

Peptides Derived from Two Separate Domains of the Matrix Protein Thrombospondin-1 Have Anti-Angiogenic Activity

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Abstract. Thrombospondin-1 (TSP1) is a large modular matrix protein containing three identical disulfide-linked 180-kD chains that inhibits neovascularization in vivo (Good et al., 1990). To determine which of the structural motifs present in the 180-kD TSP1 polypeptide mediate the anti-angiogenic activity, a series of protease-generated fragments were tested using several in vitro and in vivo assays that reflect angiogenic activity. The majority of the anti-angiogenic activity of TSP1 resides in the central 70-kD stalk region which alone could block neovascularization induced by bFGF in the rat cornea in vivo and inhibit both migration in a modified Boyden chamber and [³H]thymidine incorporation stimulated by bFGF in cultured capillary endothelial cells. Although TSP1 has been shown to bind active TGFβ1, this cytokine could not account for the inhibitory effects of the stalk region of TSP1 on cultured endothelial cells.

Peptides and truncated molecules were used to further localize inhibitory activity to two domains of the central stalk, the procollagen homology region and the properdin-like type 1 repeats. Trimeric recombinant TSP1 containing NH₂-terminal sequences truncated after the procollagen-like module inhibited endothelial cell migration in vitro and corneal neovascularization in vivo whereas trimeric molecules truncated before

this domain were inactive as was the NH₂-terminal heparin-binding domain that is present in both recombinant molecules. A series of peptides from the procollagen-like region, the smallest of which consisted of residues 303–309 of TSP1, inhibited angiogenesis in vivo in the rat cornea and the migration of endothelial cells in vitro. A 19-residue peptide containing these sequences blocked vessel formation in the granulation tissue invading a polyvinyl sponge implanted into the mouse. Nineteen residue peptides derived from two of the three type 1 repeats present in the intact TSP1 molecule blocked neovascularization in vivo in the rat cornea and inhibited the migration of cultured endothelial cells with ED₅₀'s of 0.6–7 μM. One of these peptides, containing residues 481–499 of TSP1, also inhibited vessel formation in granulation tissue invading sponges in vivo.

These results suggest that the large TSP1 molecule employs at least two different structural domains and perhaps two different mechanisms to accomplish a single physiological function, the inhibition of neovascularization. The definition of short peptides from each of these domains that are able to block the angiogenic process may be of use in designing targeted inhibitors of the pathological neovascularization that underlies many diseases.

THE growth of new blood vessels in adult organisms is rare in most tissues in the absence of wounding or disease. Although potent angiogenic factors able to stimulate new vessel growth can be isolated from many tissues (Folkman and Klagsbrun, 1987), they are ineffective

under normal physiological conditions, due in part to the presence of molecules that antagonize them and act to inhibit angiogenesis (Bouck, 1990; Klagsbrun and D'Amore, 1991; Folkman and Shing, 1992). One molecule that is particularly well positioned to play an active physiological role in maintaining the quiescence of vessels is thrombospondin. It is both a constituent of the matrix that surrounds mature vessels and a potent inhibitor of neovascularization (Good et al., 1990).

Thrombospondin-1 (TSP)¹ is a 450-kD trimeric molecule

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1. *Abbreviation used in this paper:* TSP, thrombospondin.

of mosaic construction and multiple functions that is present in platelet alpha granules and is secreted by a wide variety of other cells including endothelia and is incorporated into their matrix (for review see Lawler, 1986; Frazier, 1987; Asch and Nachman, 1989; Mosher, 1990; Frazier, 1991; Bornstein, 1992). TSP can mediate cell-cell interactions, including the attachment of malarial organisms and cells infected by them to endothelial cells. It also plays a role in cell-substrate interactions. Many cells attach, spread, and migrate on insoluble thrombospondin, although for endothelial cells TSP can be anti-adhesive. Endothelial cells can attach to TSP, but they generally spread poorly (Lahav, 1988; Taraboletti et al., 1990; Tuszyński et al., 1992) unless high calcium or DTT reduction have been used to expose the RGD site on TSP1 (Sun et al., 1992). When endothelial cells are spread on other substrates their focal contacts are broken by exposure to soluble TSP (Murphy-Ullrich and Hook, 1989; Murphy-Ullrich et al., 1991). In addition, TSP can influence the growth of a variety of cell types. When applied to endothelial cells it inhibits growth (Bagavandoss and Wilks, 1990; Taraboletti et al., 1990; Murphy-Ullrich et al., 1992), an effect shown in one case to be due to active TGF β that had bound to and copurified with platelet TSP (Murphy-Ullrich et al., 1992).

TSP was first identified as an inhibitor of angiogenesis when the amino acid sequence of an anti-angiogenic hamster protein whose secretion was controlled by a tumor suppressor gene (Rastinejad et al., 1989) was found to be similar to a sequence in human platelet TSP (Good et al., 1990). Genuine human TSP was then purified from platelets and shown to block neovascularization in vivo in the rat cornea as well as to inhibit the chemotaxis of capillary endothelial cells towards angiogenic factors in vitro (Good et al., 1990). This inhibitory role for TSP in angiogenesis is supported by its presence adjacent to mature quiescent vessels and its absence from actively growing sprouts both in vivo (O'Shea and Dixit, 1988) and in vitro (Iruela-Arispe et al., 1991); by the failure of some hemangiomas consisting of fast growing endothelial cells to make TSP (Sage and Bornstein, 1982); by the ability of antibodies to TSP to increase endothelial cell cord formation in vitro (Iruela-Arispe et al., 1991); and by the dramatic downregulation of TSP mRNA in cultures of endothelial cells that are forming tubules (Canfield et al., 1986).

The current study was undertaken to determine which domains of the complex TSP molecule are responsible for its ability to block neovascularization. Quite recently it has been found that at least four different genes encode distinct TSP-like proteins (Bornstein, 1992; Lawler et al., 1993) and isoforms of TSP exist that may be attributed to alternate splicing (Frazier, 1991). The work reported here deals with the well defined prototype molecule, now called TSP1, that was originally purified from platelets and whose cDNA has been cloned from both fibroblasts and endothelial cells (Hennessy et al., 1989; Lawler and Hynes, 1986). The TSP1 protein from platelets consists of three identical 180-kD subunits with NH₂- and COOH-terminal globular domains connected by a central stalk. The stalk region contains (a) the cysteines that form inter-chain links and adjacent regions that promote trimer formation; (b) a stretch of 96 amino acids that are homologous to sequences in the NH₂-terminal domain of the pro α -1 chains of Collagen I; (c) three

type 1 repeats also found in proteins encoded by *Plasmodium falciparum*, properdin, and proteins C6 through C9 that are active in the complement cascade; and (d) three EGF-like type 2 repeats (see reviews cited above and Fig. 1). We have found that the anti-angiogenic activity of TSP1 resides in this central stalk region of the TSP molecule. It can be reproduced by small peptides derived either from the procollagen-like region or from the properdin-like type 1 repeats. These findings define two distinct domains of the TSP1 molecule that mediate the same function and suggest testable hypotheses for the mechanism by which TSP1 inhibits neovascularization.

Materials and Methods

General Reagents

Recombinant bFGF and TGF β 1 and antibodies against purified platelet TGF β developed in the chicken or rabbit were purchased from R & D Sys. Inc. (Minneapolis, MN). Monoclonal antibody A4.1 which recognizes an epitope on the stalk region of TSP1 was prepared as previously described (Prater et al., 1991).

Thrombospondin and Its Peptide Fragments

Human thrombospondin-1 was purified from platelet releasate as described (Dixit et al., 1984) and was digested with TLCK-treated chymotrypsin in 10 mM EDTA. Fragments were purified by gel filtration and heparin-Sepharose affinity chromatography following exactly the methods described by Prater et al. (1991). Individual fragments of TSP1, one consisting of the monomeric NH₂-terminal heparin-binding domain of TSP (25 kD) and another containing two nested trimers from the central stalk region in a 1:1 ratio, one migrating on SDS-PAGE reducing gels at 50 kD, and one at 70 kD (50/70 kD), were characterized by silver-stained SDS-PAGE and Western blotting with a panel of anti-TSP monoclonal antibodies and were found to be free of intact TSP (Prater et al., 1991). A naturally occurring fragment of the hamster homologue of TSP1, a 140-kD monomer whose NH₂ terminus corresponds to amino acid 293 of the human TSP1 (hamster 140-kD, Good et al., 1990) was purified from the serum free-conditioned media of cultured hamster cells by published methods (Rastinejad et al., 1989). NH₂-terminal trimers of TSP1 truncated at residue 277 (TSP-277) or 381 (TSP-381) were produced and purified using a baculovirus expression system by Sottile and Mosher as described (Sottile et al., 1991) and generously provided by them for this study. The relationship of all these fragments to the 180-kD TSP monomer is illustrated in Fig. 1.

Peptides based on TSP amino acid sequences were synthesized by the Washington University Protein Chemistry Facility (Mark Frazier, technical director) using solid phase synthesizer (model 3804; Appl. Biosystems, Inc., Foster City, CA) and purified by reverse phase HPLC as detailed in Prater et al. (1991). To remove organic salts, peptides were dissolved in 1 mM HCl, passed over a G50 gel filtration column and lyophilized. All peptides were characterized by amino acid analysis and sequencing. As indicated in the text, some cysteines in the TSP sequence were replaced by alanines and two COOH-terminal arginines by lysines. The pro α (1) peptide was made in the same way at the Biotechnology Center at Northwestern University.

