Inhibition of Retinal Angiogenesis by Peptides Derived from Thrombospondin-1

Afshin Shafiee,1,2 John S. Penn,3 Henry C. Krutzsch,4 John K. Inman,4 David D. Roberts,4 and Diane A. Blake1

PURPOSE. Thrombospondin (TSP)-1 is a tumor suppressor with activity that is associated with its ability to inhibit neovascularization. Previous studies have mapped this antiangiogenic activity to the type 1 repeats and the amino-terminal portion of the molecule within the procollagen-like domain. The present study was performed to investigate the ability of TSP-1 and peptides derived from the type 1 repeats to inhibit retinal angiogenesis.

METHODS. TSP-1 and peptides with tryptophan-rich, heparin-binding sequences and transforming growth factor (TGF)-β1 activation sequences were evaluated in two models of retinal angiogenesis: a retinal explant assay and a rat model of retinopathy of prematurity (ROP).

RESULTS. Platelet-derived TSP-1 inhibited angiogenesis in both experimental models. Peptides from the native TSP-1 sequence, which contained both the tryptophan-rich-repeat and the TGF-β1 activation sequence, were the most potent inhibitors of endothelial cell outgrowth in the retinal explant assay. In contrast, a peptide containing only the tryptophan-rich, heparin-binding sequence was most active in inhibiting neovascular disease in the rat ROP model.

CONCLUSIONS. These results indicate that the type 1 repeats of TSP-1 contain two subdomains that may independently influence the process of neovascularization, and that peptides derived from these type 1 repeats may be promising pharmacologic agents for treatment of retinal angiogenesis. (Invest Ophthalmol Vis Sci. 2000;41:2378–2388)

Neovascular diseases of the retina collectively constitute the leading cause of blindness in developed countries.1–2 At present, retinal laser photocoagulation appears to be the most effective treatment for retinal neovascularization. However, this procedure can destroy postmitotic retinal neurons and permanently affect visual function. Pharmacologic agent(s) that inhibit angiogenesis without destroying retinal tissue could lead to new treatments for this constellation of diseases. Because angiogenesis is a multistep process regulated by an array of growth factors and extracellular matrix molecules (for reviews, see References 3–5) there are potentially many ways to interfere with its progression. Recently, a number of investigators have partially inhibited retinal and iris angiogenesis in vivo using monoclonal antibodies,6 receptor-binding chimeric proteins,7 and antisense oligonucleotides to vascular endothelial growth factor (VEGF),8 an endothelium-specific growth factor.

Thrombospondin (TSP)-1 is an extracellular matrix glycoprotein that has been shown to be both anti- and proangiogenic in various angiogenesis models.9–11 These conflicting results may be due to the complex structure of the protein and its ability to bind to a number of receptors on the same cell type (Fig. 1; for a review, see Reference 15). The regions of TSP-1 responsible for its antiangiogenic activity have been mapped to the procollagen-like domain and to the type I repeats.10 The antiangiogenic activity of these regions has been variously attributed to activation of latent transforming growth factor (TGF)-β1,17 competition for fibroblast growth factor (FGF)-2 binding to the endothelial cell surface,18,19 interaction with heparan sulfate proteoglycans,19,20 and binding to CD36, a receptor for TSP-1.21 The RGD and VVM sequences of TSP-1 also interact directly and indirectly with integrins, including both the αvβ3 and αvβ5 integrins.22 Ligation of these integrins has been shown to prevent endothelial apoptosis during angiogenesis.23,24

The therapeutic potential of the intact TSP-1 molecule is limited by its size (a trimer of 450 kDa) and its multiple biological activities; however, small peptides derived from it should provide a reasonable alternative for preventing the pathologic growth of new blood vessels. Heparin-binding peptides derived from the type 1 repeats of TSP-125 have been shown to antagonize FGF-2 binding to endothelial cell surfaces through binding to heparan sulfate and proteoglycans,19 to inhibit endothelial cell chemotaxis to FGF-2,19 and to induce apoptosis of endothelial cells.26 Stable synthetic peptide ana-
logues of these peptides suppress breast carcinoma growth in a dose-dependent manner. The present study was undertaken to evaluate the ability of TSP-1 and different regions in the TSP-1 type 1 repeats to inhibit angiogenesis in two ocular models of angiogenesis: a retinal explant assay and a rat model of ROP. We demonstrated that a small peptide from the type 1 repeats contained two active regions and that the activity of these two regions could vary, depending on the model system used to study the angiogenic process.

