Inhibition of Angiogenesis by Thrombospondin-1 Is Mediated by 2 Independent Regions Within the Type 1 Repeats

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Background—Suppression of tumor growth by thrombospondin-1 (TSP-1) has been associated with its ability to inhibit neovascularization. The antiangiogenic activity of TSP-1, as defined by cornea pocket assays, was previously mapped to the amino-terminal portion of the protein within the procollagen region and the type 1 repeats.

Methods and Results—We evaluated the specificity and efficacy of different regions of TSP-1 using recombinant fragments of the protein on chorioallantoic membrane (CAM) angiogenesis and endothelial cell proliferation assays. In both assays, fragments containing the second and third type 1 repeats but not the procollagen region inhibited angiogenesis and endothelial cell proliferation. To further define the sequences responsible for the angio-static effect of TSP-1, we used synthetic peptides. The CAM assay defined 2 sequences that independently suppressed angiogenesis. The amino-terminal end of the type 1 repeats showed higher potency for inhibiting angiogenesis driven by basic fibroblast growth factor (FGF-2), whereas the second region equally blocked angiogenesis driven by either FGF-2 or vascular endothelial growth factor (VEGF). Modifications of the active peptides revealed the specific amino acids required for the inhibitory response. One sequence included the conserved tryptophan residues in the amino-terminal end of the second and third type 1 repeats, and the other involved the amino acids that follow the CSVTCG sequence in the carboxy-terminus of these repeats. Both inhibition in the CAM assay and inhibition of breast tumor xenograft growth in nude mice were independent of the TGF-β-activating sequence located in the second type 1 repeat.

Conclusions—These results indicate that the type 1 repeats of TSP-1 contain 2 subdomains that may independently inhibit neovascularization. They also identify 2 independent pathways by which TSP-1 can block FGF-2 and VEGF angiogenic signals on endothelial cells. (Circulation. 1999;100:1423-1431.)

Key Words: angiogenesis ■ endothelium ■ vessels

Thrombospondin (TSP-1) is a matricellular protein with recognized ability to inhibit endothelial cell proliferation and to suppress angiogenesis. The region responsible for inhibition of angiogenesis has been mapped to the procollagen domain and to the type 1 repeats. The molecular mechanisms for this inhibition are not entirely understood. It is likely that the inhibition of capillary growth by TSP-1 might be multifactorial and involve competition for basic fibroblast growth factor (FGF-2) binding to the endothelial cell surface, binding to heparan sulfate proteoglycans, activation of latent transforming growth factor (TGF)-β, and/or binding to CD36, a receptor for TSP-1. More importantly, the ability of TSP-1 to suppress neovascularization has been associated with inhibition of tumor growth. We have previously demonstrated in xenograft assays that stable synthetic peptide analogues of the TSP-1 type 1 repeats suppressed breast carcinoma growth in a dose-dependent manner. The peptides used in these assays included a tryptophan-rich motif within the type 1 repeats that binds to heparin. However, these peptides did not include the sequence that binds to CD36 and elicit an antiangiogenic effect in the cornea. Additional studies have indicated that these tryptophan-rich peptides suppressed endothelial cell proliferation, inhibited chemotaxis to FGF-2, and induced apoptosis of endothelial cells. Although no direct analyses were performed to elucidate their role in angiogenesis, their suppression of FGF-2–stimulated endothelial proliferation and chemotaxis were indicative of an effect on tumor vascularization. It is not clear how potent these peptides are in relation to other recognized vascular inhibitory regions of TSP-1 or whether they might demonstrate variability in efficacy depending on the angiogenic stimulus (ie, FGF-2...
versus vascular endothelial growth factor (VEGF)). The present study was therefore undertaken to (1) evaluate the efficacy of peptides from several regions of the type 1 repeats to inhibit angiogenesis, (2) determine the effect of these peptides on FGF-2– versus VEGF-driven angiogenesis, and (3) elucidate the residues that provide a minimal functional core sequence in TSP-1.

