Platelet Thrombospondin Modulates Endothelial Cell Adhesion, Motility, and Growth: A Potential Angiogenesis Regulatory Factor

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Abstract. Components of the extracellular matrix have been shown to modulate the interaction of endothelial cells with their microenvironment. Here we report that thrombospondin (TSP), an extracellular matrix component, induces adhesion and spreading of murine lung capillary (LE-II) and bovine aortic (BAEC) endothelial cells. This TSP-induced spreading was inhibited by heparin and fucoidan, known to bind the amino-terminal globular domain of the molecule. In addition, endothelial cells were induced to migrate by a gradient of soluble TSP (chemotaxis). The chemotactic response was inhibited by heparin and fucoidan, as well as by the mAb A2.5, which also binds to the amino-terminal domain. These data are in agreement with our previous observation that the TSP amino-terminal heparin binding region is responsible for the induction of tumor cell spreading and chemotactic motility. The inhibition of chemotaxis and spreading by antibodies against the $\beta 3$ but not the $\beta 1$ chain of the integrin receptor points to a role for the integrins in the interaction of endothelial cells with TSP.

We also found that TSP modulates endothelial cell growth. When added to quiescent LE-II cells, it inhibited the mitogenic effects of serum and the angiogenic factor bFGF, in a dose-dependent manner. The inhibition of DNA synthesis detected in the mitogenic assay resulted in a true inhibition of BAEC and LE-II cell growth, as assessed by proliferation assay. This work indicates that TSP affects endothelial cell adhesion, spreading, motility and growth. TSP, therefore, has the potential to modulate the angiogenic process.

COMPONENTS of the extracellular matrix (ECM)[1] have been shown to interact with endothelial cells and to regulate such cellular functions as attachment and spreading, motility, and response to growth and transforming factors. Thus, the ECM may help maintain the integrity of the vessel wall, mediate the endothelium's interaction with platelets and other cells, regulate tissue repair and remodeling during would healing, and participate in angiogenesis and in pathological conditions, such as tumor metastasis.

Thrombospondin (TSP) is a high-molecular weight multifunctional glycoprotein (9, 19, 37). It was first described as a product of platelets, released from the alpha granules in response to activation by thrombin. Later TSP was shown to be produced in vitro by a variety of cell types including fibroblasts, smooth muscle cells, pneumocytes, macrophages, keratinocytes, monocytes, osteoblasts, and tumor cells (10, 12, 13, 19, 21, 26, 35, 37, 44). In addition, it is synthesized, secreted in the culture medium, and incorporated into the ECM by cultured endothelial cells (16, 25, 26, 31). In vivo TSP has been found in plasma, on the surface of activated platelets, in endothelial cell cytoplasm, and in basement membranes and vessel walls (45). TSP can interact with different macromolecules, including several components of the ECM (19), and with specific receptors on the cell surface (1, 2, 14, 20, 33, 41, 46), thus mediating cell-cell and cell-substrata interactions. TSP has been reported to induce attachment, spreading, motility, and growth of different cell types (22, 35, 38, 42, 43).

TSP appears to play a role in tissue neoformation, healing, remodeling, and in several pathologies. For example: (*a*) it is synthesized by tumor cells, and the concentration in the tumor mass is higher than in normal tissues (30, 35, 44); (*b*) TSP incorporation into the ECM is transient, and it is readily removed by heparitinase and heparin-like molecules (24); (*c*) TSP concentrations are also high at the site of vascular and tissue injury (45); (*d*) in vitro the production of TSP and its deposition into the ECM depends on the cell cycle, degree of confluency and level of differentiation (16, 26). It is higher under conditions of injury (heat shock) (15) and can be increased by PDGF (21), basic FGF, and IL 1 (Danoviel, J. B., and P. Bornstein. 1988. *J. Cell Biol.* 107:596a. [Abstr.]); (*e*) TSP has been shown to function as an autocrine growth regulatory factor for smooth muscle cells and to increase the mitogenic effect of EGF on these cells (22, 23).

1. *Abbreviations used in this paper*: ECM, extracellular matrix; FN, fibronectin; LM, laminin; TSP, thrombospondin.

This study was designed to investigate the interaction of TSP with endothelial cells. We analyzed the effect of TSP on endothelial cell adhesion, spreading, and motility. Proliferation and mitogenesis assays were performed to investigate whether TSP regulates cell growth. The results indicate that TSP affects important endothelial cell functions, and modulates the response to growth and angiogenic factors. These data strengthen the hypothesis that TSP has a role in wound healing and remodeling, and indicate that TSP has the potential to modulate angiogenesis.

Materials and Methods

Calcium-replete TSP was purified from the supernatant of thrombin-stimulated fresh human platelets, essentially as described (11). Laminin was purified from Engelbreth-Holm-Swarm (EHS) tumor as described (39). Fibronectin was purchased from Collaborative Research (Bedford, MA); type IV collagen and gelatin were from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antiserum against platelet GpIIbIIIa was a kind gift from Dr. E. Dejana (Istituto Mario Negri, Milano, Italy). This antibody was shown to react with both murine and bovine endothelial cell lines, as assessed by fluorescence flow cytometry, with 73.5% (BAEC) and 76.3% (LE-II) positive cells. Immunoblot analysis of endothelial cell extract was done as described (4), and showed reactivity of the antiserum with a band of 90 kD, as described for an anti-β3 antibody (4), and as expected for endothelial cells. A2.5 mAb was kindly provided by Dr. W. Frazier (Washington University School of Medicine, St. Louis, MO); the antibody against the fibronectin receptor was generously provided by Dr. R. L. Juliano (University of North Carolina, School of Medicine, Chapel Hill, NC); human recombinant basic FGF (bFGF) was kindly provided by Dr. F. Bertolero (Erbamont, Milano, Italy).