**METHODS**

Platelet-derived TSP-1 and rat tail type I collagen were purchased from Sigma (St. Louis, MO). Glucose-free Glasgow minimum essential medium (10×; GMEM) and HEPES buffer solution (1 M) were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). The peptides used in this study (Table 1) were synthesized, purified, and analyzed as described elsewhere. Peptide 246 was derived from the native sequence of TSP-1, and peptides 450 and 388 were controls prepared by systematically substituting alanine for specific amino acids known to be required for heparin binding or the activation of latent TGF-β1. Such substituted controls are usually better than scrambled peptide controls, because they retain the same secondary structure as the native sequence. D-Reverse peptide analogues were used in some experiments because of their superior resistance to most proteases. These D-reverse peptides have been shown to be active, and the stereochemical requirements for their activities have been published. For intravitreal injections, D-reverse analogues were covalently conjugated to polysucrose of molecular weight 70,000 to prevent rapid vitreous clearance. Peptides used for biological assays were purified by dialysis using Spectrapor 500-M, cutoff membranes (Spectrum, Rancho Dominguez, CA), then lyophilized and stored at −20°C. Immediately before use, the peptides were dissolved in purified water or normal saline and filter sterilized.

**Bovine Retinal Explant Assay**

Vascular endothelial outgrowth from retinal discs was assessed using modifications of a previously published procedure. Bovine eyes were processed within 2 hours of death. Excess tissue was removed and the orbits covered with gauze saturated with gentamicin (100 µg/ml in water) for 15 minutes. Under sterile conditions by using a pair of blunt curved forceps, retinas were gently peeled off the choroid, cut at the disc, and floated in a 100 × 15-mm tissue culture dish containing GMEM. Retinal discs from three to four retinas were cut from the peripheral retina with a 3-mm trephine and randomized. For the preparation of collagen gels, all procedures were performed on ice. Collagen (3.5 ml) was made up in 0.012 N HCl at a concentration of 3 mg/ml and neutralized by the addition of 1 N sodium hydroxide (40 µl). HEPES buffer solution (40 µl) and 10× GMEM (0.5 ml) were added to restore physiologic ionic strength. At this point 1× GMEM containing 10% FBS and varying concentrations of the test peptide (in 1.15 ml) were incorporated into the gel mix. Aliquots (1 ml) of the collagen mixture were then pipetted into each well of a six-well plate (Becton Dickinson, Lincoln Park, NJ) and allowed to

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932908/)

**FIGURE 1.** Structural domains of TSP-1. Diagram shows a single subunit of the TSP-1 trimer, which is covalently assembled through disulfide bonds (—S—). Selected peptide sequences with reported functional activities are positioned as indicated. The antiangiogenic activities of the boxed sequences (from peptide 246) were investigated in this study.

### TABLE 1. Peptides

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence*</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>246</td>
<td>KRFKQDGGWShWSPWSS</td>
<td>Native sequence from the second type I repeat of TSP-1; contains both heparin-binding and TGF-β1 activation sequences</td>
</tr>
<tr>
<td>416</td>
<td>Ac-cswpswshwggdkgfrk-NH₂</td>
<td>A D-reverse analogue of peptide 246</td>
</tr>
<tr>
<td>416F70</td>
<td>Ac-cswpswshwggdkgfrk-NH₂</td>
<td>Peptide 416 covalently conjugated to polysucrose (ficoll) of molecular weight 70 kDa</td>
</tr>
<tr>
<td>450</td>
<td>KRAKAAGGWSGWSPWSSCNH₂</td>
<td>Heparin binding peptide with no TGF-β activation sequence</td>
</tr>
<tr>
<td>476</td>
<td>Ac-cswpswshwggagqkfrk-NH₂</td>
<td>A D-reverse analogue of peptide 450</td>
</tr>
<tr>
<td>476F70</td>
<td>Ac-cswpswshwggagqkfrk-NH₂</td>
<td>Peptide 476 covalently conjugated to polysucrose (ficoll) of molecular weight 70 kDa</td>
</tr>
<tr>
<td>388</td>
<td>KRFKQGDGASHASP</td>
<td>Peptide with TGF-β activation sequence but no heparin-binding activity</td>
</tr>
<tr>
<td>475</td>
<td>Ac-cswspsahssagdkgfrk-NH₂</td>
<td>A D-reverse analogue of peptide 388</td>
</tr>
<tr>
<td>475F70</td>
<td>Ac-cswspsahssagdkgfrk-NH₂</td>
<td>Peptide 475 covalently conjugated to polysucrose (ficoll) of molecular weight 70 kDa</td>
</tr>
<tr>
<td>300</td>
<td>GGWSHW</td>
<td>Peptide that binds weakly to heparin; used as a nonreactive control in some experiments</td>
</tr>
</tbody>
</table>