In Vitro Assays for Angiogenic Activity

Bovine adrenal capillary endothelial cells were the kind gift of Dr. Judah Folkman, Harvard University. Cells were carried in DME with 10% donor calf serum (Flow Laboratories, McLean, VA) and 100 μ g/ml endothelial cell mitogen (Biomedical Technols. Inc., Stoughton, MA), and used between passages 10 and 15. To measure migration, cells were starved for 24 h in DME containing 0.1% BSA, harvested, suspended in DME with 0.1% BSA, and plated at 1.75×10^4 /well on the lower surface of a gelatinized 0.5 μ m filter (Nucleopore Corporation, Pleasanton, CA) in an inverted modified Boyden chamber. After 1-2 h at 37°C during which time the cells adhere to the filter, the chamber was reinverted, test material was added to the top well, and the chamber incubated 3-4 h at 37°C to allow migration. Chambers were then disassembled, membranes fixed and stained, and the

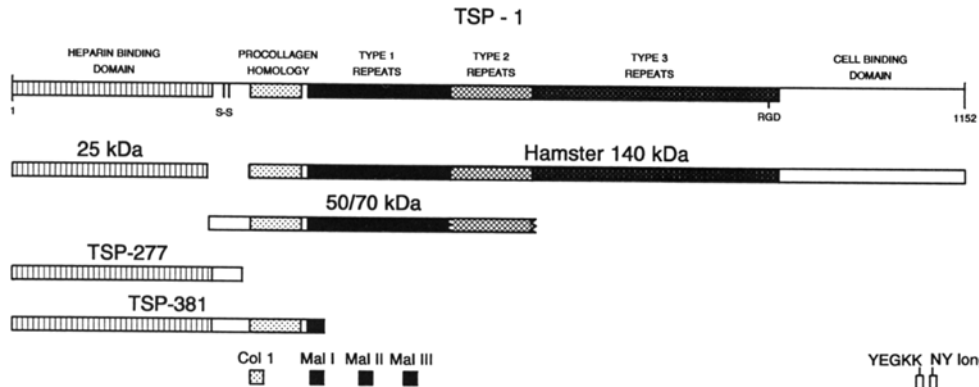


Figure 1. The relationship of various protein fragments and peptides to the 180-kD subunit of thrombospondin 1 and its previously defined domains. S-S indicates the position of the cysteine residues that join three of these 180-kD subunits in the native trimeric TSP1 molecule. Peptide fragments containing this region are also trimeric whereas fragments lacking it are monomeric. Please see Table II for exact sequences of small peptides.

number of cells that had migrated to the top of the filter in 10 high-powered fields counted (Good et al., 1990). DME with 0.1% BSA was used as a negative control and either bFGF (10 ng/ml) or conditioned media from angiogenic hamster cell lines (20 µg/ml total protein; Rastinejad et al., 1989) as a positive control. Each sample was tested in quadruplicate in a single experiment and experiments were repeated at least twice. To allow comparison of experiments performed on different days, migration data is reported as % of maximum migration towards the positive control, calculated after subtraction of background migration observed to DME plus BSA. Test compounds that depressed the random movement of endothelial cells showed a negative value for % migration when it was calculated in this way. Very high concentrations of TSP caused endothelial cells to detach from the membrane. This was detected by counting cells on the lower face of the membrane. In the rare instances when loss of cells exceeded 10%, the number of migrated cells was corrected for this loss. Such instances are marked by * in Fig. 3.

To monitor toxicity, endothelial cells were treated with each of the tested compounds at a range of concentrations that included the highest concentration for which data is reported here, under conditions identical to those used in the migration assay and cells excluding Trypan blue counted. In no case could inhibition of migration be attributed to toxicity as measured in this way. When necessary, as noted in the text, endothelial cells were pretreated for 36–48 h with peptides at 20 µM in DME with 0.1% BSA before use in migration assays. In these cases toxicity was tested over a similar time frame and found to be negligible.

Incorporation of [³H]thymidine followed by autoradiography was used to estimate the number of cells synthesizing DNA. Capillary endothelial cells were plated at 5×10^3 cells/0.25 ml chamber on 16-chamber gelatinized glass slides (Nunc, Naperville, IL) in complete media. After 24 h, the serum was lowered to 0.5% and incubation continued for 30 h. The cells were then incubated 30 h in serum-free media consisting of DME and Hank's F12 (1:1) with 1 mg/ml BSA and 5 µg/ml human transferrin (GIBCO BRL, Gaithersburg, MD). Test substances were then added to quadruplicate wells along with 1 µCi/chamber of [³H]thymidine (Amersham Corp., Arlington Heights, IL) and cultures incubated at 37°C for 30 h. TSP1 and its fragments were used at 40 nM, TGFβ at 16 pM, and bFGF at 0.6 nM. Where indicated, anti-TGFβ antibodies or anti-TSP1 antibody A4.1 were added at a final concentration of 20 µg/ml. The assay was terminated by the addition of 2.5% glutaraldehyde in PBS. Slides were treated for 30 min with 0.1 M glycine, rinsed, and after air drying were coated with NTB2 liquid emulsion (Kodak, Rochester, NY) diluted 1:1 with water, exposed for 3 d at 4°C, and developed in D-19 (Kodak). After counterstaining in Camco Quick Stain (Scientific Products, McGaw Park, IL), at least 400 nuclei were examined for each test substance and the percent nuclei labeled with more than 20 grains determined.

In Vivo Assays for Angiogenic Activity

To assess angiogenesis associated with the formation of granulation tissue, sponge implants were used as previously described (Fajardo et al., 1988). Polyvinyl-alcohol foam discs 10-mm diam × 1-mm thick were prepared by first removing a 2-mm diam central core of sponge. PBS or test peptides at 100-µM concentration were added to the sponge core which was then coated with 5 µl of sterile Hydron (Interferon Sciences, New Brunswick, NJ). After solidifying, the coated core was returned to the center of the

sponge which was then covered on both sides with 5 µm filters and secured in place with glue (Millipore Corp., Bedford, MA). One control and one test disc were then implanted subcutaneously in the lower abdomen of anesthetized Balb/c female mice where granulation tissue could invade the free perimeter of the disc. Wounds were closed with autoclips and animals left undisturbed until time of sacrifice.

Quantitative estimates of thymidine incorporation in situ into endothelial cells in the discs were obtained as previously described (Polverini et al., 1977). Sponge implants were evaluated at days 5, 7, 10, and 14 after implantation. 30 min before sacrifice, mice were injected with 1 µCi/g of [³H]thymidine in saline (specific activity 6.7 Ci/mM; New England Nuclear/Du Pont, Wilmington, DE). Sponges were removed and embedded enface to provide a uniform section of the entire circumference. Tissues were fixed in 10% neutral buffered formalin, dehydrated through a graded series of alcohols, and embedded in glycol methacrylate (Polysciences, Miles, IL). Autoradiograms were prepared by dipping sections mounted on acid-cleaned glass slides into NTB type 2 emulsion (Eastman Kodak). After exposure for 4 wk at 4°C, autoradiographs were developed in half strength D-19 developer, fixed in Kodak Rapid Fixer, and stained with hematoxylin and eosin. Quantitation of endothelial cell labeling was performed by counting all endothelial cells lining capillaries and venules that extended from the periphery to the center of the sponge by rectilinear scanning under oil immersion (×1,000). A total of 500–700 endothelial cells were counted in each of two sponges containing either PBS, TSP, or peptide fragments. Cells were considered labeled if five or more grains were detected over the nucleus. The percentage of labeled cells was calculated and a chi square analysis of data derived from control and experimental sponges performed.

The ability of experimental samples to induce and/or inhibit neovascularization in the normally vascular cornea of the rat was also assessed as previously described (Rastinejad et al., 1989; Polverini et al., 1991). Briefly, a pellet of less than 5 µl was formulated containing test ingredients in hydroxyethylmethacrylate (Hydron[®]) and implanted 1–1.5 mm from the limbus of the cornea of the anesthetized rat. Neovascularization was assessed (Polverini et al., 1991) 3, 5, and 7 d after implantation. To photograph responses, animals were perfused with colloidal carbon to label vessels and corneas were excised, flattened, and photographed.

