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Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro

(extracellular matrix/angiogenesis)

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ABSTRACT The response of endothelial cells to angiogenic stimuli has been shown to be influenced by the extracellular microenvironment. We tested whether thrombospondin, an extracellular matrix protein, modulated the spontaneous formation of cords by endothelial cells in vitro. Despite continued proliferation, a decrease in secreted thrombospondin was detected in cord-containing, as compared with subconfluent, cultures of both aortic and microvascular endothelial cells. Consistent with this trend, mRNA levels of thrombospondin decreased by factors of 16 in aortic and 60 in microvascular cultures that contained endothelial cords. Since thrombospondin was immunolocalized to fibrillar arrays that appeared to be associated with endothelial cords, we added anti-thrombospondin IgG to cord-forming cultures to limit the availability of the protein during this process. In the presence of anti-thrombospondin antibodies, there was a 33-50% increase in cord formation. These results suggest that thrombospondin is an inhibitor of angiogenesis in vitro and are consistent with its proposed roles as a destabilizer of endothelial cell focal contacts and as an inhibitor of endothelial cell proliferation.

Endothelial cells from both large vessels and capillaries demonstrate a tendency to form branching tubular networks, a process that has been termed "angiogenesis in vitro" (1). The factors that influence tube formation by endothelial cells in culture are complex and include the availability and activity of mitogens such as basic fibroblast growth factor and the synthesis and interaction of endothelial cells with components of the extracellular matrix (ECM) (2, 3). Ingber and Folkman (4, 5) have stressed the importance of an appropriate degree of adhesion of endothelial cells to components of the ECM in the formation of cords and tubes and have related this adhesivity to the transduction of mechanical forces between cells and their extracellular environment. At a molecular level, the interactions that are important in this process include the recognition of ECM macromolecules by integrins and other cell-surface receptors and the ability of such complexes to affect cytoskeletal organization and to generate intracellular chemical signals.

A conclusion that emerges from studies of angiogenesis in vitro is that cells involved in the last stages of tube formation are no longer actively proliferating (6-8). However, the incorporation of an endothelial cell into a tubular structure would appear to require the continued ability of the cell to reduce the adhesive forces that tend to keep cells spread on a substratum (5). Furthermore, the formation of capillary structures involves only a fraction of the total cellular population both in vivo and in vitro. Thus, capillary formation is likely to be a complex process resulting from an interplay of both positively and negatively acting factors.

Although the antiangiogenic effects of angiostatic steroids, heparin, and inhibitors of collagen synthesis have been explored (9, 10), little is known of the physiological factors that limit the incorporation of endothelial cells into tube-like structures in vitro or that control neovascularization in vivo (11). We have recently studied the synthesis of ECM proteins by different subpopulations of cells in a system undergoing angiogenesis in vitro (8). In this study it was noted that synthesis of the protein thrombospondin (TSP) was particularly high in cells adjacent to developing cords and tubes. TSP, a modular glycoprotein present in a granules of platelets, is synthesized by a variety of cells (12-14). TSP has been reported to support the attachment and spreading of a number of cells, including endothelial cells (15), but it has also been shown to reduce focal adhesions in endothelial cells (16). Although TSP supports the growth of smooth muscle cells (17, 18), it inhibits the mitogenic response of capillary endothelial cells to fetal calf serum (FCS) (15). In this paper we provide evidence for the ability of TSP to act as an inhibitor of angiogenesis in vitro. We suggest that, in the vicinity of developing tube-like structures, proliferating endothelial cells utilize TSP, perhaps in the process of migration (15), but that at the site of a developing cord or tube, the protein acts to limit the association of additional endothelial cells and, possibly, their proliferation.

MATERIALS AND METHODS

Cell Culture. Bovine aortic and rat vascular resistance endothelial cells (BAEC and RVEC, respectively; a gift from C. Dighi, Wayne State University, Detroit) were isolated and cultured as described (19, 20). We selected clones from BAEC expressing a sprouting phenotype (21) and RVEC clones that exhibited sprouting in vitro (44). Both cell types were cultured at 37°C in Dulbecco's modified Eagle's medium (GIBCO) containing 10% (vol/vol) heat-inactivated FCS (Flow Laboratories). Cells were used between passages 5 and 10 for BAEC and between 25 and 30 for RVEC. Spontaneous formation of endothelial cords occurred 10-15 days after confluence.

