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RESEARCH ARTICLE

Thrombospondins function as regulators of angiogenesis

Paul Bornstein

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Abstract Thrombospondins (TSPs) -1 and -2 were among the first protein inhibitors of angiogenesis to be identified, a property that was subsequently attributed to the interactions of sequences in their type I repeats with endothelial cell-surface receptors. The interactions of TSPs-1 and -2 with cell-surface receptors, proteases, growth factors, and other bioactive molecules, coupled with the absence of direct structural functions that can be attributed to these matrix proteins, qualify them for inclusion in the category of 'matricellular proteins'. The phenotypes of TSP-1, TSP-2, and double TSP-1/2-null mice confirm the roles that these proteins play in the regulation of angiogenesis, and provide clues to some of the other important functions of these multi-domain proteins. One of these functions is the ability of TSP-1 to activate the latent TGFβ1 complex, a property that is not shared by TSP-2. A major pathway by which TSP1 or TSP2 inhibits angiogenesis involves an interaction with CD 36 on endothelial cells, which leads to apoptosis of both the liganded and adjacent cells. However a homeostatic mechanism, which inhibits endothelial cell proliferation, and may be physiologically preferable under some circumstances, has also been elucidated, and involves interaction with the very low density lipoprotein receptor (VLDLR). The interaction of TSP1with its receptor, CD47, further inhibits angiogenesis by antagonizing nitric oxide signaling in endothelial and vascular smooth muscle cells. Paradoxically, there is also evidence that TSP-1 can function to promote angiogenesis. This apparent contradiction can be explained by the presence of sequences in different domains of the protein that interact with different receptors on endothelial cells. The anti-angiogenic function of TSPs has spurred interest in their use as anti-tumor agents. Currently, peptide mimetics, based on sequences in the type I repeats of TSPs that have been shown to have anti-angiogenic properties, are undergoing clinical testing.

Keywords Angiogenesis · Anti-angiogenic therapy · CD36 receptor · Homeostatic function · Knockout mice · Matricellular · Nitric oxide · Peptide mimetics · Pro-apoptotic mechanism · Thrombospondin · VLDL receptor

Abbreviations

EC	endothelial cells
ECM	extracellular matrix
FGF2	fibroblast growth factor 2
IAP	Integrin-associated protein
MMP	matrix metalloproteinase
NO	nitric oxide
TSP	thrombospondin
TGFβ1	transforming growth factor β1
VLDL	very low density lipoprotein
VLDLR	VLDL receptor

P. Bornstein (✉)
Departments of Biochemistry and Medicine,
University of Washington,
Seattle WA 98195, USA
e-mail: bornsten@u.washington.edu

Present Address:
P. Bornstein
P.O. Box 219, Tesuque, NM 87574, USA

Introduction

The term 'thrombospondin' (TSP) was coined by Jack Lawler and coworkers to identify a protein that was released and purified from thrombin-treated platelets (Lawler et al.

1977, 1978). The protein was concentrated in the 'particulate' fraction of platelets, now known to contain the α granules, and was shown to be composed of three disulfide-bonded chains, with a total molecular mass of over 4.5×10^5 daltons. These studies expanded and corrected earlier work by Baenziger et al. (1971, 1972) who had identified a 'thrombin-sensitive protein' in platelets. However, exposure of purified thrombospondin (TSP) to thrombin failed to show significant sensitivity to this enzyme (Lawler et al. (1978). Subsequently, TSP, now known as TSP-1, was shown to be synthesized and secreted by a wide variety of cells in culture, including aortic, venous, capillary, and corneal endothelial cells (EC; McPherson et al. 1981; Sage et al. 1981; Mosher et al. 1982), and by fibroblasts (Jaffe et al. 1983) and smooth muscle cells (Raugi et al. 1982).

The TSP 'family' consists of five members (Fig. 1). A second expressed gene was detected by the sequencing of a mouse genomic clone. The sequence was clearly homologous to that of the human TSP gene, but when the corresponding mouse cDNA was sequenced it was evident that the cDNA was the product of a different gene, *Thbs2*, encoding TSP-2 (Bornstein et al. 1991). A third member of the TSP family, TSP-3, was identified shortly thereafter, in both the human and mouse genomes, as a consequence of its close proximity to the *MUC1/muc1* genes, encoding human and mouse episialin, respectively. Less than 3 kilobases separate the two genes in both genomes (Vos et al. 1992).

The structures of all five TSPs are shown schematically in Fig. 1. As can be seen, TSP-3 differs from TSP-1 and TSP-2 in lacking the procollagen homology domain and the three type I repeats. Because the type I repeats play a major role in the anti-angiogenic properties of TSPs 1 and 2, it is not surprising that TSP-3 appears to lack this function (see review by Hankenson and Delany in this issue). A fourth TSP, TSP-4, was first identified by Lawler and coworkers in *Xenopus*, and subsequently in human tissues (Lawler et al. 1993). Its molecular structure is similar to that of TSP-3 (Fig. 1). While single nucleotide polymorphisms in TSP-4 have been reported to be associated with coronary artery disease in some Western populations (Stenina et al. 2004), there is currently no evidence for an anti-angiogenic function for this protein. Finally, cloning and sequence analysis of cartilage oligmeric matrix protein (COMP; Oldberg et al. 1992; Hedblom et al. 1992) revealed it to be a member of the TSP family, and homologous to TSP-3 and TSP-4 (Fig. 1). Although the term, COMP, is still used frequently in the literature, the protein is also expressed in tendon, ligament, and blood vessels, in addition to cartilage. Therefore, the term TSP-5 seems more appropriate. Despite initial published claims to the contrary, there is currently no good evidence for alternative splicing of TSP mRNAs or for heteropolymers of the trimeric TSPs. However, heteropentamers of TSP-4 and TSP-5 have been shown to exist in normal tendon and ligament (Hecht et al. 1998; Södersten et al. 2006).

