Thrombosis and Haemostasis © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 78 (1) 672–677 (1997)

# Angiogenesis: a Dynamic Balance of Stimulators and Inhibitors

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## Angiogenic stimulators

Under the influence of specific cytokines, endothelial cells (EC) change from a quiescent state in which cell cycling is measured in terms of years, to a rapidly replicating and highly invasive phenotype reminiscent of tumor cells. As a result, preexisting microvessels greatly increase in number (angiogenesis) and play a critical role in tissue remodeling. Angiogenesis may be physiological as in endometrial cycling or pathological as occurs in wound healing or in growing tumors. Cytokines that stimulate neovascularization represent a subgroup that is collectively referred to as "angiogenic growth factors."

Basic fibroblast growth factor (bFGF) was the first angiogenic cytokine to be identified (1). Although it stimulates the division of cells other than EC, bFGF established several criteria that generally characterize angiogenic growth factors. Thus, bFGF stimulates EC proliferation in vi*tro*, induces angiogenesis *in vivo*, and is frequently present at sites of capillary growth (2–4). While proteins identified by such criteria are sufficient to induce angiogenesis (see review 4), and have been found in situ at sites of pathological angiogenesis, it has been less clear that they are, in fact, necessary for determining physiological angiogenesis i*n vivo*.

Vascular permeability factor (VPF), also known as vascular endothelial growth factor or VEGF (VPF/VEGF) (5), is another protein that satisfies the criteria of angiogenic stimulator. In addition to inducing angiogenesis, VPF/VEGF is also implicated in the vasculogenesis of development. So-called "knock out" of VPF/VEGF by homologous recombination in mice results in early embryonic lethality as blood vessels fail to develop properly (6,7). Moreover, deletion of even a single copy of the VPF/VEGF gene leads to embryonic lethality, providing the first example in which haploid gene dosage is sufficient to cause lethality at the same stage as occurs in the corresponding null animal. A growing body of data speaks to the importance of VPF/VEGF in developmental angiogenesis and in the differentiation of blood vessels in general.

VPF/VEGF was discovered in the late 1970s as a mediator of vascular permeability (8). On a molar basis, VPF/VEGF induces vascular permeability with a potency many times that of histamine or bradykinin (9). Overexpression of VPF/VEGF is associated with chronic microvascular hyperpermeability that can persist for days or even years (9–11). An important consequence of VPF/VEGF-induced hyperpermeability is the leakage of plasma proteins, resulting in an immediate alteration in the extracellular matrix. Among the plasma proteins that extravasate in response to VPF/VEGF is fibrinogen which clots upon leakage into the extravascular space to form a fibrin gel. Fibrin provides a new provisional matrix which attracts and supports the growth of endothelial cells and fibroblasts during repair, leading to angiogenesis and the synthesis of mature connective tissue (9,12). It has now become clear that extravasation of plasma proteins occurs largely by a transcellular pathway, not by an intercellular or paracellular route as had been believed. Moreover, the transcellular pathway is provided by a newly recognized organelle, the vesiculo-vacuolar organelle or VVO (13,14). VVOs are grape-like clusters of interconnecting, smooth membrane-bounded vesicles and vacuoles that extend across the entire thickness of venular endothelial cytoplasm (14). Normally closed, they open to transcellular passage in response to VPF/VEGF and other vasoactive mediators so as to allow the extravasation of plasma and plasma proteins such as fibrinogen. The molecular mechanisms by which VPF/VEGF or other mediators activate VVOs is not yet understood. Whatever the mechanisms, microvascular hyperpermeability leads to a profound alteration in the extracellular matrix, transforming it from an antiangiogenic to a proangiogenic phenotype.

Structurally, VPF/VEGF is a highly conserved, disulfide-bonded, dimeric glycoprotein of 34-45 kD (reviewed in 10, 11). Alternative splicing of its mRNA results in four transcripts that encode different sized polypeptides of 206, 189, 165, and 121 amino acids. The 165 and 121 amino acid isoforms generally predominate (15).

VPF/VEGF was originally purified from tumor cell conditioned media (8); later it became evident that this cytokine is synthesized in abundance by most animal and human tumors and by most transformed cell lines that have been examined (9–11,16). Overexpression of VPF/VEGF has also been found in non-malignant pathological situations that, like tumors, are characterized by microvascular hyperpermeability, angiogenesis, and new stroma generation.

