered a vasoactive substance, responsible for endothelial cells proliferation and VEGF secretion [48]. As shown for the mare, the NO donor (spermine NONOate) was able to stimulate angiogenic activity in early CL [49]. In parallel, the expression of NO generating enzyme, the endothelial NO synthase (eNOS), was increased in equine early CL [49], supporting NO role on luteal angiogenesis promotion.

Finally, as reviewed by Shirasuna and co-workers [50] polymorphonuclear leukocytes (PMNs) invade the CL soon after the ovulation. Neutrophils infiltration of bovine early CL (days 1–4) was correlated with a high concentration of

interleukin 8 (IL-8, a neutrophil chemoattractant specific factor), suggesting that this cytokine also promotes angiogenesis in the CL [43, 44].

2.2. Luteal Endothelia Cell Migration and Proliferation. Endothelial cell migration involves its polarization towards an angiogenic stimulus, protrusion through filopodia-like structures, traction, and then retraction [51]. It is recognized that, in bovine CL, fibronectin forms a network of fibrils orientated along the axis of the capillary sprout [52], acting as a "prepatterned" guideline for endothelial cells migration. Fibronectin showed also a stimulatory effect on luteal-derived endothelial cell proliferation [53] and formation of endothelial cell networks *in vitro* [24]. Still considering the endothelial cell migration, it is believed that recently formed steroidogenic luteal cells can secrete the chemoattractants VEGF and FGF2, working on endothelial cells migration towards themselves [24].

The FGF2 also appears to be critical to endothelial network formation, since suppression of its receptor almost completely inhibited angiogenesis, by decreasing both the number of endothelial clusters and their size in the cow CL [24]. This occurred even in the presence of VEGF, emphasizing the importance of FGF2. Moreover, FGF2 expression increases during the initial stage of luteal formation, being a far more effective promoter of endothelial cell proliferation than VEGF [54]. It is suggested that both factors may have complementary rather than redundant actions on luteal angiogenesis [24].

Of particular interest for angiogenesis regulation in early CL is the modulation of FGF2 expression by prostaglandin (PG) F2α. As recently reported for early CL in cows (day 4), the so-called luteolytic PGF2α strongly increased FGF2 expression (mRNA and protein) [55]. From this standpoint, PGF2α would promote CL vascularization and support CL growth. In order to justify PGF2α putative effect on CL establishment, it was also suggested that PGF2α is able to interact with PGE_2 receptors, when present in high concentrations [56]. Admittedly, the role of PGF2α on vascularization during CL growth awaits further research. However, the present evidence of PGF2α support on VEGF, FGF2, and P4 secretion in bovine CL confirms its participation on CL growth [55, 57].

2.3. A Mature Vascular System in the Corpus Luteum. Endothelial cells need structural support. Mural cells such as pericytes vascular smooth muscular cells ensure the shape and regulate blood flow through their contractile properties. The final step of angiogenesis is the vessel stabilization, achieved with the secretion of platelet-derived growth factor beta (PDGFB), which acts on a paracrine fashion on pericytes recruitment [58]. For many years the role of pericytes on angiogenesis was neglected. However, there is growing evidence of their importance on the promotion of angiogenesis initiation. During the ovulation time, pericytes are located at what appears to be the forefront of the endothelial migratory path [59], whilst in mature CL they are closely associated with endothelial cells. Furthermore, pericytes represent a large number of proliferating cells in the early ovine CL [59]. Firstly, pericytes act as guiding structures aiding the outgrowth of endothelial cells. They produce MMPs and may promote endothelial cell invasion, by destroying ECM. Then, pericytes are recruited for vessel stabilization [24]. Activation of these cells was associated with the platelet-derived growth factor (PDGF) system. Preovulatory treatment of mice with soluble ectodomain of PDGF receptor (PDGFR) prevented the recruitment of pericytes and reduced the staining of vascular area in CL [22], while the *in vitro* inhibition of PDGFR domain decreased the vascular network formation in bovine CL [54].

