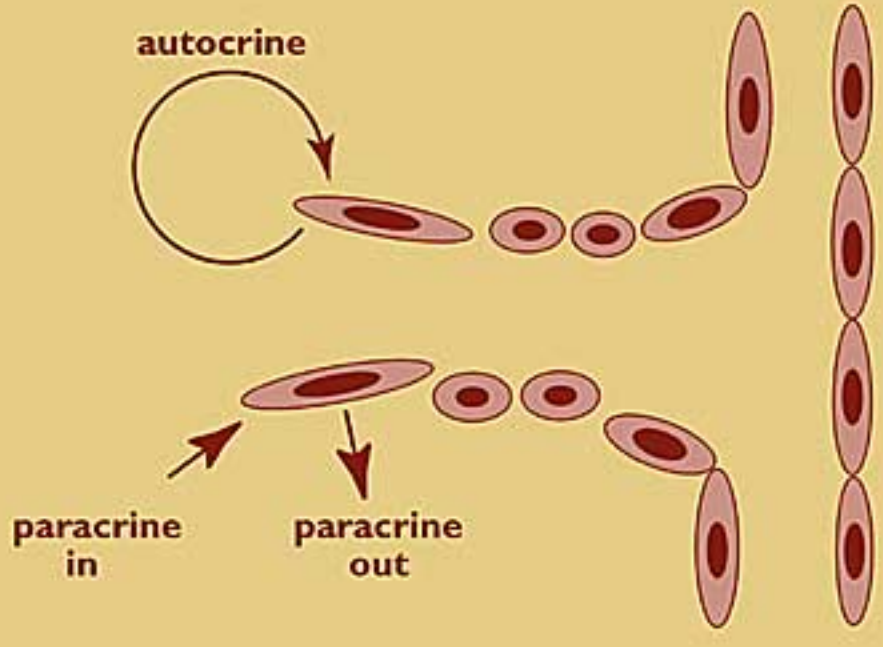
Angiogenesis in Health and Disease

Basic Mechanisms and Clinical Applications



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Antiangiogenic Function of Thrombospondin-1

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I. INTRODUCTION

Thrombospondin-1 (TSP1) is a large trimeric glycoprotein present in platelet α-granules and secreted by several cell types in the vessel wall, including endothelial and smooth muscle cells. A major function attributed to TSP1 relates to its ability to inhibit angiogenesis (1–4). The specific cellular and molecular mechanisms involved in this outcome are not entirely clear. TSP1 is known to affect migration, proliferation, and adhesion of endothelial cells (5–10), any of which could contribute to an antiangiogenic phenotype. The region of TSP1 responsible for the angioinhibitory effect has been mapped to two domains: (a) a procollagen-like sequence and (b) the type I, or properdin, repeats (4). This chapter reviews the current literature on TSP1 with emphasis on its ability to suppress blood vessel formation and, particularly, on its potential effect as an antiangiogenic agent in tumors.

Thrombospondin-1 is a member of a family of five related genes, TSP 1–4 and COMP (also known as TSP5) (reviewed in Refs. 11 and 12). Endothelial cells synthesize and secrete principally TSP1 (13). Transcripts for TSP1 and TSP2 have been observed in embryonic endothelial cells in vivo (days 9–1) (14). TSP2 has not been detected in either normal or pathological endothelium from adult animals, nor is TSP2 secreted by endothelial cells grown in vitro (13). Interestingly, no other TSP1 member has been detected in endothelium. Structurally, the presence of the type I or properdin repeats at the amino terminus is the main difference between TSP1/2 and all other TSP proteins (TSP3-5).