The identification of TSP-1 and TSP-2 as inhibitors of angiogenesis

The involvement of TSP-1 in the regulation of angiogenesis was first recognized by Good et al. (1990). These workers purified a factor, secreted by baby hamster kidney (BHK) cells in culture, that inhibited the migration of bovine adrenal capillary EC, as well as corneal neo-vascularization that was stimulated by fibroblast growth factor 2 (FGF2). Expression of the factor was linked to that of a tumor suppressor gene in BHK cells (Rastinejad et al. 1989), and its characterization revealed an identity with a C-terminal sequence in TSP-1. Good et al. also demonstrated that TSP-1 inhibited EC migration and proliferation in vitro. At about the same time, Taraboletti et al. (1990) showed that TSP-1 both

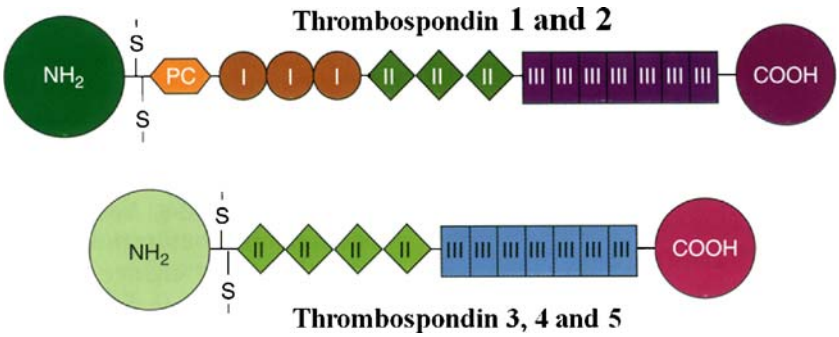

Fig. 1 A schematic representation of the structures of the individual chains in the thrombospondins. TSP-1 and TSP-2 are trimers and TSP-3, -4, and -5 are pentamers. The sizes and amino acid sequences of the NH_2-terminal domains vary considerably among the TSPs; in the case of TSP-5 this domain consists of only a few amino acids. The oligomerization domain, containing the interchain disulfide bonds, is followed by a procollagen homology domain (PC), also known as a von Willebrand type C repeat, and in the case of TSP-1 and TSP-2, by three type I (thrombospondin structural or properdin-like) repeats. All TSPs have type II (EGF-like) and type III (calcium binding) repeats, and a COOH-terminal domain. Figure and legend are reproduced with permission from Bornstein, P. 'Matricellular Proteins' in Encyclopedia of Respiratory Medicine , G.J Laurent and S.D. Shapiro, Eds. Elsevier Limited, Oxford, UK; Volume 2, pp 175–183, 2006

induced spreading of bovine aortic EC, and inhibited the mitogenic effects of serum and FGF2 on pulmonary capillary EC. These authors therefore also suggested that TSP-1 had anti-angiogenic properties. Subsequently, Tolsma et al. (1993) performed limited digestion of TSP-1 with chymotrypsin to produce fragments that inhibited rat corneal vascularization and invasion of polyvinyl sponges implanted in mice, as well as migration of EC in vitro. These peptides were derived primarily from the procollagen homology region and the second and third type I repeats in the 70 kD central 'stalk' region of the protein (see Fig. 1). In subsequent studies, Iruela-Arispe et al. (1999) used the chicken chorioallantoic membrane (CAM) and EC proliferation assays to confirm the inhibition of angiogenesis by the second and third type I repeats. However, these investigators found no activity in the first repeat or in the procollagen homology region. Importantly, the capacity of a particular sequence to serve as a binding site for an angiogenic agent depended on the nature of the angiogenic stimulus. Thus, differences in receptor sequences in TSP-1 were observed for FGF2 and vascular endothelial growth factor (VEGF). These findings were supported by the earlier work of Taraboletti et al. (1997), who showed that FGF2 bound specifically to a 140 kDa fragment of TSP-1. Many of these early studies have been ably reviewed by Chen et al. (2000). Documentation of the anti-angiogenic effects of TSP-2 has also been published (Volpert et al. 1995; Panetti et al. 1997; Noh et al. 2003; Simantov et al. 2005; see also the Section on the TSP-2-null mouse below).

Inhibition of angiogenesis is a consequence, in part, of reorganization of the actin cytoskeleton and disassembly of focal adhesions in EC by 'counteradhesive' proteins such as TSP-1 and -2, tenascin C, and SPARC (Sage and Bornstein 1991). For TSP-1, these effects are mediated by the N-terminal domain of the cell-surface protein, calreticulin (Goicoechea et al. 2000, 2002). More recently, TSP-1 has been shown to inhibit not only cell-matrix interactions, but also cell-cell interactions in EC. This function is achieved by the phosphorylation of components of the cell-to-cell adherens junction or zonula adherens, and is mediated by the activation of two protein kinases, EGFR and ErbB2 (Liu et al. 2009).