Results

Localization of Anti-Angiogenic Activity to the Stalk Region of TSP1

To determine which domains of the TSP1 molecule can block neovascularization, purified platelet TSP1 was digested with chymotrypsin in the absence of calcium and fragments corresponding to the 25 kD NH₂-terminal heparin-binding domain and to the nested 50 and 70 kD portions of the stalk region were isolated. In addition, the foreshortened hamster protein, gp140 (Rastinejad et al., 1989) that represents the COOH-terminal 140 kD of TSP1 (Good et al., 1990) was prepared. The position of these fragments within the whole

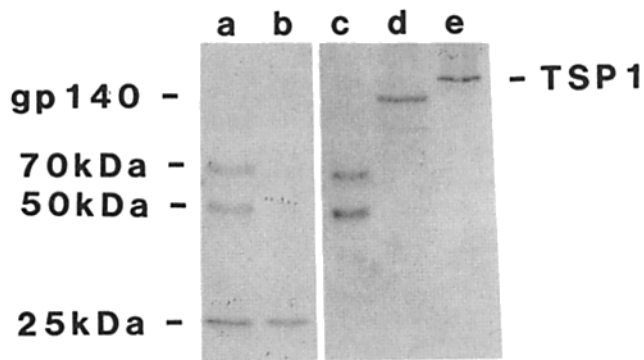


Figure 2. TSP1 and its proteolytic fragments. TSP1 purified from platelet releasates was digested with TLCK-treated chymotrypsin and proteolytic fragments isolated. In addition, the foreshortened fragment of TSP1 produced by Syrian hamster cells, gp140, was isolated from conditioned media. Proteins and fragments were run on an SDS-PAGE gel and stained with Coomassie blue. (a) total digest of TSP1; (b) purified 25-kD fragment; (c) purified 50/70-kD fragment; (d) purified hamster gp140; and (e) human platelet thrombospondin 1.

molecule is indicated in Fig. 1 and has been verified by data presented elsewhere (Good et al., 1990; Prater et al. 1991). The comparative mobility of these fragments on gels is shown in Fig. 2. Each of the protein fragments was tested for ability to induce and/or inhibit neovascularization in vivo in the rat cornea (Table I). None of the preparations stimulated neovascularization when tested alone. All of the chymotryptic fragments except the NH₂-terminal 25 kD heparin-binding region blocked neovascularization in response to bFGF, localizing the anti-angiogenic activity to the 50/70 kD stalk region. An example of inhibition of bFGF-induced neovascularization by the stalk region alone in the rat cornea is shown in Fig. 3 B.

To observe the activity of each protein fragment directly on endothelial cells in the absence of the accessory cells that are unavoidable in vivo and to quantitate their relative potencies, an in vitro migration assay was used. The ability of a compound to induce or inhibit the migration of cultured capillary endothelial cells in vitro has been found to correlate well with its ability to induce or suppress neovascularization in vivo (Zetter, 1987; Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). When tested alone at concentrations of 5 nM or less, no TSP1 fragments were able to stimulate the migration of endothelial cells (Fig. 4 a), reflecting their inability to induce neovascularization in vivo (Table I). But when higher concentrations were tested in vitro, both the entire TSP1 molecule and the 25-kD fragment containing the heparin-binding domain stimulated migration. Fragments lacking this domain, including the 50/70-kD fragment and the hamster gp140 (data not shown), remained inactive at concentrations up to 600 nM. The stimulatory activity of the 25-kD fragment is due to the stimulation of random chemokinesis rather than of directed chemotaxis for migration into the upper well of the Boyden chamber was stimulated equally if the 25-kD fragment was added to the upper well, the lower well, or to both wells (data not shown). Taraboletti et al. (1990, 1992) have also reported a dramatic stimulation of migration by intact TSP at high concentrations

Table I. The Effect of Protein Fragments of Thrombospondin on Corneal Neovascularization

Protein fragment tested	Positive corneas/number implanted	
	Alone	+ Inducer
None	0/4	4/4
TSP	0/5	0/5
TSP		
chymotryptic digest	0/6	0/3
140 kD (from hamster)	0/8	0/5
50/70 kD	0/7	0/5
25 kD	0/6	5/6
TSP-277	0/3	3/3
TSP-381	0/5	0/4

Hydron pellets containing TSP1 protein or its various fragments at 25–30 nM were formulated either with or without bFGF as an inducer at 0.15 μ M and implanted into rat corneas. Vigorous vessel ingrowth from the limbus at 7 d was recorded as a positive response (see Fig. 3 A).

and have localized this activity to the heparin-binding domain, represented here by the 25-kD fragment.

When the same protein fragments were tested for ability to block endothelial cell migration stimulated by bFGF, the results were similar to those observed in vivo. The 25-kD peptide was ineffective, but complete inhibition was obtained with the 50/70 fragment and with intact TSP1 (Fig. 4 b). The total unfractionated digest and hamster 140-kD protein were also tested over a similar concentration range and also inhibited migration, with an ED₅₀ of 1.1 nM for the digest and 0.7 nM for hamster 140 kD, values similar to the ED₅₀ of 0.9 nM seen with the 50/70-kD fragment. The 50/70 fraction differed in two days from the intact TSP1 molecule. At low concentrations it was not quite as potent an inhibitor as TSP1, possibly due to its lack of the heparin-binding domain which can mediate the attachment of TSP1 to endothelial cells (Murphy-Ullrich and Mosher, 1987). At high concentrations, the 50/70 fragment remained inhibitory whereas the intact TSP1 induced migration. This difference can also be attributed to the heparin-binding domain which, when isolated as the 25-kD fragment, is a potent inducer of migration at high concentrations (Fig. 4 a).

These data suggest that the ability of TSP1 to inhibit neovascularization in vivo and endothelial cell migration in vitro lies primarily in the stalk region of the TSP1 molecule. To further localize the residues responsible for this activity, peptides from two different stalk domains were synthesized and tested.

Peptides Derived from the Properdin Type 1 Region of TSP1 Can Inhibit Neovascularization

The properdin-like repeat region 1 of the TSP1 molecule contains a sequence of \sim 60 residues that is repeated three times. Each repeat is \sim 47% identical to six similar repeats found in the human complement protein properdin and to region II of a circumsporozoite protein common to all species of malaria parasites (Robson et al., 1988; Goundis and Reid, 1988). Protein fragments of TSP containing these repeats as well as peptides derived from them can serve as attachment factors for melanoma cells and for endothelial cells (Prater et al., 1991; Guo et al., 1992; Tuszynski et al., 1992). The

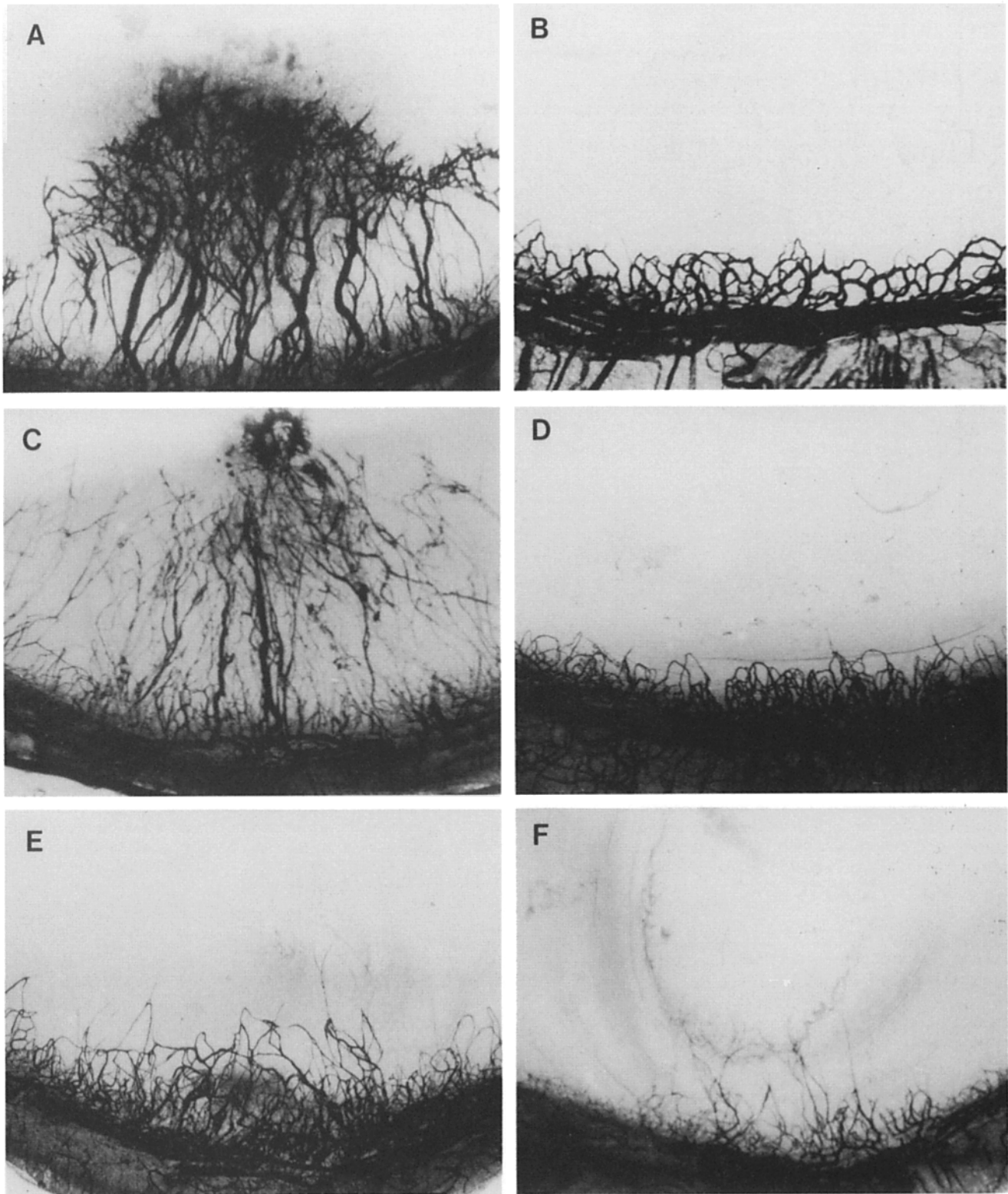


Figure 3. The effect of fragments and peptides derived from TSP1 on neovascularization induced in vivo in the rat cornea by bFGF. Hydron pellets were formulated as described in legend to Tables I and III and implanted into corneas of rats. 7 d later animals were perfused with colloidal carbon, corneas excised and vessels in the region of the pellet implant photographed. Pellets contained (A) bFGF; (B) bFGF + 50/70 kD fragment of TSP1; (C) bFGF + Mal I peptide; (D) bFGF + Mal II peptide; (E) bFGF + Mal III peptide; or (F) bFGF + Col 1 peptide.