2.4. Luteolysis Demands Vascular Regression. A fundamental question concerning regression of the CL is whether regression of vasculature plays a role on functional and structural luteolysis. It was reported in sheep [60] and guinea pig [61] that apoptosis of endothelial cells presumably originated the occlusion of blood vessels with cellular debris. This could result in subsequent apoptosis of more endothelial cells followed by apoptosis of steroidogenic cells [62]. A pitfall determining the importance of endothelial cells apoptosis on luteolysis may be the fact that the temporal association between them diverges among species. The evidence in sheep and cow that PGF2α induces apoptosis of endothelial cells, resulting in a luteolytic cascade [63], is not that obvious in primates [64]. Nonetheless, death of vascular cells undoubtedly leads to a reduction in oxygen supply and nutrients to hormonal producing cells, perhaps contributing for their death.

The main luteolytic agent, the uterine PGF2α, has been associated with *in vivo* changes on vasculature. In fact, it has been proposed that the main consequence of PGF2α is the decrease in luteal blood flow [65]. However, following PGF2α administration, different responses are seen among species. In the cow, an acute increase on luteal blood flow was verified after 30 minutes to 2 h following administration of PGF2α [63]. A similar increase in blood flow at the beginning of luteolysis was not confirmed for the mare [66], whose luteal blood flow to the mid CL decreases some days before the decline in plasma P4 [67]. Several studies have related luteal blood flow changes seen in the cow with the potent vasorelaxant NO. The NO mediated raise in blood flow in cows accelerates neutrophils infiltration of the CL, mainly resulting in the production of various inflammatory cytokines production, such as IL-8, TNF, or INFG [68]. The IL1β was also related with luteolysis via NO synthesis promotion [69]. Additionally, these factors may be determinant for further luteal infiltration with immune cells (macrophages and T lymphocytes), supporting luteolytic cascade. Still regarding PGF2α effect on cellular changes during luteolysis, it was hypothesized that pericytes may serve as a regulator of tissue remodeling and integrity maintenance of large blood vessels, allowing normal luteolysis to occur [70]. Intriguingly, the pericytes, which are known to support angiogenesis [59], appear to participate in vascular regression during luteolysis.

The involvement of NO in equine CL regulation, specifically modulating changes in the vasculature, was recently described [49]. As previously mentioned (prior Section 2.1), eNOS protein was shown to be highly expressed in the mare early CL, when NO stimulated luteal tissue for angiogenic

factors production and induced bovine aortic endothelial cells (BAEC) proliferation (used as a model to assess angiogenic factors production by luteal cells). Expression of eNOS was reduced in mid CL, and NO no longer increased BAEC mitogenic activity. In addition, participation of NO in vascular changes regulation throughout the luteal phase should be true also for the mare, since eNOS expression was increased once again in late luteal phase CL (late CL) [49]. Thus, NO is also involved in angioregulation during luteolysis.

The cytokines TNF and IFNG were shown to play a role in bovine luteal endothelial cells regulation [71]. Moreover, referred cytokines can interact with endothelin-1 (ET-1; mainly produced by endothelial cells) and PGF2α, inhibiting luteal steroidogenesis [72]. Two prior reports evidenced that TNF is cytotoxic for endothelial cells derived from bovine CL [73, 74]. Likewise, IFNG has been suggested as a locally secreted factor that may support TNF cytotoxic effect on bovine luteal endothelial cells [16]. Furthermore, cytokines TNF and IFNG can directly incite MCP-1 secretion and contribute for apoptosis of endothelial cells [61]. The present findings suggest that a cross-talk between immune and endothelial cells accounts for the increase in MCP-1 level and endothelial cell death, during PGF2α-induced luteal regression [75].

Changes in PGs and blood flow are considered necessary for local release of ET-1 and angiotensin II (ANGII), which further induce vasoconstriction and blood flow reduction [76]. Besides showing other biological functions, ET-1 is considered a potent vasoconstrictor, by acting on its receptor A [77]. Concerning the ANGII, it regulates several biological processes besides angiogenesis, including vascular tone and cellular growth. In cows, production of ANGII in the CL was associated with renin-angiotensin system [78]. Both ET-1 and ANGII can reduce luteal steroidogenesis and are considered vasoactive factors determinant for the luteolytic pathway and vascular regression [57].