Cells

BAEC bovine aortic endothelial cells were kindly provided by Dr. E. Dejana (Istituto Mario Negri, Milano, Italy). BAEC cells and LE-II murine lung capillary cells (36) were cultured in DME supplemented with glutamine and 10% FCS (LE-II) or 20% FCS (BAEC). Cells were tested and found positive by immunofluorescence for the von Willebrand factor, a marker of endothelial cells. For adhesion and motility experiments, cells were detached by brief exposure to trypsin 0.025%/EDTA 0.02%, and allowed to regenerate for 1 h in serum-containing medium.

Cell Adhesion Assay

A solution of TSP was diluted to the indicated concentrations (for routine experiments 10 μg/ml) in Dulbecco's PBS (DPBS), and 40 μl were added to each well of a 96-well plate (Falcon Pro-bind; Becton-Dickinson Co., Lincon Park, NJ). The plate was incubated at 37°C for 2 h, then washed twice with DPBS, and the nonspecific binding sites were saturated by 30 min incubation with 1% BSA in serum-free medium. Cells were washed three times in serum-free medium, resuspended at the concentration of 5 $\times$ 10^5/ml in serum-free medium supplemented with 0.1% BSA, and added to the wells (100 μl/well). The assay was incubated for 30–90 min at 37°C, since preliminary experiments showed that adhesion and spreading increased up to ~40 min, but not after that time.

The wells were then gently washed four times with DPBS and the adherent cells were fixed and stained with 0.5% crystal violet in 20% methanol, rinsed, and air-dried. The percentage of spread cells compared with the total adhered cells was calculated, by counting attached and spread cells in at least three high-power fields, then the stain was eluted with an ethanol:0.1 M sodium citrate (1:1) solution and absorbance at 540 nm was measured with a Multiscan MC (Titertek; Flow Laboratories, Milano, Italy). In some experiments, to prevent protein synthesis and secretion, cells were treated with cycloheximide (20 μM) or monensin (1 μM), as described (5). Briefly, cells were pretreated with the inhibitors for 60 min at room temperature, in serum-containing medium. The inhibitors were also maintained during the assay.

Cell Motility Assay

Chemotaxis and haptotaxis were assayed using modified Boyden chambers, with 8 (LE-II) and 5 μm (BAEC) pore size polycarbonate PVP-free Nucleopore filters.

For haptotaxis, filters were coated on one side by floating them overnight at 37°C on a solution of attractant in DPBS, as described (38). The filters were washed four times in DPBS and once in distilled water and then air-dried. Chambers were assembled with serum-free medium containing 0.1% BSA. The side of the filter with the highest protein concentration faced the lower compartment.

For chemotaxis the filters were prepared by immersing them overnight in a solution of 100 μg/ml gelatin in 0.1% acetic acid and then air-dried. Chemotaxis was conducted as described (38). Briefly, different dilutions of the attractant in DME with 0.1% BSA were added to the lower compartment of the Boyden chamber, with or without inhibitors. Cells (1–2 $\times$ 10^6/ml) in serum-free medium containing 0.1% BSA were added to the upper compartment of the chamber. After 4–5 h incubation at 37°C filters were stained with Diff Quick (Merz-Dade, Düdingen, Switzerland), and the migrated cells in 10 high-power fields were counted.

Mitogenesis and Proliferation Assay

For the mitogenesis assay, LE-II cells were seeded 10^4/well in 0.1 ml of medium supplemented with 10% FCS in the inner wells of a 96-well tissue culture plate. After 3 d, the supernatant was replaced with 0.1 ml medium containing 0.5% FCS. Cells were starved for 48 h and then serum-free medium containing the samples to be tested was put in the wells; 20 h later [^{3}H]TdR (1 μCi/well) was added. After 3 h exposure to thymidine, cells were washed three times, and the radioactivity was measured in a scintillation counter after precipitation with TCA. In some cases the wells were coated with TSP before addition of the cells, following the same procedures as for the adhesion assay.

The cell proliferation assay was performed essentially as described by Schreiber et al. (36). Briefly, 10^3 BAEC or LE-II cells were plated in each well of a 96-well cluster dish, in serum containing medium. After 24 h the supernatant was replaced with serum-free medium containing the samples to be tested. After 3 d of incubation, the cells were washed and processed as described for the adhesion assay.

Results

TSP-induced Adhesion and Spreading of Endothelial Cells

Murine lung capillary (LE-II) and bovine aortic (BAEC) endothelial cells adhered and spread on TSP-coated plastic (Figs. 1 and 2). Adhesion and spreading of the two cell types were detectable when the plastic was coated with a solution of TSP at the concentration of 5 μg/ml (10 nM) or higher. A representative experiment showing the adhesion and spreading of LE-II cells to increasing concentrations of TSP is shown in Fig. 1. Compared with FN, TSP induced a similar degree of cell attachment (not shown). Spreading was reproducible, up to 84 and 90% for BAEC and LE-II cells, respectively, at a TSP concentration of 50 μg/ml (Fig. 2, *b* and *e*). However, the degree of spreading was usually less than on a FN substrate.