The phenotypes of TSP-1 and/or TSP-2-null mice provide important clues to the functions of these TSPs in the regulation of angiogenesis

Disruption of all five TSP genes, individually, has been achieved in mice, but the phenotypes of TSP-1 and TSP-2-null (knockout) mice are most relevant to this review. The phenotype of the TSP-1 knockout mouse as reported initially by Lawler et al. (1998) was limited to increased embryonic lethality, a mild spinal lordosis, and a propensity for pulmonary infections. While the infections are thought to reflect the lack of the chemotactic activity of TSP-1 for inflammatory cells in TSP-1-null mice, these findings did not reveal the important role that this protein is now known to play in the regulation of angiogenesis. However, subsequent studies have expanded our knowledge of this phenotype considerably. Thus Wang et al. (2003) reported that retinal vascular density was increased in TSP-1-null mice, compared with wild-type mice, and that TSP-1-null mice were less sensitive to hypoxia-mediated vessel obliteration and had a reduced rate of apoptosis of endothelial cells. Furthermore, Malek and Olfert (2009) have recently demonstrated an increased density of capillaries in cardiac and skeletal muscle in TSP-1-null mice, which was manifested as an increase in heart mass and in exercise capacity. These findings are consistent with our current knowledge of the function of TSP-1 as an important inhibitor of angiogenesis.

In contrast, the phenotype of the TSP-2 null mouse (Kyriakides et al. 1998) is marked by obvious abnormalities in the extracellular matrix (ECM) of skin, tendons and ligaments, and by increased vascularity in healing wounds and in the foreign body reaction (Kyriakides et al. 1999; Kyriakides and Bornstein 2003). As a consequence of this phenotype, wound healing is accelerated and the performance of implanted sensors and delivery devices is likely to be improved. However the importance of TSP-1 was revealed in the phenotype of double TSP-1/TSP-2 null mice (Agah et al. 2002). In these mice, wound healing is delayed, and the granulation tissue of healing wounds is not excessively vascularized. We can therefore conclude that the absence of TSP-1 determines the course of wound healing in TSP-1/TSP-2 null mice. Because TSP-1 is expressed earlier than TSP-2 in the course of wound healing, and the pro-inflammatory function of TSP-1 is necessary for normal wound healing, we propose that the complex program of wound repair, including the influx of blood vessels, is delayed and disrupted in double-null mice.

The functions of TSP-1 and TSP-2 that regulate angiogenesis are determined by specific domains in these modular proteins

The complex domain structures of TSP-1 and TSP-2 are illustrated in Figs. 1 and 2. As matricellular proteins, TSP-1 and TSP-2 function as regulators of multiple cell-cell and cell-ECM interactions and also bind directly to growth factors, cytokines, and proteases (Lawler 2000; Adams 2001; Bornstein 2001; Bornstein and Sage 2002; Lawler 2002). Figure 2 depicts some of the more important interactions with macromolecules and cell-surface receptors

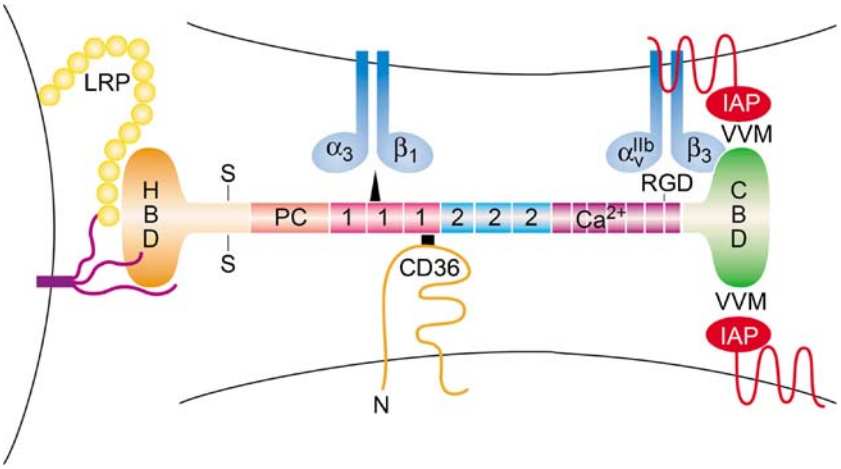

Fig. 2 A schematic representation of a generic TSP1 or TSP2 monomer together with the location of the binding sites for some of the major receptors with which the protein interacts, based on what was known in 2001. Changes resulting from more recent information are in [ ]. The structure of the chain is described in the legend to Fig. 1. The NH2-terminal heparin-binding domain (HBD) interacts with cell-surface heparan sulfate proteoglycans (purple), and the low density lipoprotein receptor-like protein,[LRP1], (yellow circles); the type 1 repeats bind to α3β1 integrin [as well as to other integrins] and CD36, [but more avidly with the HBD. Three additional integrins, α4β1, α6β1, and α9β1 also bind to the HBD]; the RGD sequence in the last Ca^{2+}-binding type 3 repeat interacts with integrins αvβ3 and α2bβ3. The COOH-terminal cell-binding domain (CBD) contains two valine-valine-methionine (VVM) sequences that may interact with two integrin-associated protein (IAP/CD47) receptors on different cells, [but these interactions are now controversial]. More information on these interactions and on interactions with other receptors is provided in the text and in Table 1. The modified figure legend and figure are reproduced from Brown and Frazier (2001), with permission from *Trends in Cell Biol*

that mediate the regulation of angiogenesis by TSP-1 and TSP-2, and Table 1 provides a more complete list of these interactions and their functions. Much of this information has been gathered from the use of synthetic sequences of TSPs that encompass one or more of these domains, and of monoclonal antibodies that recognize specific domains in these proteins (Annis et al. 2006).