Finally, vascular regression under the luteolytic context can be considered as a component of structural luteolysis. Generally, structural luteolysis implies strong ECM remodeling. As a result, MMPs participation is required once again but this time towards angioregression. It was demonstrated that, after PGF2α treatment, expression of MMPs (MMP1, MMP2, and MMP9) was increased, and this effect was potentiated by TNF [79]. Moreover, the same study showed that tissue inhibitor of metalloproteinase 1 (TIMP1, a specific inhibitor of matrix metalloproteinase) level was decreased during PGF2α-induced luteolysis, increasing the ratio of MMPs/TIMPs [79].

3. Vascular Regulation in the Equine Corpus Luteum

3.1. Angiogenic Function Characterization in the Equine CL. Since considerable differences are seen in the histology of the CL among species, the pattern of luteal vascularization should diverge. So far, few studies have described angiogenesis regulation in equine CL. Dynamic changes on vascular area in the mare CL were described throughout the luteal phase for the first time [15]. A marked increase in vascular area was seen in both early and mid CL, even though the vessel number was the highest in mid and late CL. The raise in DNA content seen from early to mid CL was associated not only with hyperplasia and luteal cell proliferation but also with endothelial cells proliferation [15]. Besides, the decrease in vascular area in the late CL might have been associated with the decrease in blood vessel lumen resultant from vessels contraction. This decrease in capillary diameter is considered determinant for blood flow fall and can initiate or accelerate luteal regression [80].

Among the various factors involved in luteal angiogenesis, VEGF appears to be the most important one for equine CL. It was evidenced that both VEGF mRNA transcription and protein expression peak in early and mid CL [81]. A direct temporal correlation with angiogenesis, blood vessels proliferation, and capillary density was established [81]. Besides, the presence in both follicular and luteal cells from equine ovary of VEGF, VEGF B, Ang1, Ang2, and the receptors VEGFR1, VEGFR2, and Tie2 was recently demonstrated [82]. A different staining was seen for VEGF, VEGFR2, and Ang2 in the periovulatory period (including the tertiary, the Graafian follicles, and early CL) [82]. These data showed their participation on equine luteal angiogenesis initiation. The Ang1 staining was mostly associated with arterioles, venules, arteries, and veins, compared with capillaries, suggesting a role on stabilization of this vasculature [83]. Regardless of the luteal stage, VEGFR1 was associated with mild expression intensity, and the complex VEGFB/VEGFR1 was not associated with proangiogenic events in the mare CL [82]. Furthermore, in the mature CL (mid CL) a more intense staining of proangiogenic studied factors could be observed specifically in the array of the vascular septa and in the CL periphery. These findings are in agreement with those from Al-zi'abi et al. [81]. In mid CL, capillary endothelial cells showed a less intense staining, mainly regarding VEGFR2 and Tie2, when compared with early CL. Also, luteal cells were characterized by a weaker immunolabeling for VEGFR2 in the mid CL [82].

3.2. Cytokines and Angiogenesis Regulation in the Equine CL

(i) Luteal Establishment. Our team described the role of cytokines TNF, IFNG, and FASL on equine CL angiogenesis regulation. It is important to indicate that the cytokine TNF showed proangiogenic properties in early CL in the mare, after (i) increasing endothelial cells BAEC viability, (ii) increasing mRNA level of proangiogenic VEGF/VEGFR2 complex, and (iii) decreasing antiangiogenic CD36 (TSP1 receptor) [84] (Figure 1). Moreover, in mid CL treated cells, TNF increased VEGF protein expression [84] (Figure 2). By assessing BAEC viability, the ability of TNF to modulate angiogenesis by equine luteal cells was characterized (Figure 1(a)). In our study, VEGF and its receptor VEGFR2 mRNA levels were increased by TNF in the early CL (Figure 1(b)). Protein analysis also showed a stimulatory role of TNF on VEGF expression by mid CL cells (Figure 2). When the inhibitory effect of TNF on the mRNA level of the antiangiogenic receptor CD36 in early CL cells is taken into consideration, these findings suggest that TNF might