We next investigated the role of protein synthesis and secretion in TSP-induced adhesion and spreading of endothelial cells. Treatment of BAEC with cycloheximide (20 μM) and monensin (1 μM) resulted in a significant reduction of TSP-induced cell attachment (56.3% inhibition for cycloheximide and 54.4% for monensin), whereas spreading was not affected (0 and 3% inhibition compared with untreated cells). LE-II spreading and, at a less extent, attachment were partially affected by treatment with cycloheximide (22.3% inhibition of spreading and 13.6% inhibition of attachment) and monensin (34.1 and 11.5% inhibition of spreading and attachment, respectively).

The inhibitory effects of the TSP-binding molecules heparin and fucoidan were tested to identify the molecular domain responsible for adhesion. Both molecules bind to the amino-terminal small globular domain of the TSP, and have

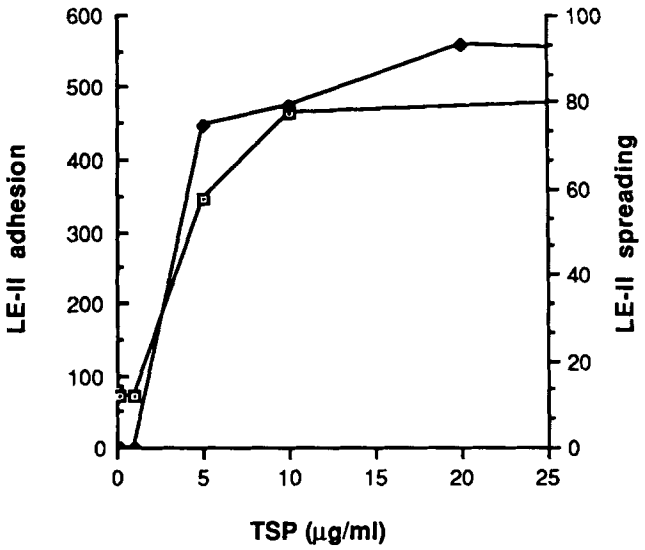

Figure 1. TSP-induced adhesion (□) and spreading (♦) of LE-II cells. Solutions at different concentrations of TSP were used to coat the plastic surface. Adhesion is expressed as absorbance ($\times 10^3$), and spreading is expressed as the percentage of spread cells, evaluated in three high-power fields. Each point is the mean of at least three values.

been shown to inhibit the attachment and spreading of human melanoma cells to TSP (35). The inhibitory effect of heparin on cell adhesion was not significant, but fucoidan, at a concentration of 100 μg/ml, partially affected the adhesion of LE-II and BAEC cells to TSP (Table I). Both heparin and fucoidan significantly inhibited endothelial cell spreading (Table I and Fig. 2), in agreement with the described role of the amino-terminal heparin-binding domain of TSP in cell spreading (35).

Antibodies against potential TSP receptors were tested for their ability to inhibit endothelial cell adhesion. An integrin receptor containing the β3 chain has been shown to interact with TSP (20, 41). An antiserum directed against the integrin receptor IIbIIIa (containing the β3 chain) partially inhibited the adhesion of LE-II to TSP, and strongly inhibited their spreading on TSP-coated substrata (Table I). In contrast, antibodies against the fibronectin receptor (α5β1) had little inhibitory effect on the adhesion or spreading of LE-II cells (Table I), at a dose that inhibited adhesion on fibronectin (not shown). These antibodies had less effect on BAEC cells: none affected adhesion, and the inhibitory effect on spreading was comparable for the anti-IIbIIIa and anti-fibronectin receptor antibody. A control nonimmune serum, and a rabbit antiserum against laminin, tested at the same dilutions, had no significant effect on attachment and spreading of LE-II and BAEC cells on TSP (not shown).

We next investigated the role of the GRGDS peptide on TSP-mediated endothelial cell adhesion and spreading. In both cell lines, the GRGDS peptide significantly inhibited cell spreading, and partially affected cell attachment (Table I). The control GRGES peptide had almost no effect in this assay (Table I).

Chemotactic Response to TSP

Thrombospondin stimulated the motility response in both endothelial cell lines. LE-II cells migrated in response to a gradient of soluble TSP (chemotaxis) in a concentration-dependent manner, reaching a plateau at 150 μg/ml (300 nM) (Fig. 3). LE-II motility response was time-dependent, reaching maximal motility after 4 h (not shown). TSP, at concentrations of 5–50 μg/ml, induced endothelial cell haptotactic migration (i.e., a motility response induced by a gradient of substrate-bound TSP) in a dose-dependent manner (not shown).