Immunocytochemistry. BAEC on Labtek chamber slides were fixed, rendered permeable by treatment with cold acetone, and incubated with anti-TSP IgG (12.8 μg/ml) (22). Cultures were then exposed sequentially to biotinylated goat anti-rabbit IgG (Vector Laboratories), avidin-biotin-peroxidase complex, and 3,3'-diaminobenzidine tetrahydrochloride (8). Control incubations were performed with normal rabbit IgG and with secondary antibody alone. Experiments were performed with an IgG fraction isolated as an ammonium sulfate precipitate of anti-TSP antisemun.

Abbreviations: BAEC, bovine aortic endothelial cells; FCS, fetal calf serum; PBS, phosphate-buffered saline; RVEC, rat vascular resistance endothelial cells; TSP, thrombospondin; TGF-β, transforming growth factor β; ECM, extracellular matrix.

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(12.8 μg/ml represented a dilution of 1:250 of the soluble IgG), and with an affinity-purified reagent prepared by adsorption of the anti-TSP IgG on a column of immobilized, purified TSP (6.4 μg/ml represents a 1:100 dilution of this preparation). At these dilutions there was no crossreactivity displayed by ELISA against serum proteins, including fibronectin, albumin, and fibrinogen, or against transforming growth factor β (TGF-β). By immunoblotting, there was no reactivity detected against 40 μg of FCS, even after lengthy exposure to x-ray film. The antibody preparations reacted principally with unreduced TSP and displayed only a low level of recognition of TSP chains. Both bovine (22) and rat (23) proteins were recognized by the anti-human TSP IgG.

**Metabolic Labeling and Immunoblot (Western) Analysis.** Metabolic labeling studies were performed with [2,3,4,5,6]H]proline (100 Ci/mmol, Amersham; 1 Ci = 37 GBq) on both subconfluent and cord-containing cultures of BAEC and RVEC as described (19). The medium was dialyzed against 0.1 M acetic acid and lyophilized. Cell numbers were determined by a hemocytometer. Lyophilized proteins were reconstituted in SDS/PAGE sample buffer containing 0.5 M urea, and volumes equivalent to 50,000 cells were resolved by SDS/PAGE on duplicate discontinuous polyacrylamide slab gels (4% stacking, 8% separating).

Proteins from the second of duplicate gels were electrophotically transferred to nitrocellulose and initially stained with a 1% solution of amido black in 10% acetic acid/20% methanol. The nitrocellulose was blocked in MT buffer [phosphate-buffered saline (PBS) (pH 7.8) containing 1% nonfat dry milk, 0.05% Tween-20] for 16 hr at 4°C. The blot was then incubated in anti-TSP IgG (6.4 μg/ml) for 1 hr at 20°C, rinsed several times in MT buffer, incubated in MT buffer containing 5 μCi of [3H]-labeled protein A (NEN), and rinsed again.

**Isolation of RNA and RNA Blot-Hybridization (Northern) Analysis.** RNA was extracted from subconfluent and cord-containing BAEC and RVEC cultures as described (24). Total RNA (7 μg) was denatured and resolved on a denaturing agarose gel. RNA was transferred to nitrocellulose under vacuum for 2 hr and crosslinked to the filter by UV irradiation. Northern blots were prehydrized at 42°C for 16 hr in 50% (vol/vol) deionized formamide/30% (vol/vol) 20× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 6.8)/50 mM sodium phosphate/4% 50× Denhard’s solution (1% Ficoll/1% polyvinylpyrrolidone/1.1% bovine serum albumin)/10 μg of yeast total RNA per ml. Hybridization was carried out under the same conditions by adding 10 cpm of heat-denatured [32P]dCTP probe per ml. The TSP probe, derived from a 1.3-kilobase (kb) fragment of human cDNA (25), was labeled with [32P]dCTP (10 cCi/ml) by nick translation and purified on a Sephacryl S-400 minicolumn (Promega). Blots were sequentially washed to a final stringency of 0.1× SSC and 0.1% SDS at 65°C. To verify the quality of RNA and the total length of the probes, the blots were probed with a bovine cDNA probe to 28S rRNA.

Slot blots were performed to verify the linearity of signals on the autoradiograms. RNA samples were denatured as described above and applied directly to nitrocellulose filters in amounts of 0.5, 5, and 10 μg. Several exposures of the autoradiograms from individual Northern and slot blot analyses were scanned with a spectrophotometer.