* Upper case letters denote L-amino acids, and lower case letters denote D-amino acids. Heparin binding sequence is shown in bold; TGF-β1 activation sequence is underlined.
polymerize at 37°C. After polymerization, four to five retinal explants were placed into each well and overlaid with a second aliquot of the gel mix (1 ml). GMEM (1 ml) containing 5 mM glucose, 10% FBS, and varying concentrations of the peptides was added, and the explants were incubated for 8 to 11 days. Medium was changed every 3 to 4 days. A phase-contrast microscope was used to count the number of outgrowths for each explant each day. The degree of inhibition in the TSP-1- or peptide-treated cultures was reported on the day that the maximal endothelial outgrowth was observed in the control cultures (no inhibitor, 10% FBS). This method of reporting results of the explant cultures controlled for variability in the response of the retinal tissue in different experiments. The variability (1–2 days) in the time that the maximal number of outgrowths was observed in control cultures of different experiments could have been due to variability in the age of the retinal tissue obtained from the abattoir, to the handling of the retinal tissue, or to the response of the tissue to the growth factors present in the culture medium.

In experiments to study the toxicity and turnover of peptides, medium containing the peptides was replaced on day 7 with GMEM containing 5 mM glucose and 10% FBS but no peptide. The explants were further incubated until day 11, and outgrowths were counted again. For some experiments, outgrowth was also expressed semiquantitatively as clock hours. The circumference of the explant was divided into 12 quadrants, and each quadrant was scored as positive if any retinal outgrowth was present in that quadrant. This analysis yielded values from 0 (no outgrowth) to 12 (outgrowth in every quadrant). Masked assessments were conducted by three independent observers whose scores were averaged for each explant. Statistical significance was determined using the Kruskal–Wallis test, a nonparametric test that does not assume a normal distribution of the data and is designed to evaluate the significance of data in a rank-order format. The clock hour and explant data presented in this study are in such a format.

**Rat Model of Retinopathy of Prematurity**

The effects of TSP-1 and peptides on retinal neovascularization were determined using a previously described rat model. All animal experiments were performed with the highest standards of care and conformed to the principles outlined in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. At birth, litters of Sprague–Dawley rats and their mothers were placed in a variable oxygen environment (cycles of 24 hours, 50% oxygen followed by 24 hours, 10% oxygen). The 10% oxygen atmosphere was produced by mixing appropriate fractions of pure nitrogen and room air. These oxygen levels were chosen because they produce blood oxygen tensions in the rat pups similar to those measured in infants in whom ROP develops. The oxygen level was alternated between 50% and 10% every 24 hours for 14 days (Fig. 2), the time required for room air–raised rats to complete retinal vascular formation. On day 14, animals were brought into room air, randomized, and injected intravitreally with the test agent (Fig. 2). Platelet-purified human TSP-1 (125 ng in 5 μl, 0.76 picomoles of TSP subunit) was injected on day 14 or 17. Polysucrose (70 kDa) or polysucrose-conjugated peptides were dissolved in physiological saline (at a final concentration of 750 μM as peptide), filter sterilized, and further diluted with physiological saline. A volume of 5 μl (3.75, 1.25, or 0.35 nanomoles per eye) was injected on day 14, and animals were killed on day 20. Abnormal neovascularization was assessed on day 20 by histochemically staining retinas for ADPase activity, a procedure that preferentially stains retinal vascular endothelium and microglia in rats of this age. Stained retinas were flattened on microscope slides using four radial cuts, marked for orientation of the superior quadrant, coverslipped in GelMount (Biomeda, Foster City, CA), and digitized using a digitizing camera (CCO-72; Dage-MTI, Michigan City, IN) and image analysis software (ImageGrabber ver. 2.0; Neotech, Hampshire, UK).

Normal vascular growth in the developing rat retinas was quantified by measuring vascular area. Retinal areas containing blood vessels were traced on the computer monitor face with an interactive stylus pen (FT Data Systems, Stanton, CA). The operator was masked to the treatment group from which each retina was derived. The area within the trace was calculated with image analysis software (Enhance 3.0; MicroFrontier, Des Moines, IA) and is reported in square millimeters. The degree of vascular disease was assessed using clock hours. A theoretical clock face was superimposed on the retinas with areas between separated quadrants omitted from the assessment. Each clock hour that was occupied by neovascular growth was counted, yielding a semiquantitative measure of severity, with values ranging from 0 (no disease) to 12 (most severe). In this context, neovascular growth included any preretinal growth and any regions of dense arterial budding within the superficial vessel plexus, with determinations confirmed at ×400 magnification. Neither of these two features is observed during normal vasculogenesis in room air–raised rats. Although not rigorously quantitative, this method was chosen because it has a clear corollary in the clinical classification of ROP and is widely published by laboratories that use ROP animal models.