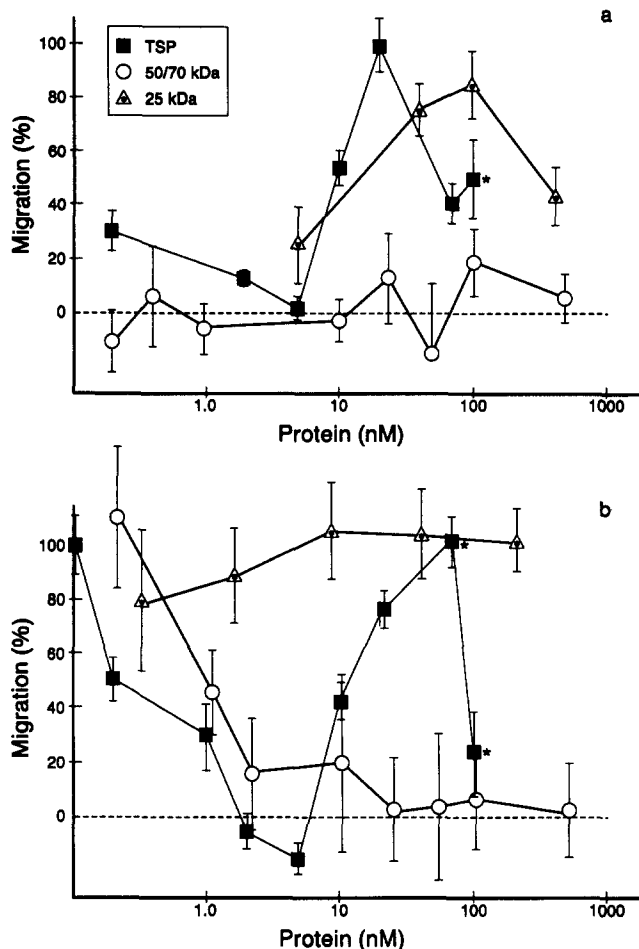


Figure 4. The effect of thrombospondin, its NH₂-terminal heparin-binding domain (25 kD) and its central stalk region (50/70 kD) on the migration of endothelial cells. Capillary endothelial cells were plated on the lower face of a gelatinized membrane in a modified Boyden chamber, the indicated compounds placed in the top well either with (a) or without (b) bFGF at 10 ng/ml and the number of cells that migrated to the top well were counted after 3–4 h at

most active of these peptides has a central motif, VTCG, that has been shown to be essential for the adhesion of malarial circumsporozoite proteins to human T cells (Rich et al., 1990). To determine if these cell-binding domains from the type 1 repeats of TSP have an effect on angiogenesis, peptides from each were synthesized (Mal peptides, Fig. 1 and Table II) and tested for their ability to block neovascularization in vivo.

In formulating these Mal peptides, alanines were substituted for cysteines in part because it is not yet clear whether the two cysteines in each Mal peptide are paired with one another or with cysteines located elsewhere in the molecule, and in part to avoid the formation of polymers under the oxidizing conditions of our assay. Free cysteines have clearly been shown to be dispensable when similar peptides serve as cell adhesion factors (Prater et al., 1991), bind CD36 (Asch et al., 1992), or block platelet aggregation (Tuszynski et al., 1992). Data reported below indicate that they are not essential for the inhibition of angiogenesis either.

Mal peptides derived from the second and third type 1 repeats were able to block neovascularization in vivo in two different assays. In the rat cornea assay, Mal II and Mal III inhibited neovascularization induced by bFGF whereas structurally similar Mal I was inactive (Table III; Fig. 3, C, D, and E). In a second in vivo assay, Mal peptides were tested for their ability to influence the neovascularization that accompanies the formation of the granulation tissue characteristic of a healing wound. Polyvinyl sponges about the size of a dime that were open only around their circumference were constructed with central pellets containing pep-

37°C. Data is reported as % of maximum migration summed over five experiments. 100% varied between experiments from 79 to 209 cells migrated/10 high-powered fields. At high concentrations intact TSP1 caused endothelial cells to detach from the membrane. Points marked with * are derived from wells where more than 10% of the cells detached and they have been corrected for this cell loss. Bars indicate SEM.

Table II. Peptide Sequences Employed in Analysis

Peptides from type 1 repeats of TSP1		Residues
Mal I	SEWTS C*STSC*GN G I QQRGK	368–386
Mal II	SPWSS C*SVT C*G DGV ITRIR	424–442
Mal III	SPWD I C*SVT C*G GGVQKR SK	481–499
Mal overlap	D I C*SVT C G	484–491
Peptides from the procollagen homology region of TSP1		
Col 1†	(L)RRPPLCYHNGVQYRNNEEWTVD S	294–317
N-Col	LRRPPLCYHNGVQYR	294–308
C-Col	GVQYRNNEEWTVDSC	304–318
Col overlap	NGVQYRN	303–309
Scrambled Col overlap	RQGN YVN	303–309 (scrambled)
Peptide from collagen I		
Proα1	N I P P I T C*V Q N G L R Y	34–47§
Control peptides		
YEGKK	Y E G K K	1108–1112
NYlong	A D S G P I Y D K T Y A G G R L G	1115–1131

* Indicates those cysteines that were replaced by alanine when peptides were synthesized.

† 90% preparation missing first L.

§ Peptide derived from human proα1(I) collagen sequence (Chu et al., 1984).

Table III. The Effect of Thrombospondin-derived Peptides on Corneal Neovascularization

	Positive corneas/number implanted	
	Peptide alone	Peptide + inducer
Peptides from repeat region 1		
Mal I	0/4	3/4
Mal II	0/5	0/4
Mal III	0/4	0/11
Peptides from procollagen like region		
Col 1	0/3	0/4
N-Col	0/8	0/3
C-Col	0/3	0/4
Col overlap	0/3	0/8
Scrambled Col overlap	0/3	3/3
Pro α -1	0/3	0/7
Controls		
TSP-1	0/5	0/4
buffer	0/4	6/6
NYlong	0/6	3/3

Hydron pellets containing peptides at concentrations ranging between 100–500 μ M with or without bFGF as an inducer at 0.15 μ M were implanted into rat corneas and neovascularization assessed 7 d later. NYlong, a peptide from the COOH-terminal region of TSP, served as a negative control.

tide and implanted into mice. The presence of TSP1 in the central pellet significantly decreased the number of vessels present in the sponge implant (Fig. 5). This effect was quantified by *in vivo* labeling with [3 H]thymidine followed by autoradiography and counting of labeled endothelial cells (Table IV). Both TSP1 and the Mal III peptide significantly decreased the number of thymidine-labeled endothelial cells present in the sponges at days 10 and 14. As in the cornea assay, Mal I was inactive. The effect of Mal II was not statistically significant *p* value of 0.001 (Table IV), but did show inhibition at day 14 that was significant at a less stringent *p* value of 0.05.

To compare the Mal peptides to one another quantitatively away from the complex environment present *in vivo*, extensive dose response curves were performed using the ability to block migration of cultured endothelial cells as an assay. Each peptide was tested over a range from 0.5 or 5 μ M to 200 or 500 μ M, stopping where toxicity became evident. From the resulting dose response curves containing 5–7 points, the concentration required to achieve half maximal response was calculated. None of the peptides induced endothelial cell migration when tested alone (Table V). When tested with inducer, the two peptides that inhibited neovascularization in the rat cornea were found to block cell migration. The Mal I peptide that was inactive *in vivo* was also inactive *in vitro*. The conditioned media used as an inducer in Table V contained low levels of gp140, the shortened hamster version of TSP1 (Rastinejad et al., 1989). This protein had no effect on the ED₅₀. When retested using pure recombinant bFGF as an inducer, Mal III inhibited migration with an ED₅₀ of 7 μ M. This value and those obtained in parallel for non-inhibitory peptides were similar to values reported in Table V. A shorter peptide containing the central region shared by inhibitory peptides Mal II and Mal III was synthesized (Mal overlap, Table II), but was inactive in the migration assay (Table V).