The best-characterized biological functions attributed to TSP1 have been its ability to activate TGF-β and to inhibit angiogenesis. It appears that the effects of TSP1 in the suppression of vascular growth are independent of those related to the activation of TGF-β, as will be discussed later. Most of the effects on angiogenesis have been demonstrated in vitro or by the use of specific in vivo assays, such as the chorioallantoic membrane (15) and cornea pocket assays (4). Systemically, only peptide mimetics to the protein have been used effectively in tumor assays (16), as the peptides have shown to reduce tumor

growth. The relative role of TSP1 as an endogenous regulator of angiogenesis is presently unknown.

A mouse model that lacks TSP1 has been generated by homologous recombination (17). Unexpectedly, the phenotype displayed by the TSP1 knockout mouse includes pulmonary chronic inflammation and pneumonia (17). No major effect in blood vessel number was reported, however, due to the significant level of homology with TSP2, functional redundancy, and compensation for the lack of TSP1 it is likely to occur. Interestingly, the TSP2-null mouse showed enhanced vascularity with an apparent twofold increase in the number of dermal capillaries (18).

Due to the likelihood of functional compensation by TSP2 and because analysis of loss of function of a vascular inhibitor might be difficult to evaluate, we decided to generate transgenic lines that overexpress TSP1 and ask whether the protein might function as a regulator or angiogenesis in a developmental setting (19). It was our premise that increased levels of an angiogenic inhibitor might be more revealing than its complete absence from the onset of embryonic development. The organ of choice for overexpression of TSP1 was the mammary gland. Upon pregnancy, the mammary gland grows significantly. Its vascular bed also expands to compensate for the increased metabolic demands of the organ upon lactation. Finally, involution is also accompanied by reduction of capillary density and vascular remodeling. Therefore this is an excellent model system in which to ask questions related to endogenous regulation of vascular growth and regression in an adult tissue.

Transgenic animals were generated by injection of blastocysts with a construct containing the mouse mammary tumor virus (MMTV) promoter driving the hTSP1 complete cDNA. The MMTV promoter directed expression primarily to the mammary gland. Four transgenic lines with varied levels of human TSP1 protein were obtained. Vascular density in mammary glands was examined upon systemic injection of FITC-dextran (19) and by immunocytochemistry of tissue sections (Fig. 1). In all four lines, increased levels of hTSP1 was associated with significant decrease in capillary density. These transgenic

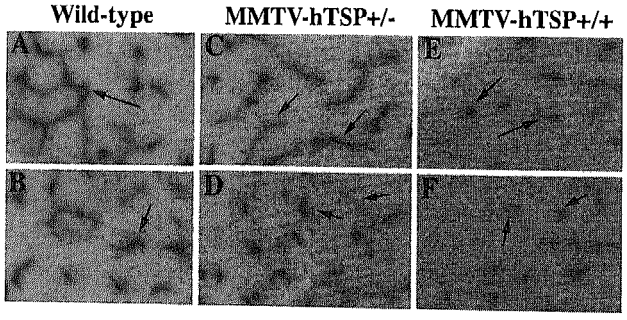

Figure 1 Overexpression of TSP1 in the mammary gland is associated with reduced capillary density. Generation of transgenic animals overexpressing TSP1 was done by injection of lincarized MMTV-hTSP1 DNA into murine blastocysts. The murine mammary tumor virus (MMTV) targeted expression of TSP1 to the mammary gland. Evaluation of the mammary gland vasculature was performed by staining 30-μm sections with *Bandeira simplicifolia* lectin. (A) and (B). Mammary glands from wild-type animals, note stained vessels (arrows); (C) and (D). Mammary vessels from heterozygous animals; and (E) and (F). Mammary vessels from homozygous transgenics. Note reduction in the vascular network in E and F when compared to other panels.

lines showed between two- to sevenfold upregulation in TSP1 protein and consistently showed a 52–73% reduction in the number of capillaries (19). To our knowledge this is the first demonstration that titration of endogenous levels of TSP1 modulates angiogenesis and capillary density in vivo.