**Treatment of Cultures with Anti-TSP IgG.** BAEC plated on 12-well Costar plates (Corning) formed cords within 2 weeks. One cord was defined as the length between two intersecting (vertex) points (see Fig. 1A). To determine the number of tubes, 10 microscope fields (using a ×10 objective and ×1 ocular lenses) were examined in a premarked plate. For each experiment, five replicate cultures were counted (day 0). The cultures were then washed three times with serum-free medium and were incubated with the following reagents: for experiment 1, anti-TSP IgG at 1 μg/ml in medium containing 1% heat-inactivated FCS [Purified normal rabbit IgG (Sigma) was added at 1 μg/ml to control dishes]; for experiment 2, anti-TSP IgG at 1 μg/ml in serum-free medium (IgG derived from an ammonium sulfate precipitate of normal rabbit serum was added at 1 μg/ml to control dishes); for experiment 3, anti-TSP IgG at 1 μg/ml in medium containing 1% heat-inactivated FCS. (IgG, derived from an ammonium sulfate precipitate of normal rabbit serum, was added at 1 μg/ml to control dishes.) Medium containing antibodies was added at time 0, and fresh medium containing a second aliquot of antibodies replaced the original medium after 24 hr. After 48 hr, the same areas of each dish were recounted.

**Statistical Analysis of the Data.** From each set of experiments, the mean number of cords ± SEM was calculated at day 0 and day 2. Values were also expressed as percentages (± SEM), with the number of cords at day 0 in each culture taken as 100%. The data were analyzed by a paired-sample t test, and the differences were considered significant when P ≤ 0.025 (26). All calculations were performed with a STATVIEW computer program (Brain Power, Macintosh).

**RESULTS**

Certain strains of cloned BAEC exhibit a propensity for sprouting (undergrowth of an otherwise contact-inhibited monolayer by elongated cells) and subsequent organization into endothelial cords and patent tubes (8, 21, 27). Examples of these structures are shown in Fig. 1A. Isolated tubes that were disaggregated with trypsin and replated as single-cell suspensions gave rise to confluent monolayers prior to initiation of sprouting (8). This experiment, as well as the expression of the endothelial protein von Willebrand factor and endocytosis of acetylated low density lipoprotein, further demonstrates the endothelial origin of the tube-forming cells. In our experience, formation of endothelial cords precedes that of endothelial tubes. Although we (8) and others (28) have documented by photomicroscopy that the tubes contain lumina, in this study we have not made a distinction between solid cords and patent tubes and therefore have referred to the structures in Fig. 1A as “endothelial cords.”

As part of a related study on the secretion of ECM proteins by BAEC at different stages of cord and/or tube formation, we noted a fibrillar staining pattern surrounding these structures after immunocytochemistry was performed with anti-TSP IgG (8). From subsequent experiments we concluded that TSP was associated preferentially with mature endothelial cords and was predominantly extracellular (Fig. 1B). Although these data indicated a potential role for TSP in the process of angiogenesis in vitro, we were unable to distinguish among several possibilities ranging from (i) TSP is secreted by endothelial cells that participate in cord formation and facilitates the organization and progression of an extensive tubular network to (ii) TSP is precipitated by cells that are not involved in the assembly of cords, and its presence on mature cords serves to inhibit further cord formation.

To study the synthesis of TSP by distinct populations of cells, we measured the levels of TSP mRNA and protein in cultures of BAEC and RVEC undergoing angiogenesis in vitro. The biosynthetic profile reflects an induction of type I procollagen in tube-forming cultures of BAEC (Fig. 2, lane 1) and microvascular endothelial cells (not shown). The identities of type III procollagen (a major biosynthetic product of BAEC) and fibronectin were confirmed by Western blotting (data not shown). On reduced SDS/polyacrylamide gels, TSP appeared to diminish in tube-forming cultures; however, quantitation of this apparent decrease was not possible because the TSP monomer comigrated in this system with the procollagen chain. Therefore, we performed SDS/PAGE in
the absence of a reducing agent and confirmed the identity of TSP on an equivalent Western blot (Fig. 2, lanes 2–5). TSP was clearly diminished in tube-containing cultures of BAEC (compare lanes 2 and 3 of Fig. 2) and RVEC (compare lanes 4 and 5 of Fig. 2). Values averaged from three independent experiments showed a decrease in TSP protein by a factor of 7.7 in BAEC and 15.6 in RVEC, when equal numbers of cells in subconfluent and tube-forming cultures were compared.

mRNA levels of TSP were measured by blot hybridization of total cellular RNA and scanning densitometry (Fig. 3). Consistent with the decrease in TSP protein observed in cord-containing versus subconfluent cultures (Fig. 2), there was substantial diminution of TSP mRNA associated with cultures undergoing cord formation (Fig. 3A). This trend was confirmed over a total of eight independent experiments in BAEC and four independent experiments in RVEC. From quantitative scanning densitometry (Fig. 3B), we calculated a decrease in TSP mRNA by a factor of 16 in BAEC cultures and 60 in RVEC cultures. Slot blots were also performed, which confirmed the linearity of the hybridization signals and the data obtained from the Northern blots (data not shown).

