Matrix-Bound Thrombospondin Promotes Angiogenesis In Vitro

Roberto F. Nicosia* and George P. Tuszynski‡

* Departments of Pathology and Laboratory Medicine, and ‡ Department of Medicine, Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

Abstract. Thrombospondin (TSP) is a multidomain adhesive protein postulated to play an important role in the biological activity of the extracellular matrix. To test this hypothesis, TSP-containing fibrin and collagen matrices were evaluated for their capacity to support angiogenesis and cell growth from explants of rat aorta. This serum-free model allowed us to study the angiogenic effect of TSP without the interference of attachment and growth factors present in serum. TSP promoted dose-dependent growth of microvessels and fibroblast-like cells. The number of microvessels in TSP-containing collagen and fibrin gels increased by 136 and 94%, respectively. The TSP effect was due in part to cell proliferation since a 97% increase in [^{3}H]thymidine incorporation by the aortic culture was observed. The effect was TSP-specific because TSP preparations adsorbed with anti-TSP antibody showed no activity. TSP did not promote angiogenesis directly since no TSP-dependent growth of isolated endothelial cells could be demonstrated. Rather TSP directly stimulated the growth of aortic culture-derived myofibroblasts which in turn promoted microvessel formation when cocultured with the aortic explants. Angiogenesis was also stimulated by myofibroblast-conditioned medium. Partial characterization of the conditioned medium suggests that the angiogenic activity is due to heparin-binding protein(s) with molecular weight >30 kD. These results indicate that matrix-bound TSP can indirectly promote microvessel formation through growth-promoting effects on myofibroblasts and that TSP may be an important stimulator of angiogenesis and wound healing in vivo.

THROMBOSPONDIN (TSP)[1] is a 450,000 dalton glycoprotein secreted by platelets in response to such physiological activators as thrombin and collagen (11). TSP comprises 3% of the total platelet protein and 25% of the total platelet secreted protein (43). TSP is also synthesized and secreted by fibroblasts (9), smooth muscle cells (29), endothelial cells (15), and tumor cells (28, 30, 45). In most tissues examined thus far, TSP has been found in the extracellular matrix (48). The structure of TSP is conserved among various animal species since antibody against the human protein cross-reacts with TSP from mouse, rat, pig, cow, sheep, dog, and turkey (36). Like fibronectin, TSP is composed of linear polypeptide domains that specifically interact with a number of macromolecules such as heparin (50), fibrinogen (43), collagen (19), and plasminogen (4).

Although the precise biological role of TSP has yet to be fully established, it is generally accepted that TSP plays a major role in cell adhesion and cell–cell interactions. For example, TSP was found to promote the cell-substratum adhesion of a variety of cells, including platelets, melanoma cells, smooth muscle cells, endothelial cells, fibroblasts, and epithelial cells (42). In addition, cells with very specialized functions such as keratinocytes (44) and osteoblasts (31) attach to TSP. Finally, TSP promotes the irreversible aggregation of platelets (41).

Recently, TSP has been implicated in the mechanisms of cellular proliferation. For example, it has been shown that (*a*) TSP potentiates the mitogenic activity of epidermal growth factor on smooth muscle cells (13), (*b*) platelet-derived-growth factor induces smooth muscle cell TSP synthesis (12), (*c*) TSP stimulates activation of smooth muscle cell S_6 kinase, a protein kinase involved in the transition of cells from the quiescent to the proliferative state (37), (*d*) anti-TSP antibodies inhibit the growth in culture of smooth muscle cells (14, 15), and (*e*) TSP promotes the proliferation of human fibroblasts in culture (27). In addition, Murphy-Ullrich and Hook (21) observed that TSP inhibited focal adhesion plaque formation by bovine aortic endothelial cells. These authors suggested that TSP by destabilizing cell matrix contacts facilitates mitosis and migration during wound healing and angiogenesis.

Since a considerable amount of evidence as summarized above suggests that TSP promotes cellular proliferation, it seemed reasonable to postulate that TSP incorporated in the extracellular matrix might play a role in angiogenesis. To test

Address all correspondence to Dr. G. P. Tuszynski, Department of Medicine, Medical College of Pennsylvania, 2900 Queen Lane, Philadelphia, PA 19129.

1. *Abbreviations used in this paper*: BAE, bovine aortic endothelial cells; ECGS, endothelial cell growth supplement; RAE, rat aortic endothelial cells; TSP, thrombospondin.

this hypothesis, we used the serum-free rat aorta model which allows study of angiogenesis without the confounding effects of serum factors (23) which like TSP might affect endothelial cell behavior. We report here that matrix-bound TSP promotes rat aortic angiogenesis by stimulating growth of myofibroblasts. These cells in turn stimulate microvessel formation by secreting soluble heparin-binding angiogenic factor(s).

Materials and Methods

Materials

All reagents, unless specified otherwise, were purchased from Sigma Chem. Co. (St. Louis, MO). Tissue culture supplies were purchased from ICN Flow (Irvine, CA). MCDB 131 serum-free medium was purchased from Clonetics (San Diego, CA). Reagents for SDS-PAGE were obtained from Bio-Rad Labs. (Richmond, CA). CN-activated Sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Interstitial collagen was prepared from rat tails according to Elsdale and Bard (6). Purified bovine basic fibroblast growth factor (bFGF) was obtained from R&D Systems (Minneapolis, MN).

TSP Purification

TSP was purified from Ca^{+2} ionophore A23187-activated platelets (43). Purified TSP preparations contained no detectable levels of fibronectin, vitronectin, von Willebrand factor, and less than 0.1% fibrinogen as previously described (42).

Angiogenesis Assay

Rings of rat aortas were embedded in gels of fibrin or interstitial collagen and grown in serum-free MCDB 131 medium as previously described (23). TSP was added to the gel at a final concentration of 0.5–60 μg/ml. Fibrin gels were prepared from a 3-mg/ml solution of fibrinogen chromatographed on gelatin agarose to remove contaminating fibronectin, and lysine agarose to remove contaminating plasminogen (23). For fibrin gel cultures, epsilon aminocaproic acid (300 μg/ml) was added to the medium to prevent fibrinolysis by the aortic explant. Gels of interstitial collagen were prepared from a 1-mg/ml solution of rat tail collagen (23). The culture medium was changed every other day. Developing microvessels were viewed and counted under an inverted light microscope according to published criteria (23). The length of the microvessels in the living cultures was measured by digitizing morphometry with a Bioquant IV image analysis system (24).

Cell Culture

Bovine aortic endothelial cells (BAE) were a gift from Denis Gospodarowicz, University of California, San Francisco. Rat aortic endothelial cells (RAE) were isolated from aortic rings by a nonenzymatic method (16) which has recently been modified in our laboratory to avoid contamination by fibroblasts and other nonendothelial cells (manuscript submitted for publication). Briefly, aortic rings were cultured on a 35-mm plastic dish to obtain endothelial outgrowths. After mechanically removing any contaminating nonendothelial spindle-shaped cells, the endothelial outgrowths were segregated in cloning rings, trypsinized, and subcultured in MCDB 131 medium supplemented with 10% FBS, 100 μ/ml endothelial cell growth supplement (ECGS, Collaborative Biomedical Products, Bedford, MA), and 200 μg/ml heparin. Both BAE cells and RAE cells immunostained for the endothelial marker factor VIII-related antigen (FVIII-RAg) and took up 1-1'-dioctadecyl-3,3,3',3'-tetramethyl indocarboxyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies Inc., Stoughton, MA). Myofibroblasts were isolated nonenzymatically from the primary outgrowths of serum-free collagen gel cultures of rat aorta. Fragments of the aortic outgrowths were detached from the collagen gel with a 22 gauge needle and isolated under a dissecting microscope with a pulled glass pipette. They were then washed in medium, transferred to collagen-coated 18-mm dishes (Nunc, Interlab, Thousand Oaks, CA), and grown in MCDB 131 medium supplemented with 10% FBS and 50 μg/ml gentamicin. As cultures were passed at a split ratio of 1:3, cells were characterized by immunohistochemistry and electron microscopy. After repeat passages the cultures were composed almost exclusively of myofibroblasts. At this stage the myofibroblasts were cloned and further characterized immunohistochemically. BAE cells and rat aorta-derived myofibroblasts were cultured either in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin sulfate or in MCDB 131 medium supplemented with 10% FBS and 50 μg/ml gentamicin sulfate.