Multiple transverse sections were impractical for the samples sizes required by this study; in addition, because of the asymmetry of the disease, such sections are prone to sampling errors. In other studies, when these sampling errors were controlled by serially sectioning entire eyes in a similar ROP model, the number of preretinal nuclei was highly correlated...
RESULTS

Dose- and Time-Dependent Inhibition of Retinal Neovascularization by Platelet-Derived TSP

A newborn rat model of ROP was used to assess the antiangiogenic effects of platelet-derived TSP. Preliminary experiments indicated that TSP-1, when injected immediately after the rats had been transferred to room air, caused a dose-dependent decrease in retinal neovascularization. Maximal response was observed when between 75 and 750 ng of TSP-1 was injected per eye (data not shown), and 125 ng/eye (0.76 picomoles TSP-1 subunit per eye) was chosen as the dose for subsequent experiments. Figure 3 shows representative retinas from 20-day-old rats that were subjected to 14 days of alternating oxygen tension and then maintained in room air for an additional 6 days. The left side of the figure presents a retina from an animal injected with vehicle (PBS) immediately after return to room air. The retina exhibited severe abnormal neovascularization, including the growth of many discrete vascular tufts immediately posterior to the advancing front of vessel formation (Fig. 3, left, arrowheads). The right side of the figure shows the contralateral retina from the same rat. This eye was injected with 0.76 picomoles TSP-1 immediately after removal from the exposure chamber. Although some abnormal neovascularization was observed, overall, this retina exhibited fewer vascular tufts than retinas from vehicle-injected eyes. Retinas from animals injected with TSP-1 3 days after return to room air had an appearance that was intermediate between that of vehicle-injected eyes and eyes injected with TSP-1 immediately after removal from the exposure chamber (data not shown).

The severity of abnormal neovascularization in untreated retinas and those injected with TSP-1 or vehicle alone was quantified as shown in Figure 4A. Injection of PBS immediately after transferring the rats to room air caused a small but significant ($P < 0.0106$) decrease in retinal neovascularization, when platelet-derived TSP-1 was injected immediately after the animals were removed from the exposure chamber, however, neovascularization was reduced by 48% compared with the vehicle-injected control ($P < 0.0001$). If the injection of TSP was delayed for 3 days after transfer of the animals to room air, it had less inhibitory effect on the neovascularization response (29% inhibition, $P = 0.0435$, compared with the vehicle-injected control). Injection of TSP or PBS did not significantly alter normal intraretinal vessel development (vasculogenesis) at either time of injection, as shown in Figure 4B.

Inhibition of Angiogenesis in a Retinal Explant Assay by TSP-1 and Synthetic Peptides Derived from the Type 1 Repeats

A retinal explant assay was used to evaluate the relative potency of intact TSP-1 and three synthetic peptides derived from the type 1 repeats of TSP-1. This assay is based on the outgrowth of endothelial cells from retinal tissue into a collagen gel. When intact TSP-1 was tested in this assay, low concentrations of TSP-1 (1.5 nM) slightly stimulated endothelial outgrowth, whereas higher concentrations (15 and 150 nM) significantly decreased endothelial outgrowth when compared with a serum-stimulated positive control (data not shown). The antiangiogenic properties of the TSP-1 molecule were further examined using synthetic peptides derived from the second type I repeat of TSP (Fig. 1). Four peptides were initially tested for their ability to inhibit endothelial cell outgrowth in the retinal explant model (Table 1). Peptide 388 (KRFKQDG-
GASHASPASS) contained the KRFK sequence previously reported to activate latent TGF-β1, peptide 450 (KRAKAGGWSHWSPWSSC) contained the GWXXWSPW sequence required for maximal heparin binding, and peptide 246 (KRFKQDGGWSHWSPWSS) derived from the native sequence of TSP-1, contained both the heparin-binding and TGF-β1-activating sequences. Peptide 300 (GGGWSH) contained the minimal-consensus heparin-binding WXSW sequence; however, previous studies have shown that this sequence binds very weakly to heparin compared with peptides containing the WSPW sequence, and peptide 300 was used as a nonreactive control in the present experiments. Incubation of retinal explants in medium containing 10% FBS or 10% FBS and peptide 300 at 200 μM yielded cultures of identical appearance, with many retinal vascular outgrowths (>30, data not shown). In subsequent experiments, medium containing 10% FBS was used as the positive control.

