

Invited Review

Thrombospondin-1, PECAM-1, and regulation of angiogenesis

N. Sheibani and W.A. Frazier

Washington University School of Medicine, Department of Biochemistry and Molecular Biophysics, St. Louis, MO, USA

Summary. Thrombospondin-1 (TSP1) is a multidomain glycoprotein expressed by many cell types. It is a multifunctional protein with important roles in regulation of vascular cell functions. Mutation or loss of tumor suppressor genes results in down regulation of TSP1 expression during malignant transformation. Thus, suggesting that down regulation of TSP1 may contribute to development of the tumor angiogenic phenotype and perhaps tumor metastasis. TSP1 was demonstrated to be a natural inhibitor of angiogenesis. Peptides from procollagen-like domain and type 1 repeats of TSP1, like whole TSP1, inhibit the angiogenic response to a variety of angiogenic stimuli *in vivo* and endothelial cell (EC) migration *in vitro* by directly acting on ECs. The molecular mechanisms which mediate these inhibitory effects of TSP1 and its peptides are not understood. TSP1 expression is down regulated in the Polyoma middle T transformed mouse brain ECs (bEND.3). This may remove the TSP1 inhibitory effects allowing ECs to rapidly proliferate in culture and form hemangiomas *in vivo*. Re-expression of TSP1 in bEND.3 cells restores a normal phenotype and suppresses their ability to form hemangiomas. This is mediated by modulating expression of several genes in concert favoring a differentiated state of endothelium. TSP1 transfected bEND.3 cells down regulate expression of PECAM-1, a multifunctional endothelial cell adhesion molecule with essential roles in angiogenesis. A similar phenotype to that of TSP1 transfected cells was observed when endogenous PECAM-1 levels were down regulated by anti-sense transfection of bEND.3 cells. The anti-sense PECAM-1 transfected cells turn on expression of endogenous TSP1 and its angioinhibitory receptor, CD36. Expression of other genes with potential roles in regulation of EC phenotype were also affected in patterns very similar to those observed in TSP1 transfected bEND.3 cells. Therefore, it appears that a reciprocal relationship exists between TSP1 and

PECAM-1 such that they are constituents of a "switch" that regulates in concert many components of the angiogenic and differentiated phenotype of ECs.

Key words: CD31, Endothelial cells, Thrombospondins

Introduction

Angiogenesis, the process of new capillary formation, is tightly regulated by a balanced production of stimulatory and inhibitory factors (Folkman and Shing, 1992; Hanahan and Folkman, 1996). It is the alteration in this balanced production of positive and negative regulators which results in angiogenesis. Angiogenesis is a multistep process involving the destabilization of differentiated endothelium, activation of proteases which digest the basement membrane components allowing endothelial cells (ECs) to migrate toward a gradient of angiogenic factor, proliferation of ECs, alignment of ECs to cord-like structures, lumen formation, and stabilization of the differentiated endothelium. Angiogenesis rarely occurs in normal adults but is essential for development, wound healing, and cyclic expansion of the corpus luteum and endometrium. Unregulated angiogenesis contributes to pathogenesis of many diseases of the eye, psoriasis, arthritis and cancer. Therefore, development of agents which can block neovascularization will have great impact on treatment of these diseases (Folkman, 1995). This requires a clear understanding of the naturally occurring factors which stimulate angiogenesis in normal quiescent endothelial cells, as well as those factors which normally serve to limit the extent of the angiogenic response to these stimuli *in vivo*. One such factor which is widely expressed by many cells, in both vascular and non-vascular compartments, is thrombospondin (TSP), or more appropriately, the family of thrombospondins (Adams et al., 1995).

Platelet TSP, now called TSP1, is the most abundant component of α -granules. TSP1 is released upon platelet activation (Baenziger et al., 1971) thus indicating an important role for TSP1 in hemostasis. It is now

Offprint requests to: Nader Sheibani, Ph.D., Washington University School of Medicine, Department of Biochemistry and Molecular Biophysics, 660 South Euclid Avenue, Box 8231, St. Louis, MO 63110, USA. Fax 1-314-362-4153. e-mail: sheibani@biochem.wustl.edu