Effect of Myofibroblasts on Angiogenesis

The effect of myofibroblasts on angiogenesis was studied either by incorporating these cells in collagen gel cultures of rat aorta or by evaluating the stimulatory activity of their conditioned medium. For the coculture experiment, myofibroblasts were trypsinized, mixed with MCDB 131 containing 10% FBS to neutralize the trypsin, centrifuged, and resuspended in serum-free medium. They were then washed extensively in serum-free medium to remove any residual serum, centrifuged, and resuspended in the collagen solution at increasing densities. Then, aortic rings were embedded in myofibroblast-containing collagen gels and cultured under serum-free conditions in MCDB 131 growth medium. For the conditioned medium experiment, serum-free MCDB 131 medium was conditioned by a 24-h incubation with exponentially growing subconfluent cultures of myofibroblasts. Collagen gel cultures of rat aorta were fed on alternate days with a 1:1 mixture of fresh MDCB 131 and conditioned medium. For partial characterization of myofibroblast angiogenic activity, conditioned medium was filtered through Amicon centricon concentrators (Amicon, Beverly, MA) having molecular weight exclusion limits of 100,000, 30,000, and 10,000.

Filtrate fractions were assayed for their angiogenic activity in the rat aortic model as described above. Conditioned media was also applied to a 3-ml heparin-agarose column (Sigma Chem. Co.) equilibrated in Tris-buffered saline and the flow-through fraction assayed for angiogenic activity.

Immunohistochemical Studies

For immunohistochemistry, cell cultures were fixed in buffered formalin, rinsed in PBS, permeabilized in Triton X-100, blocked with nonimmune serum, reacted with anti-FVIII-RAg rabbit polyclonal antibody (1:250, Dako) or anti-alpha-smooth muscle actin mouse monoclonal antibody (1:2,000, Sigma Chem. Co.), rinsed in PBS, incubated with the appropriate biotinylated secondary antibody, rinsed in PBS, and reacted with the avidin-biotin-peroxidase complex (Vector, Burlingame, CA). After washing with PBS, the cells were incubated with a diaminobenzidine solution containing H_2O_2, rinsed in distilled water, and counterstained with Harris' hematoxylin. Collagen gel cultures of rat aorta were fixed in buffered formalin, embedded in paraffin, and serially sectioned. Histologic sections were deparaffinized and processed for immunohistochemical studies as described above.

Transmission Electron Microscopy

For ultrastructural studies, myofibroblast cultures and collagen cultures of rat aorta were fixed in 1% glutaraldehyde 4% formaldehyde in phosphate buffer, pH 7.4, and embedded in EPON. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10A transmission electron microscope.

Cell Proliferation Assays

The effect of TSP on endothelial and myofibroblast cell proliferation was studied by the MTT assay for cell proliferation according to the procedure provided by Chemicon (Temecula, CA) and as reported (18). The principle of the assay is that MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), a non-toxic pale yellow substrate, is taken up by living cells to yield a dark blue formazin product. The process requires active mitochondria so dead cells will not form formazan. After 4 h of incorporation as little as 200 cells can be detected. The formazan color at 570 nm is directly proportional to the number of cells and the assay can be performed in a microtiter dish and quantitated with a microplate reader. A calibration curve relating absorbance to cell number was determined for each cell line studied. For this assay, cells were maintained in MCDB 131 medium supplemented with 1% FBS for several days and then harvested with EDTA. Approximately 10,000–30,000 cells suspended in 300 μl of serum-free MCDB 131 medium were plated per well of a 96 well microtiter plate and allowed to attach overnight. The medium was then changed to serum-free MCDB 131 medium containing no TSP or 30 μg/ml TSP. Enough replicate wells were plated so that cells could be counted on days 0, 2, and 4 and that the average value of triplicate cultures could be determined per time point. The culture medium was changed every other day.

The MTT assay was also used to determine if TSP affected the capacity of endothelial cells to respond to exogenous growth factors. For this experiment, RAE cells were cultured on TSP-collagen-coated microtiter plates in serum-free MCDB 131 with or without bFGF. For coating, each well was filled with 50 μl of MCDB 131 containing 50 μg/ml TSP and 50 μg/ml collagen and kept at room temperature for 20–30 min. Then the solution was aspirated and the plates were incubated for 30 min at 37°C to induce collagen fibrillogenesis on the bottom of the wells. After rinsing with MCDB 131 medium, each well received 10,000 RAE cells suspended in 100 μl of serum-free MCDB 131 with or without 10 ng/ml bFGF. In a separate experimental group designed to test the effect of soluble TSP according to a previously published protocol (3), RAE cells were seeded on uncoated wells and fed with MCDB 131 medium supplemented with 1% FBS and 10 ng/ml bFGF with or without 50 μg/ml TSP.

The effect of TSP on DNA synthesis by endothelial cells was studied by incubating serum-free cultures of BAE cells with 1 μCi/ml [^{3}H]thymidine for 5 h. Cultures were prepared by seeding 4-well Nunc culture dishes with 24,000 cells per well. After labeling, the cultures were rinsed with PBS, dissolved in tissue solubilizer, added to a scintillation fluid, and measured for radioactivity in a scintillation counter. The effect of TSP on growth of microvessels in matrix culture of rat aorta was monitored by the uptake of [^{3}H]thymidine. DNA synthesis by aortic outgrowths containing either 30 μg/ml BSA or increasing concentrations of TSP in the gel was determined by a 5-h pulse with 5 μCi/ml of [^{3}H]thymidine. The cultures were then rinsed in PBS, dissolved in tissue solubilizer, sonicated, added to a scintillation fluid, and counted for radioactivity.

Western Immunoblotting

Rabbit anti-TSP antibody was characterized by Western immunoblotting using Pharmacia's Phast gel electrophoresis system. Approximately 100 ng of TSP was separated under reducing conditions on an 8–25% polyacrylamide gradient SDS gel. The gel-separated TSP was electrophoretically transferred onto nitrocellulose paper and silver stained or immunostained. For immunostaining, the paper was blocked with 1% BSA in PBS, containing 0.05% Tween 20 (PBS-T) and treated with either 5 μg/ml of purified rabbit anti-TSP or rabbit IgG for 1 h, washed with PBS-T, and developed according to the instructions provided with the VECTASTAIN ABC immunoperoxidase system (Vector).

Statistical Analysis

Data were analyzed statistically by Student's t test or analysis of variance followed by Scheffé's test of significance. Statistical significance was set at $p < 0.05$.

Results

Effect of TSP on Angiogenesis

The angiogenesis assay used in this study is based on the observation that rings of rat aorta form microvessels when cultured in three-dimensional gels of fibrin or collagen (22, 23). In this system microvessels develop in serum-free medium and do not require addition of exogenous growth factors (23). Since TSP binds fibrin (20) and collagen (19), the aortic ring model is ideal to evaluate the effect of matrix-bound TSP on angiogenesis. Addition of TSP to fibrin and collagen gel cultures of rat aorta stimulated angiogenesis. Angiogenesis occurring in the presence of TSP was characteristically preceded by a marked increase in the outgrowth of fibroblast-like cells. Fig. 1 shows micrographs of aortic rings after 8 d of growth in fibrin gels containing either no TSP or 50 μg/ml TSP. Fig. 2 shows the same experiment performed in a collagen gel. Both TSP- and non-TSP-containing cultures developed microvessels that proliferated, branched, and anastomosed with each other forming a network around the aortic explant. However, TSP-treated cultures displayed a marked increase over control cultures in the number, length, and branching of the microvessels as well as in the number of fibroblast-like cells. All microvessels were positive for FVIII-RAg as previously reported (23, 25). The fibroblast-like outgrowth was negative for FVIII-RAg and included 15–20% alpha-smooth muscle actin-positive cells. Alpha-smooth muscle actin positive cells were also seen around microvessels (25). The expression of alpha-smooth muscle actin by these cells was low during early stages of angiogenesis and increased over time as the outgrowth matured and differentiated.

The potentiating effect of TSP on angiogenesis was dose-dependent in both fibrin and collagen gels with little or no stimulation at concentrations at or below 5 μg/ml and maximal stimulation at concentrations of 50–60 μg/ml. The time