Peptide 246 inhibited endothelial cell outgrowth from the retinal tissue explant in a dose-dependent fashion, as detailed in Table 2. Between four and five explants were examined at four peptide concentrations on days 9 and 11 after the cultures were established. In the absence of the inhibitory peptide, the growth factors in the culture medium stimulated exuberant endothelial cell outgrowth (see photomicrograph in Fig. 5A) in four of five of the explants by 9 days of culture and in five of five explants after 11 days in culture. Because of the three-dimensional nature of the explant cultures, an accurate count of endothelial sprouts from the explanted retinal tissue was very difficult to perform when the response was robust, and explants with more than 30 outgrowths were denoted as “more than 30.” The data in Table 2 was representative of three independent experiments with peptide 246 (data not shown).

Peptide 246 was a potent inhibitor, significantly (P < 0.01) inhibiting outgrowth at 100 and 200 μM (see Table 2 and photomicrograph in Fig. 5B). When peptide 246 was withdrawn from the cultures, explants initially maintained in 100 and 200 μM peptide, which had previously shown minimal endothelial cell sprouting, started to form tube-like structures (see day 11 data in Table 2). These data suggest that peptide 246 inhibited endothelial cell sprouting without irreversibly damaging the cellular machinery required for the outgrowth response. Peptides 388 and 450 were less active than 246 in inhibiting endothelial cell outgrowth, as shown in Table 3. These data are representative of three independent experiments (data not shown). Peptide 388, which contained the TGF-β1 activating sequence, reduced the number of outgrowths to less than 10 per explant at the highest concentration tested (200 μM, P < 0.01; Fig. 5C). Peptide 450 at 100 μM significantly reduced (P = 0.05) the response in the explant assay. It reduced the number of outgrowths on three of five

Peptide 246 at concentrations of 20 to 200 μM was tested in the retinal explant assay. Medium with the indicated concentration of peptide was replaced on day 3. On day 7, the peptide inhibitor was removed from the culture medium. The number of outgrowths was determined on days 9 and 11. Statistical significance was determined using the Kruskal–Wallis test.

**Table 2.** Effect of Peptide 246 on Endothelial Cell Outgrowth from Retinal Explants

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Explant Outgrowth (Day 9)</th>
<th>Outgrowth (Day 11)</th>
<th>Significantly Different from 0 μM at Day 9</th>
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<tr>
<td>246</td>
<td>0</td>
<td>10</td>
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Peptide 246 at concentrations of 20 to 200 μM was tested in the retinal explant assay. Medium with the indicated concentration of peptide was replaced on day 3. On day 7, the peptide inhibitor was removed from the culture medium. The number of outgrowths was determined on days 9 and 11. Statistical significance was determined using the Kruskal–Wallis test.
potency of δ-reverse analogues and their respective polysucrose conjugates was therefore compared with that of the L-forward peptides, as shown in Figure 6. Potency was more readily compared in the retinal explant assays when assay response was reported as clock hours of endothelial outgrowth. In this analysis, each one twelfth of the explant circumference was examined for endothelial outgrowth and scored as positive if any outgrowth occurred in that quadrant, as shown in Figure 6. As previously noted, peptide 246 at a concentration of 100 μM almost completely inhibited endothelial outgrowth in the retinal explant assay (Fig. 6A). When peptide 416 (the δ-reverse analogue of 246) was tested at a concentration of 25 μM, two of four explants showed some endothelial outgrowth; peptide 416 at a concentration of 50 μM showed a pattern of response identical with that of peptide 246 at 100 μM. Addition of 100 μM peptide 450 produced a pattern of endothelial outgrowth similar to that observed in previous experiments (Table 3), and the inhibition observed with 50 μM of the δ-reverse analogue of 450 (peptide 476) was not significantly different. We estimate from these data that the δ-reverse analogues of 246 and 450 were approximately twice as potent as the L-forward peptides in the retinal explant assay.

The potency of the δ-reverse polysucrose-conjugated analogue of peptide 246 and 450 were also tested (Fig. 6B); the δ-reverse polysucrose-conjugated 246 analogue (416F70) was estimated to be approximately 40 times more potent than the corresponding L-forward peptide. Conjugation to polysucrose had a lesser effect on the potency of the conjugated heparin-binding peptide (476F70). At concentrations of 1 and 5 μM, these peptides did not significantly inhibit endothelial cell outgrowth in the explant assay.

Inhibition of Retinal Angiogenesis in the Rat Model of ROP by Synthetic Peptides Derived from TSP

For in vivo experiments, the δ-reverse polysucrose-conjugated analogues of the peptides tested were used to reduce inactivation and clearance from the vitreous. Three peptide-polysucrose conjugates were tested in these studies (Table 1); peptide 475F70, a δ-reverse analogue with the TGF-β1 activation sequence; peptide 476F70, an analogue with heparin-binding activity; and peptide 416F70, an analogue with both activities. The peptides were conjugated to polysucrose with a molecular weight of 70 kDa. Polysucrose (70 kDa) at a concentration equivalent to that in the peptide-polysucrose conjugates was used as the vehicle control.