recognized that TSPs have much broader roles in vascular biology. In addition to modulation of angiogenesis (Good et al., 1990; Tolsma et al., 1993), they augment platelet aggregation (Leung, 1984; Dixit et al., 1985; Chung et al., 1997), condition the provisional matrix of clot at wound sites (Raugi et al., 1987), facilitate appropriate cellular migration such as leukocyte extravasation (Huber et al., 1992) and chemotaxis (Mansfield and Suchard, 1994; Mansfield et al., 1990; Suchard, 1993), phagocytosis of apoptotic neutrophils (Savill et al., 1993), and regulation of smooth muscle cell proliferation and migrations (Majack et al., 1986, 1988; Botney et al., 1992; Yabkowitz et al., 1993; Wang and Frazier, 1998). In recent years, the family of TSPs has grown and currently contains four additional members (Adams et al., 1995). TSP2 is structurally very similar to TSP1 (Bornstein, 1992; Bornstein and Sage, 1994), while TSP3, 4, and COMP (cartilage oligomeric matrix protein) or TSP5 are missing two domains of TSP1, the procollagen-like domain and the type 1 or "malaria-like" repeats, which appear to be responsible for the antiangiogenic effects of TSP1 and TSP2. Various TSP isoforms are expressed during development in an acutely regulated temporal and spacial fashion (Iruela-Arispe et al., 1993; Bornstein and Sage, 1994; Adams et al., 1995; Bornstein, 1995). The wide distribution and the diversity of the isoforms expressed will present a challenge in determining the role and mechanisms of action of this family of proteins. Here we will present our current model for the role of TSP1 in regulation of neovascularization.

TSP1 and angiogenesis

The domain structure of TSP1 or platelet thrombospondin is shown in Fig. 1. It consists of a heparin

binding globular domain, followed by a region of homology to the proregion of collagen, the type 1 or "malaria like" repeats, the type 2 or "EGF like" repeats, the type 3 or calcium binding repeats the last of which contains an RGD sequence, and the carboxyl terminal or cell binding domain (CBD). The TSP1 core fragment along with the TSP1 domains and peptides which exhibit antiangiogenic activity are illustrated in Fig. 1. TSP1 interacts with more than a dozen cell surface receptors that are expressed in various combinations on different cell types. These include heparan sulfate proteoglycans, CD36, $\alpha v \beta 3$ and other integrins, and recently the integrin associated protein or CD47 (IAP) (Asch, 1993; Lawler, 1993; Gao et al., 1996b). The downstream signaling events that emanate following engagement of these receptors remain largely unknown.

TSP1 expression was shown to be regulated by a tumor suppressor gene whose inactivation with tumor progression results in decreased TSP1 expression (Rastinejad et al., 1989; Volpert et al., 1995a). This suggested that TSP1 may be a negative regulator of angiogenesis whose loss of expression favors an angiogenic phenotype. Angiogenesis is essential for tumor growth beyond a small nodule and for metastasis to distant sites (Folkman and Shing, 1992; Hanahan and Folkman, 1996). In collaboration with Dr. Bouck, we have shown that TSP1 is a natural inhibitor of angiogenesis in several *in vivo* models and have identified the domains of TSP1 that are responsible for this activity (Good et al., 1990; Tolsma et al., 1993). These domains include the TSP1 procollagen-like domain and the type 1 repeats (see Fig. 1). A peptide as short as 7 amino acids from the procollagen-like domain (NGVQYRN) was shown to inhibit angiogenesis *in vivo* (Tolsma et al., 1993). These peptides also inhibited endothelial cell migration *in vitro*. The action of TSP1