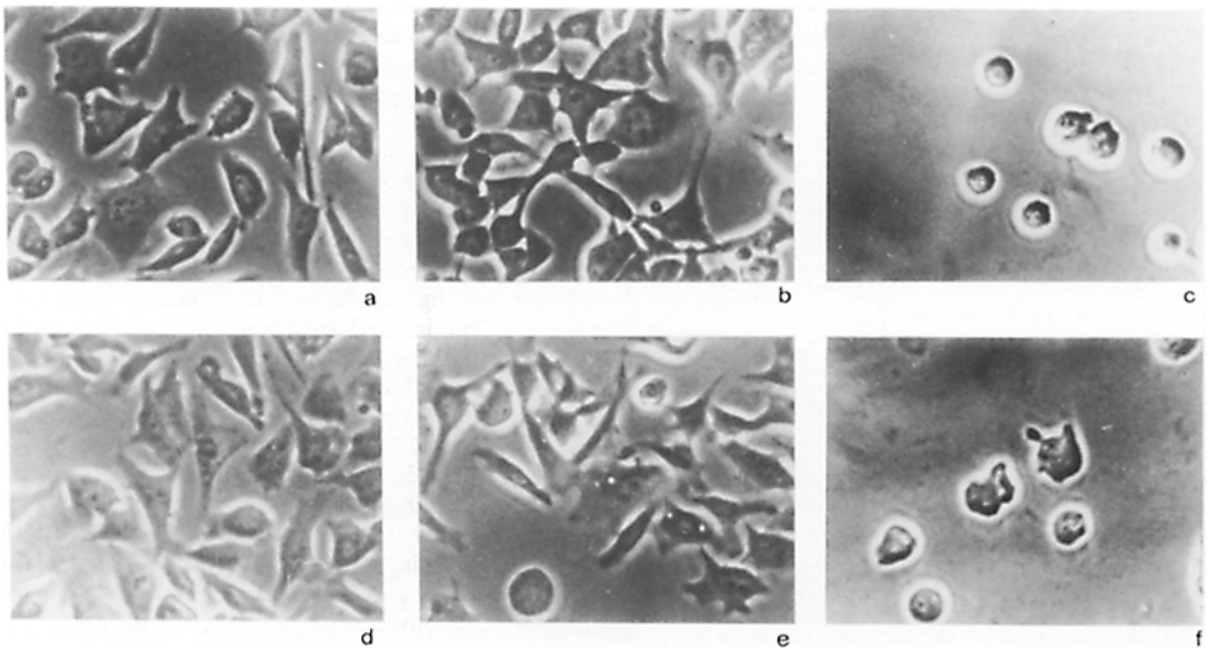

Figure 2. Adhesion and spreading of LE-II (*a*, *b*, and *c*) and BAEC (*d*, *e*, and *f*) to fibronectin (*a* and *d*), thrombospondin (*b* and *e*) and thrombospondin in the presence of 100 μg/ml fucoidan (*c* and *f*). Substrata were prepared as described in Materials and Methods, using a solution of 10 μg/ml of fibronectin or 20 μg/ml TSP. Cells were fixed with 2.5% glutaraldehyde in DPBS. 400×.

Table I. TSP-induced Endothelial Cell Attachment and Spreading: Effect of Inhibitors

Inhibitor	Dose	LE-II		BAEC	
		Adhesion	Spreading	Adhesion	Spreading
	μg/ml				
Heparin	200	81.5 ± 6.5	49.8 ± 7.6*	107.7 ± 6.0	72.7 ± 3.5‡
	100	93.5 ± 4.6	68.0 ± 9.1*	84.2 ± 21.7	71.3 ± 7.2*
	10	85.4 ± 5.2	83.0 ± 9.0	89.6 ± 14.8	83.0 ± 3.0*
	1	112.6 ± 7.2	91.5 ± 3.5	95.6 ± 6.5	88.0 ± 1.0
Fucoidan	100	71.8 ± 2.2*	45.7 ± 9.6‡	60.4 ± 5.5*	38.0 ± 10.7‡
	10	97.8 ± 20.9	62.5 ± 4.5*	88.4 ± 4.1	75.0 ± 2.0‡
	1	104.4 ± 8.8	87.0 ± 14.0	94.7 ± 4.7	85.0 ± 4.0*
Anti-IIbIIIa	1:40	75.2 ± 12.3	36.7 ± 14.1*	99.5 ± 1.5	76.0 ± 12.0
Anti-FN Rec	30	88.3 ± 0.7	86.0 ± 5.0	100.7 ± 3.5	64.0 ± 4.0
GRGDS	100	71.5 ± 5.8*	45.4 ± 5.0‡	63.0 ± 3.9‡	4.1 ± 2.6‡
	10	81.2 ± 6.1	58.7 ± 5.5‡	62.8 ± 1.9‡	70.0 ± 5.7*
	1	84.7 ± 3.6	96.8 ± 5.5	68.9 ± 1.7‡	95.4 ± 7.0
GRGES	100	103.1 ± 3.6	85.5 ± 5.9*	87.5 ± 0.7	105.2 ± 3.1
	10	101.2 ± 2.1	97.7 ± 7.7	90.1 ± 3.9	99.5 ± 1.9
	1	100.3 ± 1.2	90.9 ± 1.7	87.6 ± 2.2	90.4 ± 12.3

Results are expressed as percentage of response obtained with TSP (20 μg/ml), without any inhibitor (control = 100). Data are mean ± SE of triplicate of one experiment representative of two to six experiments.
* $P \leq 0.05$ and ‡ $P \leq 0.005$ compared with control (*t* test).

Most of the studies to characterize the motility response to TSP were done with LE-II cells, although TSP also induced a concentration-dependent motility response in BAEC cells. In this case, the peak of activity was observed at TSP concentrations of 10–25 μg/ml, and there was less response at higher doses (not shown).