Methods

Protein, Recombinant Fusion Protein, and Peptides

Thrombospondin was purified from human platelets. Recombinant fusion proteins of TSP-1 were prepared by use of the pGEX vectors. Vascular permeability factor (VPF)/VEGF was obtained from Peprotech; FGF-2 was a generous gift from Dr Gera Neufeld (Technion University, Israel).

The peptides used in this study were synthesized on a Biosearch model 9600 peptide synthesizer using standard Merrifield solid-phase synthesis protocols and Fmoc chemistry. Peptides were analyzed by reverse-phase high-performance liquid chromatography and further purified by dialysis with Spectrapor 500 MWCO tubing, gel permeation chromatography, or reverse-phase high-performance liquid chromatography and further purified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Polysucrose conjugates of some peptides were prepared as previously described. Before use on chorioallantoic membrane (CAM) assays, peptides were also filtered on Centricron P100 to eliminate traces of endotoxin.

Endothelial Cells and Proliferation Assays

Chicken endothelial cells (CECs) were isolated from the brain of day 7 chicken embryos. Endothelial cells were purified and characterized by standard techniques, as described.

For proliferation assays, quiescent endothelial cells were seeded in 24-well plates in EBM medium supplemented with 0.1% FCS and 50 ng/mL of VEGF (PeproTech Inc) and/or 2 ng/mL of FGF-2 in the presence of TSP-1, fusion proteins, peptides, or vehicle control. During the last 8 hours of the treatment, the cells were pulsed with 1 µCi/well of [3H]thymidine (DuPont-NEN). Trichloroacetic acid–precipitable counts of [3H]thymidine were measured as previously described. The significance of inhibition was assessed by a 2-tailed t test.

CAM Assays

The effect of TSP-1, fusion proteins, and peptides on angiogenesis was evaluated with a modified CAM assay. The method is based on the vertical growth of new capillary vessels into a collagen gel pellet placed on the CAM. The collagen gel was supplemented with an angiogenic factor such as FGF-2 (50 ng/gel) or VEGF (250 ng/gel) in the presence or absence of test proteins/peptides. The extent of the angiogenic response was measured by use of FITC–dextran (50 µg/mL) (Sigma) injected into the circulation of the CAM. The degree of fluorescence intensity parallels variations in capillary density; the linearity of this correlation can be observed with a range of capillaries between 5 and 540. Morphometric analyses were done by acquisition of images with a Sony, single-chip CCD camera. Images were imported into NIHimage 1.59, and measurements of fluorescence intensity were obtained as positive pixels. Each data point was compared with its own positive and negative controls present in the same CAM and interpreted as percentage of control, considering the positive control to be 100%.

Results

To validate the use of the chicken CAM for assessing angiogenic responses to human TSP-1, we first determined whether human TSP-1 inhibited the proliferation of CECs. Figure 1 shows that human TSP-1 inhibited the proliferation of CECs to a degree similar to that of human dermal microvascular endothelial cells. The possible contamination of platelet TSP-1 preparations with TGF-β, raises concerns about the contribution of TGF-β to the antiproliferative activities of platelet TSP-1. TGF-β has been shown to bind and to copurify with TSP-1. Furthermore, TGF-β is a potent inhibitor of endothelial cell proliferation. The specificity of TSP-1–mediated suppression of endothelial cell proliferation was validated with anti–TSP-1 blocking antibodies (Figure 1). A polyclonal TSP-1 antibody raised in guinea pig neutralized the TSP-1–mediated inhibitory effect, whereas antibodies alone had a mild but statistically insignificant effect on proliferation (data not shown). Preimmune guinea pig IgG in the presence of TSP-1 did not ameliorate endothelial growth inhibition. We also examined the levels of TGF-β, present in the TSP-1
preparations. In general, TGF-β ranged between 5 and 90 pg/mL in our preparations of TSP-1 from platelets. Further dilution in tissue culture media reduced this value 100- to 500-fold in the proliferation assays. Addition of purified TGF-β to the CECs suppressed proliferation only at 5 ng/mL (data not shown). Taken together, the neutralization of the antiproliferative effect mediated by anti–TSP-1 antibodies and the limited levels of TGF-β contamination confirm that the suppressive effects on endothelial proliferation were mediated by TSP-1.