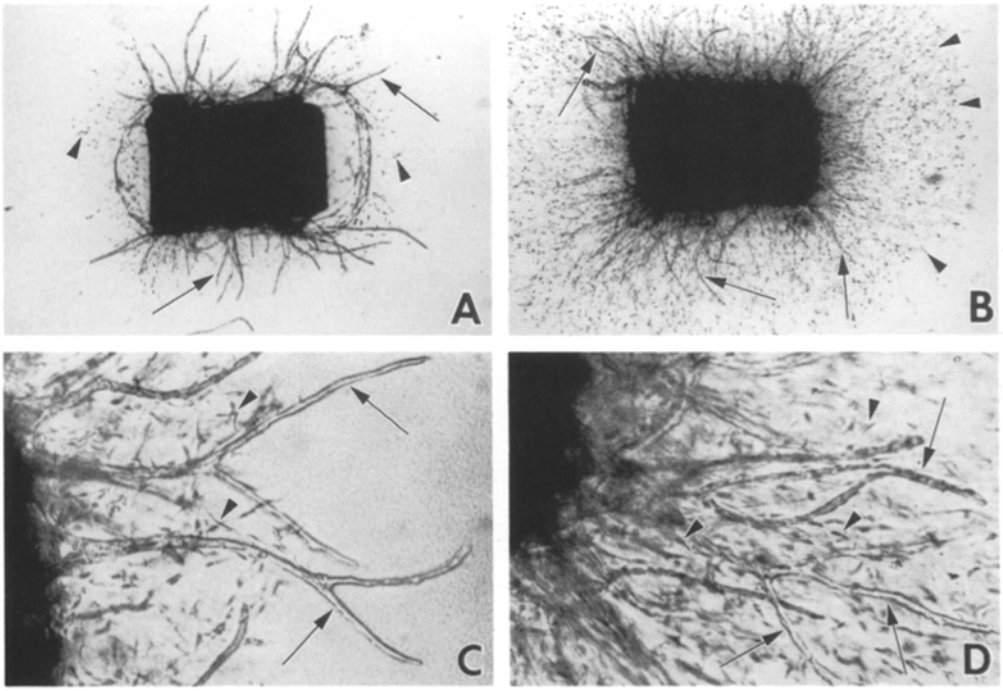

Figure 1. Serum-free cultures of rat aorta in fibrin gel (*A* and *C*) and fibrin gel supplemented with 50 μg/ml TSP (*B* and *D*). Aortic rings exposed to TSP (*B* and *D*) gave rise to a denser vascular outgrowth containing more microvessels and fibroblast-like cells than aortic rings not treated with TSP. Arrows and arrowheads indicate microvessels and fibroblast-like cells, respectively. Magnifications: *A* and *B*, ×25; *C* and *D*, ×140.

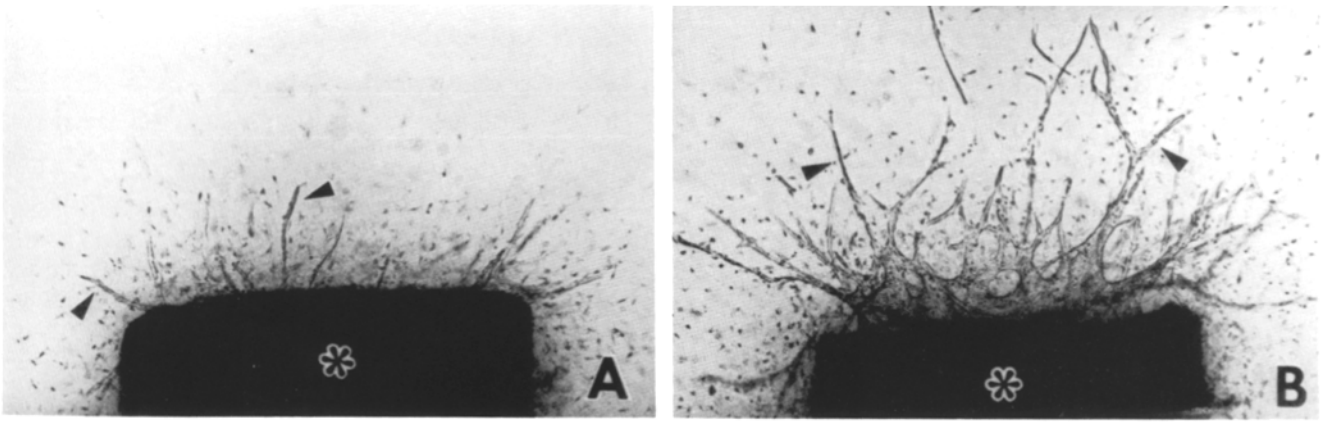

Figure 2. Serum-free cultures of rat aorta in control collagen gel (*A*) and collagen gel supplemented with 60 μg/ml TSP (*B*). TSP stimulated growth of microvessels and fibroblast-like cells. The aortic explants are marked by asterisks. Arrowheads indicate microvessels. Magnification: ×45.

course for TSP stimulation of angiogenesis revealed that TSP began to promote microvessel formation on day 5 of culture. On day 9, a 94% increase in microvessel formation was observed in TSP-containing fibrin gels as compared to untreated controls (Fig. 3). In collagen gel cultures the TSP effect was even more pronounced with a 137% stimulation on day 9 (Fig. 3). Fibrin and TSP had an additive effect on angiogenesis, and, in the presence of both molecules, microvessel formation continued to increase over time so that after the ninth day of culture there were too many microvessels and fibroblast-like cells to accurately quantitate. In addition to promoting vascular proliferation, TSP stimulated the elongation of the newly formed microvessels. The effect of TSP on microvascular length was dose-dependent and saturable (Fig. 4). Increased cellular proliferation induced by TSP was further demonstrated by showing that TSP-treated cultures incorporated ~97% more thymidine than controls when pulsed with [^{3}H]thymidine after 8 d of culture (Fig. 5). These experiments provide quantitative evidence that TSP promotes microvessel formation and proliferation of fibroblast-like cells in serum-free fibrin and collagen gel culture of rat aorta.

Specificity of the Angiogenesis-promoting Activity of TSP

The TSP used in these studies was electrophoretically pure as judged by silver-stained SDS-gels (Fig. 6). TSP, reduced by beta-mercaptoethanol, analyzed as a major band of 180,000 daltons. Previously, we demonstrated that our TSP preparations contained no detectable laminin, fibronectin, vitronectin, or von Willebrand factor (42). However, the possibility could not entirely be ruled out that our TSP contained trace amounts of growth factors that could contribute

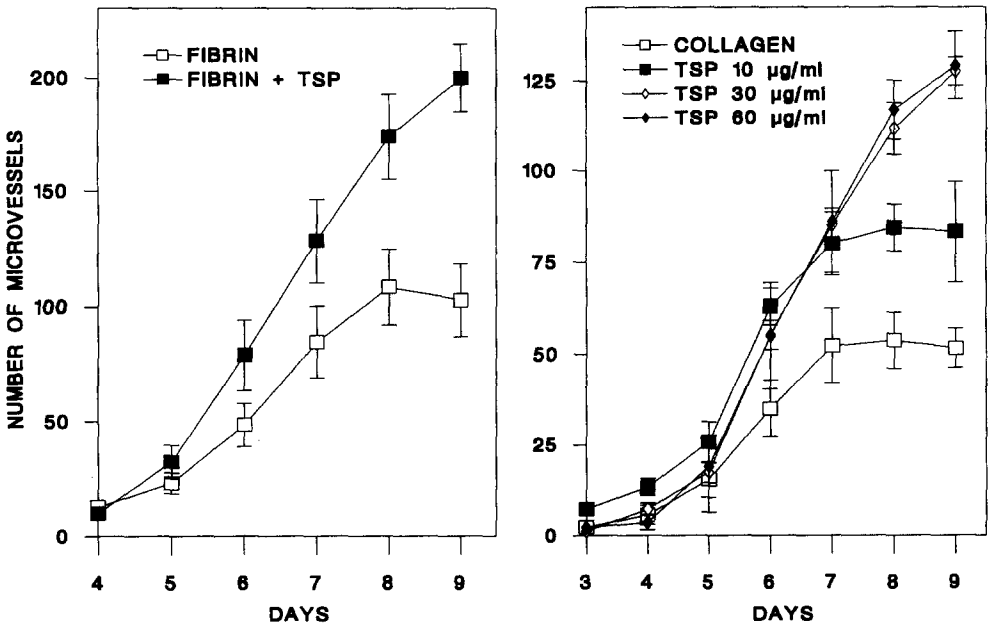

Figure 3. Effect of matrix-bound TSP on the growth curve of microvessels in fibrin (*left*) and collagen (*right*) gel cultures of rat aorta. TSP caused dose-dependent stimulation of angiogenesis. 50 μg/ml TSP induced a 94% increased of microvessels in fibrin ($p < 0.01$). 30–60 μg/ml TSP stimulated angiogenesis in collagen by 136% ($p < 0.01$). n = 8–11 (fibrin); 3 (collagen). The error bars represent the standard error of the mean (SEM).