Inhibitory action of the peptides on retinal neovascularization is summarized in Figure 7A. Those eyes receiving no injection had significantly more vascular disease \(P = 0.0351\) than those injected with vehicle. Such findings are consistent with previously published results using this animal model.\(^{42}\) Peptide 476F70, the heparin-binding peptide with no TGF-β1-activating activity, was the most potent antiangiogenic peptide in vivo, significantly reducing pathologic neovascularization at both concentrations tested (1.25 and 3.75 nanomoles per eye, \(P = 0.0117\) and 0.0003, respectively). Peptide 416F70 (which contains both heparin-binding and TGF-β1 activating sequences) was less effective at inhibiting retinal neovascularization than equimolar concentrations of peptide 476F70 and showed statistically significant inhibition only at the highest concentration tested (3.75 nanomoles per eye, \(P = 0.0397\)). In contrast, the highest concentration of peptide 475F70, which contains only the TGF-β1-activating sequence, significantly

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**Figure 5.** Retinal explant assay for antiangiogenic activity. Discs from bovine retinas (R) were sandwiched between type I collagen gels and overlaid with GMEM containing various test agents. (A) Positive control with 10% FBS; (B) 10% FBS and 200 μM peptide 246; (C) 10% FBS and 200 μM peptide 388. Photomicrographs were taken on day 8; magnification, ×160.
(P = 0.0383) enhanced neovascularization when compared with vehicle-injected control eyes. At the lower concentration of 475F70, there was no difference in neovascularization compared with the vehicle control. As shown for TSP, the injected peptides had no significant effect on normal intraretinal vessel development (vasculogenesis data shown in Fig. 7B). The morphologic differences among eyes injected with peptide 416F70 (Fig. 8, left), peptide 476F70 (Fig. 8 middle), or 457F70 (Fig. 8 right) mirrored the results of the more quantitative data presented in Figure 7A.

**DISCUSSION**

There is growing evidence for the role of TSP-1 in inhibition of angiogenesis both in vitro and in vivo.10–12,16,43–45 TSP-1 has been shown to inhibit FGF-2–induced migration and growth in bovine aortic endothelial cells,18 reduce angiogenesis in transfected cell lines,12,46 and inhibit rat corneal neovascularization, independent of TGF-β activity.45,47 The reported proangiogenic action of TSP-1 appeared to be indirect and as a result of leukocyte or myofibroblast infiltration of the biological assays used.14,48

The ability of TSP-1 to suppress angiogenesis appears to reside solely in the procollagen-like domain and the second and third type 1 repeats of the molecule. Tolmsa et al.16 showed that the central 70-kDa region of TSP-1 could block angiogenesis induced by FGF-2 in the rat cornea and inhibit migration and [3H]thymidine incorporation stimulated by FGF-2 in cultured bovine adrenal capillary endothelial cells. Further studies have revealed two subdomains that appear to act independently to suppress angiogenesis: a tryptophan-rich motif that binds to heparin20 and the GVQXR motif that binds CD36.21 The second type 1 repeat of TSP-1 also contains a basic sequence KRFK that binds to and activates TGF-β1 in vitro and in vivo.17,49 TGF-β has been reported to be a potent inhibitor of endothelial proliferation in vitro,50 whereas it has been reported to stimulate angiogenesis in vivo.51,52

In this study, we examined the effect of TSP-1 and peptides derived from the second type 1 repeat on two models of retinal neovascularization: an in vivo model of ROP and an in vitro model of retinal endothelial cell outgrowth. Injection of 0.76 picomoles of the intact TSP-1 molecule in the rat ROP model inhibited pathologic neovascularization in the retinas of treated animals. TSP-1 was more effective in vivo when administered immediately after removal of the animals from the oxygen pressure chamber. Thus, this extracellular matrix molecule most likely inhibited an early step in the angiogenic process. Previous reports that platelet-derived TSP may be contaminated with TGF-β1 raised concerns about the contribution of TGF-β1 to the antiangiogenic activities of TSP.