To assess the random or directional nature of TSP-induced motility, we made a checkerboard analysis. In this assay cells are exposed to different gradient conditions, obtained by varying the amounts of TSP added to the upper and lower compartment of the Boyden chamber (Fig. 4). A motility response is considered random when it is not gradient dependent; a true chemotactic response occurs only in the presence of a positive gradient of the attractant. The diagonal in the figure represents the null gradient, when the TSP concentrations on both sides of the filter are identical. This diagonal separates the conditions of positive gradient (higher doses in the lower compartment of the chamber, below the diagonal) from the negative gradient (higher doses added to the compartment with the cells, above the diagonal). We found that a positive gradient gave the strongest stimulation of migration. However, positive (*bottom left*), negative (*top right*), and null (*bottom right*) gradients all induced cell migration, indicating that TSP-induced endothelial cell motility has both directional and random components.

Effect of Heparin, Fucoidan, and A2.5 Antibody on TSP-induced Chemotaxis

Heparin, fucoidan, and mAb A2.5 bind to the amino-terminal domain of TSP, and inhibit the TSP-induced chemotac-

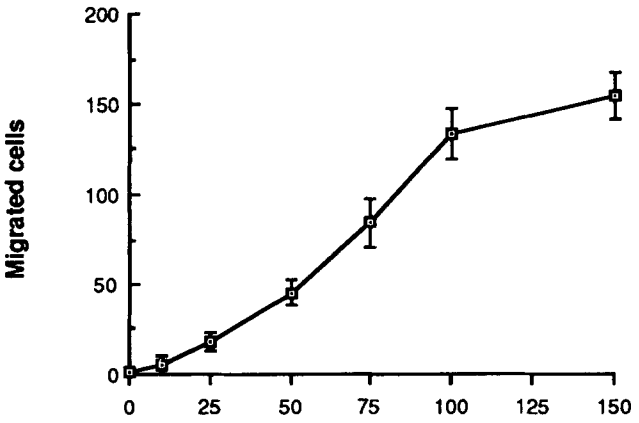

Figure 3. LE-II chemotactic response to TSP. Increasing doses of TSP were added to the lower compartment of the Boyden chamber. Data are mean ± SE of the number of migrated cells counted in 10 high-power fields.

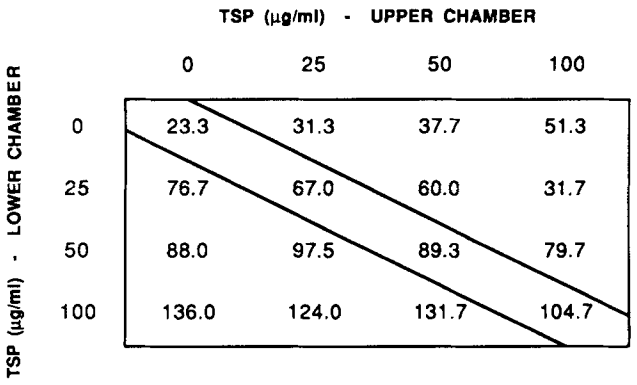

Figure 4. Checkerboard analysis of the chemotactic activity of TSP on endothelial cells. Different gradient conditions were created by adding various concentrations of soluble TSP to the upper and lower compartments of the chamber, as indicated. Motility response is expressed as the mean of triplicate values (standard errors were <15% of the mean).

tic response of human melanoma cells (38). Both heparin and fucoidan, when added to the lower compartment of the Boyden chamber together with TSP, strongly inhibited the chemotactic response of LE-II cells to TSP (Table II). Fucoidan was a more potent inhibitor of TSP-induced chemotaxis of LE-II cells (81% inhibition at 100 μg/ml) than heparin (56% at the same dose). In both cases the inhibitory effect was dose dependent, and concentrations as low as 1 μg/ml (fucoidan) and 10 μg/ml (heparin) significantly reduced the motility response. Similarly, the mAb A2.5, directed against the amino-terminal heparin binding domain of TSP, strongly inhibited (73% at 50 μg/ml) the chemotactic response of LE-II cells to TSP (Table II). This indicates that in endothelial cells, as in melanoma cells, the amino-terminal domain of TSP is responsible for the induction of chemotaxis.

Role of Integrin Receptors in TSP-induced Chemotaxis

The antibody against the β3 integrin receptor IIbIIIa significantly inhibited the chemotactic response of LE-II cells to TSP (Table II), whereas no effect was seen when a rabbit nonimmune serum or an antiserum against laminin were used as control (not shown). Therefore, a β3 integrin receptor appears to mediate TSP-induced chemotaxis. Antibodies against the FN receptor did not inhibit cell migration (Table II) at a dose that caused 50% inhibition of fibronectin-induced LE-II chemotaxis (not shown). The same results were obtained when chemotaxis was performed using a laminin-coated filter, thus showing that the interaction of antibodies with the gelatin substrate was not responsible for the inhibition. It was not possible to establish a role for the GRGDS peptide in TSP-induced chemotaxis, since the addition of the peptide in the assay prevented endothelial cell attachment to the filter, even in the absence of a chemoattractant or using filters coated with different substances.