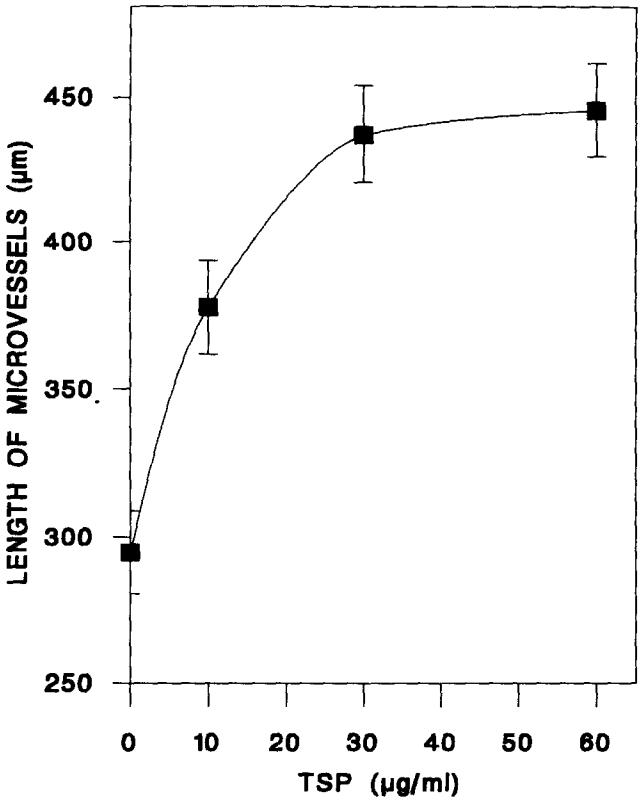

Figure 4. Effect of TSP on the length of microvessels formed in collagen gel culture of rat aorta. TSP promoted microvascular elongation in a dose-dependent and saturable manner. n = 122–243 microvessels per data point. Error bars indicate SEM.

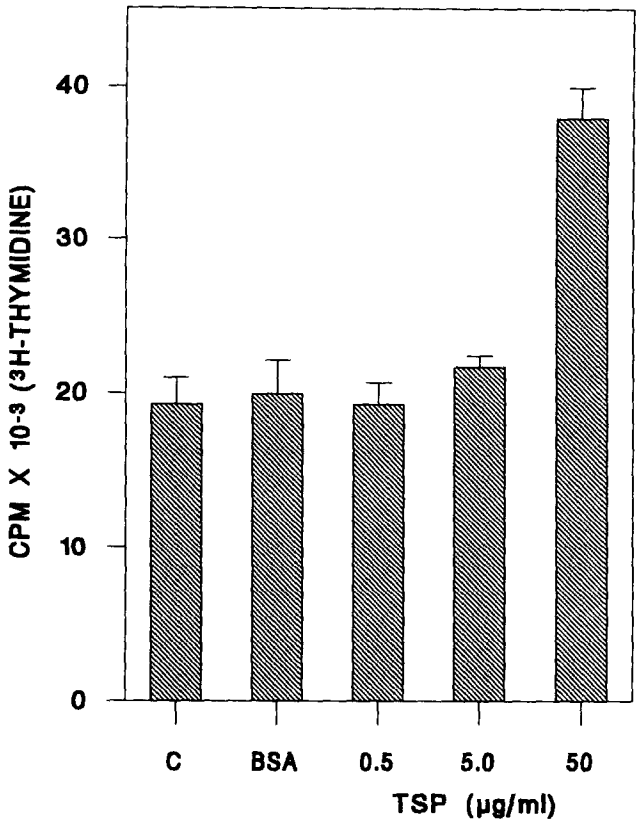

Figure 5. Effect of TSP on DNA synthesis in fibrin gel cultures of rat aorta. 8-d-old cultures were incubated with 5 μCi/ml [^{3}H]thymidine for 5 h and measured for incorporation of radioactivity. 50 μg/ml TSP stimulated DNA synthesis as compared to buffer control (*C*) and 50 μg/ml BSA ($p < 0.01$). Each data point represents the mean of triplicate cultures ± SEM.

to its observed angiogenic activity. To address this question, TSP was adsorbed with a monospecific rabbit anti-TSP antibody (Fig. 6) and tested for angiogenic activity. When 80% of the TSP was removed from a 50-μl solution of TSP, the angiogenic effect was abolished (Fig. 7). These experiments suggest that TSP and not some contaminant promotes angiogenesis in our assay system.

Effect of TSP on Endothelial Cell Proliferation

To investigate the mechanism of the angiogenesis-promoting activity of TSP, the effect of TSP on the growth of endothelial cells in culture was determined. We initially found that 30 μg/ml of TSP, which was the same concentration that promoted angiogenesis in the rat aorta model, had no stimulatory effect on the growth of BAE cells as measured by DNA synthesis (Fig. 8) and by cell counts (Fig. 9). Indeed previous studies have shown that soluble TSP inhibits the response of endothelial cells to bFGF (3). However, there are no reports on the effect of matrix-bound TSP on endothelial proliferation. Since rat aortic angiogenesis is in part mediated by endogenous bFGF (46), we decided to test the effect of collagen-bound TSP on the proliferative response of RAE cells to this growth factor. RAE cells grown on TSP-collagen substrate were unable to grow. However, they responded to bFGF which produced a threefold increase in endothelial

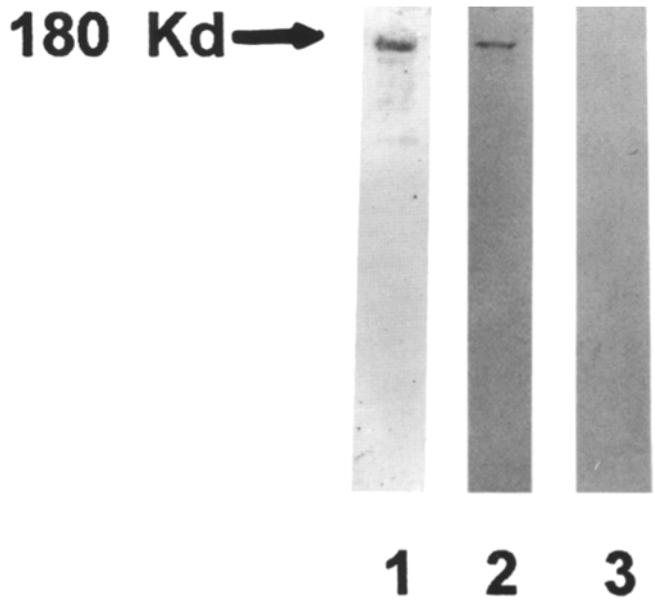

Figure 6. Western blot analysis of anti-TSP antibody. TSP was analyzed on an 8–25% polyacrylamide gradient SDS gel. (Lane *1*) silver-stained TSP; (lane *2*) anti-TSP-stained TSP; (lane *3*) nonimmune IgG-stained TSP.

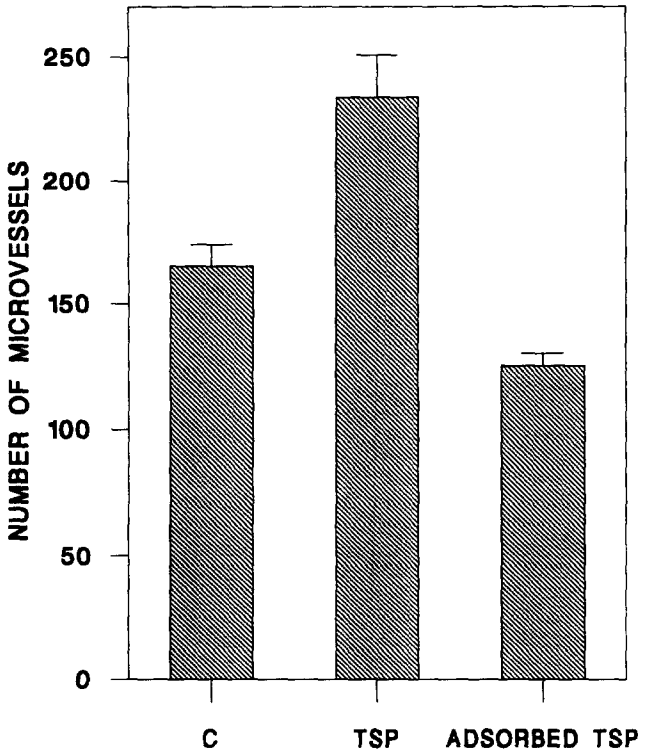

Figure 7. Growth of microvessels in fibrin gels treated with 50 μg/ml TSP, TSP adsorbed with Sepharose gel-coupled anti-TSP antibody, or no TSP (*C*). The total TSP antigen of the anti-TSP adsorbed TSP solution was reduced by 80% as determined by protein assay. Adsorption of TSP abolished the angiogenic effect of the TSP preparation ($p < 0.01$). $n = 3$ (TSP, ANTI-TSP), 9 (*C*). The error bars represent the standard deviation (SD).