**Table 3. Effect of Peptides 388 and 450 on Endothelial Cell Outgrowth from Retinal Explants**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Concentration (μM)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide 388</td>
<td>Explants responding/explants tested</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Extent of response (number of outgrowths)</td>
<td>≥30</td>
<td>≥30</td>
<td>20</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Significantly different from 0 μM</td>
<td>No</td>
<td>No</td>
<td>P = 0.05</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Peptide 450</td>
<td>Explants responding/explants tested</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>Extent of response (number of outgrowths)</td>
<td>≥30</td>
<td>≥30</td>
<td>&lt;10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Significantly different from 0 μM</td>
<td>No</td>
<td>No</td>
<td>P = 0.05</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

Peptide 388 and 450 at concentrations of 50 to 200 μM were tested in the retinal explant assay. Medium containing the indicated concentration of peptide was replaced on days 3 and 7. The number of outgrowths was determined on day 8. Statistical significance was determined using the Kruskal–Wallis test.29

**Figure 6.** Comparisons of the potency of L-forward peptides, D-reverse peptides, and D-reverse polysucrose-conjugated peptides in the retinal explant assay. Each one-twelfth of the explant circumference was examined for endothelial cell outgrowth and scored as positive if any outgrowth occurred in that quadrant. (A), Individual datum for each explant; (+), the mean. (A) Comparison of the activities of the L-forward and D-reverse peptides with heparin-binding activity (peptides 450 and 476, respectively) and with both heparin-binding and TGF-β1–activating activity (peptides 246 and 416, respectively); (B) potency of the L-forward peptide 246 was compared with the D-reverse polysucrose-conjugated analogue with both heparin-binding and TGF-β1 activating activity or the analogue with only heparin-binding activity (peptides 416F70 and 476F70, respectively).
TGF-β1 was undetectable by enzyme-linked immunosorbent assay in the platelet-purified TSP-1 used in these experiments (data not shown). Because levels in the nanogram per millimeter range are required for the suppression of endothelial cell proliferation,\textsuperscript{50} it is unlikely that the antiangiogenic responses observed with the injected TSP were due to contamination with TGF-β1.

Peptides derived from the native sequence of TSP-1 (containing both the WSXW heparin-binding domain and the KRFK TGF-β1 activation domain) and mutant versions in which these domains were abrogated were first tested in a bovine retinal explant assay. This model is a complex system allowing multiple cellular interactions in a three-dimensional type I collagen gel. The cells that grow out from the explant have been shown by surface markers to be of endothelial origin.\textsuperscript{28} The system also contains a large number of activated and proliferating macrophages, a cell type that exhibits very high levels of oxygen consumption.\textsuperscript{53} In cultures with high oxygen consumption, the rate of diffusion usually limits oxygen delivery to the explanted tissue.\textsuperscript{54} The low oxygen tensions that result from limited oxygen delivery are likely to be responsible for the tissue damage and subsequent endothelial outgrowth observed in the explanted retinal tissue.\textsuperscript{28} Peptide 246, derived from the native sequence of TSP, significantly inhibited retinal vascular outgrowth in this assay at concentrations of 100 and 200 μM. This pronounced inhibition of vascular outgrowth did not appear to be due to endothelial cell toxicity at high peptide concentrations. If the peptide in the explant cultures was not replenished, small outgrowths from explants were observed in previously inhibited cultures 4 days after the last addition of peptide (Table 2). These data suggest that the explants had the ability to metabolize the inhibitory peptide and that cells capable of forming vascular outgrowths were still present in the cultures. The enhanced potency of the D-reverse peptides and their polysucrose conjugates in the explant assay (Fig. 6) may thus be a result of their enhanced half-life in the vicinity of the explant.

The active sequence motifs of peptide 246 (WSXW and KRFK) were further investigated using mutated versions of the peptide. Peptide 388, containing the KRFK domain responsible for activating TGF-β1, was active only at the highest concentration tested (200 μM; Table 3 and Fig. 3B). Peptide 450 and 476F70, which bind to heparin through tryptophan-rich se-
quences, were more inhibitory than peptides carrying only the KRFK sequence and inferior to the peptides carrying both sequences. Studies indicate that heparin and heparan sulfate are required for the stabilization and oligomerization of FGF-2 and VEGF that lead to receptor activation and signaling. Binding of tryptophan-rich motif to cell surface heparan sulfate proteoglycans may therefore deplete their availability for growth factor receptor binding. A heparin-binding peptide from type I collagen has recently been shown to inhibit endothelial tube formation in collagen gels, presumably by disrupting cell interactions with collagen fibrils, and it is possible that the heparin-binding, tryptophan-rich sequences in the current study had a similar effect. In the retinal explant assay, peptides 246, 416, and 416F70 (containing both the WSSX and KRFK sequences) were the most potent of the three peptides, possibly because of the synergism of growth factor antagonism and TGF-β1 activation. Experiments with pure populations of vascular endothelial cells have demonstrated that peptide 246 inhibits migration and proliferation of these cells while increasing the rate of apoptosis.