It has been our premise that potent and tissue-specific inhibitors of angiogenesis are constantly expressed in tissues providing vascular surveillance. Such molecules would function to regulate the pattern of new vessel growth and to maintain homeostasis of established tissues. Our recent results on mammary gland–targeted expression of TSP1 clearly demonstrate that this molecule has considerable angiostatic/antiangiogenic activity and that it is likely an endogenous modulator of physiological angiogenesis. This concept is also supported by the pattern of expression displayed by TSP1 in the human endometrium (20). The primate endometrium is characterized by recurrent cycles of growth during the proliferative phase (first half of the menstrual cycle) and growth inhibition in the secretory phase (second half of the menstrual cycle). We found that TSP1 is expressed in the secretory phase and is induced by progesterone (20). In fact, conditioned medium from progesterone-treated stromal cells suppresses endothelial cell proliferation in vitro. The inhibition appears to be mediated by increased levels of TSP1 in the conditioned medium, since neutralizing antibodies to TSP1 block the inhibitory effect (20).

II. EFFECTS OF TSP1 ON ENDOTHELIAL CELLS

Addition of TSP1 protein to endothelial cells in culture has been shown to inhibit proliferation (5), to induce attachment but not necessarily spreading (6,10), and to disrupt focal adhesions (6).

The domains of TSP1 that account for its antiproliferative and antiadhesive activity are located at the amino-terminal end. Based on inhibition by monoclonal antibodies, the heparin-binding domain might regulate endothelial cell proliferation (20,21); however, a 140-kDa fragment of TSP1 lacking the heparin-binding domain also suppresses endothelial cell growth (1).

The effects of TSP on endothelial cells are further complicated by its interaction with a variety of other extracellular macromolecules and growth factors (11,12). In the presence of type I collagen, TSP1 appears to facilitate rather than inhibit tube formation in vitro (22), and TSP1 binds and activates TGF-β (23). Therefore, the nature of the extracellular environment or the state of TSP1 (i.e., bound or in solution) might modulate the responses of TSP1 on capillary morphogenesis through the activation of different receptor subsets.

Currently three domains of TSP1 have been implicated in interactions with endothelial cells: (a) $\alpha v\beta 3$, binds to an RGD sequence located in the carboxyl-terminus of TSP1 (last type III repeat) (7), (b) CD-36, binds to the second and third type I repeats (24–26), and (c) a partially characterized receptor that binds to the type I repeats (27). Four additional receptor activities have been characterized in other cells (Table 1); however their relevance to cells of the vascular wall has not been established.

The antiproliferative effect mediated by TSP1 is specific to endothelial cells; in fact, smooth muscle cells spread and proliferate in response to TSP1. The role of TSP1 in smooth muscle cell proliferation was initially demonstrated by Majack (31). In these studies, monoclonal antibodies directed against several regions of TSP1 inhibited the proliferation of aortic smooth muscle cells. We have been able to confirm Majack's experiments

Table 1 Binding Sites Present in TSP1, Receptors, and Cell Types in Which These Receptors Have Been Shown to Bind to TSP1

Binding sequence	Receptor	Cell type	Reference
Type I repeats and heparin-binding	Glycoconjugates (includes heparin, fucoidin, and sulfatide)	Melanoma cells	34
TSP1 (site not defined)	LRP	Fibroblasts, smooth muscle cells	28
CSVTCG	CD-36	Endothelial cells, platelets, monocytes adipocytes, tumor cells, epithelial cells	24–26
GRDS	$\alpha_4\beta_1$ and $\alpha_5\beta_1$	Lymphocytes,	29
	$\alpha_v\beta_1$	Endothelial, smooth muscle	7
RFYVVMWK and FIRVVMYEGKK	Carboxyl-terminal receptor (52-kDa)	K569 cell line	30

with purified TSP1 in the absence of TGF-β1 as well as with recombinant truncated fragments corresponding to the TSP1 amino-terminal region.