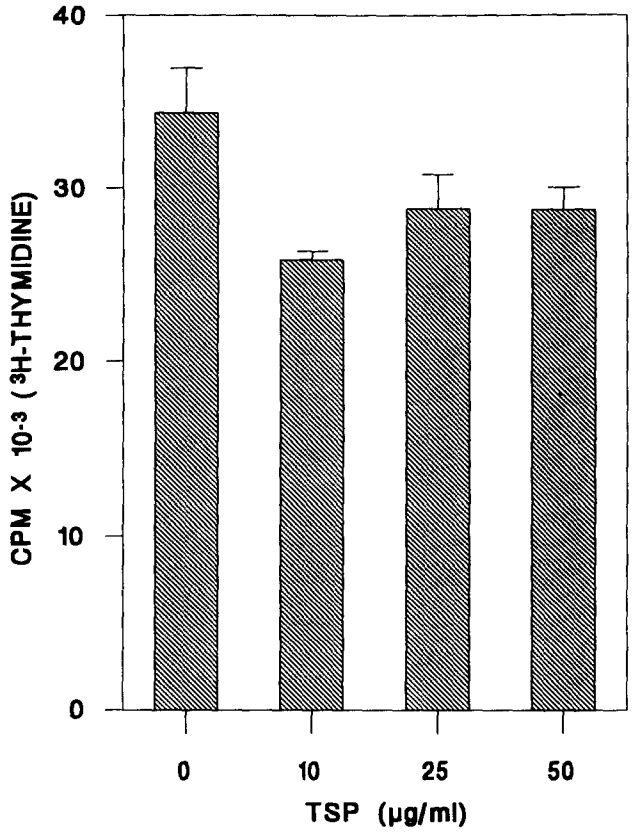

Figure 8. Effect of TSP on DNA synthesis by endothelial cells. Bovine aortic endothelial cells (BAE) were grown in serum-free medium containing increasing concentrations of TSP and labeled with 1 μCi/ml[^{3}H]thymidine for 5 h. TSP had no stimulatory effect on endothelial proliferation. Each data point represents the mean of four replicate cultures ± SEM.

cell number at day 4 (Fig. 10). Conversely, soluble TSP added to the medium of RAE cells cultured on uncoated dishes abrogated the stimulatory effect of bFGF (data not shown) as previously reported by others (3). These results indicate that TSP does not stimulate angiogenesis directly by promoting the growth of endothelial cells. They also show that matrix-bound TSP, unlike soluble TSP, does not affect the proliferative response of endothelial cells to bFGF. Since TSP does not stimulate endothelial cell proliferation directly, its angiogenic effect must be mediated by some other component in the system.

Isolation and Characterization of Myofibroblasts from Rat Aorta Cultures

The formation of microvessels from the aortic explants was always preceded by an outgrowth of fibroblast-like cells (Figs. 1 and 2). Immunohistochemical analysis showed that this outgrowth contained alpha-smooth muscle actin-positive cells. Rat aorta culture-derived nonendothelial cells were isolated, subcultured, cloned, and characterized immunohistochemically as myofibroblasts, based on a positive reaction for alpha-smooth muscle actin and negative FVIII-RAg stain. The rat aorta culture-derived myofibroblasts were able to modulate the alpha-smooth muscle actin, expressing this antigen in larger amounts in postconfluent cultures (Fig. 11). Ultrastructurally, myofibroblasts showed peripherally condensed myofilaments, abundant rough endoplasmic reticulum, pinocytotic vesicles, and were surrounded by abundant extracellular matrix (Fig. 12).

Effect of TSP on Proliferation of Rat Aorta Culture-derived Myofibroblasts

To investigate if the increased growth of fibroblast-like cells in collagen gel culture of rat aorta was due to TSP, we tested the effect of TSP on the proliferation of myofibroblasts. After 4 d, myofibroblasts grown in serum-free MCDB 131 medium containing 30 μg/ml TSP approximately doubled in number whereas those grown in the absence of TSP showed no growth (Fig. 9). These results indicate that TSP has a direct proliferative effect on rat aorta-derived myofibroblasts.

Effect of Rat Aorta Culture-derived Myofibroblasts on Angiogenesis

To investigate if the angiogenic response of the rat aorta to TSP was mediated by accessory nonendothelial cells which were sensitive to TSP stimulation, aortic explants were cultured under serum-free conditions in collagen gels contain-

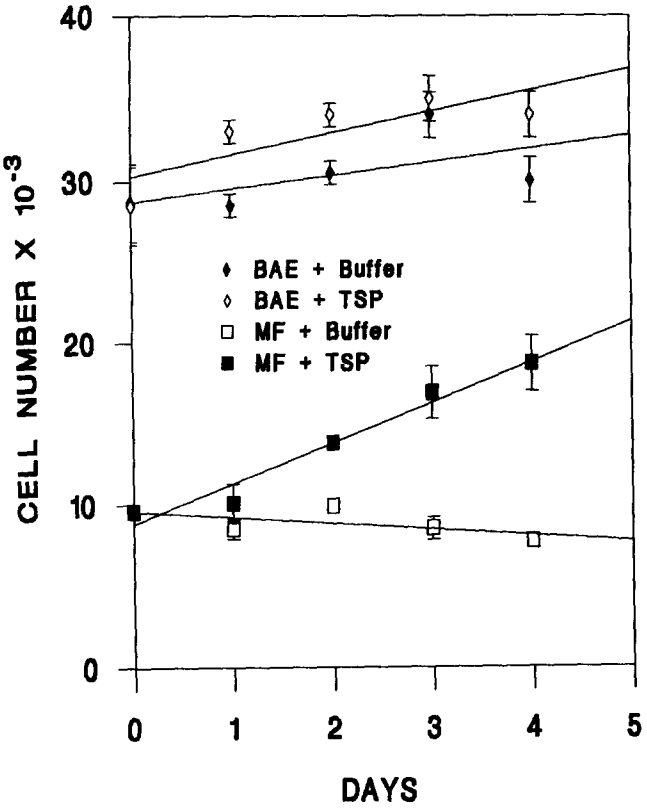

Figure 9. Effect of TSP on proliferation of bovine aortic endothelial cells (*BAE*) and rat aorta culture-derived myofibroblasts (*MF*). Cells were cultured in serum-free medium and their number was determined at daily intervals by the MTT cell proliferation assay. TSP stimulated the proliferation of myofibroblasts but had no effect on BAE cells. Each data point is the mean of 3 cultures. Error bars represent the SD.

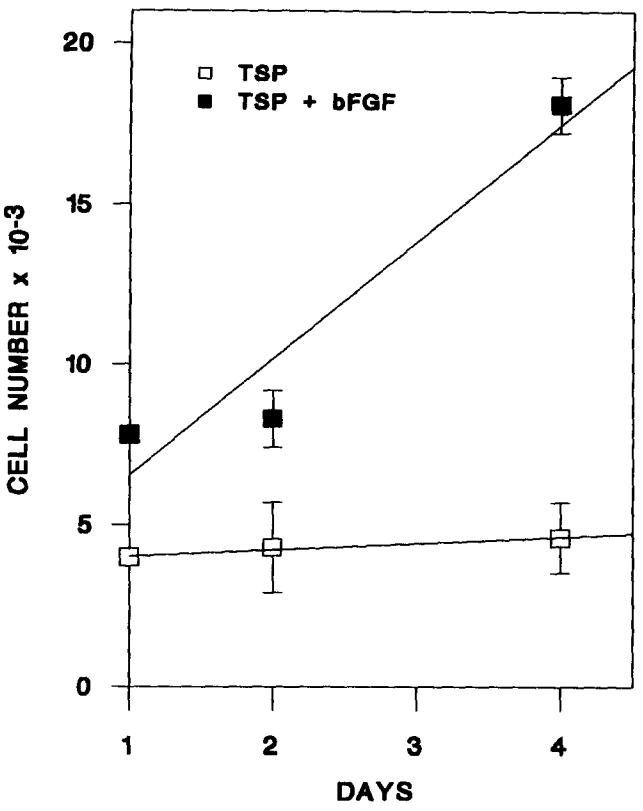

Figure 10. Effect of bFGF (10 ng/ml) on the proliferation of RAE cells cultured on collagen-TSP substrate in serum-free medium. bFGF promoted a threefold increase in the number of RAE cells on day 4 ($p = 0.0007$). RAE cells cultured on collagen-TSP in the absence of bFGF were unable to grow.

ing increasing concentrations of exogenously added myofibroblasts. Myofibroblasts stimulated angiogenesis by 100–115% as compared to controls containing no exogenous cells (Fig. 13). An even greater stimulation (167%) was obtained with myofibroblast cell clones. In addition, microvessels formed in the myofibroblast-containing gels were more stable and survived longer than control microvessels, which tended to regress at a faster rate. The angiogenic stimulation by myofibroblasts was dose-dependent with maximum effect obtained at 50,000 cells/ml of collagen. Therefore, myofibroblasts can directly promote angiogenesis. To determine whether the angiogenic activity of the myofibroblasts was mediated by soluble factor(s), we evaluated the effect of myofibroblast-conditioned medium on angiogenesis. After 7 d, collagen gel cultures of rat aorta treated with myofibroblast-conditioned medium showed a 105% stimulation of angiogenesis as compared to untreated controls. Taken together these results suggest that matrix-bound TSP promotes angiogenesis indirectly by stimulating the proliferation of myofibroblasts, which in turn secrete angiogenic factor(s).