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Thus, myocytes in hypoxic myocardium overexpress VPF/VEGF after myocardial infarction. Keratinocytes and a subpopulation of macrophages also increase VPF/VEGF synthesis during wound healing, psoriasis and delayed-type hypersensitivity (10). Lastly, VPF/VEGF is also overexpressed in placenta, fetal tissues, and in adult tissues subjected to recurrent cycles of angiogenesis (e.g., proliferating endometrium, corpus luteum).

Almost ten years after this protein was purified, VPF was cloned and renamed VEGF because of its mitrogenic activity for cultured endothelial cells (5,9). Mitogenesis, and presumably all other effects of VPF/VEGF, are mediated by two high-affinity receptors, flt-1 and KDR. These transmembrane proteins are members of the tyrosine kinase superfamily, and, with only a few exceptions, are expressed exclusively by endothelial cells (9–11).

Although it has become clear that VPF/VEGF is an important mitogen and chemoattractant for endothelial cells, its most potent activity, and one not yet attributable to any other angiogenic growth factor, is that of rendering the microvasculature hyperpermeable. Data from a number of different laboratories have demonstrated that hyperpermeability precedes and/or occurs simultaneously with angiogenesis in all cases where both functions have been assessed; e.g., in tumors, wound healing, rheumatoid arthritis, psoriasis, delayed hypersensitivity, corpus luteum formation, etc. (reviewed in 9–11).

Recently, several additional members of the VPF/VEGF family have been discovered: VEGF-B and VEGF-C, and placenta growth factor (PlGF) (17,18). Both VEGF-B and -C are strongly expressed during development at sites of angiogenesis. VEGF-C has been shown to bind to flk-1 (the mouse version of KDR) and to promote proliferation of vascular endothelial cells (17). In contrast, VEGF-B binds to flt-4, a tyrosine kinase receptor present only in lymphatic endothelium (18). At this time, very little information is available about these cytokines, and as is typical of science, there are more questions than answers. Nevertheless, the fact that VEGF-B could not compensate for the lack of VPF/VEGF in null animals or in heterozygotes suggests that these proteins do not have overlapping functions.

A final group of "potential" angiogenic factors are the ligands for the endothelial cell-specific receptor tyrosine kinases tie-1 and tie-2 (or tek) (19–22). Suppression of both receptors by homologous recombination results in neonatal death, in the case of tie-1, or early embryonic lethality, in that of tie-2 (23, 24). Examination of both types of null mice revealed severe vascular abnormalities, particularly in blood vessel architecture. Recently, one of the ligands for tie-2 has been identified and named angiopoetin (25,26). Although the direct effect of angiopoetin in angiogenesis has not been determined, null animals displayed a phenotype very similar to that of tie-2 mice (26). These findings suggest that angiopoetin function is concerned with events that follow the early morphogenesis of capillaries, but that are also necessary for the maintenance and architecture of mature capillary beds.

## Angiogenic inhibitors

The search for angiogenic inhibitors has followed two independent paths: 1) identification of inhibitors in tissues that normally lack blood vessels; and 2) identification of synthetic or natural substances that antagonize the effect of stimulators. The first approach led to the isolation of inhibitors of vessel growth that were present in cartilage (27–29); the second pathway identified several molecules that suppress blood vessel formation. Among the latter, inhibitors of prostaglandin synthesis have been shown to block the angiogenic response (30). Protamine, an antagonist of heparin, has also been shown to suppress angiogenic response (31). These inhibitors, however, do not specifically target endothelial cells and therefore offer little prospect as specific blockers of vessel growth. In 1989 Ingber and colleagues identified fumagillin (or AGM1470), a secretory product of certain fungi that effectively blocked endothelial cell migration and proliferation (32). The effect of AGM1470 appeared to be specific for endothelium, and the drug is currently undergoing clinical trials.