It is likely that cell-specific effects of TSP1 reflect differences in the type and/or number of cell-surface receptors utilized by endothelial and smooth muscle cells to bind to TSP1. To date, seven TSP1 receptors have been identified (Table 1), but little of their signal transduction pathways or functions has been elucidated. One might anticipate that occupancy of different TSP1 receptors would result in different metabolic changes, such as protein phosphorylation, with alternate consequences on gene expression in different cell types. The search for signaling pathways and also for the identification of the minimal region responsible for TSP1 effect in the vasculature has engendered a great deal of interest and led to the identification of the region(s) that effectively inhibit angiogenesis.

III. ANTIANGIOGENIC DOMAINS IN THROMBOSPONDIN

Two regions of TSP1—the procollagen-homology sequence and the type I repeats—were initially identified as domains that could independently suppress angiogenesis (4). Subsequently, a more detailed series of structure-function analyses indicated that the amino acid sequence associated with the CD36 binding domain, located in the second and third type I repeats, was the specific region that conferred angiosuppressive features to TSP1 (32). In addition, the same study implicated CD36 as the signaling receptor for the antiangiogenic effects. We and others have found that the procollagen region is not effective in suppressing angiogenesis in the chorioallantoic membrane or in endothelial cell proliferation (15).

The relative contribution of the type I repeats to the inhibitory signals mediated by TSP1 has been studied in the cornea pocket (4) and chorioallantoic membrane (15); both assays concur that this region of the molecule is effective in blocking angiogenic signals by growth factors. TSP1 contains three tandem type I or properdin repeats; nevertheless,

only the second and third repeats appear relevant to the suppressive vascular effect of TSP1 in the cornea pocket assay (4). We have examined the role of the second and third type I repeats in the inhibition of tumor growth and vascularization using tumor xenographs. Briefly, HT1080 human tumor cell lines were transfected with a truncated construct containing either all three or only the first type I domains. The resulting stable lines were tested for secretion of recombinant protein and injected into the dorsal flanks of nude mice. The results of these experiments showed that the second and third type I repeats but not the first repeat are essential to the antiangiogenic response mediated by TSP1 (Fig. 2). In addition, peptide mimetics of the type I repeats as well as truncated recombinant proteins containing these regions were able to antagonize growth factor–mediated angiogenesis in the chorioallantoic membrane (15,33) and suppress growth of tumors in nude mice (16).

The type I repeats of TSP1 have been characterized extensively (4,23,34). The second type I repeat of TSP1 and shown to (a) bind heparin in three regions (KRFK, WSPW, CSVTCG), (b) activate latent forms of TGF-β via a nonproteolytic pathway (KRFKR), (c) bind sulfatide, (d) promote cell adhesion, and (e) inhibit angiogenesis through the CSVTCG sequence.

Recently, much interest has focused on the role of TSP1 as an activator of latent TGF-β. In fact, the pulmonary phenotype displayed by the TSP1 knockout mouse can be compensated and reverted by systemic injection of peptides containing the TGF-β activating sequence (KRFK) (35). However, it is important to note that peptides that activate TGF-β do not appear to have a bearing on the antiangiogenic functions of TSP1. Peptides mimetics to the amino-terminal region of the second type I repeats containing mutations in the KRFK sequence are as efficient as wild-type peptides in suppressing tumor growth in nude mice (16) and inhibiting angiogenesis in the chorioallantoic membrane (33).

Additional dissection of the second and third type I domains has been performed by several laboratories using nonoverlapping assays. These studies have generated more specific information as to which amino acids, within the type I repeats, are responsible for the antiangiogenic effect of TSP1. Using the cornea pocket assay, Bouck and col-