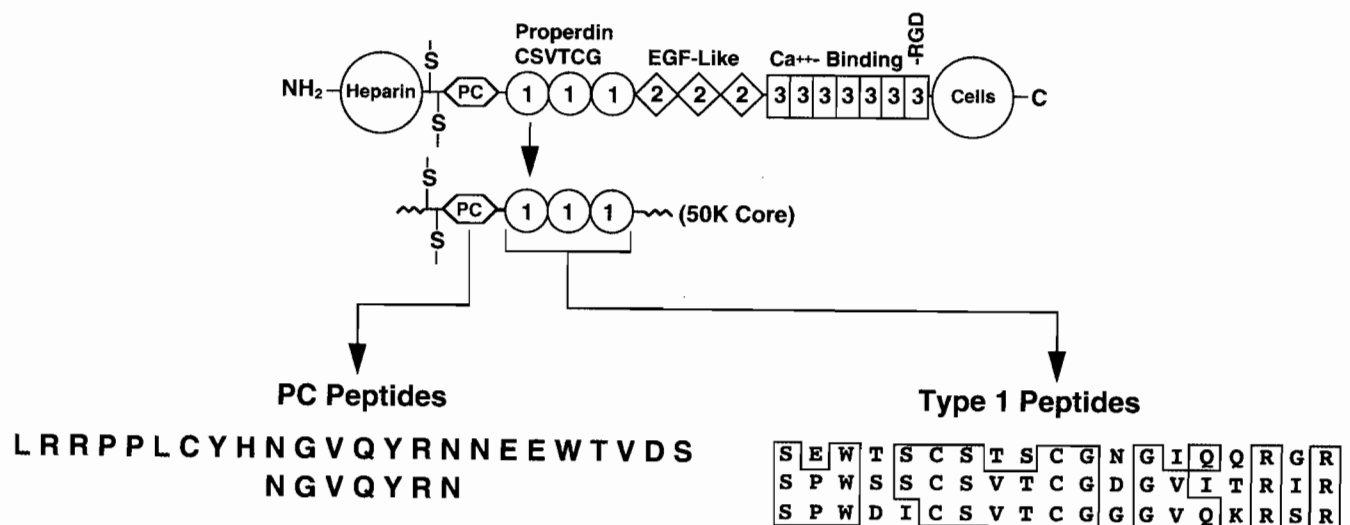


Fig. 1. Structural and functional domains of TSP1. The diagram schematically illustrates the domain organization of a single subunit of the TSP1 trimer. The antiangiogenic domains and active peptide sequences from these domains are depicted.

appears to be general and can inhibit angiogenic responses to not only bFGF, but also aFGF, VEGF, IL-8, TGF- β , and tumor fragments in the cornea bioassay (Good et al., 1991; Tolsma et al., 1993; Volpert et al., 1995b). TSP1 and its peptides can inhibit the chemotactic migration of endothelial cells toward angiogenic factors such as FGFs, VEGF, PDGF, TGF- β , IL-8, and PGE1 in a classical Boyden chamber assay (Good et al., 1991; Tolsma et al., 1993). TSP2, which contains sequences in its type 1 repeats homologous to the angioinhibitory sequences from TSP1, but lacks a good homologue of the procollagen-like domain's inhibitory sequence, also inhibits the response of EC to most of these inducers of angiogenesis. However, a higher concentration of TSP2 is required to achieve same level of inhibition (Volpert et al., 1995b). COMP or TSP5 which lacks angioinhibitory domains (Oldberg et al., 1992) failed to inhibit angiogenesis in the rat cornea and EC migration in vitro (Volpert et al., 1995b). These experiments indicated that TSP1 and TSP2 act directly on the EC themselves. Another in vitro assay of angiogenesis is the formation of tubes or capillary like structures in three dimensional gels, usually Matrigel (Cockerill et al., 1995). TSP1 and its inhibitory peptides block migration, alignment, and formation of capillary like tubes by microvascular endothelial cells on Matrigel (our unpublished observations and Tolsma et al., 1997).

TSP1 is reported to inhibit proliferation of many types of EC in vitro (Bagavandoss et al., 1990; Taraboletti et al., 1990; Iruela-Arispe et al., 1991; RayChaudhury et al., 1994). However, unlike the chemotaxis assays, or the tube formation assays, this effect of the TSP1 protein on proliferation is not mimicked by the angioinhibitory peptides (our unpublished observations). Thus suggesting that other structures within the TSP1 protein are needed to exert an inhibitory effect on proliferation. One possibility is that the site of TSP1 which activates TGF- β (Schultz-Cherry et al., 1995) may be required for inhibition of EC proliferation in vitro. The RFK sequence of TSP1 which is responsible for activation of TGF- β is absent from the angioinhibitory peptides. However, Panetti et al. (1997) have recently shown that inhibition of endothelial cell mitogenesis stimulated by LPA, or bFGF is inhibited by recombinantly produced TSP1 and TSP2 independent of modulation of TGF- β activity. This is consistent with the lack of TSP2 ability to activate TGF- β . Therefore, the in vitro activation of TGF- β by TSP1 may not contribute to the in vivo role of TSP1 as an inhibitor of angiogenesis (see below).

Mechanism of TSP1 action on EC

The mechanisms by which the angioinhibitory effects of TSP1 and/or its peptides are mediated have not been delineated. The in vitro angiogenic assays indicate a direct effect of TSP1 and its peptides on ECs. Therefore, the identity of the receptor or receptors on the surface of ECs which mediate the inhibitory effects of

TSP1 has been investigated. One of these receptors is platelet gpIV or CD36 which was thought to interact with a region of TSP1 that contains the CSVTG sequence (Asch et al., 1992, 1993; Li et al., 1993). This sequence is from the type 1 repeats of TSP1 and is present (in a modified form) in the antiangiogenic peptides such as Mal III from the third type 1 repeat. However, several peptides containing the sequence VTTCG including the native CSVTCG have no angioinhibitory activity (Tolsma et al., 1993). Therefore, the CSVTC sequence is ineffective and its presence in the longer Mal III peptide was not necessary to inhibit EC functions. The active sequence from the procollagen like domain, NGVQYRN is very similar to the C-terminal region of Mal III (GVQKRS), suggesting that it is this sequence of Mal III which contains the inhibitory activity. The C-terminal portion of Mal III (GGGVQKR SK) does indeed inhibit EC migration (Tolsma et al., 1994).