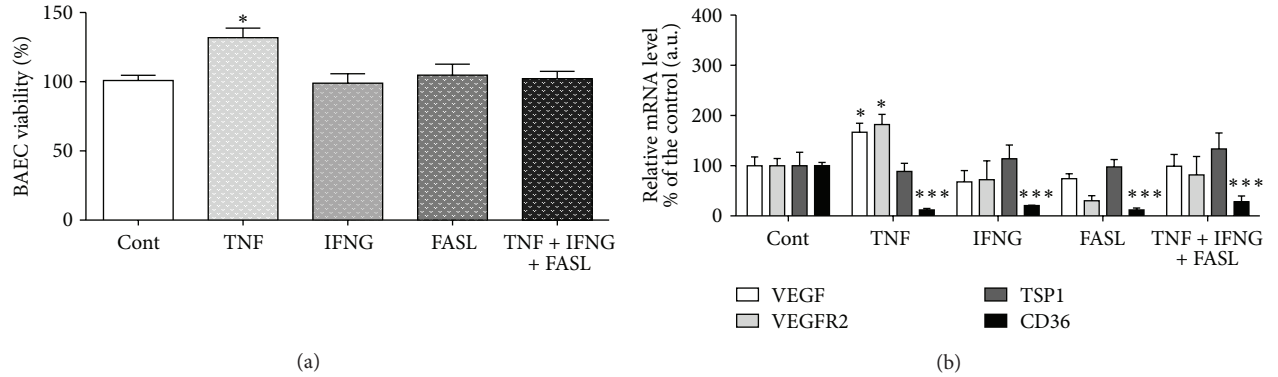

Figure 1: Figure adapted and modified from Galvão et al. [84]. (a) Bovine aortic endothelial cell (BAEC) proliferation rate, after incubation with conditioned media from luteal cells obtained from early CL (cytokines treatment for 24 h). (b) Relative quantification of VEGF, VEGFR2, TSP1, and CD36 mRNA transcription by real time PCR in early CL luteal cells (cytokines treatment for 24 h). Transcription normalized with the housekeeping gene—B2MG. Bars represent mean ± SEM. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{***}P < 0.001$), regarding the control values.

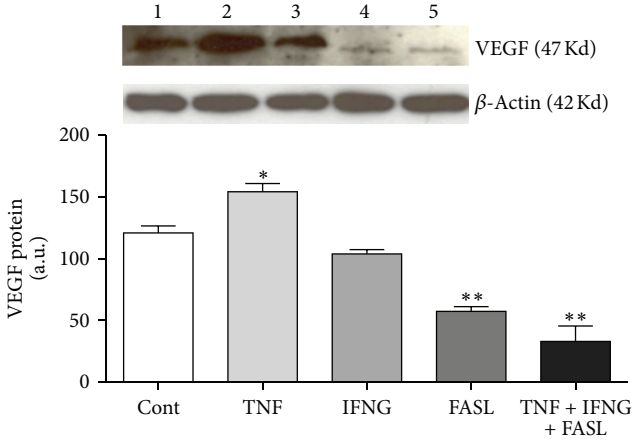

Figure 2: Figure adapted and modified from Galvão et al. [84]: VEGF protein expression in equine mid CL. Upper panels depict representative Western blot ($n = 4$). Lanes: (1) control; (2) TNF; (3) IFNG; (4) FASL; and (5) TNF + IFNG + FASL. Data normalized against β-actin density values. Bars represent mean ± SEM. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{**}P < 0.01$).