Onset of ischemia is one process that may initiate the cascade of events leading to retinal neovascularization, although nonischemic conditions as acidosis and bacterial infection can also lead to new blood vessel formation. In our in vivo study, ischemia was induced by exposing newborn rats to variable hyperoxia. The reverse peptide analogue containing the tryptophan-rich heparin-binding sequence but without the TGF-β1 activation sequence was most active in inhibiting neovascularization in the ROP model. In contrast, injection of the peptide containing only the TGF-β1 activation sequence actually stimulated the extent of pathologic neovascularization over that observed in vehicle-injected controls. The peptide with both WSSX and KRFK sequences also significantly reduced the neovascularization in the ROP model but was not as potent as the peptide containing only the heparin-binding domain. This could be due to opposing activities of the KRK and WSXW sequences. The action of peptides in the ROP model varied significantly from that of retinal explant assay. The difference in the responses observed in the two angiogenic assays was not due solely to the use of D-reverse peptide analogue but was not as potent as the peptide containing only the KRFK sequence and inferior to the peptides carrying both sequences, were more inhibitory than peptides carrying only the KRK sequence and inferior to the peptides carrying both sequences. Studies indicate that heparin and heparan sulfate are required for the stabilization and oligomerization of FGF-2 and VEGF that lead to receptor activation and signaling. Binding of tryptophan-rich motif to cell surface heparan sulfate proteoglycans may therefore deplete their availability for growth factor receptor binding. A heparin-binding peptide from type I collagen has recently been shown to inhibit endothelial tube formation in collagen gels, presumably by disrupting cell interactions with collagen fibrils, and it is possible that the heparin-binding, tryptophan-rich sequences in the current study had a similar effect. In the retinal explant assay, peptides 246, 416, and 416F70 (containing both the WSSX and KRFK sequences) were the most potent of the three peptides, possibly because of the synergism of growth factor antagonism and TGF-β1 activation. Experiments with pure populations of vascular endothelial cells have demonstrated that peptide 246 inhibits migration and proliferation of these cells while increasing the rate of apoptosis.

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The peptides used to inhibit neovascularization in the rat ROP model were polycysnose-conjugated molecules of relatively high molecular weight (>80 kDa). The high molecular weight of these conjugates prevented their penetration through the inner limiting membrane of the retina; the molecular weight limit for the penetration of proteins from the vitreous into the retina appears to be approximately 60 kDa. Because the pathologic neovascularization has infiltrated the inner limiting membrane, these vessels would be susceptible to the action of the protein and peptide inhibitors injected into the vitreous, whereas normal vasculogenesis would not. Thus, under the conditions of these experiments, both TSP and the TSP-derived peptides appear to inhibit only the neovascularization. Experiments are in progress to determine whether the size of the peptide administered intravitreally may influence the type of inhibition observed in the rat model.

In the ROP model, the heparin-binding peptide was most active in inhibiting neovascularization. Previous studies from our laboratories have shown that that peptide 246 antagonizes basic (b)FGF through binding to heparan sulfate and proteoglycans. These data include inhibition of binding of radiolabeled bFGF to endothelial cells, heparin, and heparan sulfate. The interaction of the peptides with cells is inhibited by heparanase treatment. Thus, the essential role of heparan sulfate proteoglycans in growth factor signaling has also been extensively documented. The primary mode of inhibition by the peptides appeared to be interference in the binding and activation of heparin-binding growth factors to their receptors. Ischemia in the retina stimulates the release of VEGF and FGF-2 as a result of tissue damage or due to hypoxic induction of Müller cells and leukocytes.

The variable effect of the TGF-β1 activation domain in the two angiogenic assays may relate to the levels of latent TGF-β1 present in the two model systems. TGF-β1 at low levels has been shown to inhibit endothelial migration and proliferation in vitro, and low levels of TGF-β have been reported in the vitreous of human donors without ocular complications. However, vitreoretinal diseases have been previously shown to cause a breakdown of the blood–ocular barrier and this breakdown could subsequently lead to increased vitreous concentrations of TGF-β1. Activation of these higher levels of TGF-β1 by the KRK domain of the type 1 repeats might then stimulate the angiogenic process.

TSP-1 has long been known as a natural regulator of the angiogenic process, and recent studies by Suzuki et al. have demonstrated that ischemic retina and VEGF-stimulated retinal neovascular cells synthesize and secrete TSP-1. The authors suggest that the VEGF-mediated induction of TSP-1 may be a negative feedback mechanism to control retinal neovascularization. In those cases in which neovascularization is not controlled by this process, the administration of peptides derived from the antiangiogenic type 1 repeats of TSP-1 may be a safe and effective therapy. In the context of the ROP model, it is notable that normal intraretinal vessel growth was unaffected by TSP-1 or peptide injection. The in vivo effectiveness of these simple peptides suggests that agents that compete with heparin-binding growth factors for cell surface heparan sulfate proteoglycans may be promising pharmacologic agents for treatment of ocular angiogenesis.

References

Inhibition of Angiogenesis by Peptides from TSP-1