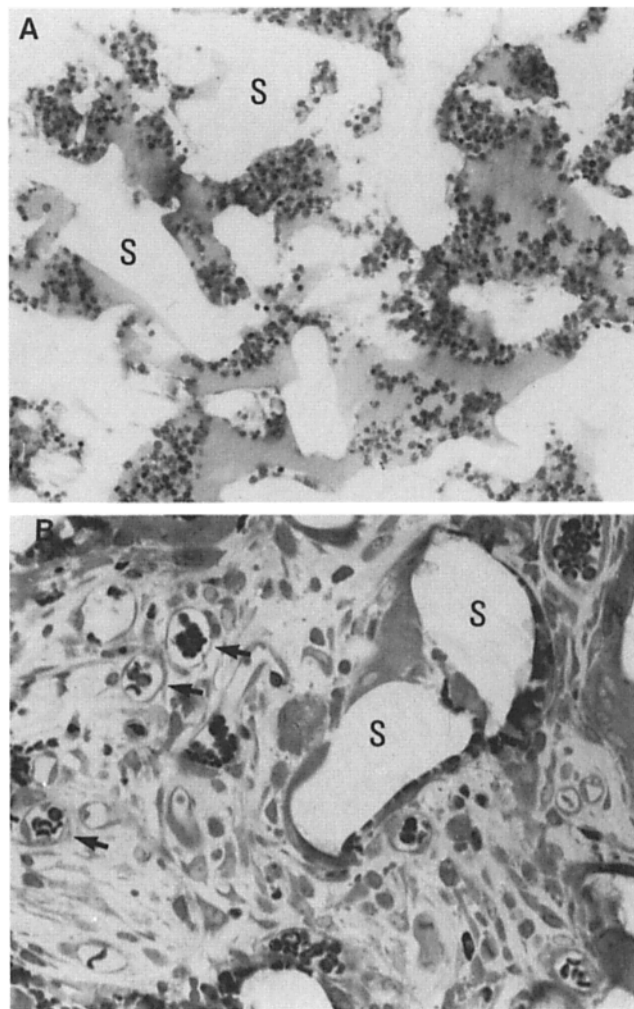


Figure 5. Neovascularization associated with wound healing is severely limited by TSP1. Enface sections through polyvinyl sponges recovered 10 d after implanting in the mouse were photographed midway between central pellet and periphery of the sponge disc. (A) Sponge containing central pellet formulated as in legend to Table I containing TSP1. Note the presence of inflammatory cells but the absence of organized capillaries. (B) Sponge containing a central pellet containing buffer. Note luxuriant ingrowth of new vessels. S indicates sponge material; arrows mark some of the vessels. Magnification 225 \times .

Peptides Derived from the Procollagen Homology Region of TSP1 Block Neovascularization

The procollagen-like region of TSP1 consists of \sim 90 residues, including 10 cysteines, that are homologous to the 10.7-kD propeptide that is cleaved from the NH₂ terminus of human collagen I during maturation of the collagen fibril (Vuorio and de Crombrugge, 1990). This collagen propeptide can selectively downregulate synthesis of the pro α 1 and pro α 2 chains of collagen I (Wiestner et al., 1979). The fact that many diverse inhibitors of angiogenesis have in common the ability to interfere with collagen synthesis and/or deposition (Ingber and Folkman, 1988; Maragoudakis et al., 1988) led us to test the procollagen homology region of TSP1 for inhibitory activity. A series of peptides (Col peptides, Table II) were synthesized and tested.

Table IV. Modulation of Wound Neovascularization by Thrombospondin and Its Peptides

Day after implantation	% Thymidine-labeled endothelial cells							
	PBS control	TSP	Mal I	Mal II	Mal III	NYLONG	YEGKK	Col 1
5	3.84 ± 1.15	2.27 ± 1.89	4.23 ± 2.01	5.01 ± 1.11	2.11 ± 0.98	2.99 ± 1.99	4.55 ± 2.48	2.98 ± 2.35
7	5.36 ± 0.56	1.98 ± 2.18	6.17 ± 1.11	6.15 ± 2.17	2.38 ± 1.98	3.25 ± 2.75	7.19 ± 3.29	3.29 ± 1.18
10	8.13 ± 1.38	3.76 ± 2.08*	8.89 ± 2.39	8.81 ± 2.10	4.47 ± 2.39*	7.38 ± 2.29	10.18 ± 2.54	4.89 ± 2.15*
14	13.67 ± 2.25	7.49 ± 2.10*	12.18 ± 2.66	10.21 ± 1.19	6.19 ± 1.79*	14.92 ± 3.65	15.16 ± 3.08	7.88 ± 3.38*

One-half centimeter diameter, 1-mm thick discs of polyvinyl sponge were modified to release test substances from pellets at their center and implanted subcutaneously in the lower abdomen of Balb/c female mice. Animals were given a 30-min pulse of [³H]thymidine before being sacrificed on various days after implantation. Thymidine incorporation by endothelial cells lining capillaries and venules were counted on autoradiograms. Values reported are means ± SD.

* Significantly different (*P* < 0.001) when compared to controls.

The 24 residue Col 1 peptide as well as overlapping peptides from its NH₂ and COOH terminus and a short 7-residue peptide from the central overlap region were all able to block neovascularization in vivo in the rat cornea (Table III; Fig. 3 F). When the sequence of the seven amino acids in the Col overlap peptide was scrambled, activity was lost. The large Col 1 peptide was also effective at inhibiting angiogenesis in vivo in the sponge model (Table IV).

Despite their in vivo activity, the Col peptides were inactive when they were first tested in vitro for ability to inhibit endothelial cell migration. Reasoning that the time frame of the migration assay, where endothelial cells are exposed to inducers and/or inhibitors for only 3–4 h may be too short to register the effect of inhibitors that are working by indirect means, such as regulation of other secreted molecules, the assay was modified. Endothelial cells were pretreated for 36–48 h with peptide, and then harvested, plated into migration assay in a Boyden chamber, and their ability to migrate towards an inducer in the presence or absence of test peptides measured. The effect of peptides alone was also examined. Assayed in this way all the Col peptides that were active inhibitors in vivo in the rat cornea were able to inhibit migration towards bFGF in vitro (Figs. 6 and 7). The scrambled peptide was inactive in vitro as it had been in the cornea. Pretreatment by the Col 1 peptide but not by a control peptide could sensitize the endothelial cells to inhibition of migration by other Col peptides. Inhibition by the Mal III

Table V. The Effect of Peptides from the TSP Malarial Repeat Region 1 on Endothelial Cell Migration

	ED ₅₀ for endothelial cell migration (μM)	
	Induction of migration Peptide alone	Inhibition of migration Peptide + inducer
TSP	0.01	0.0002
Mal I	>100	>100
Mal II	>300	0.6
Mal III	>400	5
Mal overlap	>200	>200
NYlong	>400	>400

The ability of peptides to induce migration of capillary endothelial cells was measured by estimating from dose response curves the concentration in μM required to stimulate migration of endothelial cells by 50% of maximum. The ability of peptides to inhibit migration was measured by estimating from dose response curves the dose required to reduce capillary endothelial cell migration toward inducer by 50%. Inducer used was angiogenic conditioned media from transformed hamster cells. NYlong, a peptide from the COOH-terminal region of TSP1 served as a negative control. When no effect was observed, the > symbol was used to indicate the highest concentration at which it was possible to test each peptide within the limits of toxicity and solubility.

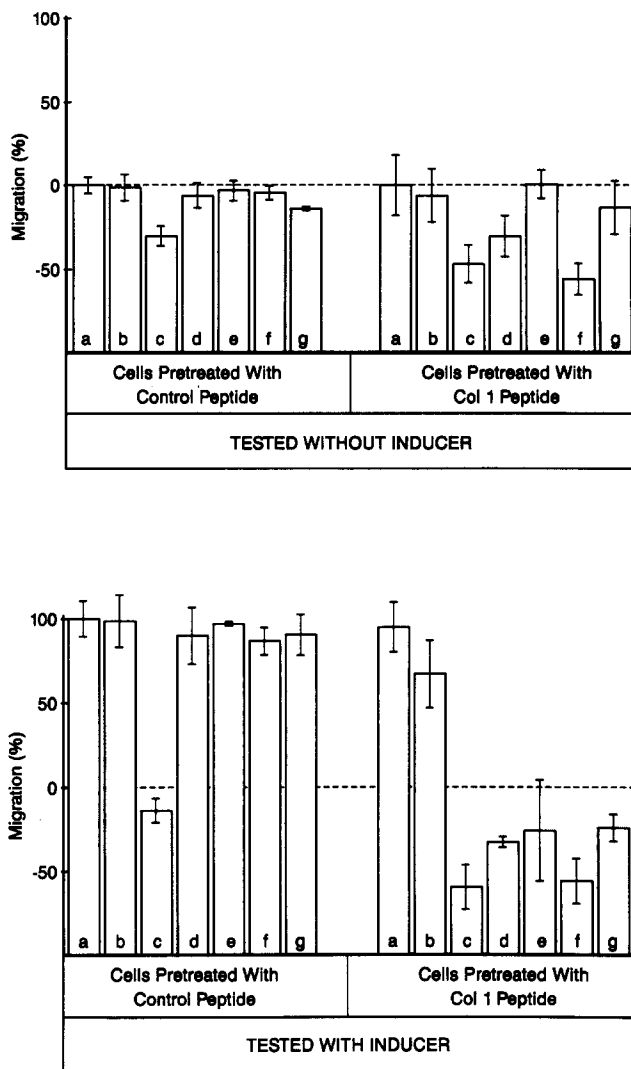


Figure 6. The effect of peptides from the procollagen homology region of thrombospondin on endothelial cell migration. Endothelial cells were pretreated for 36–48 h with control peptide (Mal I) or with Col 1 peptide at 20 μM, and then harvested and tested in a migration assay (as described in legend to Fig. 4) for ability to migrate to a chamber containing various peptides at 20 μM either with or without an inducer, bFGF at 10 ng/ml. Peptides used were (a) none, (b) Mal I, (c) Mal III, (d) Col 1, (e) N-Col, (f) C-Col, and (g) Proα1. Data is reported as % maximum migration to bFGF where 100% varied from experiment to experiment between 60 and 146 cells migrated/10 high power fields. Negative percentages reflect an inhibition of the random movement of endothelial cells.