Partial Characterization of Rat Aorta Culture-derived Myofibroblast Conditioned Medium

The stimulatory activity of medium conditioned by myofibroblasts isolated from the aortic cultures was retained by a heparin-agarose affinity chromatography column (Fig. 14) and was destroyed by proteolysis with trypsin. The activity was due to a protein >30 kD since the flow-through fractions of 10 kD and 30 kD Amicon filters were ineffective whereas fractions of larger molecular weight retained partial activity.

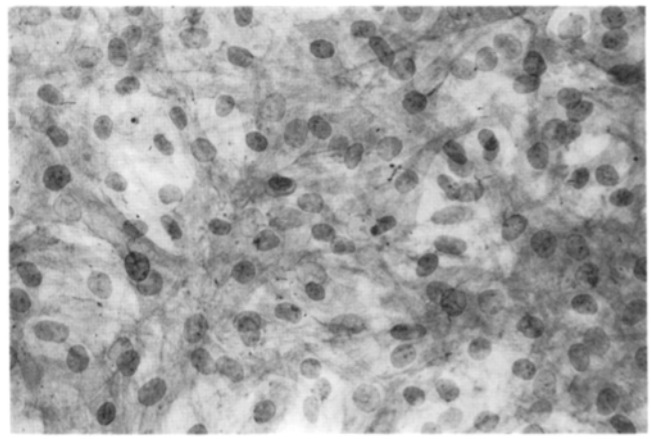

Figure 11. Light micrograph of myofibroblast cell culture immunostained by the ABC method for alpha-smooth muscle actin. Note the positive staining of the actin cytoskeleton. Magnification: ×470.

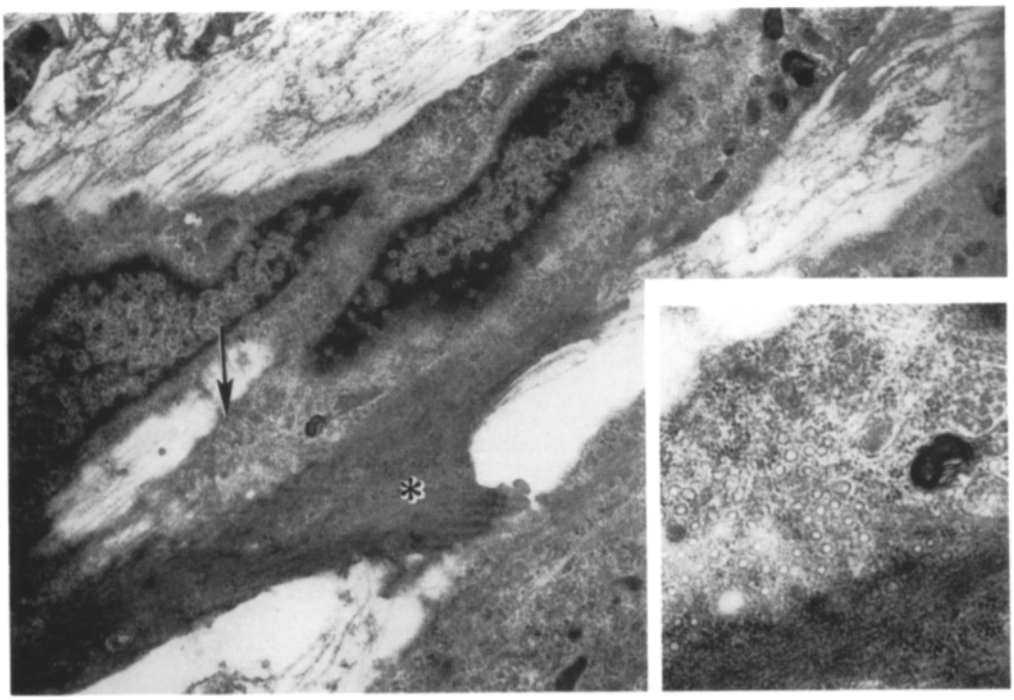

Figure 12. Electron micrograph of cultured myofibroblasts. Note the abundant peripheral microfilaments (*) and pinocytotic vesicles (*arrow* and *inset*). Magnification: ×19,200; inset, ×43,000.

Discussion

The experiments presented in this study provide evidence that TSP incorporated in a fibrin or collagen gel promotes angiogenesis from explants of rat aorta. TSP appears to stimulate angiogenesis indirectly through its growth-promoting effect on myofibroblasts derived from the aortic wall. TSP promoted the growth of fibroblast-like cells from the aortic explants before any microvessels had developed. Some of these cells stained for alpha-smooth muscle actin and, when isolated, showed morphological features of myofibroblasts. Consistent with the observations that expression of TSP is enhanced by fibroblasts and smooth muscle cell mitogens (10, 12), that antibodies against TSP inhibit growth of rat aortic smooth muscle cells (14), and that TSP stimulates the growth of fibroblasts (27), we find that TSP promotes the growth of myofibroblasts isolated from rat aortic cultures. These cells in turn stimulate angiogenesis by secreting

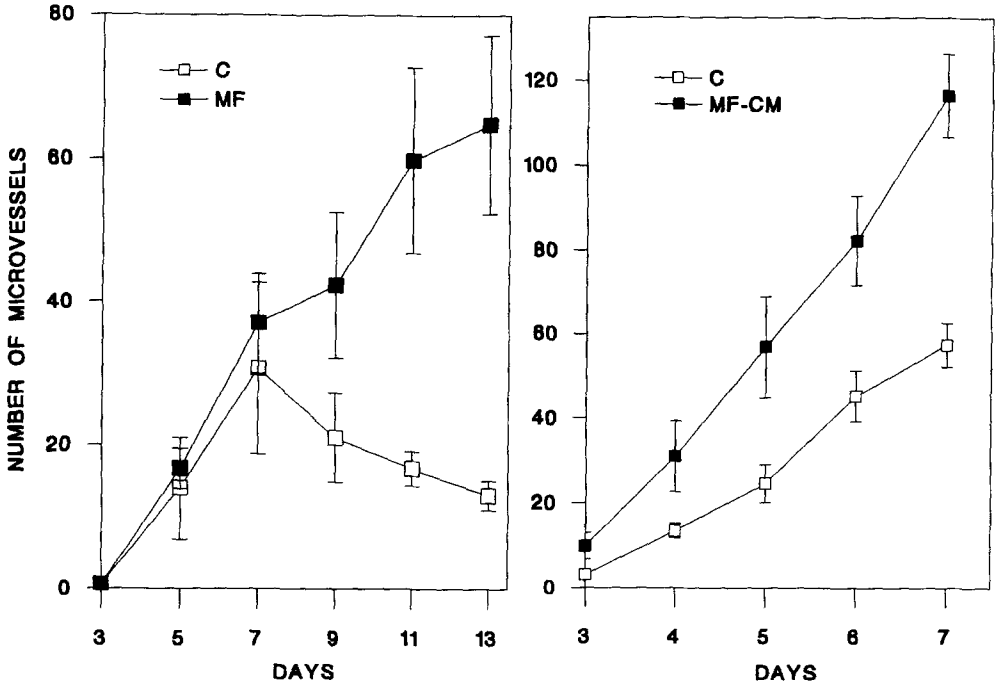

Figure 13. Effect of myofibroblasts (*left*) and myofibroblast-conditioned medium (*right*) on rat aortic angiogenesis in collagen gel culture. Myofibroblasts (*MF*) stimulated angiogenesis by 115% as compared to the control (*C*) containing no exogenous cells (day 13, $p = 0.015$; $N = 3$). Myofibroblast-conditioned medium (*MF-CM*) stimulated the angiogenic response by 105% (day 7, $p = 0.007$; $N = 5$). Error bars indicate SEM.

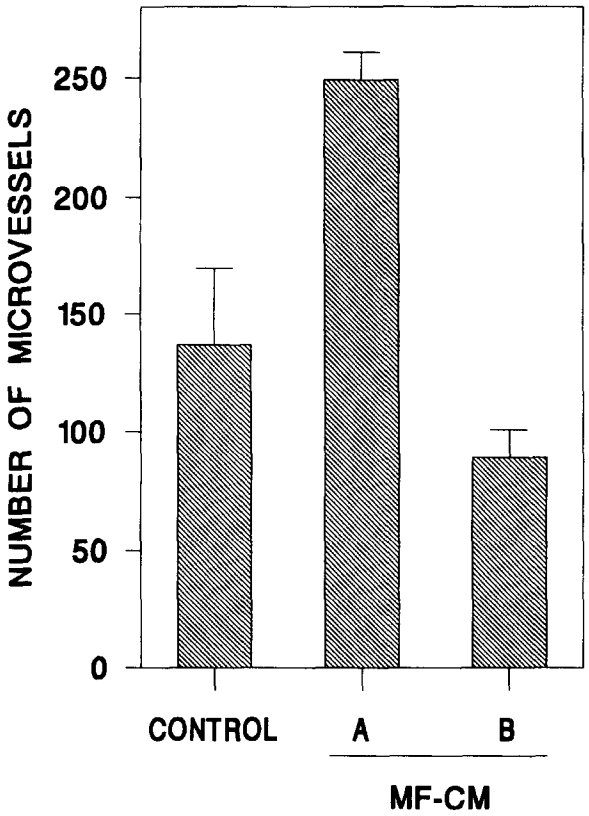

Figure 14. Maximum number of microvessels formed in control cultures and in cultures treated with rat aortic myofibroblast-conditioned medium (*MF-CM*) before (*A*) and after (*B*) adsorption on a heparin affinity chromatography column. Error bars indicate SEM, $n = 3$.

growth factor(s) as previously described (32, 33). Myofibroblasts may promote angiogenesis also by producing extracellular matrix molecules required by the endothelial cells (32).