Modulation of bFGF-induced Chemotaxis by TSP

Basic FGF is known to induce a chemotactic response in endothelial cells (32). We investigated whether TSP could modulate the chemotactic response of LE-II cells to bFGF. Basic FGF (10 ng/ml), in the lower compartment of the Boyden chamber, induced LE-II cell motility (2.2 times the background). The addition of high concentrations of TSP (100 μg/ml) to the constant amount of bFGF (10 ng/ml) further stimulated LE-II motility that was higher (260.3 migrated cells) than that induced by either TSP (134.7 cells) or bFGF (84.7 cells) alone (Fig. 5). When TSP was used at nonchemotactic concentrations (0.1 and 0.01 μg/ml) we found inhibition of bFGF-induced cell migration (Fig. 5). This was not complete (15–61% inhibition) but was reproducible in six experiments.

Inhibition of Growth Response by Soluble TSP

To test the effect of TSP on LE-II cell growth, two assays were used. In the mitogenic assay LE-II cells were grown to confluency, TSP with or without growth factors was added to quiescent cells, and incorporation of [^{3}H]thymidine was evaluated. In the proliferation assay, cells were counted after three days of growth from nonconfluent cultures. We were not able to stimulate [^{3}H]-thymidine incorporation in confluent and quiescent BAEC (mitogenic assay); BAEC growth could only be measured using the proliferation assay.

Table II. TSP-induced LE-II Chemotaxis: Effect of Inhibitors

Inhibitor	Concentration	Percentage of response* (±SE)
	μg/ml	
Heparin	100	31.7 ± 6.1‡
	10	45.0 ± 8.2‡
	1	70.2 ± 11.2‡
Fucoidan	100	20.8 ± 3.9‡
	10	30.5 ± 4.5‡
	1	65.8 ± 14.0‡
A2.5	50	16.8 ± 3.2‡
	10	88.3 ± 11.8
	1	93.1 ± 5.9
Anti IIbIIIa	1:40	4.3 ± 3.2‡
Anti FN Rec.	30	116.6 ± 28.1

* Results are expressed as a percentage of the migration induced by TSP (50 μg/ml) without any inhibitor.
Data represent mean ± standard error of 2 to 5 different experiments.
Random motility ranged from 0 to 22% of maximal response.
‡ $P \leq 0.001$ (*t* test).

The presence of TSP alone had no effect on the LE-II mitogenesis. However, the addition of soluble TSP to either FCS or the angiogenic factor bFGF, resulted in dramatic inhibition of the mitogenic response of LE-II cells to the two growth factors. The inhibitory effect of soluble TSP was dose dependent, reaching maximal activity at 100 μg/ml (Fig. 6 *a*), whereas laminin and fibronectin did not affect the mitogenic response at equimolar concentrations. This inhibition was always observed in 10 different experiments, and ranged

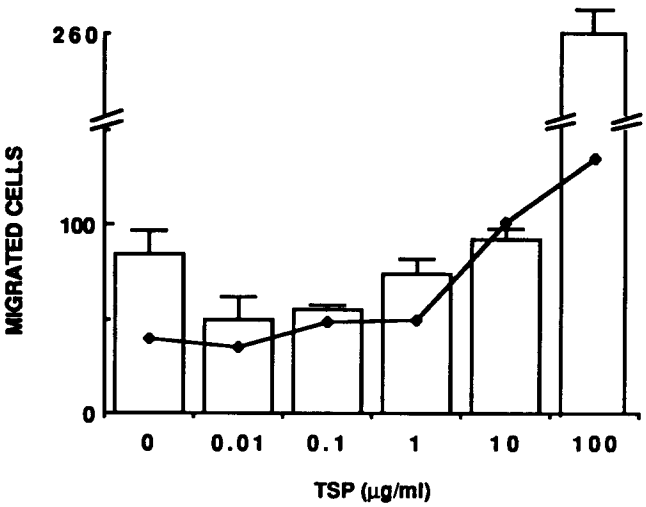

Figure 5. Effect of TSP on bFGF-induced chemotaxis of LE-II cells. Different amounts of TSP were added to a constant amount of bFGF in the lower compartment of the chamber. Columns represent the chemotactic response induced by 10 ng/ml bFGF alone (*left column*) or in the presence of increasing concentrations of soluble TSP. The line indicates the motility response induced by each amount of TSP alone, without bFGF. Data are expressed as number of migrated cells in 10 high-power fields.

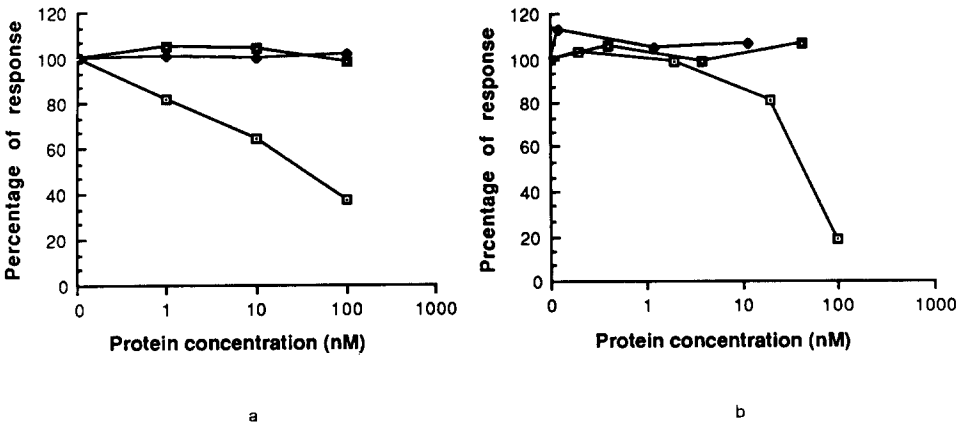

Figure 6. Effect of increasing amounts of TSP, LM, and FN on FCS- and bFGF-induced mitogenesis of LE-II cells. Different concentrations of TSP (⊡), LM (□), and FN (♦) were added to FCS (1%, *a*) or bFGF (10 ng/ml, *b*). Data are expressed as percentage of the response given by FCS or bFGF alone.

from 25 to 77% reduction of the response obtained with 1% serum alone.