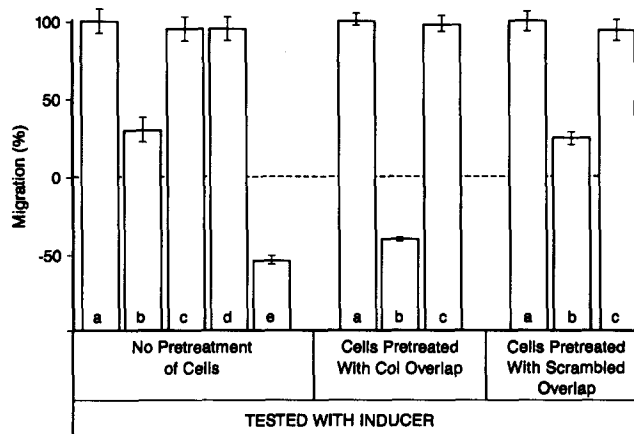
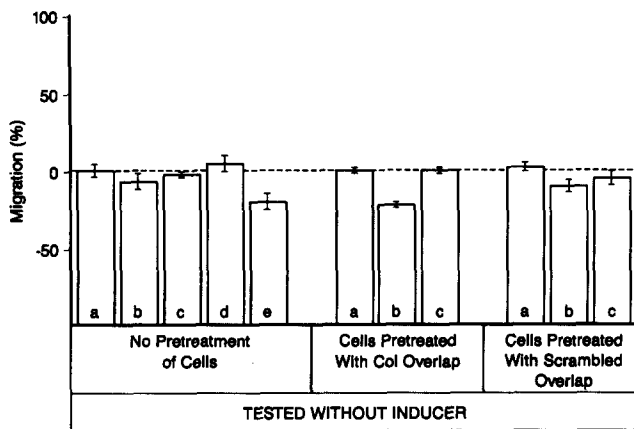


Figure 7. The effects of protein fragments and a seven amino acid peptide from the procollagen like region of the thrombospondin on endothelial cell migration. Experiment performed as in Fig. 6. Endothelial cells were either not pretreated, or were pretreated with the collagen overlap peptide or with a peptide derived from its scrambled sequences, and then tested for ability to migrate in the presence or absence of inducer (bFGF) to wells containing (a) no added peptide, (b) collagen overlap peptide, (c) scrambled amino acids from the collagen overlap peptide, (d) TSP-277, or (e) TSP-381. Peptides were used at 25 μ M, fragments at 5 nM; pretreatment was for 48 h.

peptide did not require such sensitization (Fig. 6 c) and sensitization was not sufficient to allow the inactive Mal I peptide to inhibit migration (Fig. 6 b). In additional experiments not shown it was found that both C-Col and N-Col were effective at sensitizing cells. Only the Col overlap peptide was able to significantly reduce migration without pretreatment of endothelial cells although pretreatment did increase its effectiveness (Fig. 7 b).

To determine if the homology between the procollagen region of TSP1 and the propeptide of authentic collagen I is functionally significant, a peptide was synthesized based on the published sequence of human pro α 1(I) collagen that covered that region homologous to the Col 1 peptide. This peptide (pro α 1, Table II) was found to inhibit neovascularization in the rat cornea in vivo (Table III) and to block migration towards bFGF in vitro when cells were pretreated with Col 1 (Fig. 6 g) or with the Pro α 1 peptide itself (data not shown).

Seeking evidence that the region of TSP1 from which the Col peptides are derived is exposed and thus active when TSP is in its native conformation, recombinant truncated TSP1 molecules secreted by insect cells were tested, one of which contained the procollagen homology region (TSP-381, truncated at amino acid 381) and one of which was lacking it (TSP-277, truncated at amino acid 277). The TSP-381 protein includes part of the inactive Mal I region, but none of the active Mal II or Mal III regions of the type 1 repeats. Both of the truncated proteins assume the trimer configuration characteristic of the native molecule (Sottile et al., 1991). When tested in vivo in the cornea assay (Table I) as well as when tested in vitro for ability to block endothelial cell migration (Fig. 7, d and e), only the fragment that retained the procollagen-like region was able to inhibit, suggesting that this region is exposed in this glycosylated version of the native trimeric molecule. In contrast to most Col peptides, TSP-381 could inhibit migration efficiently without pretreatment of the endothelial cells (Fig. 7 e). Unlike the peptides, TSP-381 does have a trimeric heparin-binding domain that may facilitate its interaction with cultured cells.

The In Vitro Anti-Angiogenic Activity of the 50/70 Stalk of TSP1 Is Not Due to Contaminating TGF β

It has recently been shown that active TGF β can bind to and copurify with TSP (Murphy-Ullrich et al., 1992), raising the possibility that contaminating TGF β could be responsible for a portion of the anti-angiogenic activity of intact TSP and its fragments. Although TGF β is usually found to induce angiogenesis when tested in vivo (Rastinejad et al., 1989; Roberts and Sporn, 1989; Yang and Moses, 1990), it can inhibit a number of endothelial cell functions in vitro, including endothelial cell mitogenesis, migration, proteolysis, and tube formation (Muller et al., 1987; Roberts and Sporn, 1989; Pepper et al., 1990). Two separate preparations of the purified TSP1 used in this work were tested in a bioassay for the presence of active TGF β by Dr. Joanne Murphy-Ullrich of the University of Alabama at Birmingham and found to contain 0.96–2.8 fmol of active TGF β per 2.2 pM of TSP1 trimer, or about one molecule of TGF β for every 1,000 molecules of TSP1 (Murphy-Ullrich, J., personal communication).

Two in vitro assays were used to test whether or not this small amount of TGF β played a role in the anti-angiogenic activity of TSP1. To test the influence of TGF β on the migration assay, an extensive TGF β dose response curve was generated (Fig. 8). At very low doses of <10 pg/ml TGF β 1 induced endothelial cell migration and this induction was additive with that stimulated by bFGF. At concentrations above 10 pg/ml, as has been observed previously (Muller et al., 1987), TGF β 1 inhibited endothelial cell migration. Similar biphasic responses have been seen for TGF β effects on the induced growth of small vessel endothelial cells (Plouet and Gospodarowicz, 1989; Myoken et al., 1990). The concentration of anti-TGF β 1 antisera sufficient to block both the inducing and inhibitory effects of TGF β 1 was determined, and then the ability of antibodies used at this concentration to relieve inhibition of migration caused by TSP1 was tested. Antibodies able to neutralize the effects of TGF β had no effect on inhibition of migration by TSP1 (Table VI).

As the biological effect of TSP-associated-TGF β was first detected using a mitogenesis assay (Murphy-Ullrich et al.,

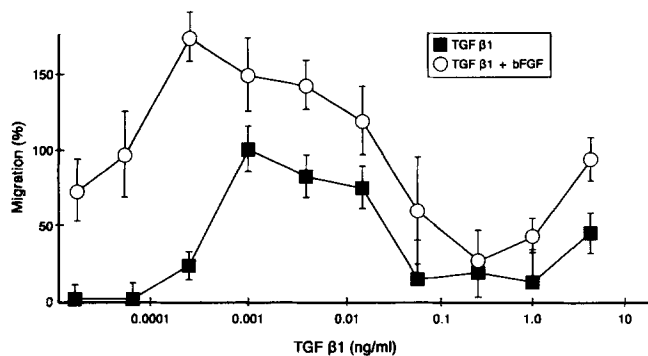


Figure 8. The induction and the inhibition of endothelial cell migration by TGFβ1. Methods as in legend to Fig. 4. 100% migration was taken as the maximal migration in response to TGFβ1 and is equivalent to an average of 138 cells migrated/10 high-powered fields.

1992), the ability of TSP1 and its fragments to influence bFGF-stimulated [³H]thymidine incorporation into capillary endothelial cells was also examined (Fig. 9). The inhibition of [³H]thymidine incorporation by TSP1 in this assay was similar to the inhibition of cell proliferation by TSP1 previously reported by Murphy-Ullrich et al. (1992) in that it was reversible partially by anti-TGFβ and partially by anti-TSP1. However, inhibition by the 50/70-kD stalk fragment of TSP1 was insensitive to anti-TGFβ and fully reversed by anti-TSP1, suggesting that inhibition by the stalk region of TSP1 is not due to associated TGFβ. A modest reduction in the number of nuclei incorporating thymidine was seen in the presence of the 25-kD fragment of TSP1. This fragment lacks the epitope recognized by the anti-TSP1 monoclonal used and was insensitive to both this antibody and to anti-TGFβ.