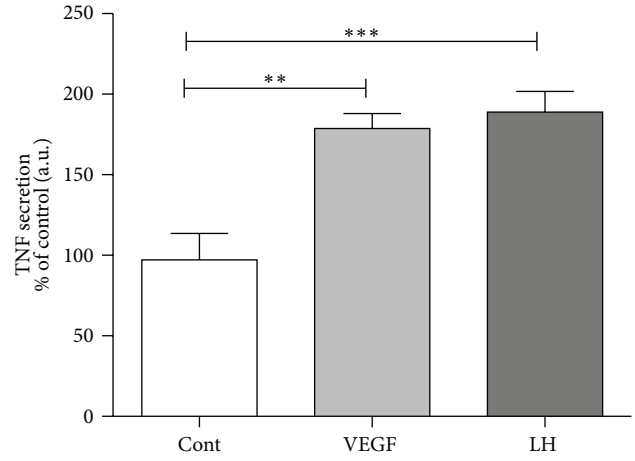

Figure 3: VEGF and LH action on TNF production by mid CL cells, after a 24 h stimulation. Stimulation dose for VEGF 50 ng/mL and for LH 10 ng/mL. Bars represent mean ± SEM. Asterisks indicate significant differences ($^{**}P < 0.01$; $^{***}P < 0.001$). Luteal tissue and venous blood from jugular vein were collected after mortem at the local abattoir from randomly designated cyclic Lusitano mares. The luteal structures were classified in different luteal stages (early, mid, and late CL) as previously described [72, 82]. All methodologies for luteal cells isolation and culture, culture medium analysis by enzymatic immuno assay, and statistical analysis were recently described in detail [72].

participate in angiogenesis at the time of luteal formation in the mare.

In spite of not being the only factor involved in endothelial cells promotion, VEGF is a crucial promoter of ovarian angiogenesis [20]. Thus, the interaction between TNF and VEGF may be determinant for luteal vasculature establishment. For instance, VEGF, which is secreted by macrophages in human ovary [28], was shown to be chemotactic for the same immune cell type, inducing neovascularization in mice [85]. Our group recently concluded that equine mid CL isolated cells treated with VEGF presented a raise in TNF secretion (Figure 3). This suggests the existence of a luteotrophic intraluteal loop, where TNF increases the VEGF production by equine luteal cells, and, in turn, VEGF synergically acts on TNF secretion. The notorious interaction between both immune and vascular systems here characterized is in agreement with previous findings in bovine CL [86]. Still considering the TNF/VEGF loop, the LH action should also be discussed. We demonstrated that mid CL LH treated cells presented an increase in TNF output, compared with control (Figure 3). As a matter of fact, LH is a major regulator of angiogenesis in several species [27], but its exact effect conducting luteal vascularization is not known. The *in vitro* stimulatory effect of LH on VEGF secretion by granulosa