Recent experimental evidence strongly suggests that CD36 is the receptor that mediates the inhibitory effects of TSP1 and its peptides on EC migration in vitro (Dawson et al., 1997). Soluble fragments of CD36 were utilized to investigate whether they could interfere with the inhibition of bFGF chemotaxis by TSP1. The region of CD36 previously shown to interact with TSP1(aa 93-120) was able to block TSP1 inhibition of chemotaxis to bFGF. Other regions of CD36 were ineffective. In addition, the TSP1 mediated inhibition of EC chemotaxis was found to be reversed by an antibody to CD36 (mAb OKM5), while SMO (an IgM) antibody was able to mimic the action of TSP1 and the peptides, and is itself an inhibitor of chemotaxis toward bFGF (Dawson et al., 1997). This is perhaps due to the ability of the multivalent IgM antibody to cluster and activate the CD36 receptor. In addition, TSP1 inhibited the chemotaxis of microvascular EC which express CD36, but not of HUVEC which do not express CD36, to bFGF (Swerlick et al., 1992; Dawson et al., 1997). Expression of CD36 in HUVEC made these cells susceptible to inhibition by TSP1. These observations are further supported by the inability of TSP1 to inhibit bFGF mediated angiogenesis in cornea of CD36 mutant mice (CD36^{-/-}) (Lennon et al., 1998). The downstream signaling events which mediate the inhibitory effects of TSP1 and/or its peptides via CD36 are under investigation. Understanding the mechanisms by which a natural inhibitor of angiogenesis regulates endothelial cell phenotype will not only provide insight into the regulatory mechanisms which keep angiogenesis in check but also will aid in design of effective agents to block angiogenesis under pathological conditions such as cancer and various types of eye diseases.

TSP1 and tumorigenesis

There is great interest in inhibition of angiogenesis as a novel method to treat many diseases with a neovascular component. Several inhibitors of angiogenesis

have proven effective as anti-tumor agents in animal models and some are in clinical trials (Folkman, 1995). A large number of naturally occurring inhibitors of angiogenesis have been identified that are produced by various cells in the body (O'Reilly et al., 1997). However, the regulation of these molecules and their mechanism(s) of action are not well characterized. It is now apparent that TSP1 expression is regulated in a number of cell types by tumor suppressor genes in a manner consistent with its involvement in physiologic control of angiogenesis (Volpert et al., 1995a; DiPietro, 1997). This was initially illustrated by Bouck and colleagues who demonstrated a decrease in expression of TSP1 with malignant progression of BHK cells which lose a tumor suppressor gene (Rastinejad et al., 1989). Loss of the second p53 allele in fibroblasts from Li-Fraumeni patients coincides with malignant transformation and decreased expression of TSP1 (Dameron et al., 1994). TSP1 expression in glial cells is also shown to be regulated by a tumor suppressor gene on chromosome 10 (Volpert et al., 1995a). These cases suggest that a function of tumor suppressor genes is to maintain the angiogenic balance by production of endogenous inhibitors of angiogenesis. Transformation of normal cells, which express high levels of TSP1, with oncogenes such as *ras*, *src*, *jun*, and middle T results in a dramatic down regulation of TSP1 (Mettouchi et al., 1994; Sheibani and Frazier, 1995, 1996). Expression of some of these oncogenes is also reported to upregulate expression of angiogenic factors (Rak et al., 1995). Therefore, an essential part of the program of oncogenesis is tipping the angiogenic balance toward angiogenesis.

TSP1 and regulation of EC phenotype

To begin studying the mechanisms of TSP1 action on endothelial cells we have utilized a Polyoma middle T transformed mouse brain endothelial cell line (bEND.3). These cells express little or no TSP1, rapidly proliferate in culture, have high levels of fibrinolytic activity, and rapidly form hemangiomas in mice, primarily by recruiting host EC (Williams et al., 1989; Montesano et al., 1990; RayChaudhury et al., 1994; Sheibani and Frazier, 1995). bEND.3 cells express many endothelial cell markers and respond to TSP1 and TGF- β in a normal way (Mueller et al., 1987; Williams et al., 1989; RayChaudhury et al., 1994). Addition of exogenous TSP1 (or TGF- β) inhibited proliferation of these cells in culture (RayChaudhury et al., 1994). However, the results of these studies are complicated by the fact that TSP1 may bind and activate TGF- β (Schultz-Cherry et al., 1995). Our hypothesis was that down regulation of TSP1 in these cells allows them to rapidly proliferate and maintain an invasive angiogenic phenotype. To test this hypothesis we expressed the human TSP1 cDNA in bEND.3 cells. TSP1 expression restored a normal phenotype in bEND.3 cells, and most importantly suppressed their ability to form heman-

giomas (Sheibani and Frazier, 1995). The TSP1 expressing cells grew much slower, exhibited a normal proteolytic balance by down regulating urokinase plasminogen activator and enhancing production of its inhibitor (PAI-1), and organized into cord structures on Matrigel. These effects of TSP1 were independent of changes in the level of TGF- β activity (Sheibani and Frazier, 1995). Together these results indicated that TSP1 may be a major regulator of endothelial cell phenotype involved in maintaining a differentiated state of endothelium.