The NH_2-terminal, heparin-binding domains of TSP-1 and TSP-2 bind heparan sulfate proteoglycans, which function as co-receptors for the low density lipoprotein receptor-related protein (LRP1). LRP1 is a scavenger receptor that functions to clear complexes of TSP-1, or TSP-2, with MMP2, MMP9, or VEGF from the pericellular environment of mesenchymal cells (Yang et al. 2001; Hahn-Dantona et al. 2001; Greenaway et al. 2007). The reduction in protease activity has an inhibitory effect on angiogenesis (Rodriguez-Manzaneque et al. 2001) and influences the structure of the ECM (Agah et al. 2005). As shown in Table 1, the NH_2-terminal domains of TSP-1 and TSP-2 also interact with a number of integrins that have varied effects on the angiogenic response (see Table 1 for references). For example, the interaction with α3β1 integrin has been implicated in the angiogenic functions of TSP-1 (Chandrasekaran et al. 2000; see below)

The type I repeats mediate important interactions of TSP-1 and TSP-2 that include those with the small latent TGFβ1 complex (Schultz-Cherry et al. 1995; Crawford et al. 1998; Ribeiro et al. 1999) and CD36 (see below and Table 1). TSP-1 has been shown to promote the mobilization of matrix-bound FGF-2 by interaction of FGF2 with its type I repeats, thus inhibiting the proliferation of EC (Margosio et al. 2003). It is important to note that the activating sequence, KRFK, in TSP-1 is replaced in TSP-2 by KRIR, which prevents the activation of latent TGFβ1 by TSP-2 (Schultz-Cherry et al. 1995). This difference between the two paralogs accounts for a significant fraction of the differences in their properties. Additional interactions of the type I repeats are summarized in Table 1. It is of interest that subdomains of the type I repeats have been identified that can discriminate, in their inhibitory capacity, between the stimulation of angiogenesis caused by FGF2 or VEGF, (Iruela-Arispe et al. 1999).

The type II and type III repeats were thought initially to be responsible for the adhesion of TSPs to EC, a property that was attributed chiefly to the RGD sequence that is present in the third type III repeat in both TSP-1 and TSP-2 (see Fig. 2). However, more recent studies (see Chandrasekaran et al. 2000) have not confirmed these findings and suggest that the interaction of β-1 integrins on the surface of endothelial cells with the N-terminal domain represents the major mechanism for their adhesion to TSP-1

The COOH-terminal domain of TSP-1 contains two sequences that share the adhesion motif, VVM, and are recognized by CD47, also known as integrin-associated protein (IAP); Kosfeld and Frazier 1993; Brown and Frazier 2001). However, only the second VVM sequence

Table 1 Interactions and functions of specific domains in TSP-1 and TSP-2 in the regulation of angiogenesis*

Domain	Binding Receptors or Molecules	Functions	References
NH_2-Terminal	HSPGs	Adhesion of EC; co-receptor for LRP1	[Adams, 2001; Yang et al, 2001; Lawler, 2000]
	LRP1	Clearance of MMPs 2 and 9	[Yang et al, 2001; Hahn-Dantona et al, 2001] [Chandrasekaran et al, 2000]
	α3β1	Stimulates angiogenesis; adhesion, spreading and chemotaxis of cells**	[Calzada et al, 2003]
	α6β1		
	α9β1	Adhesion of HUMVEC to immobilized TSPs; chemotaxis of EC to soluble TSPs Stimulates angiogenesis	
	α4β1	Supports adhesion of venous EC and mediates chemotaxis of microvascular EC	[Staniszewska et al, 2007] [Calzada et al, 2004a]
Type I Repeats	Small latent TGFβ1 complex	Activation of latent TGFβ1**	[Schultz-Cherry et al, 1995; Ribeiro et al, 1999]
	CD36	Induces apoptosis in EC in vitro and in tumor-derived blood vessels in vivo	[Jimenez et al, 2000; Dawson et al, 1997]
Types I and II Repeats	pan-β1 integrins		
Type III Repeats	αvβ3, αIIbβ3	Modulate EC adhesion, inhibit EC migration	[Calzada et al, 2004b; Short et al, 2005]
		Adhesion of EC	[Adams, 2001; Lawler & Hynes, 1989]
COOH-Terminal	CD47/IAP	Antagonizes NO-mediated vasodilation	[Isenberg et al, 2006]
	αvβ3, αIIbβ3	Adhesion and spreading of EC and platelets	[Brown and Frazier, 2001]

HSPGs heparan sulfate proteoglycans; *HUMVEC* human umbilical vein endothelial cells; *EC* endothelial cells

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** Function not shared with TSP2

is exposed in the crystal structure of TSP-1. CD47 in TSP-1 associates with a number of integrins to promote the spreading of platelets, EC, and other cells. Recently, CD47 has been shown to be involved in the inhibition of vascular cell responses to nitric oxide (NO) by TSP-1 (Isenberg et al. 2006; see section on nitric oxide). Since TSP-2 lacks the second VVM sequence present in TSP-1, the functions resulting from the interaction of TSP-1with CD-47 may not be shared by TSP-2.