To determine whether TSP functioned in angiogenesis in vitro, we added anti-TSP IgG to cord-containing cultures of BAEC and measured the change in the number of cords 2 days later. A total of five independent experiments was performed over a period of 6 months with different cultures of BAEC (representing two clonally derived strains) and different preparations of antibody. A statistically significant increase in the number of endothelial cords was seen when cord-containing cultures of BAEC were incubated for 2 days with 1–5 μg of anti-TSP IgG per ml (Fig. 4 and Table 1). This effect was dose dependent; antibody at 76 ng/ml produced no change and at 1–5 μg/ml provided a maximal response. Qualitatively similar results were obtained with the affinity-purified anti-TSP reagent. In no case was there a significant change in the number of cords in the three different control cultures. Nonimmune rabbit IgG or anti-TSP IgG produced no overt deleterious effects on BAEC morphology or change in adhesive properties after 1 or 2 days in culture (data not shown).

We have shown data from three independent experiments with three different reagents as controls (Table 1 and Fig. 4) in which the increase in cord number varied from 33% to 50%. In a fourth experiment, there was a 52% decrease in the number of cords in control cells that were cultured in purified normal rabbit IgG in the absence of FCS. In this case, the increase in cord number in the presence of anti-TSP IgG (and no FCS) was 80%.

DISCUSSION

The experiments reported in this study show that cultures of subconfluent endothelial cells synthesize substantial amounts of TSP and contain high levels of TSP mRNA prior to the formation of cords and tubes. On the other hand, cultures that form capillary-like structures contain substantially reduced levels of TSP mRNA and also synthesize less
of the protein. Since endothelial cells that are incorporated into tubes tend to be less active mitotically than the population from which they are derived (6-8), these findings are consistent with earlier data indicating an inverse relationship between the rate of growth and TSP production by endothelial cells in culture (29). The findings are also consistent with the more recent studies of Canfield et al. (30), who reported that the level of TSP synthesis by BAEC correlated more precisely with cell shape than with rate of growth. In particular, these workers showed that BAEC cultured within collagen gels, a condition that promoted an elongated morphology similar to that displayed by sprouts, produced low levels of TSP mRNA and protein, regardless of the proliferative state of the cells.

It was difficult to judge, based on the distribution of the protein in tube-forming endothelial cells, whether TSP played a positive or a negative role in cord formation or whether the protein was indeed functional in this process. Thus, on the one hand, TSP might be produced by endothelial cells participating in cord formation and facilitate the progression of a tubular network; alternatively, the presence of TSP on morphologically distinct structures could serve to inhibit further cord formation.

The available information on the role of TSP in cellular processes that could be relevant to capillary formation in vitro and in vivo is conflicting. TSP has been reported to support the adhesion of both lung capillary and aortic endothelial cells (15, 31) and to reduce the attachment of endothelial cells to a glass surface (32). In favor of an antiadhesive component to its mode of action are studies showing that TSP reduces focal adhesions in BAEC (16) and that human

![Figure 3](image1.png)

**Fig. 3.** Levels of TSP mRNA are modulated during the organization of endothelial cords in culture. (A) Northern blots of total RNA. RNAs were subjected to electrophoresis under denaturing conditions, transferred to nitrocellulose, and hybridized to a TSP probe. Lanes: 1, subconfluent BAEC cultures; 2, cord-containing BAEC cultures; 3, subconfluent RVEC cultures; 4, cord-containing RVEC cultures. A 28S RNA probe was used to normalize for loadings and transfer. (B) Histogram showing levels of TSP mRNA. Values were determined by scanning autoradiograms of Northern blots, and the numbers were normalized for loadings by using the signal from 28S RNA. Each bar represents the mean ± SEM of three independent experiments. Numbers 1, 2, 3, and 4 correspond to those in A.