TSP1 expression in bEND.3 cells affected expression of several genes with potential roles in regulation of endothelial cell phenotype. Fig. 2 is a panel of Northern blots of poly A⁺ RNA prepared from parental, vector control, and several clones of TSP1 transfected bEND.3 cells probed for expression of some of these genes. As illustrated here, we observed changes in the expression of many of them. Some are upregulated such as TSP2, TSP3, CD36, fibronectin, α 1 (III) collagen, α v integrin, β 1 integrin, β 5 integrin, TIMP-2, PAI-1, stromelysin-1, and collagenase. Some are down regulated such as uPA, α 1 (IV) collagen, β 3 integrin, flk-1,flt-1, and PECAM-1 (Fig. 2; also see Sheibani and Frazier, 1995, 1998; Sheibani et al., 1997). Expression of TGF- β , c-myc, SPARC, E-selectin, P-selectin, tPA, PAI-2, and α 5 integrin was unchanged (Sheibani and Frazier, unpublished data). The enhanced expression of endogenous mouse TSPs and CD36 as well as down regulation of vascular endothelial growth factor receptors, flk-1 and flt-1, contribute to the slower growth rate and perhaps reduced survival. However, the enhanced expression of fibronectin and its receptor (α 5 β 1) are consistent with the phenotype of non-transformed cells and may provide survival signals and stability to differentiated endothelium. The down regulation of β 3 integrin is also consistent with the phenotype of differentiated endothelium (Brooks, 1996). The reduced fibrinolytic activity and perhaps balanced production of metalloproteinases and their inhibitors may provide a more controlled and limited proteolytic activity. The role of other gene products in regulation of angiogenesis are not well characterized and require further investigation.

We have focused our attention on PECAM-1/CD31 (platelet endothelial cell adhesion molecule-1) whose expression is completely suppressed following TSP1 expression in bEND.3 cells (Sheibani et al., 1997). Hemangiomas are formed by the ability of bEND.3 cells to recruit and interact with host EC (Williams et al., 1989). Therefore, we speculated that PECAM-1, an EC adhesion molecule, may take part in hemangioma formation by parental bEND.3 cells and that its loss might contribute to changes in the morphology of TSP1-transfected cells and their lack of ability to form anastomosed cords on Matrigel.

PECAM-1 is a multifunctional vascular cell adhesion molecule and is a member of the immunoglobulin gene superfamily (Newman, 1997; DeLisser et