TSP1 and TSP2 inhibit angiogenesis by a pro-apoptotic mechanism that is mediated by their interaction with CD36

Guo et al. (1997) were the first investigators to document that TSP-1, and peptides derived from its type I repeats, cause apoptosis in cultured EC. A major source of anti-angiogenic peptides is the 36 kDa fragments generated by cleavage of either TSP-1 or TSP-2 by the ADAMTS1 proteinase (Lee et al. 2006). Apoptosis, assessed by morphological changes and DNA fragmentation, did not depend on activation by TGF-β1, and was reduced when cells were confluent or were plated on fibronectin. At about the same time, Dawson et al. (1997) showed that the inhibitory effect of TSP-1 on angiogenesis was dependent on the level of expression of the transmembrane receptor, CD36, in human umbilical vein endothelial cells. An 88 kDa glycoprotein, CD36 was known to be an adhesion receptor for TSP-1 (Asch et al. 1993) and serves as a scavenger receptor for anionic phospholipids, apoptotic cells, and rod outer segments in the retina (see Dawson et al. 1997 for references). The clearest evidence that TSP-2 also interacted with CD36 to inhibit angiogenesis has been provided by Simantov et al. (2005). These authors also showed that histidine-rich glycoprotein (HRGP), interacted with TSP-1 and -2, thus acting as a decoy receptor and an inhibitor of the anti-angiogenic functions of the TSPs. It is of interest that HRGP contains a so-called CLESH domain, a region homologous to the binding site for TSPs in CD36. Some of the earlier history of the pro-apoptotic effects of TSP-1 has been described in previously published reviews (Lawler 2002; Armstrong and Bornstein 2003). Comprehensive reviews of the molecular mechanisms involved in the pro-apoptotic functions of TSP-1 and TSP-2, including the role of CD36 in these processes, have recently been published (Silverstein and Febbraio 2007; Mirochnik et al. 2008)

The signaling pathway that leads from engagement of CD36 by TSP-1 or TSP-2 on one cell to the activation of

caspases, both in that cell and in adjacent cells, was elucidated by Jimenez et al. (2000). An abbreviated scheme of this pathway is presented in Fig. 3. The binding and activation of CD36, a process inhibited competitively by histidine-rich glycoprotein, leads to its interaction with $p59^{fyn}$, a member of the Src-family of tyrosine kinases. In a subsequent step, $p59^{fyn}$ activates p38MAPK and Jun N-terminal kinase (JNK); this step requires the action of caspases 3 and 8, members of the group II caspaces that are associated with apoptosis. An apoptotic cascade is subsequently triggered by the interaction of Fas ligand (FasL) on one cell with a Fas receptor on another (Fig. 3). Recently TSP-1 has been shown to induce apoptosis in brain microvascular EC by a different pathway that requires tumor necrosis factor (TNF) receptor 1 and caspases 3 and 8 (Rege et al. 2009). This mechanism entails the induction of TNFα mRNA and protein. Although both TSP-1 and TSP-2 have been shown to be capable of causing apoptosis in cells in culture, and in tumor cells *in vivo*, the ability of these proteins to function as pro-apoptotic agents in normal cells *in vivo* remains to be confirmed.