TSP also inhibited the mitogenic effect of bFGF (10 ng/ml) on LE-II cells in a dose-dependent manner (Fig. 6 *b*), the reduction ranging from 67 to 84% in three different experiments. The inhibitory effect of TSP on serum-induced mitogenesis was also observed at higher concentrations of serum (Fig. 7). To preclude the possibility that this inhibition by TSP could be the result of cell detachment, parallel experiments were done as above, except that the cells were washed twice with PBS, stained, and counted as for the adhesion assay. The various treatment groups gave similar results, suggesting that soluble TSP does not induce cell detachment by competing for adhesive receptors on the cell surface. The addition to the assay of either an antiserum against TSP or against the IIbIIIa integrin receptor, resulted in cell detachment from the surface. Thus, it was not possible to measure the mitogenic response accurately.

TSP-inhibition of FCS- or bFGF-induced mitogenesis resulted in a true inhibition of cell growth, as assessed by the proliferation assay, done on both LE-II and BAEC cells. The cell count after three days of culture was significantly lower (33 and 61% inhibition, respectively) when soluble TSP (50 μg/ml) was added to the serum (1% for LE-II and 5% for BAEC cells) in the culture medium. In addition, TSP inhibited the proliferative response of both cell lines to bFGF (not shown).

We found that the GRGDS peptide not only failed to prevent TSP-mediated inhibition of endothelial cell proliferation, but the peptide itself had an inhibitory effect on cell growth, probably by interfering with adhesive mechanism of the cells.

The above results indicate an inhibitory effect of soluble TSP on endothelial cell growth. In a limited series of experiments, however, we found that endothelial cell mitogenic and proliferative behavior was not significantly affected by substrate-bound TSP (not shown).

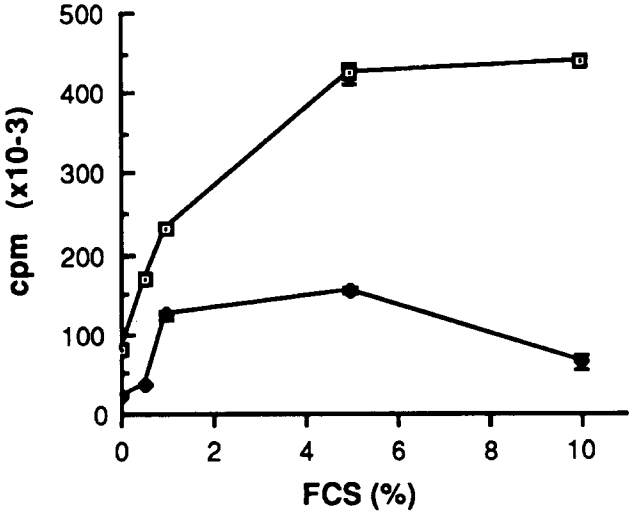

Figure 7. Effect of soluble TSP on LE-II mitogenic response to FCS. Different amounts of serum were added to quiescent LE-II cells, without (⊡) or with (♦) soluble TSP (50 μg/ml). After 20 h, the mitogenic response was evaluated by [^{3}H]-thymidine incorporation.

Discussion

Endothelial cell attachment, spreading, directed migration, and proliferation are basic steps in processes such as angiogenesis, wound healing, and tissue remodeling. This study reports that TSP can induce adhesion, spreading, and motility of murine lung capillary and bovine aortic endothelial cells. In addition, TSP inhibits the mitogenic and proliferative response of endothelial cells to growth and angiogenic factors.

The adhesive properties of TSP for several cell types have been described (9, 14, 20, 34, 35, 42, 43). However, different studies have produced conflicting data on whether TSP modulates attachment and spreading of endothelial cells. Tuszynski et al. described the adhesion of endothelial cells on TSP (40). This was confirmed by Lawler et al., who reported that human umbilical vein and arterial endothelial cells adhered on TSP (20). However, these authors found that <5% of the attached cells spread on the TSP substrate (20). TSP was also reported to inhibit both adhesion and spreading of BAEC, on either a TSP-coated glass surface, or a fibronectin surface treated with TSP (17). The amino-terminal domain of TSP causes loss of focal adhesion plaques on adherent endothelial cells (27). In our assays, TSP induced both adhesion and spreading of LE-II cells and BAEC, though spreading was never as complete as on FN-coated substrate. The contrasting data could be related to different sources of endothelial cells or differences in methodology. For example, the sensitivity of the TSP receptor(s) to proteases should

be considered. Treatment of cells with trypsin (without recovery) prevents cell attachment to TSP (40).

Endothelial cell adhesion on TSP could be influenced by de novo synthesis and secretion of other adhesive molecules, (i.e., fibronectin). Our data show that for BEAC, but not for LE-II cells, endogenous proteins can be responsible for TSP-induced cell adhesion. Spreading, however, is only partially affected by inhibitors of protein synthesis and secretion. These data suggest that endogenous molecules play a minor role in TSP-induced cell adhesion and spreading.