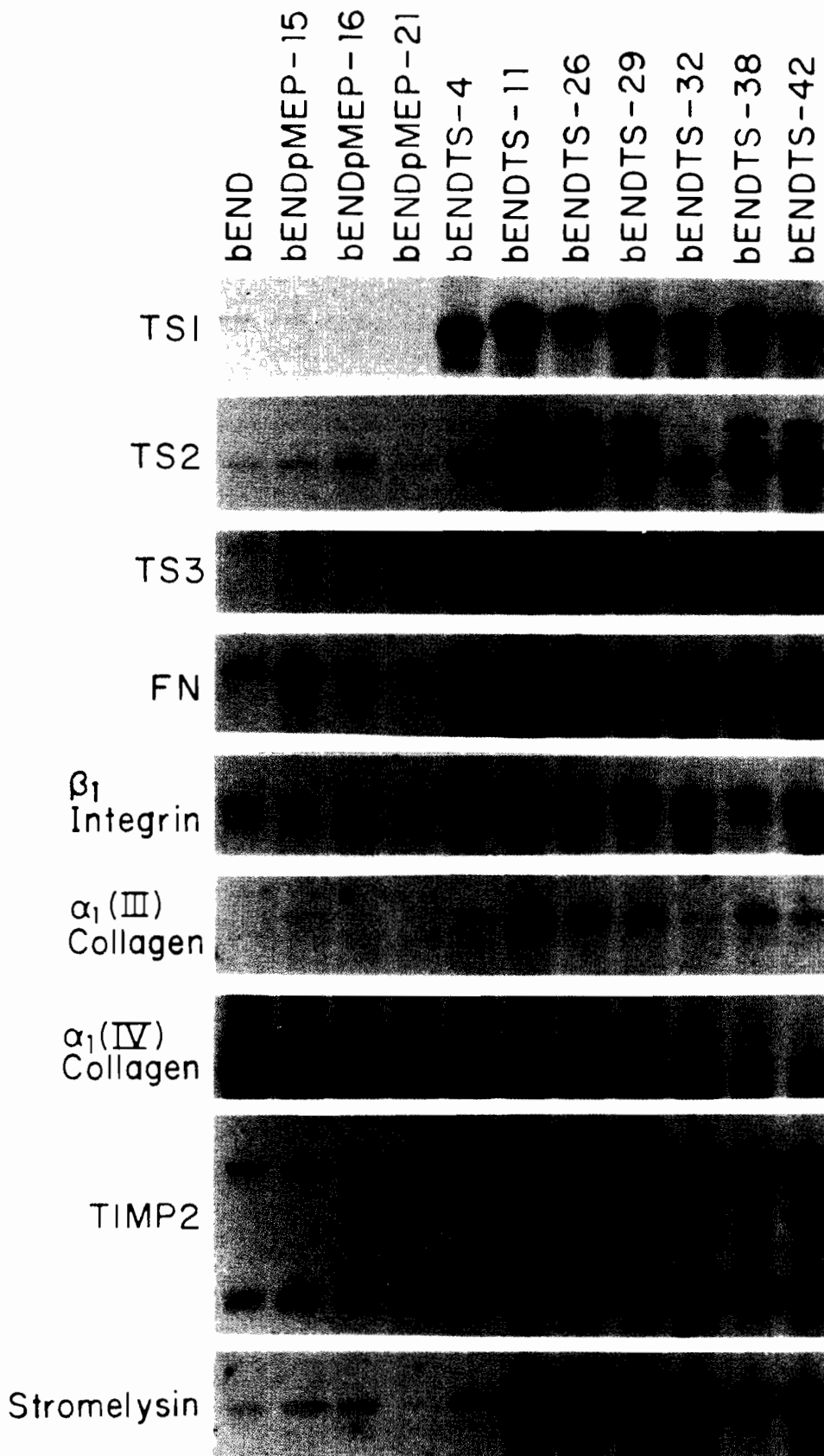


Fig. 2. Analysis of the steady-state TSP1, TSP2, TSP3, fibronectin (FN), β_1 integrin, α_1 (III) collagen, α_1 (IV) collagen, tissue inhibitor of metalloproteinases II (TIMP-2), and stromelysin mRNA levels in TSP1 transfected bEND.3 cells. Poly A⁺ RNA (5 μ g) was size fractionated on 1.2% agarose-formaldehyde gel, transferred to membrane, and probed with specific cDNAs for desired genes. The GAPDH probe of this blot indicated equal loading of RNA in all lanes (not shown). bEND are the parental cells, pMEP 15, 16, and 21 are three clones of vector transfected controls. TS-4, -11, -26, -29, -32, -38, and -42 are TSP1-transfected clones. Please note that TS-4 clone expresses a shorter mRNA which is not translated and these cells behave very similar to parental or vector control transfected cells in many of their characteristics (Sheibani and Frazier, 1995; Sheibani et al., 1997).

Table 1. PECAM-1 isoforms detected in mouse tissues and endothelial cell lines

ISOFORMS	bEND.3	MOUSE BRAIN
Full	+	+
$\Delta 12$	+	ND
$\Delta 14$	+	+
$\Delta 15$	+	+
$\Delta 12,14$	ND	+
$\Delta 12,15$	+	+
$\Delta 14,15^*$	+	+
$\Delta 12,14,15$	ND	+

Isoforms of PECAM-1 were identified by cloning and sequencing RT/PCR products from total RNA isolated from various mouse tissues or endothelial cell lines. Not all the isoforms were expressed in different tissues or endothelial cell lines. The isoforms detected in bEND.3 cells and mouse brain are indicated by a "+". *: most frequently detected isoform; ND: not detected.

al., 1997a). It is highly expressed on the surface of endothelial cells and at lower levels on platelets, monocytes, and a subset of T cells. PECAM-1 plays an important role in endothelial cell-cell and cell-matrix interactions, transendothelial migration of leukocytes, and cardiovascular development. Incubation of subconfluent cultures of human umbilical cord endothelial cells (HUVEC) with anti-PECAM-1 prevents typical tight monolayer formation. However, addition of antibodies to intact monolayers has no effect (reviewed in DeLisser et al., 1997a). This suggests that PECAM-1 is essential for initial endothelial cell-cell interactions and its localization at sites of EC contact may help to stabilize these interactions. We have demonstrated that antibodies to PECAM-1 block tube formation of HUVEC in Matrigel (Sheibani et al., 1997). Matsumura et al. (1997) recently shown that antibodies to human VE-cadherin (an endothelial cell cadherin) along with anti-PECAM-1 can block neovascularization in vivo and tube formation in vitro. DeLisser et al. (1997b) found that inhibition of PECAM-1 is sufficient to block angiogenesis in rat and murine cornea. The exact role of PECAM-1 during angiogenesis has not been delineated. However, these results strongly suggest that PECAM-1 engagement on the surface of EC is essential for EC migration and morphogenesis.