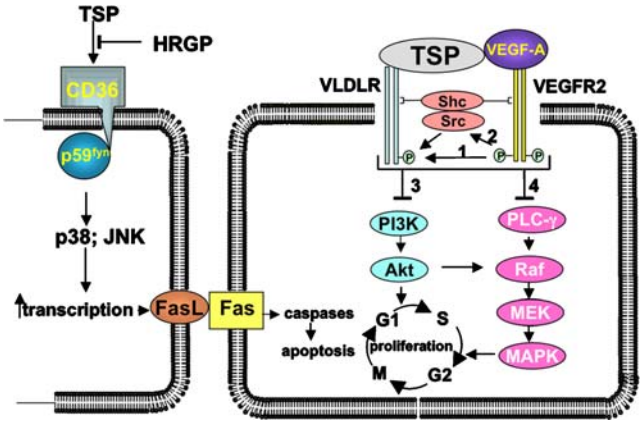

Fig. 3 A scheme that describes the apoptotic (left) and homeostatic (right) functions of the TSPs**. Left**: the mechanism, described by Jimenez et al. (2000), and extended by Rege et al. (2009), for the apoptotic function of TSP-1 or -2. Activation of CD36 also leads directly to an increase in transcription of caspaces and the TNF-R. **Right**: the coordinate and integrated interaction of TSP-1 or -2 with theVLDLR and VEGF-bound VEGFR, together with the activation of Src and the adapter protein, Shc, leads to an inhibition of the PI3K and MAPK pathways, and consequently, of cell cycle progression in EC. The bridging of the VLDLR and the VEGFA-ligated VEGFR by TSP-1 or -2 is conjectural. The mechanisms encompassed by the bracket are based on analogous pathways recently described for the activated PDGF receptor-beta and LRP1 (see Newton et al. 2005). As indicated by arrow 1, the activated VEGFR phosphorylates an NPXY sequence on the VLDLR. Arrow 2 indicates that the kinase domain of the VEGFR also activates Src, which phosphorylates the VLDLR, and Shc, which bridges the two receptors by virtue of its two docking domains, SH2 and PTB. These reactions lead, by mechanisms that have not yet been defined, to the inhibition of the P13K/Akt (3) and MAPK (4) pathways, and the consequent inhibition of cell cycle progression. The modified and updated figure legend and the figure are reproduced from Oganesian et al. Mol Biol Cell 19: 1563, 2008 with permission from The American Society for Cell Biology

TSP-1 and TSP-2 also serve a homeostatic function, mediated by the VLDL receptor, in the regulation of angiogenesis

The requirement for a homeostatic function for TSP-1 and TSP-2 in a normal adult animal was suggested by the following considerations. 1) The rate of replication of aortic EC in healthy adult rats ranges from 2 to 10 months, depending on their anatomical location, and longer life spans might be expected in humans (see references in Oganesian et al. 2008 for this and subsequent points). 2) This quiescence exists despite the fact that plasma contains sufficient VEGF to stimulate EC growth. 3) In the event of trauma, it would seem preferable for ECs in the periphery of a wound to become quiescent rather than to undergo apoptosis in response to TSP-1 released from platelets, because the exposure of additional sub-endothelial matrix could lead to more extensive and undesirable thrombosis.

Initial work by Armstrong et al. (2002) indicated that both TSP-1 and TSP-2 can inhibit the proliferation of human microvascular EC (HMVEC) in the absence of cell death, and that the mechanisms responsible for inhibition of cell cycle progression differed from those leading to apoptosis. More recently, Oganesian et al. (2008) have established that the homeostatic function of both TSP-1 and TSP-2 is mediated by the VLDL receptor (VLDLR), a member of the LRP receptor family. Additional information regarding the VLDL receptor can be found in Oganesian et al. (2008). As shown in Fig. 3, the coordinated interaction of TSP-1 or -2 bound to the VLDLR, and VEGF bound to the VEGFR, leads to an inhibition of the P13K and MAPK pathways, and consequently to the inhibition of cell cycle progression in EC. This scheme is analogous to the co-receptor function of the platelet-derived growth factor receptor and LRP1 (Newton et al. 2005).

Recently, the work of Blake et al. (2008) has confirmed the interaction between the VLDLR and TSP-1. These workers showed that this interaction induces phosphorylation of Dab1 in the subventricular zone of the brain, and that the consequent signaling during the postnatal migration of neuronal precursors from the subventricular zone to the olfactory bulb stabilizes subventricular neuronal chains. Jiang et al (2009) have also reported signaling from the VLDLR in retinal vascular EC. Although the role of TSPs was not examined in this study, the fact that angiogenesis was increased in VLDLR-null mice suggests that TSPs could inhibit angiogenesis in the retinas of wild-type mice.

TSP-1 inhibits signaling by nitric oxide (NO): consequences for vascularity and angiogenesis

NO is a bioactive gas that is produced by conversion of L-arginine to L-citruline, a reaction catalyzed by three NO synthases. NO relaxes vascular smooth muscle cells and thus increases blood vessel diameter and blood flow (Isenberg et al. 2009a). However, in the context of the regulation of angiogenesis by TSPs, NO also markedly increases the inhibitory potency of TSP-1 (see reviews by Isenberg et al. 2008a, 2009a, b, for details of the function of NO). TSP-1, by binding to CD47, also known as IAP, reduces the dilatation of blood vessels by NO. Thus, Isenberg et al. (2008b) have shown that gene silencing of CD47, by use of CD47antisense morpholino oligonucleotides, or antibody blockade of TSP-1, increases the patency of blood vessels and can therefore increase the survival of ischemic tissues. Similar results were achieved in mice that lacked CD47 (Isenberg et al. 2007a). Since TSP-1 also limits vascular smooth muscle cell relaxation (Isenberg et al. 2007b), its expression can therefore contribute to tissue ischemia by different mechanisms.

Recently, the interactions of the 'signature domains' of TSPs-1, -2 and -4 (comprised of the types III repeats and the C-terminal domain) with CD47, and their capacity to modulate cGMP signaling, were compared (Isenberg et al. 2009a, b). In these assays, TSP-1was found to be more effective than either TSP-2 or TSP-4 in the inhibition of NO-stimulated cGMP synthesis in vascular smooth muscle cells. Based on these and other experiments, Isenberg et al. concluded that TSP-1 is the dominant regulator of CD47-mediated NO/cGMP signaling, and that its role in limiting responses to acute ischemic injuries is not shared by TSP-2.