The specificity of TSP-1 to inhibit CEC proliferation was also determined by use of glutathione S-transferase (GST) fusion proteins expressing the procollagen, type 1, type 2, type 3, and C-terminal domains of TSP (Figure 2). These bacterially expressed proteins lack any TGF-β contamination and can be used to define the functional domain(s) within TSP-1 responsible for the inhibitory effect. Interestingly, only the type 1 repeat recombinant fragment inhibited endothelial cell proliferation. TSP-1 10 μmol/L inhibited proliferation by 35% on CECs (P=0.009). At the same molar concentration, the recombinant GST type 1 domain inhibited CEC proliferation by 48% (P=0.0009). The GST control alone had no effect. Other recombinant fragments tested in this assay did not show inhibition of either bovine aortic endothelial cell (data not shown) or CEC proliferation, and the partial inhibition by the carboxy-terminal fusion protein (83% of control) was not statistically significant (P=0.21). It was interesting that the entire TSP-1 molecule had a more moderate effect on proliferation than that of the GST fusion protein containing the type 1 repeats.

The effects of intact TSP-1 and GST fusion proteins on angiogenesis were evaluated on a mesh CAM assay. Growth of capillaries in this assay is stimulated vertically, against gravity, by VEGF or FGF-2 cast into a polymerized collagen gel. Figure 3A shows the effect of TSP-1 and fusion proteins on neovascularization of the acellular collagen matrix. The presence of angiogenic growth factors induces the growth of a thin vasculature in the acellular gel as early as 24 hours (Figure 3A, arrows) from the thicker vessels located under the nylon mesh (larger vessels out of focus). In the absence of angiogenic growth factors, no network was observed (Figure 3B). The ability of inhibitors to suppress the stimulatory signal of growth factors was then evaluated by inclusion of these proteins in the polymerized vitrogen gel. Both TSP-1 and the GST type 1 repeat fusion protein were effective at suppressing the angiogenic response mediated by growth factors. TSP-1 was able to block VEGF-mediated angiogenesis by 35%, whereas the GST type 1 repeat fusion protein was more effective at the same molar ratio (57%) (Figure 3B). No significant effects were detected with any of the proteins alone, ie, in the absence of VEGF, or with proteins other than the type 1 repeats in the presence of VEGF or FGF-2. The effect was reproducible with several preparations of TSP-1 and of recombinant protein and was performed at least 4 separate times with each treatment in triplicate (total of 12 assays). These results are consistent with data obtained from the proliferation experiments and again indicate that at an equivalent molar ratio, the type 1 repeats of TSP-1 appear to be more effective than the intact protein. To this end, it has recently been postulated that the carboxy-terminal end of TSP-1 might exert a positive effect on angiogenesis by its ability to interact with integrin-associated protein.16 Interestingly, we observed a slight but reproducible increase in angiogenic rate with the carboxy-terminal end (Figure 3B). The entire TSP-1 protein might therefore contain regions that elicit both positive and negative signals on endothelial cell proliferation and angiogenesis, thus providing amelioration to the suppressive growth signals. A careful dissection of these areas is required to clearly elucidate the potential function of each domain.

We also found that the first repeat of the type 1 domain had no effect on suppressing angiogenesis in the CAM assay (Figure 3B). The ability of TSP-1 to inhibit vascular growth therefore appears to be located within the last 2 type 1 repeats.