The functional analysis of PECAM-1 has become more complex in light of the presence of multiple PECAM-1 isoforms. The PECAM-1 transcript undergoes alternative splicing, generating at least eight different isoforms which differ in the length of their cytoplasmic domains. Table 1 demonstrates the different isoforms of PECAM-1 detected in mouse tissues and EC lines. We have examined the distribution of various PECAM-1 isoforms in different mouse tissues and EC lines by RT-PCR. Multiple isoforms of PECAM-1 were detected in every tissue and EC line examined albeit at different frequencies. The isoform which lacked exons 14 and 15 ($\Delta 14,15$) was most frequently detected in all cases. The isoforms detected in mouse brain and

bEND.3 cells are shown in Table 1. We identified a new isoform of PECAM-1 ($\Delta 12,14$) in RNA prepared from brains of three week old mice (Sheibani and Frazier, 1997) that was not previously detected in RNA prepared from early mouse embryos (Yan et al., 1995). However, determination of the expression levels of the protein products of these isoforms awaits development of antibodies which can specifically recognize each isoform. The majority of PECAM-1 antibodies available recognize the extracellular domains.

The lack of an appropriate EC model has limited structural and functional studies of PECAM-1 isoforms to non-endothelial cells. Such studies may not accurately assess the functional characteristics of PECAM-1 and its contribution to regulation of EC phenotype. TSP1 transfected bEND.3 (bEND/TS) cells which lack PECAM-1 provide a suitable cell line to study PECAM-1 functions in EC. To gain insight on the role of PECAM-1 in regulation of EC phenotype, we have expressed the full length, the $\Delta 15$ and the $\Delta 14,15$ PECAM-1 isoforms in bEND/TS cells. Expression of all these isoforms resulted in an enhanced ability of bEND/TS cells to undergo morphogenesis in three dimensional Matrigel cultures (Sheibani et al., 1997). This morphogenesis was specifically blocked by antibodies to PECAM-1. Interestingly, none of these PECAM-1 isoforms localized to sites of cell-cell contact, a characteristic of PECAM-1 expressed in vivo and in most cultured EC (Newman, 1997). This is inconsistent with the proposed role of exon 14 whose absence, mutation, or phosphorylation was found to favor homotypic interactions of PECAM-1 in L-cells (Yan et al., 1995; Famiglietti et al., 1997). However, the contribution of alterations in cytoskeletal organization and/or alterations in formation of adherens junctions in TSP1 transfected cells requires investigation. Reorganization of the cytoskeleton can modulate gene expression (Rosette and Karin, 1995) and distribution of PECAM-1 (Newman et al., 1992; Romer et al., 1995). In addition, adherens junctions are necessary to stabilize endothelial cell-cell interactions (Rabiet et al., 1996) and may be required for stabilization and/or promotion of junctional localization of PECAM-1 in EC. However, the contribution of VE-cadherin to junctional localization of PECAM-1 remains unknown. The antibody studies described above along with our transfection data strongly suggest that PECAM-1 mediated endothelial cell-cell interactions and migration are essential during EC morphogenesis and interference with these interactions can block angiogenesis. Therefore, downregulation of PECAM-1 may be an important mechanism by which the antiangiogenic activity of TSP1 is mediated.

PECAM-1 and Regulation of EC phenotype

bEND.3 cells express very high levels of PECAM-1 that localizes to sites of cell-cell contact (Sheibani et al., 1997; Sheibani and Frazier, 1998). To further explore the role of PECAM-1 in regulation of EC phenotype we have down regulated PECAM-1 levels by anti-sense

transfection of bEND.3 cells. We obtained several clones which expressed little or no PECAM-1 and some that expressed moderate levels of PECAM-1. Complete lack of PECAM-1 expression had a dramatic effect on proliferation, survival, and morphology of the cells. The clones which completely lacked PECAM-1 grew much slower and exhibited a decrease in cell survival (Sheibani and Frazier, 1998). This was consistent with complete down regulation of vascular endothelial growth factor receptors, flk-1 and flt-1, and decreased expression of $\beta 3$ integrin subunit in anti-sense transfected cells. These cells also organized better in Matrigel when compared to bEND.3 cells, comparable to the bEND/TS cells which lack PECAM-1. The anti-sense transfected cells expressing moderate levels of PECAM-1 exhibited a growth rate and morphology intermediate to bEND.3 and cells that completely lacked PECAM-1. Very few dead cells were detected in cultures of these cells and they exhibited an enhanced ability to differentiate in Matrigel. In fact, they were very similar to bEND/TS cells that we transfected with PECAM-1 cDNA. These results suggest that an optimal level may exist for PECAM-1 expression such that high levels of PECAM-1 interfere with migration and organization of EC, while moderate levels of PECAM-1 enhance EC migration, and organization. Unlike parental bEND.3 cells, the anti-sense transfected cells failed to form hemangiomas in nude mice.