More recently, a fragment of plaminogen, named angiostatin (33), and a C-terminal fragment of collagen XVIII, named endostatin (34), have been found to suppress vessel growth by directly inhibiting endothelial cell proliferation. Both angiostatin and endostatin were first identified as tumor cell products. Angiostatin was found in the urine or blood of mice carrying a large tumor burden. Endostatin was isolated from cultures of hemangioma cells, tumors of endothelial cell origin, and was not present in the conditioned media of normal EC. Thus, tumor cells apparently secrete factors that provide negative "feedback" regulation and serve to suppress vascular growth; they may therefore serve to restrain the growth of secondary tumors or metastases. Although this is an intriguing possibility (34), negative dominance of the primary tumor over secondary tumors is extremely rare in vivo (35). Future studies will be necessary to determine how these factors mediate their effects at the molecular level, how they are regulated, and whether they act exclusively on tumor vasculature as compared with other vascular beds.

Thrombospondin -1 (TSP-1) has recently been identified as a negative regulator of the angiogenic response (36–38). We have devoted considerable effort to determine: 1) whether TSP-1 is indeed a physiological inhibitor of the angiogenic response; and 2) the molecular mechanisms by which TSP-1 exerts its effect. TSP-1 differs from endostatin and angiostatin in that it is found in a variety of normal adult tissues; e.g., bone, endometrium, ovary and mammary gland (39 and unpublished observations), all tissues that regularly undergo physiological remodeling and angiogenesis.

TSP-1, a high molecular weight trimeric glycoprotein originally identified in platelet a-granules, was later described as a major secretory product of several cells of the vessel wall, including smooth muscle and EC (40,41). As a component of the extracellular milieu, TSP has been shown to have autocrine functions, increasing the mitogenic response of smooth muscle cells to epidermal growth factor (42). In endothelial cells, however, TSP has been shown to inhibit proliferation (43) and to inhibit the formation of focal adhe-

sions (44). *In vivo*, TSP-1 has been found to inhibit bFGF-mediated angiogenesis in the cornea pocket assay (37) and the potential of TSP-1 to suppress capillary formation has also been addressed *in vitro* (38). An example of the anti-angiogenic effect mediated by TSP-1, as well as the balance between stimulators and inhibitors, is presented in Fig. 1. Neovascularization in this assay was driven by VPF/VEGF cast into collagen gels; TSP-1 blocked vessel progression in a concentration-dependent manner.

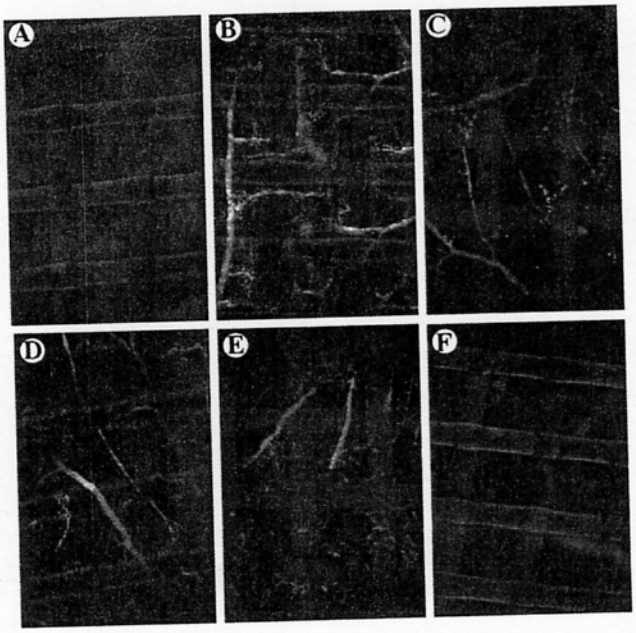


Fig.1. Angiogenic response induced by VPF/VEGF and inhibited by TSP-1. Mesh CAM assays were prepared as follows: unpolymerized type I collagen (Vitrogen) was diluted and neutralized with Hanks' balanced salt solution at a final concentration of 750 μg/ml. To aliquots of 150 μl of Vitrogen, either VPF/VEGF and/or increasing concentrations of TSP-1 were added as indicated below. Samples were mixed and 50 μl of sample was placed onto nylon meshes of 100 μm2. Each experimental sample was performed in triplicate. Samples were allowed to polymerize at 37°C for 2 h, followed by incubation at 4°C for 1h. After polymerization was complete, meshes were placed onto the chorioallantoic membrane of 12d old chick embryos and returned to the incubator for 24 h. After this time, meshes were evaluated under a dissecting microscope. The embryo was subsequently injected with FITC-dextran (0.1 mg/ml) and the meshes fixed, dissected from the CAM, mounted on histological slides and observed under a fluorescent microscope. Images were imported to a 7100 AV Power Macintosh computer for evaluation of capillary density using a Sony CCD camera. Capillary density was assessed using the program NIH Image 1.61. All counts were normalized to the negative control in each CAM (Vitrogen alone); results are expressed as percentage of control. A. **Vitr**ogen alone; B. VPF/VEGF 100 ng; C. VPF/VEGF 100 ng and 10 μg; D. VPF/VEGF 100 ng and 100 μg of TSP-1; E. VPF/VEGF 100 ng and 500 μg of TSP-1; F. TSP-1 alone, 500 ug.