Our data also indicate that matrix-bound TSP acts as a permissive substrate allowing endothelial cells to proliferate in response to growth factors such as bFGF. This is in contrast to soluble TSP, which has anti-proliferative effects on endothelial cells (3, 8, 38). In fact, RAE cells cultured on a collagen matrix containing TSP increased threefold after 4 d of treatment with bFGF. The same cells were unable to respond to bFGF when grown on uncoated plastic in the presence of soluble TSP, as previously reported (3). These results are consistent with the data of Morandi et al. (17) who showed that endothelial cells in culture require TSP as an attachment factor.

The growth-promoting effects of TSP do not appear to be due to growth factor contaminants since anti-TSP adsorbed preparations had no effect on angiogenesis. In addition, the fact that our TSP had no effect on endothelial cell proliferation rules out contamination by angiogenic cytokines such as bFGF, which directly stimulate endothelial cell growth. Moreover, bFGF is already present in the system as an endogenous component released by injured aortic endothelial and smooth muscle cells (46). It is also unlikely that the TSP effect was due to contamination with transforming growth factor beta-1 since this cytokine has anti-proliferative and anti-migratory effects on endothelial cells in vitro (51) and inhibits rat aortic angiogenesis when added to the collagen gel in its active form (unpublished observations). The angiogenic effect of TSP may be due in part to the capacity of this molecule to stimulate endothelial motility (38) and to modulate endothelial cell adhesion (21). In fact, elongation of microvessels, such as the one induced by TSP, can be promoted by a proliferation-independent migratory recruitment of endothelial cells (24).

TSP stimulated growth at concentrations similar to those of TSP present in serum or fibrin after blood coagulation. We have observed that tissue culture growth medium supplemented with 5% FBS contains 60 μg/ml of TSP as determined by indirect immunoadsorbent assay using anti-human TSP antibody and human TSP as a standard. Therefore, the similarity in our cultures of the effect of TSP with that of serum suggests that TSP may be one of the growth components of serum.

The previous report that TSP inhibits the angiogenic effect of bFGF in the rabbit cornea model is not inconsistent with our results (7). The rabbit cornea model depends on the capacity of molecules to diffuse from polymeric implants placed in the cornea, which is avascular, toward the limbus, which is richly vascularized. TSP diffuses poorly even when placed into a well of an agarose gel used for double immunodiffusion (unpublished observations). Therefore, it is not surprising that TSP, which has been shown to bind bFGF (39), does not promote angiogenesis in the cornea model but rather acts as a sink to sequester soluble angiogenic factors, impeding their diffusion toward their target vessels in the limbus. In our system the TSP effect on angiogenesis does not depend on diffusion since TSP is incorporated in the same matrix that contains the endothelial cells and the angiogenic factors, including bFGF (46), which are released by the aorta.

Our observation that TSP promotes angiogenesis indirectly by promoting the proliferation of myofibroblasts which in turn stimulate angiogenesis is intriguing since these cells may have derived from fibroblasts, smooth muscle cells, or pericytes (1, 5). The possible origin of myofibroblasts from fibroblasts is supported by the observation that fibroblasts can express alpha-smooth muscle actin (5) and stimulate angiogenesis in vitro (manuscript in preparation). Conversely, smooth muscle cells and pericytes have been shown to inhibit endothelial migration and proliferation (26, 51). However these cells, which are potential sources of myofibroblasts, may modulate their activity depending on their state of differentiation since they require physical contact to inhibit the endothelium (26, 51). Thus, angiogenesis may be promoted when pericytes and smooth muscle cells de-differentiate becoming myofibroblasts and inhibited when the same cells mature and establish contacts with the endothelium (35). Alternatively, as proposed by others (34), the aortic wall may contain different subpopulations of smooth muscle cells some of which respond to TSP and stimulate angiogenesis. This dynamic interpretation of smooth muscle cell function would reconcile apparently conflicting reports that have attributed either angiogenic (2) or anti-angiogenic roles (26, 51) to these cells. It is also consistent with the observations that the progressive increase in the number of pericytes in aortic cultures (Nicosia, R. F.,

and E. Bonanno. 1992. *Lab. Invest.* 66:22A) and in other angiogenesis systems (36) is eventually followed by cessation of vascular proliferation and maturation of microvessels.

Partial characterization of the myofibroblast conditioned medium suggests that the angiogenic activity is due to heparin-binding protein(s) having a molecular weight in excess of 30 kD. However, more studies are needed to further characterize these factor(s) and determine their relation to TSP.

In summary, our observations suggest that matrix-bound TSP may play an important role in stimulating the response of vascular connective tissue to injury at sites of fibrin and platelet deposition. Our studies provide evidence that TSP may mediate the proliferative response of the aortic wall to injury and the vascularization of the atherosclerotic plaque by recruiting myofibroblasts which in turn stimulate angiogenesis. These observations also suggest that TSP may promote angiogenesis and myofibroblast proliferation during wound healing. Preliminary studies sowing that TSP directly promotes healing of full thickness skin wounds in a pig model support this interpretation (unpublished data). Finally, TSP may potentiate tumor cell metastasis not only by promoting the adhesion of tumor cells and their subsequent sequestration in the lungs as previously reported (40), but also by stimulating angiogenesis. Recent reports from our laboratories (Tuszynski, G. P., V. L. Rothman, M. Papale, B. Zangwill, and R. F. Nicosia. 1992. *Mol. Biol. Cell.* 3:20*a*) and by others (28, 49) showing that malignant breast tumors, which are richly vascularized (47), contain a high level of stromal-associated TSP, are consistent with the conclusion that TSP promotes angiogenesis.

The authors wish to thank Drs. Antonio Ottinetti and Elena Bonanno for their assistance during the early stages of this work and Dr. Sergio Villaschi for helpful discussions. The authors gratefully acknowledge Vicki Rothman, Marion Smith, and James Diven for their excellent technical assistance.

This work was supported by Public Health Service grants HL43392 (R. F. Nicosia) and HL28149 (G. Tuszynski), and a grant from the Southeast Pennsylvania Chapter of the American Heart Association (G. Tuszynski).

Received for publication 1 December 1992 and in revised form 8 October 1993.