We next examined the activities of synthetic peptides derived from the procollagen and second and third type 1 repeats of TSP-1, as well as the carboxy-terminal end of TSP-1 (Figure 4 and Table). The procollagen region and the last 2 type 1 repeats have previously been shown to have antiangiogenic activity in the cornea pocket assay.3 Evaluation of the procollagen region in the CAM assay, however, did not result in angiogenic suppression, in contrast to peptides from the second (508) and third (616) type 1 repeats,
Figure 3. Effect of TSP-1 fusion proteins on angiogenesis. A, Mesh-CAM assays for evaluation of TSP-1 and GST fusion proteins were performed on day 11 chicken embryos. Each pellet contained vitrogen 50 \( \mu \)g/mL, VEGF 250 ng/mesh, and FGF-2 50 ng/mL (a), except negative control (no growth factors were added) (b), in addition to TSP-1 (c), GST-procollagen region (d), GST type 1 repeats (e), GST type-2 repeats (f), GST type-3 repeats (g), GST carboxy-terminus (h), or GST control (i). Fusion proteins were used at 20 \( \mu \)mol/L and TSP-1 at 10 \( \mu \)g. B, Quantification of angiogenic response. Evaluation was determined 24 hours after application of pellets to CAM surface. Systemic injection of FITC-dextran revealed vessels with patent lumens. Ten squares of 250 \( \mu \)m\(^2\) were evaluated per mesh. Three meshes in independent embryos were performed per time point. In each case, extent of angiogenesis suppression/stimulation was determined by direct comparison to control meshes (VEGF alone=100%) in same CAM. As in Figure 2, only TSP-1 and type 1 repeat fusion proteins were significantly different from control (\( P<0.01 \)).
which were antiangiogenic. Interestingly, a peptide from the carboxy-terminal domain (458) showed a slight but reproducible proangiogenic effect. The region has been shown to interact with integrin-associated protein and enhance attachment and migration. The specificity of this positive response was supported by concurrent experiments performed with 2 mutated versions (604 and 605) of the carboxy-terminal peptide. Substitution of the 2 Val residues was sufficient to suppress the proangiogenic effect (Figure 4).

On the basis of these studies, it appears that in the CAM assay, only peptides derived from the second and third type 1 repeats are angioinhibitory. We therefore focused subsequent studies on these domains.

Figure 5 shows the sequence of the last 2 TSP-1 type 1 repeats and correlates previously identified functions to specific sequences within these domains. A region of interest, because of its demonstrated ability to suppress tumor growth, is located at the amino-terminal end of these repeats. The direct effect of this region on angiogenesis has not yet been tested. Our results indicated that peptides from this region at 1 μmol/L suppress vascular growth induced by a mixture of FGF-2 and VEGF (Figure 6A). The effect was dose-dependent, and retro-inverso analogues or polysucrose conjugates of the peptides were more potent than the native TSP-1 peptide. These modifications are known to increase the half-life of polypeptides by reducing degradation in vivo. Assays were also performed using mutated versions of the peptides as well as deletion mutants. Mutation of the 3

Figure 5. Amino acid sequence of second and third type 1 repeats of TSP-1. Amino acids are given in single-letter code. Regions with identified activity are indicated. References are provided (superscript numbers).
tryptophan residues to alanines (peptides 597 and 598) completely abolished inhibitory activity, indicating that these residues are critical to the antiangiogenic response (Figure 6B). A peptide with 2 instead of 3 tryptophan residues was partially effective (peptides 493 and 530) (Figure 6B). The latent TGF-β-activating sequence (RFK) was not required for the suppressive effect on neovascularization, because mutation of the essential phenylalanine residue to alanine (compare peptides 545 and 596) did not have any deleterious effect on the activity of the peptide. This mutation abrogates the ability of this peptide to activate latent TGF-β1.

We next examined the effects of the second half of the type 1 repeats, a region previously concluded to be responsible for the antiangiogenic effects promoted by TSP-1 in cornea pocket assays.3 Our results support those observations and demonstrate activity of the same peptides in the CAM assay (Figure 7A). In agreement with recent reports,7 the active region appears to be carboxy-terminal to the CSVTCG region, because this sequence alone was inactive, and deletion of the first 2 residues of this motif did not affect the antiangiogenic activity of peptide 205 (VTCGDDGVTIR) from the second type 1 repeat or peptide 245 (VTCGGVQKRSRL) from the third type 1 repeat (Figure 7A). However, the VTCG sequence without these flanking sequences completely lacked antiangiogenic activity. This flanking sequence has been shown to act through CD36, a receptor for TSP-1.7 We have verified that CECs and vessels in the CAM do express this receptor by Northern blot analysis (data not shown). Therefore, it is likely that the mechanism of action is similar.