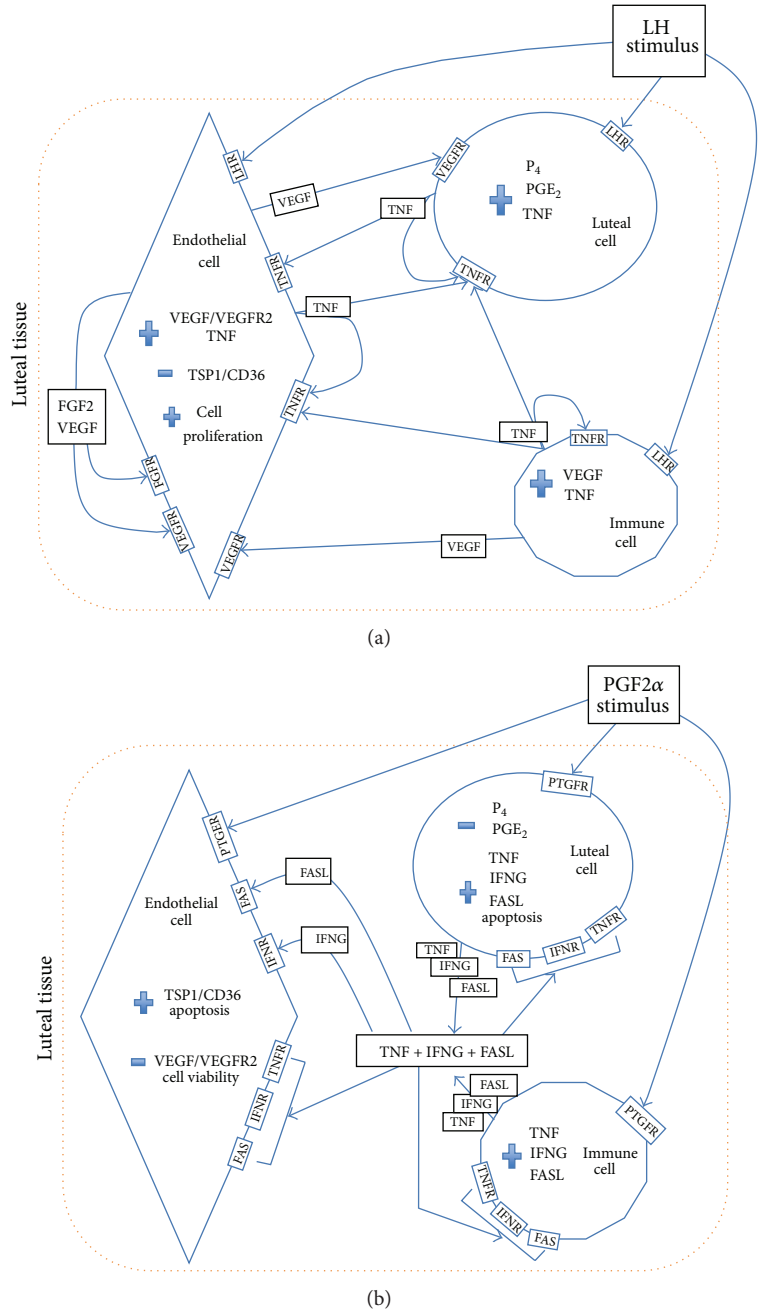

Figure 4: Schematic proposed interaction between endothelial, immune, and steroidogenic luteal cells in equine CL: (a) in early CL LH triggered luteotrophic loop between TNF, VEGF, and other factors during luteal growth; (b) in late CL PGF2α triggered luteolytic loop between cytokines FASL and cytokines synergic action TNF + IFNG + FASL towards angioregression and luteolytic cascade. Thick arrows indicate synergistic action of cytokines TNF + IFNG + FASL. The symbol + means increase in transcription/translation level; the symbol—means decrease in transcription/translation level. LH: luteotrophic hormone; LHR: LH receptor; PG: prostaglandin; PTGF: PGF2α receptor; P4: progesterone; TNF: tumor necrosis factor α; TNFR: TNF receptor; IFNG: interferon gamma; IFNR: IFNG receptor; FASL: Fas ligand; FAS: FASL receptor; FGF2: fibroblast growth factor 2; FGFR: FGF2 receptor; VEGF: vascular endothelial factor A; VEGFR2: VEGF receptor 2.

cells has been described in cows [86] and women [87]. In view of the present data, one may suggest that LH triggers TNF production during equine luteal angiogenesis promotion (Figure 4(a)), acting on different cellular departments, such as luteal cells, immune cells, or endothelial cells. As a result, TNF auto-, paracrine action (mainly on endothelial cells) stimulates VEGF production. The stimulus for vessels proliferation is then maintained with the VEGF action on luteal steroidogenic cells, via TNF transcription activation and transduction. Furthermore, TNF is a potent leukocyte chemoattractant factor, increasing this way the immune cells population in the developing CL (T cells, neutrophils,