References

1. Adler, K. B., R. B. Low, K. O. Leslie, J. Michell, and J. N. Evans. 1989. Contractile cells in normal and fibrotic lung. *Lab. Invest.* 60:473–485.
2. Alpern-Elran, H., N. Morog, F. Robert, G. Hoover, N. Kalant, and S. Brem. 1989. Angiogenic activity of the atherosclerotic carotid artery plaque. *J. Neurosurg.* 70:942–945.
3. Bagavandoss, P., and J. W. Wilks. 1990. Specific inhibition of endothelial cell proliferation by thrombospondin. *Biochem. Biophys. Res. Comm.* 170:867–872.
4. DePoli, P., T. Bacon-Baguley, S. Kendra-Franczak, S. T. Cederholm, and D. A. Walz. 1989. Thrombospondin interaction with plasminogen. Evidence for binding to a specific region of the kringle structure of plasminogen. *Blood.* 73:976–982.
5. Demoulière, A., L. Rubbia-Brandt, A. Abdiu, T. Walz, A. Macieira-Coelho, and G. Gabbiani. 1992. Alpha-smooth muscle actin is expressed in a subpopulation of cultured and cloned fibroblasts and is modulated by gamma-interferon. *Exp. Cell Res.* 201:64–73.
6. Elsdale, T., and J. Bard. 1972. Collagen substrata for studies on cell behavior. *J. Cell Biol.* 54:626–632.
7. Good, D. J., P. J. Polverini, F. Rastinejad, M. M. LeBeau, R. S. Lemons, W. A. Frazier, and N. P. Bouck. 1990. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc. Natl. Acad. Sci. USA.* 87:6624–6628.
8. Iruela-Arispe, M. L., P. Bornstein, and H. Sage. 1990. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. *Proc. Natl. Acad. Sci. USA.* 88:5026–5030.
9. Jaffe, E. A., J. T. Ruggiero, L. L. K. Leung, M. J. Doyle, P. J. McKeown-Longo, and D. F. Mosher. 1983. Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into the extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 80:999–1002.
10. Janat, M. F., and G. Liau. 1992. Transforming growth factor beta 1 is a powerful modulator of platelet-derived factor action in vascular smooth muscle cells. *J. Cell. Physiol.* 150:232–242.
11. Lawler, J. 1986. The structural and functional properties of thrombospondin. *Blood.* 67:112–123.
12. Majack, R. A., S. C. Cook, and P. Bornstein. 1985. Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. *J. Cell Biol.* 101:1059–1071.
13. Majack, R. A., S. C. Cook, and P. Bornstein. 1986. Regulation of smooth muscle cell growth by components of the extracellular matrix: an autocrine role for thrombospondin. *Proc. Natl. Acad. Sci. USA.* 83: 9050–9054.
14. Majack, R. A., L. V. Goodman, and V. M. Dixit. 1988. Cell surface thrombospondin is functionally essential for vascular smooth muscle cell proliferation. *J. Cell Biol.* 106:415–422.
15. McPhearson, J., H. Sage, and P. Bornstein. 1981. Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture: apparent identity with platelet thrombospondin. *J. Biol. Chem.* 256: 11330–11336.
16. Merrilees, M. J., and L. Scott. 1981. Culture of rat and pig aortic endothelial cells. Differences in their isolation, growth rate, and glycosaminoglycan synthesis. *Atherosclerosis.* 30:19–26.
17. Morandi, V., F. Fauvel-Lafeve, C. Legrand, and Y. J. Legrand. 1993. Role of thrombospondin in the adhesion of human endothelial cells in primary culture. *In Vitro Cell. Dev. Biol.* 29A:585–591.
18. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival. *J. Immuno. Methods.* 65:55–63.
19. Mumby, S. M., G. J. Raugi, and P. Bornstein. 1984. Interactions of thrombospondin with extracellular matrix proteins: selective binding to type V collagen. *J. Cell Biol.* 98:646–652.
20. Murphy-Ullrich, J. E., and D. F. Mosher. 1985. Localization of thrombospondin in clots formed in situ. *Blood.* 66:1098–1104.
21. Murphy-Ullrich, J. E., and M. Hook. 1989. Thrombospondin modulates focal adhesions in endothelial cells. *J. Cell Biol.* 109:1309–1319.
22. Nicosia, R. F., and J. A. Madri. 1987. The microvascular extracellular matrix, developmental changes during angiogenesis in the aortic ring-plasma clot model. *Am. J. Path.* 128:78–90.
23. Nicosia, R. F., and A. Ottinetti. 1990. Growth of microvessels in serum-free matrix culture of rat aorta: a quantitative assay of angiogenesis in vitro. *Lab. Invest.* 63:115–122.
24. Nicosia, R. F., E. Bonanno, and M. Smith. 1993. Fibronectin promotes the elongation of microvessels during angiogenesis in vitro. *J. Cell Physiol.* 154:654–661.
25. Nicosia, R. F., E. Bonanno, and S. Villaschi. 1992. Large-vessel endothelium switches to a microvascular phenotype during angiogenesis in collagen gel culture of rat aorta. *Atherosclerosis.* 95:191–199.
26. Orlidge, A., and P. D'Amore. 1987. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J. Cell Biol.* 105:1455–1462.
27. Phan, S. H., R. G. Dillon, B. M. McGarry, and V. M. Dixit. 1989. Stimulation of fibroblast proliferation by thrombospondin. *Biochem. Biophys. Res. Commun.* 163:56–63.
28. Pratt, D. A., W. R. Miller, and J. Dawes. 1989. Thrombospondin in malignant and non-malignant breast tissue. *Eur. J. Cancer & Clin. Oncol.* 25:343–350.
29. Raugi, G. J., S. M. Mumby, D. Abbott-Brown, and P. Bornstein. 1982. Thrombospondin: synthesis and secretion by cells in culture. *J. Cell Biol.* 95:351–354.
30. Riser, B. L., J. Varani, K. O'Rourke, and V. M. Dixit. 1988. Thrombospondin binding by human squamous carcinoma and melanoma cells: relationship to biological activity. *Exp. Cell Res.* 174:319–329.
31. Robey, P. G., M. F. Young, L. W. Fisher, and T. D. McClain. 1989. Thrombospondin is an osteoblast-derived component of mineralized extracellular matrix. *J. Cell Biol.* 108:719–727.
32. Sato, N., Y. Sawasaki, A. Senoo, Y. Fuse, Y. Hirano, and T. Goto. 1987. Development of capillary networks from rat microvascular fragments in vitro: the role of myofibroblastic cells. *Microvasc. Res.* 33:194–210.
33. Satoh, T., M. Kan, K. Masuko, and M. Obinata. 1989. Characterization of a new endothelial cell growth factor (f-ECGF) partially purified from the supernatant of human fibroblast cells. *Cell Struct. Funct.* 14:731–740.
34. Schwartz, S., R. L. Heimark, and M. W. Majeski. 1990. Developmental mechanisms underlying pathology of arteries. *Physiol. Rev.* 70:1177–1209.
35. Sims, D. 1991. Recent advances in pericyte biology – implications for health and disease. *Can. J. Cardiol.* 7:431–443.
36. Switalska, H. I., S. Niewiarowski, G. P. Tuszynski, B. Rucinski, A. Schmaier, and T. A. Morinelli. 1985. Radioimmunoassay of human platelet thrombospondin: different patterns of b-thromboglobulin antigen

secretion and clearance from the circulation. *J. Lab. Clin. Med.* 106: 690–700.
37. Scott-Burden, T., T. J. Resink, U. Bauer, M. Burgin, and F. R. Buhler. 1988. Activation of S_6 kinase in cultured vascular smooth muscle cells by submitogenic levels of thrombospondin. *Biochem. Biophys. Res. Commun.* 150:278–286.
38. Taraboletti, G., D. Roberts, L. A. Liotta, and R. Giavazzi. 1990. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *J. Cell Biol.* 111: 765–772.
39. Taraboletti, G., D. Belotti, and R. Giavazzi. 1992. Thrombospondin modulates basic fibroblast growth factor activities on endothelial cells. *Exs (Exper. Suppl.).* 61:210–213.
40. Tuszynski, G. P., T. B. Gasic, V. L. Rothman, K. A. Knudsen, and G. J. Gasic. 1987. Thrombospondin potentiates tumor cell metastasis. *Cancer Res.* 47:4130–4141.
41. Tuszynski, G. P., V. L. Rothman, A. Murphy, K. Siegler, and K. A. Knudsen. 1988. Thrombospondin promotes platelet aggregation. *Blood.* 72: 109–115.
42. Tuszynski, G. P., V. Rothman, A. Murphy, K. Siegler, L. Smith, S. Smith, J. Karczewski, and K. Knudsen. 1987. Thrombospondin promotes cell-substratum adhesion. *Science (Wash. DC).* 236:1570–1573.
43. Tuszynski, G. P., S. Srivastava, H. I. Switalska, J. C. Holt, C. S. Cierniewski, and S. Niewiarowski. 1985. The interaction of human platelet thrombospondin with fibrinogen purification and specificity of interaction. *J. Biol. Chem.* 260:12240–12245.
44. Varani, J., B. J. Nickoloff, B. L. Riser, R. S. Mitra, K. O'Rourke, and V. M. Dixit. 1988. Thrombospondin-induced adhesion of human keratinocytes. *J. Clin. Invest.* 81:1537–1544.
45. Varani, J., B. L. Riser, L. A. Hughes, T. E. Carey, S. E. G. Fligiel, and V. M. Dixit. 1989. Characterization of thrombospondin synthesis, secretion and cell surface expression by human tumor cells. *Clin. & Exp. Metastasis.* 7:265–276.
46. Villaschi, S., and R. F. Nicosia. 1993. Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury. *Am. J. Pathol.* 143:1–10.
47. Weidner, N., J. P. Semple, W. R. Welch, and J. Folkman. 1991. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *New Engl. J. Med.* 324(1):1–8.
48. Wight, T. N., G. J. Raugi, S. M. Mumby, and P. Bornstein. 1985. Light microscopic immunolocalization of thrombospondin in human tissues. *J. Histochem. Cytochem.* 33:295–302.
49. Wong, S. Y., A. T. Purdie, and P. Han. 1992. Thrombospondin and other possible related matrix proteins in malignant and benign breast disease. *Am. J. Path.* 140:1473–1482.
50. Yabkowitz, R., J. B. Lowe, and V. M. Dixit. 1989. Expression and initial characterization of a recombinant human thrombospondin heparin binding domain. *J. Biol. Chem.* 264:10888–10896.
51. Yasufumi, S., and D. B. Rifkin. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta1-like molecule by plasmin during co-culture. *J. Cell Biol.* 109:309–315.