In an effort to understand whether TSP-1 is indeed a natural regulator of angiogenesis, we investigated its expression and regulation in the human endometrium, a tissue that undergoes recurrent periods of vessel growth and inhibition. We found that TSP-1 is indeed temporally regulated during the endometrial cycle (39). TSP-1 expression was restricted to the secretory phase, a time in the endometrial cycle when cellular proliferation ceases. TSP-1 protein at this time was preferentially localized to the basement membrane of small capillaries of the functional endometrium (tissue that is discharged during menstruation), but not to the basal endometrium or myometrium. The specific distribution of TSP-1 is consistent with its role as a suppressor of remodeling vessels, but not of mature, non-growing vessels. In addition, we demonstrated that TSP-1 mRNA was regulated by progesterone in a dose and time-dependent manner, and that the levels of TSP-1 induced by this steroid were sufficient to inhibit EC migration and proliferation (39).

More recently, we have examined the kinetics of TSP-1 expression in the mammary gland at various stages of growth and regression. Here as well TSP-1 expression was increased significantly at times coincident with the hypothesis that TSP-1 is involved in vascular regression, since the vascular density of regressing mammary glands in TSP-1 knock-out mice was significantly higher than control animals (L. Iruela-Arispe and J. Lawler, in preparation). Together, these observations support the view that TSP-1 functions as a physiological regulator of vessel growth. Nevertheless, it is clear that TSP-1 is not the only molecule with this inhibitory effect; otherwise, the outcome of TSP-1 null mice would have been far more severe.

Now, if TSP-1 is in fact a suppressor of angiogenesis, what are the mechanisms that mediate its inhibitory effect? The biological responses of EC to TSP-1 are controversial. Paradoxically, expression of both TSP-1 mRNA and protein are increased in proliferating endothelial cells in vitro (38), whereas exogenous TSP-1 inhibits the growth of cultured EC (43). TSP-1 has been shown to mediate cell attachment (45); however, it decreases focal adhesions in EC (44). An important point to consider is the experimental conditions under which each of these studies was performed. It is well documented that the expression of several extracellular matrix proteins, as well as the cellular response to cytokines, is dependent upon the biological context in which the cell is placed. Indeed, synthesis of TSP-1 by EC is modulated by cell shape and by the nature of the substrate used in *vitro* (46).

One consistent observation has been the inhibitory effect of TSP-1 on angiogenesis *in vivo* and *in vitro* (36-38). Clearly, at this time, it will be important to determine the intermediate cellular event(s) that contribute to this inhibition, the receptor(s) involved, and the intracellular signaling pathways that ultimately mediate TSP-1's effects. The multifunctional features attributed to TSP on EC could be attributed to interactions with different TSP-receptors. In fact, six different TSP-1 receptors have been described (45,47–51). Hypothetically, unique TSP-receptor associations could account, at least in part, for the multiplicity of functions attributed to TSP, with different receptors mediating specific cellular responses. In this regard, it was essential to specify the region of TSP-1 that was responsible for the anti-angiogenic features of the protein. Dr. Bouck's group was able to determine that ~9 amino acids in the procollagen-like region and 12 amino acids in the type I-like (or properdin) repeat region conferred TSP-1's anti-angiogenic function (52). The anti-angiogenic region in the type I repeats has been shown to

bind to CD-36, a rather controversial receptor for TSP-1, expressed by a large number of cell types, including EC. At this point, there is little information regarding intracellular signaling mediated by CD-36 in EC and there is no strong evidence that CD-36 is the receptor that mediates the anti-angiogenic function of TSP-1.