TSP-1 and TSP-2 also subserve an angiogenic function

Despite the extensive experimental data that point to an anti-angiogenic function for TSP-1 and -2, evidence has gradually accumulated that, paradoxically, also supports an angiogenic function for these proteins. In 1994, Nicosia and Tuszynski described experiments in which rat aortic rings were implanted in collagen and fibrin matrices containing TSP-1. In these experiments they observed a concentration-dependent outgrowth of microvessels from these aortic explants and concluded that TSP-1 stimulated the outgrowth of myofibroblasts from the aortic rings, and thereby promoted the formation of microvessels. However, the mechanisms that led to the increase in proliferation of the EC, and that would be required for blood vessel formation in these experiments, were not characterized. Subsequently, Qian et al. (1997) showed that the capacity of bovine aortic EC to invade and form micro-vessel-like tubes in collagen gels was increased at low concentrations of exogenous TSP-1, but was inhibited at higher concentrations. This biphasic effect was correlated with the stimulation of matrix metalloproteinase-9 (MMP-9) activity by TSP-1. Thus Qian et al. proposed that TSP-1, via activated MMP-9, was responsible for gel invasion and tube formation by EC, because these functions were inhibited by antibodies against either protein. However, these results are subject to the reservation that bovine aortic EC, rather than microvascular EC, were used in the experiments. In support of an angiogenic function for TSP-1, Taraboletti et al. (2000) used thrombin to digest human platelet TSP-1 into NH_2-terminal heparin-binding fragments of 25 kDa and COOH-terminal fragments of 140 kDa. In a rabbit corneal assay, both intact TSP-1 and the 25 kDa fragment promoted the angiogenesis induced by FGF2, whereas the 140 kDa fragment did not induce angiogenesis and inhibited the angiogenic response to FGF2. The authors concluded, on the basis of these and other experiments, that intact TSP-1 could be the source of smaller peptides that functioned in a manner opposite to that of the intact protein.

Additional support for an angiogenic function of TSP-1 has been contributed by Roberts and coworkers. Chandrasekaran et al. (2000) showed that the interaction of $\alpha3\beta1$ integrin with immobilized TSP-1 stimulated angiogenesis. This interaction was inhibited by a soluble peptide from TSP-1 and by the interaction of the $\alpha3\beta1$ integrin with VE cadherin, but was stimulated by the interaction of the $\alpha3\beta1$ integrin with CD98. The outcome of these interactions is further complicated by whether or not the peptide is part of a larger fragment of TSP-1. More recently, Staniszewska et al. (2007) showed that TSP-1 also interacts with $\alpha9\beta1$ integrin via its N-terminal domain. $\alpha9\beta1$ is expressed on microvascular EC in several organs and its interaction with sequences from the N-terminal domain of TSP-1 induced neovascularization in quail chorioallantoic membranes and in a matrigel plug formation assay in mice. $\alpha4\beta1$ and $\alpha6\beta1$ have also been shown to interact with TSP-1 (Calzada et al. 2004a, b), but the evidence for an angiogenic function for these interactions is incomplete.

The regulation of angiogenesis by TSP-1 and TSP-2 in tumors

There is ample evidence for inhibition of both tumorigenic (Hawighorst et al. 2001; see Kazerounian et al. 2008 for a review) and non-tumorigenic (Cursiefen et al. 2004) angiogenesis by endogenous TSP-1 and TSP-2. Because the effects of TSP-1 and TSP-2 on the proliferation of ECs are predominantly inhibitory, there has been considerable interest in their potential, and in that of peptides from the two proteins, as anti-tumor agents. However, in keeping

with the evidence that TSP-1 can also stimulate angiogenesis, both inhibitory and stimulatory effects have been reported (see Roberts 1996 for a review of the early literature). Furthermore, there are several different sources of endogenous TSP-1 in tumors, including the malignant cells themselves and the host-derived vasculature and stroma, and each of these cellular compartments is subject to different and complex regulation. The outcome of the addition of exogenous TSP-1 can therefore be difficult to predict.

Nevertheless, recent advances in molecular and cell biology have suggested novel means by which the manipulation of TSP levels can be used in the regulation of angiogenesis. Thus studies have established that prosaposin, a precursor form of the lipid hydrolase activators, saposin A-D, stimulates the expression of TSP-1 in both tumors and in normal tissues in a p53-dependent manner (Kang et al. 2009). These studies confirm earlier findings by Dameron et al. (1994), that established a role for p53 in the regulation of expression of TSP-1. Furthermore, there is now evidence that the expression of microRNAs can be used to regulate angiogenesis (Kuehbacher et al. 2007; Dews et al. 2006) Thus, it is plausible that manipulation of microRNA levels could be used eventually to selectively increase TSP levels in tumors and surrounding tissues.

In 1997, Castle et al. showed that transfection of cDNA for intact TSP-1, or for its N-terminal domain, into *src*-NIH 3 T3 cells suppressed angiogenesis and tumor formation when the transfected cells were injected into nude mice. Subsequently, Tokunaga et al. (1999) quantified expression of TSP-1 and TSP-2 by RT-PCR in patients with colon cancer. More than half of the patients' cancers expressed TSP-2, and these patients exhibited a significantly reduced incidence of hepatic metastases and tumor vascularity in comparison with patients whose tumors were negative for TSP-2. A similar correlation with expression of TSP-1 was not found. Along these lines, de Fraipont et al. (2001) conducted a survey of 18 published papers in which the level of expression of TSP-1 or -2 was correlated with clinical status and outcome. Despite the variables in these studies, for example studies that used biochemical methods to quantify TSPs did not distinguish between stromal *versus* tumoral sources of the TSPs, the authors concluded that, for most of the tumors, there was an inverse correlation between the level of TSP in the tumor and the degree of its malignancy. However, in keeping with evidence for an angiogenic function for TSP-1, Tuszynski and Nicosia (1996) have also summarized the evidence that TSP-1 can actually promote tumor progression. Additional information concerning the effects of TSP-1 and TSP-2 in tumor progression is provided in a review by Lawler and Detmar (2004).