The migration of endothelial cells induced by ECM components is known to occur in vitro in the process of wound healing, and in vivo during angiogenesis. Both LE-II cells and BAEC migrated toward a gradient of soluble TSP (chemotaxis). TSP also induced migration of these cells to a gradient of substrate-bound attractant (haptotaxis), with TSP providing both the stimulus and the substrate for cells to migrate upon. These data indicate that a gradient of either soluble TSP, as in plasma, or substrate-bound TSP, embedded in the basement membrane or tissues, could induce endothelial cell migration. The chemotactic properties of TSP for human melanoma cells have been described (38).

TSP contains several possible cell binding domains (9, 19, 35, 37, 38). In previous studies we found that the amino-terminal heparin binding domain was responsible for TSP-induced spreading and chemotactic motility of tumor cells, whereas the carboxy-terminal domain mediated tumor cell attachment and haptotactic motility, but not spreading (35, 38). In this study, we found that heparin and fucoidan inhibited spreading of endothelial cells in response to TSP. These data confirm that the heparin binding amino-terminal domain of TSP is responsible for the induction of cell spreading. In addition, the fact that heparin and fucoidan did not completely inhibit attachment indicates the presence of another cell attachment domain on TSP, as already described for tumor cells. Heparin, fucoidan, and the mAb A2.5, which all bind the amino-terminal small globular region of TSP, inhibited LE-II chemotaxis, in agreement with our previous findings on tumor cells. These data confirm that different domains of the molecule are responsible for the induction of specific functions of tumor cells, like those of endothelial cells.

This conclusion leads to the hypothesis that at least two receptors are involved in the interaction of TSP with cells. One receptor would bind to the amino-terminal end of the TSP molecule, mediating cell adhesion, spreading, and chemotactic response, and could be a heparan sulfate proteoglycan or a sulfated glycolipid, as described (14, 27, 33). Another receptor would bind to the carboxy-terminal end, and would mediate cell attachment, but not spreading, and haptotactic motility. This receptor could be the integrin receptor related to the IIbIIIa, that is reported to bind to the RGDA sequence present in the carboxy-terminal region of the molecule (20, 41).

However, the unexpected significant inhibition of adhesion, spreading, and chemotaxis by the anti-IIbIIIa antibody indicates that the integrin receptor might be involved in functions that are not mediated by the RGDA-containing region of TSP. A direct interaction between the amino-terminal region of TSP and the integrin receptor can be ruled out by the findings of Lawler et al. (20), who showed that the interaction between TSP and its integrin receptor is mediated by the RGDA sequence, thus indicating that the receptor binds to the carboxy-terminal region. Accordingly, we found that the GRGDS peptide affects TSP-mediated endothelial cell adhesion.

Another possibility is that the different TSP receptors on the cell surface are closely associated, so that binding of the Ab to the IIbIIIa results in steric hindrance of the interaction between the amino-terminal domain of TSP and its receptor. However, we have no direct indication that this association of receptors does in fact occur on the cell surface.

ECM components are known to modulate cell response to growth factors. TSP has been described as necessary for the proliferation of smooth muscle cells (22, 23). The surprising countereffect of TSP on FCS and bFGF might have very important implications. Endothelial cells proliferate during vessel formation, in embryonic development and in several pathologies, including tumor-induced angiogenesis. Several agents have been described as mitogenic for endothelial cells, and thus possibly important in angiogenesis (7). TSP is reported to be present in the blood vessel wall, in higher concentrations at the site of vessel injury. Different authors report that TSP is usually associated with undifferentiated tissues or cells. However, immunocytochemical studies of the mouse embryo by O'Shea (29) indicated that little TSP is found in the early forming vessel, and that it becomes detectable only at later stages. TSP production by cultured cells is reported to be increased by growth factors, including bFGF (6). On the basis of these data, and on our finding that TSP inhibits endothelial cell response to growth factors, one could speculate that soluble TSP acts as a regulatory agent during the angiogenic process, allowing a feed-back mechanism. At the site of the lesion, the angiogenic agent (bFGF) stimulates the production of TSP (6) which in turn limits the angiogenic effect of bFGF.

The mechanism of inhibition of growth response by TSP is not known. The possibility that soluble TSP competes with growth factors for similar receptors is supported by the fact that TSP and several growth factors (like bFGF) can bind to heparin and heparan sulfate proteoglycans (3, 8, 9, 28, 32). However, there may be some direct interaction of TSP with the growth factor molecules. The two possible mechanisms are currently under investigation.

In a preliminary experiment, we found that mAbs directed against different domains of the TSP molecule inhibited endothelial cell growth response to serum and bFGF. A similar effect has been considered a proof that cell surface TSP is required in smooth muscle cells proliferation (23). In our case, we can hypothesize that endothelial cell growth requires endothelial-derived cell surface TSP, that might be displaced by an excess of platelet-derived soluble TSP. Together with the finding that substrate-bound TSP had no significant effect on endothelial cell growth, this seems to confirm the hypothesis of Lahav et al., that TSP in different compartments (associated to cells or matrix, or soluble) could have different physiological functions (18).

In conclusion, we have shown that TSP stimulates different endothelial cell functions potentially related to angiogenesis. It also appears to modulate the activity of angiogenic factors. Further investigations are necessary to elucidate the physiological role of TSP in angiogenesis.

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