To further elucidate the mechanism of action of these 2 subregions, CAM experiments were performed with either VEGF or FGF-2 as stimulator of the angiogenic response (Figure 8). Interestingly, we observed a clear distinction between the tryptophan repeat peptides and the CD36-binding domain peptides. Peptides 508 and 599, which have
the tryptophan motif, suppressed the angiogenic response only to FGF-2–mediated angiogenesis but had no effect on VEGF-driven vascular growth. In contrast, the CD36-binding sequences blocked both VEGF- and FGF-2–induced angiogenesis. Interestingly, the tryptophan domain has previously been shown to prevent FGF-2 binding to endothelial cells.\(^4\,5\) Therefore, inhibition of binding or further sequestration of FGF-2 is the most likely mechanism of action of this amino-terminal portion of the type 1 repeats.

A surprising result was the lack of antiangiogenic activity of the TGF-\(\beta\) activating sequence. To determine whether the CAM assay has predictive value for inhibition of tumor angiogenesis, we assessed the role of the TGF-\(\beta\)-activating sequence in inhibition of breast carcinoma tumor growth in vivo using orthotopic xenografts of MDA435 breast carcinoma cells in athymic mice (Figure 9).\(^9\) Reverse analogues of the native TSP-1 sequence (peptide 599) and a modified sequence lacking TGF-\(\beta\)-activating activity (peptide 596)\(^\text{10}\) both strongly suppressed tumor growth when administered intravenously to the mice beginning 25 days after implantation of the tumor cells in the mammary fat pad. The data are in agreement with our findings in the CAM assay and provide further support that the suppression of tumor growth results from the ability of TSP-1 to suppress angiogenesis independently of latent TGF-\(\beta\) activation.

**Discussion**

In this study, we evaluated both TSP-1 fusion proteins and synthetic peptides to provide a comparative assessment of the antiangiogenic activity displayed by the different domains of TSP-1. We combined these data with analysis of deletion and point mutants to identify the minimal sequences with vascuoinhibitory properties. One result of these structure/function analyses demonstrated that the ability of TSP-1 to suppress angiogenesis resides solely in the second and third type 1 repeats. The data are in agreement with previous findings by Tolsma and coworkers.\(^3\) In addition, these studies revealed 2 subdomains in the type 1 repeats that act independently to suppress angiogenesis and identified essential residues responsible for these effects: the tryptophan-rich WSXW motifs and the CD36-binding region. These 2 sequences also display growth factor selectivity in that the tryptophan-rich domain preferentially suppresses FGF-2 angiogenic signals, whereas the CD36-binding region inhibits capillary formation driven by either FGF-2 or VEGF. These findings contribute to understanding the antitumor activity of some TSP-1 peptides and suggest that these peptides might act, at least in part, by suppressing tumor-mediated angiogenesis.

The neovascular suppression displayed by TSP-1 has previously been attributed to the second (amino acid [aa] 424 to 442) and third (aa 481 to 246) type 1 repeats of TSP-1.\(^3\) The type 1 domain of TSP-1 consists of 3 polypeptide repeats that have complete conservation of the cysteine and tryptophan residues and that have been identified in a variety of other proteins, including properdin, F-spondin, BAI, and metallospindins.\(^18\,\text{–}\,21\) Nevertheless, the antiangiogenic potential of the type 1 repeats is not shared by all these proteins, indicating that context-specific primary sequences and/or secondary structure influence the function of the type 1 repeats.