A striking and unanticipated finding in the anti-sense PECAM-1 transfected bEND.3 cells was the enhanced expression of endogenous mouse TSP1 and its angioinhibitory receptor CD36 (Sheibani and Frazier, 1998). This is consistent with the reduced cell proliferation and survival we recently observed in CD36 transfected HUVEC which normally express TSP1 but not CD36 (Dawson et al., 1997). These results are also consistent with the role of CD36 as the receptor which mediates the angioinhibitory activity of TSP1. The simultaneous expression of TSP1 and CD36 has a dramatic impact on expression of EC specific growth factor receptors, integrins, metalloproteinases and their inhibitors which provide migratory, proliferative and survival signals. The detailed mechanisms of regulation of these genes by TSP1 and/or PECAM-1 is under investigation.

The effect of TSP1 is complex and may involve interaction of TSP1 with multiple receptors on EC. The heparin binding domain can labilize focal adhesions of EC spread on other matrix components (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1993). It is not clear whether this facilitates or inhibits migration in vivo or if this effect could reduce EC survival which requires that matrix contact be maintained via certain integrins (Brooks, 1996). Furthermore, the heparin binding domain may directly bind and/or compete for binding of the angiogenic factors such as VEGF and FGF which utilize heparin sulfate containing proteoglycans as low affinity receptors of their biological action (Roberts et al., 1985; Vogel et al., 1993; DiPietro, 1997). The cell

binding domain of TSP1, and probably all the other TSP family members, binds IAP/CD47 and thus modulates signaling from $\alpha v\beta 3$, $\alpha IIb\beta 3$, and probably other integrins which are subject to activation by inside-out signaling (Schwartz et al., 1995; Gao et al., 1996a; Chung et al., 1997). IAP/CD47 is widely distributed and found on all types of EC. Expression of $\alpha v\beta 3$ during angiogenesis is important in EC migration and in EC survival (Brooks, 1996). TSP1 can be a ligand for both IAP/CD47 (via CBD) and $\alpha v\beta 3$ (via the RGD sequence upstream of the CBD). Therefore, we speculate that TSP1 may have a role in reestablishing the differentiated or vessel phenotype of EC as they undergo the phenotypic switch from the active migratory state back to the quiescent, differentiated state. This may depend on the local concentration of TSP1, the expression pattern of its receptors on EC, and perhaps other factors, such as expression of PECAM-1.

In summary, it appears that down regulation of PECAM-1 or expression of TSP1 in bEND.3 cells results in concerted regulation of many genes that are important regulators of EC phenotype including CD36, the vascular endothelial growth factor receptors, the $\alpha v\beta 3$ integrin, fibronectin, $\beta 1$ integrin, collagens, metalloproteinases and their inhibitors. There is a reciprocal relationship between TSP1 and PECAM-1 expression. We believe TSP1 and PECAM-1 are components of a regulatory switch that controls the angiogenic or differentiated phenotype of endothelium. Understanding the regulation of this reciprocal relationship will reveal important functions of these proteins in regulation of angiogenesis. Examination of the pattern of expression of these genes in vivo may provide insight into the regulatory factors involved. PECAM-1 expression is detected very early in development (E7.5) (DeLisser et al., 1997a). However, TSP1 expression is not detected until much later time after the vasculature is well established (Reed et al., 1995). The mutant mice which lack TSP1 (TSP1^{-/-}) develop normally and exhibit no defect in their vasculature suggesting that TSP1 may not be involved in regulation of embryonic angiogenesis (Lawler et al., 1998). However, TSP1 may play a role in repair and adult associated angiogenesis since TSP1^{-/-} mice exhibit a delay in wound healing (DiPietro et al., 1996). TSP2 mutant mice exhibit extensive abnormalities in development of their connective tissues along with a bleeding problem and high blood vessel densities in several organs (Kyriakides et al., 1998). Therefore, TSP2 may be involved in regulation of embryonic angiogenesis. Much work remains to be done to understand the role of TSP and PECAM-1 isoforms in developmental, repair associated, and reproductive angiogenesis.

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