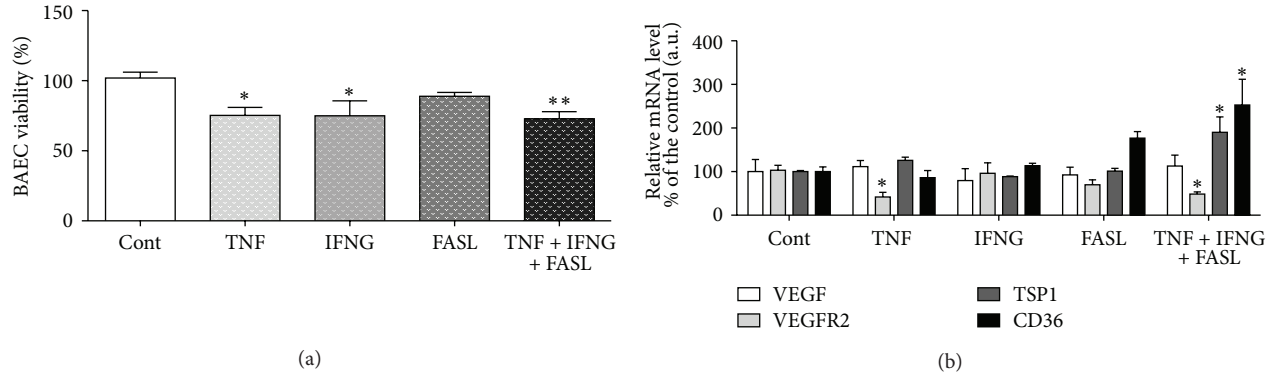

Figure 5: Figure adapted and modified from Galvão et al. [84]. (a) Bovine aortic endothelial cell (BAEC) proliferation rate, after incubation with conditioned media from luteal cells obtained from late CL (cytokines treatment for 24 h). (b) Relative quantification of VEGF, VEGFR2, TSP1, and CD36 mRNA transcription by real time PCR in late CL luteal cells (cytokines treatment for 24 h). Transcription normalized with the housekeeping gene—B2MG. Bars represent mean ± SEM. Asterisks indicate significant differences ($^{*}P < 0.05$; $^{***}P < 0.01$), regarding the control values.

eosinophils, and macrophages are leukocytes present in the developing CL of several species—revised by Shirasuna et al. [50]). As mentioned before, VEGF is also chemotactic for macrophages [28], cooperating with TNF on CL immune cells infiltration.

(ii) Luteal Regression. In the mare, late CL conditioned media treated with PGF2α reduced mitogenic activity of BAEC [15]. In another study, after luteolytic PGF2α *in vivo* treatment, the expression of proangiogenic factors in the CL was reduced, and antiangiogenic factors production increased [88]. Also, after 12 h of induced luteal regression with PGF2α, on day 10 of the luteal phase, signs of swelling and apoptosis in equine luteal endothelial cells, as well as detachment from the blood vessels, were observed [88]. Active caspase-3 was also identified in large luteal cells and endothelial cells [74, 78]. In both steroidogenic and endothelial cells, the increase in caspase-3 expression was on day 14 of the luteal phase or 36 h after PGF2α administration [89]. Another important finding is the relationship between the onset of caspase-3 expression in endothelial cells on day 14 of luteal phase (or after luteolysis induction) and the decrease in mRNA and protein expression of VEGF in steroidogenic cells [81]. Nevertheless, in the mare there is no evidence that luteal endothelial cell death is the trigger for luteolysis, since death of endothelial cells is temporarily associated with death of steroidogenic cells.

Regarding the immune-vascular interaction at the time of luteolysis, as previously reported in equine CL, macrophages population mainly increases in the late luteal phase [90]. Moreover, an influx of neutrophils was seen during spontaneous regression in hamsters CL [91]. Although neutrophils have been largely associated with phagocytosis, their participation in luteolysis has been also ascribed to cytokines secretion [91]. Hence, our recent work clearly stated the cytokines role on angiogenesis downregulation in equine CL [84]. In the late CL, a startling rise in antiangiogenic factors production after TNF treatment shows the stage specific role of this cytokine (Figure 5(a)) [84]. This definitely indicates TNF pleiotropy, suggesting that its modulation of angiogenic-signaling pathways depends on the local microenvironment and auto-, paracrine interactions with other factors.