Discussion

TSP1 and peptides derived from two distinct regions of its central 70 kD stalk were able to block the growth of new blood vessels in two *in vivo* systems. In the rat cornea assay, considered the gold standard in the field, TSP1, the 50/70-kD stalk and peptides derived from it inhibited new vessels that are induced to grow into the normally avascular cornea in the absence of inflammation. TSP1 and its peptides also prevented neovascularization in the polyvinyl sponge assay, a model of normal wound healing where new vessels grow into the sponge material without added inducer in response to natural inflammatory signals as part of healing granula-

Table VI. Inhibitory Activity of Thrombospondin on Endothelial Cell Migration Is Not Sensitive to Neutralizing Antiserum against TGFβ

Additions to upper chamber	Migration of endothelial cells to upper chamber (%)	
	Control	+ anti-TGFβ
None	<1 ± 15	10 ± 16
TGFβ (1 pg/ml)	98 ± 17	<1 ± 17
bFGF (10 ng/ml)	100 ± 17	94 ± 19
bFGF + TGFβ (0.2 ng/ml)	28 ± 16	79 ± 16
bFGF + TSP (2.5 μg/ml)	<1 ± 15	<1 ± 18

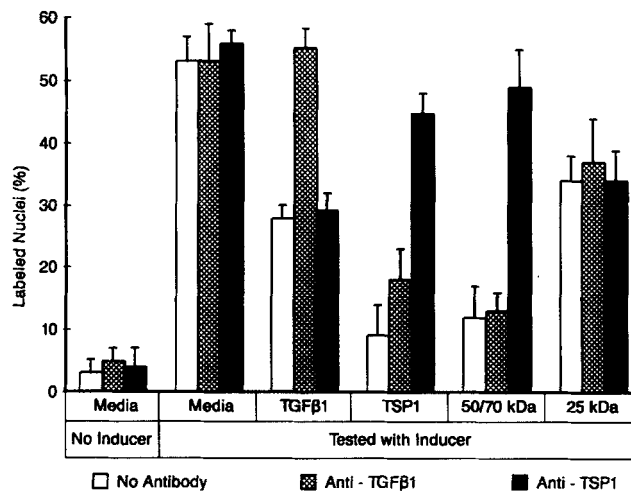


Figure 9. Inhibition of the incorporation of thymidine into capillary endothelial cells by the stalk region of TSP1 is independent of TGFβ. Capillary endothelial cells were starved, incubated for 30 h in quadruplicate with the indicated test substances in the presence of [³H]thymidine, and the % labeled nuclei determined after autoradiography. bFGF was used as an inducer at 0.6 nM, TGFβ1 was present at 16 pM, and TSP1 and its fragments at 40 nM. Data from one of three similar experiments is presented. An independent *t* test showed that the number of labeled nuclei was significantly different ($P < 0.05$) from the number labeled in the relevant positive control (media + inducer) for all samples tested except those where the effects of TGFβ and the 50/70-kD stalk of TSP1 were blocked by anti-TGFβ and anti-TSP, respectively.

tion tissue. The fact that TSP1 and its derivatives were also inhibitory in *in vitro* assays using isolated cultured capillary endothelial cells suggests that they may inhibit *in vivo* by interacting directly with the endothelial cells. In these *in vivo* assays it is not yet clear how the large, "sticky" TSP1 molecule can diffuse from the pellet to nearby vessels rapidly enough to prevent activation of the angiogenic response. It may well be broken down by proteolysis into smaller, more mobile, active fragments after implantation.

It is quite remarkable that the small peptides derived from TSP1 are active in these *in vivo* assays that allow prolonged exposure of the peptides to physiological fluids. The activity of the peptides suggests that they may have a very high affinity for whatever receptors they interact with on the endothelial cell. The two *in vivo* assays correlated well, with the exception of Mal II which blocked neovascularization in the cornea, and migration *in vitro*, but showed only borderline activity in the sponge, although the Mal III peptide which is composed of very similar residues was very effective in all assays. This discrepancy might be explained by differential sensitivity of these two peptides to proteases encountered *in vivo* in the sponge. The sponge assay not only requires a longer time *in vivo* than the cornea assay, but also exposes the peptide to products of migrating fibroblasts and inflammatory cells that are abundant in the sponge environment but rare in the cornea assay. Mal II but not Mal III contains a central aspartic acid residue that can be a cleavage site for several bacterial proteases that cleave at glutamic or aspartic acid residues (Kakudo et al., 1992, and references therein). Endoprotease activities with similar specificities have been identified in mammalian inflammatory cells

(Caughey et al., 1990; Salvesen and Enghild, 1990), raising the possibility that such proteases, to which Mal II might be particularly sensitive, are present in the inflammatory wound environment of the sponge and may account for the failure of Mal II to block vessel formation efficiently in this assay.

In vitro assays with cultured endothelial cells are designed to dissect out and assay separately the various activities required of endothelial cells in vivo during an angiogenic response. Of the common in vitro assays, the migration assay has most consistently correlated with in vivo angiogenic activity (Folkman and Klagsbrun, 1987; Klagsbrun and D'Amore, 1991). As performed here it requires that endothelial cells digest their way through the gelatin that fills pores of a membrane and move to the other side of the membrane where a test compound has been placed. It measures the sum of chemokinesis and chemotaxis. TSP and the hamster 140-kD fragment inhibit both activities (Taraboletti et al., 1990; Rastinejad et al., 1989). All fragments and peptides of TSP1 that blocked in vivo neovascularization in the rat cornea assay also blocked endothelial cell migration in vitro. Intact TSP1, however, was inhibitory in vitro only at lower concentrations. At high concentrations it stimulated migration (Fig. 4), as has been observed by others (Taraboletti et al., 1990; 1991). As previously shown by Taraboletti et al. (1990), the stimulatory activity localized to the 25-kD heparin-binding domain and appeared in large part to be the result of activation of random movement of endothelial cells rather than of directed chemotaxis. The mechanism underlying this paradoxical effect of TSP1 has not been explored, but may relate to the ability of the heparin-binding domain to reverse focal adhesions formed by endothelial cells (Murphy-Ullrich and Hood, 1989; Murphy-Ullrich et al., 1991).

The data presented here localize the majority of the anti-angiogenic activity of the multidomain TSP1 protein to the central 70-kD stalk region of the 180-kD subunit. This is consistent with the ability of the stalk to bind to endothelial cells (Dardik and Lahav, 1991) and with the ability of monoclonal antibodies whose epitopes lie in the stalk region to block anti-angiogenic activity (Good et al., 1990). The data presented cannot rigorously eliminate the possibility that some anti-angiogenic activity resides in the COOH-terminal cell-binding globular domain of TSP1 for it was not tested directly. However, two peptides from this region that show a high affinity for endothelial cells in cell attachment assays (Kosfeld, M. D., M. Finn, and W. A. Frazier, unpublished data) and thus might be expected to influence angiogenesis, were unable to inhibit endothelial cell migration (Tolsma, S. S., and N. Bouck, unpublished data).

The anti-angiogenic stalk region of TSP1 contains three distinct domains each of which could play a role in blocking neovascularization. Anti-angiogenic activity has been identified in two of these, the procollagen homology region and in the properdin-like repeat region 1. The third domain consists of three type 2 EGF-like repeats similar to those found in a wide variety of other molecules where they may serve to facilitate protein-protein interactions, respond to calcium or pH changes, or act as receptors in cell-cell communication networks (Davis, 1990; Rebay et al., 1991). Our observation that the 50/70-kD fragment of TSP1, in which half the molecules lack the EGF repeats, has an ED₅₀ that is similar to that of gp140, which retains the EGF repeats, suggests that

these repeats are not major determinants of the anti-angiogenic activity of the stalk region. Their activity can be tested when purified TSP3 and TSP4 proteins become available, for they retain EGF repeat regions that are highly homologous to those present in TSP1 and lack the procollagen homology region and the type 1 repeats that are anti-angiogenic (Bornstein, 1992).

Active TGF β copurifying with platelet TSP1 can be responsible for up to 60% of the inhibitory activity of TSP1 on endothelial cell mitogenesis (Murphy-Ullrich et al., 1992). Our TSP preparations do contain some TGF β , hence there was some concern that inhibition of in vitro assays for angiogenesis attributed to TSP1 and its fragments could be due to contaminating TGF β . Previously published evidence argues against this possibility (summarized in Frazier, 1991) and is supported by data presented here. Antibodies that effectively neutralize TGF β 1 failed to neutralize the inhibitory activity of TSP1 in migration assays or the activity of its 50/70-kD fragment in [³H]thymidine incorporation assays. Furthermore, synthetic peptides containing no TGF β reproduced the angiostatic effects of TSP1 in vitro and in vivo.

TGF β induces angiogenesis in several in vivo models (Rastinejad et al., 1989; Roberts and Sporn, 1989; Yang and Moses, 1990), yet when tested in vitro at nanomolar concentrations, it blocks activities of endothelial cells usually correlated with angiogenesis. This dichotomy has suggested that induction of angiogenesis in vivo by TGF β may be indirect and due to the release of angiogenic factors by inflammatory cells attracted by the TGF β (Roberts and Sporn, 1989). But the bimodal dose-dependent effects of TGF β 1 on endothelial cell migration reported here suggest that at low doses of TGF β may be stimulatory in the absence of accessory inflammatory cells. Concentrations from 0.04–0.4 pM stimulated migration. Although this concentration is well below that at which TGF β affects a range of other biological activities (5–35 pM, Cheifetz et al., 1988), similar low concentrations have been shown to induce the migration of monocytes (Wahl et al., 1987) and of human fibroblasts (Postlethwaite et al., 1987), and could be physiologically relevant in vivo.