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**Table 1. Effect of anti-TSP IgG on the number of endothelial cords in cultures of BAEC**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Condition</th>
<th>Day 0</th>
<th>Day 2</th>
<th>% increase</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-TSP/1% FCS</td>
<td>19 ± 1.1</td>
<td>25.2 ± 1.4</td>
<td>33</td>
<td>P ≤ 0.0005</td>
</tr>
<tr>
<td></td>
<td>NR IgG/1% FCS</td>
<td>19 ± 1.3</td>
<td>19.8 ± 1.9</td>
<td>0.1</td>
<td>&lt;P ≤ 0.37</td>
</tr>
<tr>
<td>2</td>
<td>Anti-TSP</td>
<td>6.8 ± 0.51</td>
<td>9.2 ± 0.73</td>
<td>35</td>
<td>0.0005 &lt;P ≤ 0.0005</td>
</tr>
<tr>
<td></td>
<td>NR serum</td>
<td>6.2 ± 0.58</td>
<td>6 ± 0.8</td>
<td>0.1</td>
<td>&lt;P ≤ 0.37</td>
</tr>
<tr>
<td>3</td>
<td>Anti-TSP/1% FCS</td>
<td>6.4 ± 0.6</td>
<td>9.6 ± 1.5</td>
<td>50</td>
<td>0.01 &lt;P ≤ 0.025</td>
</tr>
<tr>
<td></td>
<td>NR serum/1% FCS</td>
<td>4.4 ± 0.51</td>
<td>5.2 ± 0.73</td>
<td>0.025</td>
<td>&lt;P ≤ 0.05</td>
</tr>
</tbody>
</table>

BAEC cultures containing cords were measured (day 0) as described. Cultures were incubated with anti-TSP IgG (1 μg/ml), normal rabbit (NR) IgG (1 μg/ml), or NR serum (1 μg/ml). Experiments were performed with and without 1% FCS as indicated. After 24 hr, the medium was replaced by new medium supplemented with the corresponding antibodies. After a total of 2 days, the cords were recounted. Numbers represent the mean of five values ± SEM.

*Paired t test was performed to evaluate significance. The differences were considered significant when P ≤ 0.025.
umbilical vein and arterial cells attach but do not spread on a TSP-coated surface (33). TSP has also been reported to stimulate chemotaxis by endothelial cells (15) but it is far from obvious how this property relates to the propensity of a subpopulation of cells in culture to alter their shape and associate into structures that eventually acquire a lumen.

A recent clue to the role of TSP in angiogenesis has come from the work of Bouck and coworkers (34, 35). These investigators have identified a truncated form of TSP in the conditioned medium of hamster cells and hamster–human cell hybrids that suppresses neovascularization, induced by basic fibroblast growth factor, in the rat cornea. This inhibitory activity correlated with the presence of a tumor suppressor gene in transformants, revertants, and segregating hybrids of these cells (34). Support for the role of TSP as an inhibitor of angiogenesis is also provided by the recent finding that TSP mRNA levels are very low in MCF7 human breast cancer cells but significantly higher in a normal immortalized human mammary epithelial cell line and in somatic cell hybrids between this line and mammary tumor cells (36). Therefore, it is possible that the ability of some tumor cells to grow is, at least in part, to their failure to produce sufficient amounts of TSP, which would ordinarily function to limit capillary ingrowth into the vicinity of a growing tumor.

In an attempt to address a direct role for TSP in the angiogenic process in vitro, we initially added human platelet TSP, purified by molecular sieve and heparin-Sepharose chromatography, to cord-forming cultures of BAEC. Although we observed a substantial reduction in cord formation (unpublished observations), the interpretation of the experiments was complicated by the subsequent finding that preparations of platelet TSP were contaminated by small, but significant, quantities of both active and inactive TGF-β (unpublished observations). Therefore, we used highly active, monospecific, polyclonal antibodies to human platelet TSP, which are known to cross-react with both rat (23) and bovine TSP (22). As a further control, a series of experiments was conducted, in which TGF-β was added to cord-containing cultures of BAEC at concentrations of 0.05, 0.5, and 5 ng/ml. We found that TGF-β decreased to a very slight extent the cord formation over a 2-week period (data not shown). The results of these experiments provide strong evidence for the conclusion that TSP plays an antiangiogenic role in cord formation in vitro and that TGF-β does not participate directly in this process.

TSP has been reported to facilitate the growth of smooth muscle cells (17) and fibroblasts (37), and monoclonal antibodies to TSP inhibit smooth muscle cell growth (18). On the other hand, TSP has also been shown to inhibit the growth of several types of endothelial cells (38) and to inhibit the mitogenic effects of serum and basic fibroblast growth factor on murine lung capillary endothelial cells (15). The ability of TSP to function as an inhibitor of angiogenesis could provide an explanation for the known activity of heparin in potentiating the angiogenic response of limiting amounts of angiogenic factors (39, 40). Although the effects of heparin on cells are complex and varied, heparin is known to inhibit the adhesion of CHO cells to TSP (41) and to reduce the association of TSP with the surface of smooth muscle cells (23) and porcine aortic endothelial cells (42). Furthermore, at least one form of association of TSP with the cell surface is through an interaction with cell surface-associated heparin sulfate proteoglycans (43). The ability of heparin to potentiate angiogenic stimuli could therefore derive, in part, from its ability to inhibit the binding of TSP to cells.

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