As mentioned in Section 2.4, TNF deleterious action on bovine endothelial cells appears to be supported by IFNG [16]. In our work, the synergic action of these two cytokines on equine CL angiogenesis was not considered. Nevertheless, IFNG alone was able to decrease angiogenic activity in the late CL (Figure 5(a)), but no other changes were observed [84]. It should be also indicated that in the cow IFNG was associated with senescence and antiproliferative effects on specific luteal endothelial cell types [74].

Another interesting finding by this team was the demonstration of the negative effect played by FASL on VEGF protein expression (Figure 2). Our conclusions concerning FASL role on equine CL regulation show its importance besides the so-well characterized participation in structural luteolysis and apoptosis [92]. Initially, we have characterized FASL participation in luteal secretory impairment at the luteolysis time [93]. Since luteolysis is a dynamic process, we hypothesized that FASL could play a role in angiogenesis regulation. This cytokine is known to diminish angiogenesis in different organs [94]. Also in the mare, we have shown FASL specific downregulation of VEGF in the CL (Figure 2), which may trigger angioregression.

We have also demonstrated that the cytokine association TNF + IFNG + FASL decreases VEGF protein expression in mid CL cells (Figure 2). In addition, cytokines association adequately restricted angiogenesis, after (i) increasing TSP1 and CD36 mRNA level, (ii) decreasing VGEFR2 mRNA level, and (iii) reducing BAEC proliferation in the late CL (Figure 5). When all the cytokines were tested together (TNF + IFNG + FASL), angiogenesis restriction was very effective in late CL cells (reduction of BAEC viability—Figure 5(a)). In late CL isolated cells, both TSP1 and CD36 mRNA levels were increased, while VEGFR2 was reduced (Figure 5(b)). Although no changes were seen in mRNA level,

the same cytokine combination also reduced VEGF protein expression in mid CL cells (Figure 2).

The proteomic profile of these three cytokines (TNF, IFNG, and FASL) show that the increase in their expression [69, 82], and especially their synergic action, might be associated with functional luteolysis and consequently with angiogenesis downregulation (Figure 4(b)). Their combined action on angiogenesis regression was also demonstrated in the cow [16]. Thus, a temporal association between PGF2α, cytokines (TNF, IFNG, and FASL) increased expression, and VEGF reduced expression may be a major factor determining angioregression in the equine CL (Figure 4(b)).

4. Conclusion

A subnormal CL from the immediate previous estrous cycle will not prepare the uterus optimally for that gestation, ending in abortion. Despite the seriousness of this problem, the physiologic relevance of most studies on ovarian function in woman is questionable, since they are based on knowledge generated from abnormal tissue or granulosa cells collected from *in vitro* fertilization in women subjected to exogenous supraphysiological doses of gonadotropins [10]. Therefore, several investigators have proposed that tissues of female reproductive organs could serve as a model to study tissue growth/regression and angiogenesis in general [13]. Moreover, proximities on ovarian physiology between mares and women have been recently recognized [9]. Indeed, demonstrated similarities between those species in the dynamics of follicles during the interovulatory interval and during the ovulatory follicular wave endow the equine ovary with the best experimental model for studying ovarian function regulation [9]. Thus, we expect that produced data on ovarian angiogenesis modulation in the mare will significantly contribute for a better knowledge on the molecular mechanisms regulating luteal vascular growth and regression. With a short-term goal of a supportive application on assisted reproductive technologies, generated insights are likely to contribute for fertility improvement. Moreover, angiogenesis in general is better elucidated, with special impact on vascular diseases and tumorigenesis [3].

Many of the studies here cited represent a milestone in the depiction of this complex process of angiogenesis in the CL. It is now well established how crucial the interactions between different luteal cellular departments are. To be precise, immune cells, through their secreted cytokines, target gene expression and cell viability of both endothelial and steroidogenic luteal cells. This is a tight interaction where cytokines, through their auto-, paracrine actions, are seen as the main players. Hence, immune-vascular cross-talk was shown to be determinant for both luteal establishment and regression. Understanding the molecular regulation of these interactions will contribute for a better knowledge on angiogenesis regulation in general and luteal function in particular.

Acknowledgments

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