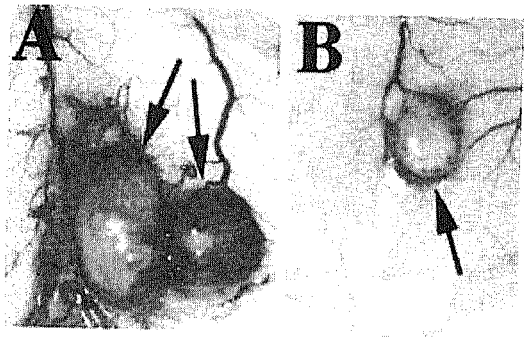

Figure 2 Effect of TSP1 overexpression on tumor growth. Truncated TSP1 mammalian expression constructs expanding from the amino-terminus until the first type I repeat (A) or containing all three type I repeats (B) were used to transfect HT1080 fibrosarcoma cell lines. The resulting stable lines were used to generate tumors in immunocompromised mice. Dorsal injection of 10^5 cells was allowed to grow as a tumor for 5 weeks. Examination of the resulting tumors revealed that tumors from cells transfected with all three type I repeats were significantly smaller (B) than the ones resulting from cells containing the first type I repeat alone (A).

leagues found that the CD-36 binding sequence (located in middle region of the second and third type I repeats) is the most active angioinhibitory region (32). Roberts and colleagues have generated a series of peptide mimetics to the amino-terminal region of the second and third type I repeats. By injecting these peptides into nude mice bearing tumors, they demonstrated that this region is also effective at suppressing vascularization and tumor growth (16). Since the assays are quite different, it is difficult to compare the results directly and evaluate if these regions are equally effective at inhibiting angiogenesis. In collaboration with Roberts, we set out to perform structure-function analysis of the type I repeats utilizing recombinant protein and peptides in the chorioallantoic membrane (33). The objective was to evaluate side by side and in the same assay the efficacy of these subregions within the type I repeats. Our results were clear and revealed that both regions are equal contributors to the antiangiogenic effect mediated by TSP1. The findings also revealed growth-factor selectivity. While the amino-terminal end of the type I repeats suppressed angiogenesis driven by bFGF, the sequence carboxyl-terminal to that (the region that binds to CD36) was able to suppress angiogenesis driven by either bFGF or VEGF (33).

IV. TSP1 AS AN INHIBITOR OF ANGIOGENESIS IN TUMORS

The dependency of tumor growth on neovascularization was demonstrated over three decades ago by Folkman and colleagues (reviewed in Refs. 36 and 37). The initial studies demonstrated that tumors maintained in organ culture grew to approximately 1–2 mm in diameter, after which no further increase in total mass was observed regardless of time in culture (38). In contrast, tumors introduced into the rabbit cornea within 6 mm of the iris promoted neovascularization, grew exponentially, and metastasized (39). These findings implicated an absolute requirement for angiogenesis in the progression of tumor growth. Thirty-five years later, this original hypothesis has gained solid acceptance, and the basic observation has been validated in a variety of tumors including cutaneous malignant melanomas (40), breast carcinomas (41), and brain tumors (42), among others. Indeed, tumor-mediated angiogenesis has been identified as a significant and independent prognostic indicator of early-stage breast carcinoma, among several other tumor types, as well as a prerequisite for the expansion and metastatic progression of malignant disease (43,44).

The possibility that tumor progression could be regulated by pharmacological and/or genetic suppression of blood vessel growth has engendered a long-standing interest in the identification of molecules or synthetic compounds that block angiogenesis.

It has been our premise that endogenous physiological regulators of vascular growth are also likely to be effective at suppressing the vascularization of tumors, offer no resistance, and perhaps add tissue-selectivity to the process. TSP1 and related molecules/genes could likely be among such candidates. Injection of TSP1, truncated proteins, and peptide mimetics has proven effective in the suppression of vascular growth of tumors and the inhibition of tumor growth itself (16). Questions that still remain relate to tissue-specificity of the effect mediated by TSP1 and its relative potency as compared with other angiogenic inhibitors. Finally, the unraveling of TSP1-mediated signal transduction pathways in endothelial cells will be fundamental to our understanding of the effects mediated by this protein in the suppression of vascular growth.

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