Anti-angiogenic activity was localized to two separate domains of TSP1. Peptides derived from type 1 repeats were able to block endothelial cell migration in vitro and neovascularization in vivo. These repeats contain adhesive domains that when adsorbed to plastic can mediate the adherence of a variety of cells (Prater et al., 1991; Tuszynski, 1992) and when soluble can block cell-cell, cell-TSP, and TSP-heparin or -sulfatide interactions (Tuszynski, 1992; Asch et al., 1992; Guo et al., 1992). Peptide sequences from this region bind to a receptor on a tumor cell line (Tuszynski et al., 1993) and to the CD36 (GPIV) cell receptor (Asch et al., 1992; Catimel et al., 1992), a molecule that has been shown to be present on endothelial cells derived from small vessels (Knowles et al., 1984; Swerlick et al., 1992) and is thought to serve as a receptor for TSP. Anti-angiogenic activity was found in peptides derived from the second and third type 1 repeats, but not from the first repeat. This first repeat was also inactive as an attachment factor for human melanoma cells (Prater et al., 1991), although a shorter peptide derived from this same first type 1 repeat is active as an attachment factor for mouse melanoma cells (Tuszynski et al., 1992). The two active Mal peptides have in common a central

Table VII. Small Peptides Able to Inhibit Angiogenesis In Vivo

Peptide	Source	Reference
C D P G Y I G S R-NH ₂	Laminin	Sakamoto et al., 1991; Grant et al., 1989, 1992
L Y K K I I K K L L E S	Platelet factor-4	Maione et al., 1990
F C Y W K V C W	Somatostatin	Woltering et al., 1991
F C F W K T C T	Somatostatin	Woltering et al., 1991
G R G D	Fibronectin, others	Eijan et al., 1991
S P W S S C* S V T C* G D G V I T R I R	TSP Mal II	This paper
S P W D I C* S V T C* G G G V Q K R S K	TSP Mal III	This paper
N G V Q Y R N	TSP, procollagen region	This paper
N I P P I T C* V Q N G L R Y	Collagen I	This paper

* See Table II.

CSVTCG, but an 8-mer including these residues was inactive in migration assays, although a 6-mer nested within this peptide can block platelet aggregation (Tuszynski et al., 1992; Asch et al., 1992). Perhaps the stability of this short peptide is adequate for the platelet aggregation assay that is complete within minutes but insufficient for the three hour migration assays. The two active Mal peptides also have in common the sequence GVXXR (Gly-Val-X-X-Arg) which is also present in all active Col peptides except pro α 1. The sequence G/AVQ (Gly/Ala-Val-Gln) is present in the active Mal III and all active Col peptides but not in active Mal II. Additional work is needed to clarify active residues within these peptides. Peptides able to block in vivo neovascularization have also been derived from molecules unrelated to TSP, see Table VII. No clear and informative homologies have yet been detected between these peptides and angioinhibitory peptides derived from TSP1.

A series of peptides able to inhibit angiogenesis could also be derived from amino acid sequences present in the procollagen-like region of the central stalk of TSP1. These Col peptides appear to affect the endothelial cell differently than do the Mal peptides for their ability to block endothelial cell migration increased with and usually required the pretreatment of the cells with peptide, supporting a delayed mechanism of action. A peptide derived from the homologous NH₂-terminal propeptide region of the genuine human procollagen I sequence was also inhibitory in vitro and in vivo. The NH₂-terminal propeptides that are cleaved from the pro α 1 chains of collagens I and III during maturation of the collagen fibril are able to specifically downregulate the production of pro α 1 and pro α 2 chains of collagen I (Wiestner et al., 1979; Paglia et al., 1979; Wu et al., 1986). Endothelial cells do synthesize collagen I (Canfield et al., 1992a,b). The perturbation of collagen synthesis can halt neovascularization (Ingber and Folkman, 1988) and could provide a delayed mechanism by which the Col peptides might inhibit angiogenesis. The finding that a peptide derived directly from human procollagen can block angiogenesis raises the intriguing possibility that one of the in vivo functions of the NH₂-terminal collagen propeptides liberated from developing fibrils of collagens I, III, and sometimes II, is to depress angiogenesis. This would be consistent with high levels of NH₂-terminal propeptide in tissues with limited angiogenic activity like developing bone and cartilage (Fisher et al., 1987; Ryan and Sandell, 1990).

If data derived from peptides is to be relevant to the function of the intact parental molecule, the regions containing

the active residues must be exposed on the surface of the mature molecule. Evidence that repeat region 1 from which the active Mal II and Mal III peptides are derived is accessible to the environment is strong. Polyclonal antiserum raised to Mal II which recognizes Mal II and Mal III but not Mal I inhibits attachment of melanoma cells to TSP1, to the 50/70-kD stalk and to the peptides themselves (Prater et al., 1991). In addition, antibodies raised to the central residues in these peptides block platelet aggregation and platelet-dependent metastasis (Tuszynski, 1992). The availability of this region is consistent with biophysical studies on homologous regions of the properdin molecule that show each repeat consists of a separately folded elongated beta sheet accessible to the aqueous environment (Smith et al., 1991, 1992). Data suggesting that the procollagen-like region is exposed and active in the intact trimeric TSP1 molecule comes from the observation that the baculovirus-expressed truncated trimeric TSP-381 which carries the procollagen-like region but not the angioinhibitory properdin-like repeats is an active inhibitor.

It is not yet clear whether proteins encoded by other members of the growing family of TSP genes can block neovascularization. Mouse TSP2 cDNA (Laherty et al., 1992; Bornstein, 1992) shows poor homology to TSP1 in the procollagen region, but good matches in the type 1 repeats. Residues contained in inhibitory Mal II and Mal III peptides are better conserved than those in inactive Mal I, but whether this represents conservation of TSP's role as a solid state attachment factor or as a soluble anti-angiogenic factor, or both is unclear. Type 1 repeats are also present in the neural cell adhesion protein F-spondin, the newest TSP family member (Klar et al., 1992). Both the type 1 repeats and the procollagen homology region are absent from TSP3 and TSP4 (Lawler et al., 1993; Bornstein, 1992), and the thrombospondin-like protein, COMP (Oldberg et al., 1992).

A number of mechanisms can be envisioned by which TSP could act to limit an angiogenic response. (a) TSP1 and its peptides could interfere with positive angiogenic factors, sequestering or inactivating them, or interfering with their binding to cells. Arguing against this possibility is the observation that the TSP1 molecule and/or its 140-kD hamster homologue can block angiogenesis induced in vivo by diverse stimuli including bFGF, tumor necrosis factor, TGF β , and conditioned media from active macrophages, carcinoma, and sarcoma tumor cell lines (Rastinejad et al., 1989; Bouck, N., and P. J. Polverini, unpublished data). It seems unlikely that TSP1 could effectively sequester each of these

stimulators or that the very different Col and Mal peptides, both of which block bFGF-induced angiogenesis, could both sequester bFGF. (b) TSP might disrupt endothelial cell adhesion to stroma thereby blocking migration. This possibility is rendered less likely by the observation that although the anti-adhesive activity of TSP1 has been localized to the heparin-binding domain (Murphy-Ullrich et al., 1989), the isolated protein fragment carrying this activity, the 25-kD fragment, was not inhibitory in our angiogenic assays. In addition, the Mal peptides known to be adhesive for melanoma cells serve as very poor attachment factors for cultured capillary endothelial cells (Finn, M., and W. A. Frazier, unpublished data). (c) The proteolytic activity essential for neovascularization (Berman et al., 1982; Avery et al., 1990) could be sensitive to TSP inhibition. TSP binds plasminogen, plasminogen activator and plasminogen activator inhibitor (Lawler et al., 1986; Silverstein et al., 1986), and may influence plasmin activity (Hogg et al., 1992). Plasminogen also binds to the 70-kD stalk fragment of TSP (Lawler et al., 1986) that inhibits angiogenesis, but it is unknown if any of the active peptides from this fragment retain this activity and if the sum of such interactions enhance or inhibit proteolytic activity in vivo. (d) Many angioinhibitory molecules have in common the ability to block collagen synthesis or deposition (Ingber and Folkman, 1988; Maragoudakis et al., 1988), and TSP may act similarly. It does bind collagens I, II, III, and V, possibly via its procollagen-like region (Galvin et al., 1987; Frazier, 1987), and could act as does the pro α (1) NH₂-terminal peptide of collagen I (from which we could also derive an angioinhibitory peptide) to downregulate collagen synthesis and thereby angiogenesis. (e) TSP may bind to a receptor on endothelial cells, possibly via sequences present in repeat region 1 that can interact with CD36, producing an intracellular change that interferes with the positive signals generated by stimulatory angiogenic factors. The low concentration at which both TSP and its peptides act supports such a receptor-mediated mode of action.

Testing each of these mechanisms should result in a clear understanding of how and possibly why two different structural domains of TSP1 are able to influence a single physiological function, the inhibition of neovascularization. In addition, the definition of anti-angiogenic domains and peptides enables the design of molecules containing single domains that may express only one of TSP's many functions and thereby specifically block only the angiogenic process. Such targeted inhibitors could be of therapeutic use in combating pathological conditions from vascular disease to solid tumors that are dependent upon unregulated neovascularization.

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