Previous publications have indicated that the first type 1 repeat of TSP-1 has no antiangiogenic activity.\(^3\) We confirmed these results in the CAM angiogenic assay. Recent studies have further mapped a subregion within the second and third type 1 repeats including and carboxy-terminal to the CSVTCG sequence with angiobinhibitory effects.\(^7\) The relevant region has been found to bind to CD36 and to be responsible for the intracellular events related to the suppression of several mitogenic signals on endothelial cells.\(^7\) Our data are consistent with the observations of Dawson et al\(^7\) but do not correlate with evidence that the CSVTCG sequence alone,
responsible for binding of TSP-1 to CD36, has an effect. Further structure-function and mutagenesis analysis will be required to resolve these discrepancies.

Although activity of the CD36-binding peptides from the carboxyl end of the second and third type 1 repeats provides an explanation for some of the activity of the type 1 repeats, other regions within the type 1 repeats have also been shown to reduce tumor growth and display potential angiostatic or antiangiogenic activities. Because these small fragments of the protein were tested in different laboratories and angiogenesis assays, we felt that it was necessary to analyze TSP-1 fragments and peptides side by side and in a single in vivo assay. Our observations demonstrate that the tryptophan-rich motif contains a second angioinhibitory region with activity similar to that of the CD36-binding sequence. We would predict that the potential of this region to inhibit FGF-2-mediated angiogenesis relates to its ability to bind to heparan sulfate and thereby block FGF-2 receptor signaling. The minimum sequence necessary for heparin-binding activity is the pentapeptide WSPWS, although if the preceding positively charged residues are added to the SHWSPWSS sequence, the heparin-binding activity of the peptide can be enhanced up to 10-fold. Our results indicate that the GGWSHWSPWSS worked better for inhibiting angiogenesis than the SHWSPW sequence alone.

The interaction of the type 1 repeats with heparin in the tryptophan-rich region lacks stereospecificity, because forward and inverse peptide analogues (L-forward, L-reverse, and D-reverse) displayed equivalent ability to interact with heparin, and in the CAM angiogenesis assay, the retro-inverso analogue was better in blocking angiogenesis. Thus, the polypeptide backbone is not involved in this response. Conjugation of the type 1 repeat peptides to polysucrose did not significantly affect their antiangiogenic function, although conjugation increased their potency in vitro for inhibiting proliferation of endothelial and breast carcinoma cells stimulated by FGF-2. The D-reverse analogues are resistant to proteases, and we have shown in xenograft assays that retro-inverso analogues are effective when administered intravenously in mice. The enhanced activity of the D-reverse peptides in the CAM assay may therefore result from an enhanced half-life in the gel or chick embryo circulation.

Studies by Tolsma and coworkers have demonstrated that in addition to the type 1 repeats, a region in the procollagen domain (aa 294 to 317) inhibits angiogenesis in the cornea pocket assay. Our results, however, did not support these findings by use of either the fusion protein or the synthetic peptide. It is possible that unlike with the type 1 repeats, the procollagen region is not effective across species; in fact, the amino-terminal end of TSP-1 differs more significantly than the carboxy-terminal end. Nevertheless, we also were not able to see any effect on proliferation or migration using the procollagen region fusion protein on human dermal microvascular endothelial cells.

TSP-1 has been shown to bind and activate latent TGF-β, because TGF-β modulates endothelial cell function, some involvement of the latent TGF-β-activating sequence was expected in the activity of TSP-1. Although TGF-β inhibits endothelial cell proliferation, angiogenesis assays in vitro as well as injection of TGF-β in vivo have demonstrated proangiogenic activity. Peptides with KRFK sequences might therefore be predicted to induce angiogenesis by activating endogenous latent TGF-β. Perhaps this is why some of the KRFK peptides without the tryptophans seem to stimulate angiogenesis (eg, 597). In any case, our studies demonstrate that at least the antiangiogenic activity of type 1 repeats was independent of latent TGF-β activation, because substitution of an Ala residue for the essential Phe residue did not affect the antiangiogenic potential of the peptide.

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References


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