A new and important consideration in the interpretation of effects mediated by TSP-1 has been the recent description of at least four novel genes in the TSP family (TSP-2, TSP-3, TSP-4, and COMP or TSP-5), all of which are homologous to platelet TSP (TSP-1) (53–59). Overall, amino acid identity is high among all members of the family and increases toward the carboxyl-terminus in all five proteins. Given the high degree of homology among the TSPs, it is possible that many of the functions initially attributed to platelet TSP (TSP-1) are shared by other members of the family, contingent on cell- or site-specific expression. In particular, TSP-1 and -2 share identical structural features and a very high degree of conservation (for a review, see 53). With regard to EC, we demonstrated that TSP-1, -2, but not -3, were expressed in in vivo. TSP-1, however, was the only protein that was secreted in the conditioned media of cultured EC and the only TSP isoform that accumulated in the basement membrane of capillaries *in vivo* (60). Treatment of endothelial cells with neutralizing antibodies specific for TSP-1 resulted in an increased number of cord-like structures *in vitro* (38). These results, along with findings in the TSP-1 null mouse mentioned previously, have favored TSP-1, among all members of the TSP family, in inhibition of capillary growth; nevertheless, they do not exclude the possibility of functional redundancy mediated by TSP-2 in the absence of TSP-1.

## Concluding remarks

This is a very exciting time for vascular biologists. The central role of blood vessel growth in normal physiology, in repair, and in pathological conditions is widely accepted. Several major players in the orchestration of neovascularization have been identified and mediators of intracellular signaling are being unraveled. A more complete understanding of this scenario will surely develop within the next five years. Questions that are now appropriate to address will relate to the specific effects of the tie-1 and -2 ligands and their functions in vascular morphogenesis, the molecular mechanisms by which VPF/VEGF promotes vascular permeability, intracellular signaling pathways initiated by angiogenic growth factors, and specific effects of the novel members of the VPF/VEGF superfamily of proteins.

With regard to the inhibitors of angiogenesis (TSP-1, angiostatin, endostatin), much more remains to be learned. Their mechanism of action and specificity (i.e., physiological and/or pathological angiogenesis) is yet to be determined. Additional inhibitory proteins and small molecules will probably be discovered, since a large effort is currently being applied to this area of research. It seems likely that organ- and endothelial cell-specificity will be of some importance in the effectiveness of various stimulators and inhibitors of angiogenesis.

The past 20 years has witnessed the identification of several major regulators of blood vessel formation and an appreciation for the interactive role of both positive and negative signals in this process. It appears that a broad understanding of the "general" mechanisms that govern angiogenesis is close at hand and that our concerns will soon turn to identification of "specific" processes involved in particular pathological or physiological settings.

## Summary

Angiogenesis, the formation of new blood vessels from a pre-existing vasculature, is tightly regulated in normal adults. Under physiological circumstances, angiogenesis occurs in only a few instances; e.g., the female reproductive system in response to ovulation or gestation, the normal hair cycle, etc. In these examples, growth of new capillaries is tightly controlled by an interplay of growth regulatory proteins which act either to stimulate or to inhibit blood vessel growth. Normally, the balance between these forces is tipped in favor of inhibition and consequently capillary growth is restrained. Under certain pathological circumstances, however, local inhibitory controls are unable to restrain the increased activity of angiogenic inducers. Thus, in wound healing, inflammation and tumors, to name just a few examples, angiogenesis is integral to the pathology, engendering the hope that these pathological entities could be regulated by pharmacological and/or genetic suppression (or enhancement) of blood vessel growth. This hope, in turn, has fostered interest in the molecular mechanisms that regulate angiogenesis. In this chapter, we have reviewed the current literature regarding some angiogenic stimulators and inhibitors, emphasizing vascular permeability factor (VPF, also known as vascular endothelial growth factor or VEGF), as a major angiogenic inducer, and thrombospondin (TSP) as the best known example of a natural inhibitor of vessel growth.

## Acknowledgements

The authors thank Ms. Carol Foss for the preparation of the manuscript. Studies presented in this manuscript were funded by NIH grants R29 CA63356-01 and Department of Defense Award #DAMD 17-94-J-4346 (to LI-A) and by NIH grants CA-50453 and HL-54465 (to HFD).

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