The potential for use of TSP-1-derived peptides in clinical anti-angiogenic therapy

There are a number of ways in which the properties of TSP-1 and -2 could be used to inhibit the vascularity and/or growth and metastasis of tumors. For example, TSP-1 binds VEGF directly, and the complex can be endocytosed by the scavenger receptor, LRP1 (Greenaway et al. 2007). This clearance function is analogous to that by which TSPs reduce the levels of MMP2 (Yang et al. 2001) and MMP9 (Hahn-Dantona et al. 2001) in the pericellular environment. Similarly, other anti-angiogenic functions of TSP-1 and -2, described in this review, could be exploited. However, approaches that require administration of intact TSP are clearly not feasible in a clinical setting, and neither are cell-based therapies or therapies that depend on the use of viruses as delivery agents (Hahn et al. 2004; see review by Mirochnik et al. 2008, for a more complete review of the limitations of therapy with TSP-1).

On the other hand, peptides that mimic sequences in the type I repeats of TSP-1 show considerable promise as anti-tumor agents. Haviv et al. (2005) synthesized a series of peptides based on a sequence in the second type I repeat of human TSP-1. Selected peptides, modified to increase their stability in vivo, were then tested for their anti-angiogenic properties by several criteria: inhibition of tube formation by human microvascular EC; induction of apoptosis; inhibition of EC migration; and inhibition of capillary sprout formation. Two of the peptides, ABT-526 and ABT-510, which showed the most promise, were then tested for their effectiveness as inhibitors of angiogenesis and tumor progression in animals. ABT-526 was found to be effective in the inhibition of neovascularization in a rat cornea model, and ABT-510 reduced the growth of a syngeneic Lewis lung carcinoma in mice (Haviv et al. 2005).

The type I TSP repeats are also present in a number of related proteins other than the TSPs, such as pigment epithelium-derived factor (PEDF), WISP-1, and brain angiogenesis inhibitor-1(BAI-1). PEDF has been shown to be a potent inhibitor of angiogenesis in the mammalian eye (Dawson et al. 1999). WISP-1 contains an 18 amino acid sequence, termed wispostatin-1, which is very similar to the Mal II and Mal III sequences in the type I repeats of TSP-1. Cano et al. (2009) found that wispostatin-1 inhibited the migration and proliferation of human retinal EC, abolished FGF-induced retinal neo-vascularization in a corneal micropocket assay, and inhibited laser-induced choroidal neovascularization in mice. BAI-1, which contains five TSP type I repeats, can be cleaved at a conserved proteolytic cleavage site to release a soluble anti-angiogenic fragment termed vasculostatin, which contains these repeats (Kaur et al. 2005). Vasculostatin suppresses the growth of malignant gliomas in rats and inhibits the migration of human dermal

microvascular EC, but not that of human umbilical vein EC. These effects are dependent on the presence of CD36 on EC (Kaur et al. 2009). Most recently, Ren et al. (2009) have evaluated the ability of a 'double hit,' consisting of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and a TRAIL receptor 2 agonist antibody (Lexatumumab), which is directed to the type I repeats in TSP-1, to inhibit the progression of human colon cancer in nude mice and to induce apoptosis in human dermal microvascular EC. Favorable results were observed in both assays.

Perhaps the most encouraging studies that set the stage for the use of TSP peptide mimetics in the treatment of tumors in patients, involve the TSP-1-derived peptides, ABT-526 and ABT-510, developed by Haviv et al. (2005). Hoekstra et al. (2006), in a Phase I study, reported that the inclusion of ABT-510 in a course of therapy with 5-fluorouracil and leucovorin showed no ill effects in patients with solid tumors. Two studies in dogs are also encouraging. ABT-510 and ABT-526 were used to treat malignant tumors in 242 pet dogs. No dose-limiting toxicity was observed in any of the animals, and 42 of the dogs showed a greater than 50% reduction in tumor size (Rusk et al. 2006a). In a second study of pet dogs with relapsed non-Hodgkin's lymphoma, ABT-526 was added to chemotherapy with lomusine. Whereas no difference was observed in the rate of response, there was a significant increase in its duration, with no attendant toxicity (Rusk et al. 2006b). In a different animal model, Greenaway et al. (2009) showed that ABT-510 was effective in the induction of apoptosis of syngeneic mouse epithelial cancer cells, introduced into the bursa of C57BL/6 mice. Finally, in a recent phase II study, Baker et al. (2008) treated patients with advanced soft tissue sarcoma with ABT-510. Although a favorable safety profile was observed, and the rate of disease control and overall survival were encouraging, the authors concluded that compelling evidence for the efficacy of ABT-510 as a single agent in this disease had not been achieved.

Additional trials with these two peptide mimetics are clearly necessary, and are likely to be performed. Hopefully, a reduction in the morbidity of some human cancers that results from a combinational therapy, including TSP derivatives, will be forthcoming.

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