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Cell Contact–dependent Activation of α3β1 Integrin Modulates Endothelial Cell Responses to Thrombospondin-1

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Thrombospondin-1 (TSP1) can inhibit angiogenesis by interacting with endothelial cell CD36 or proteoglycan receptors. We have now identified α3β1 integrin as an additional receptor for TSP1 that modulates angiogenesis and the in vitro behavior of endothelial cells. Recognition of TSP1 and an α3β1 integrin–binding peptide from TSP1 by normal endothelial cells is induced after loss of cell–cell contact or ligation of CD98. Although confluent endothelial cells do not spread on a TSP1 substrate, α3β1 integrin mediates efficient spreading on TSP1 substrates of endothelial cells deprived of cell–cell contact or vascular endothelial cadherin signaling. Activation of this integrin is independent of proliferation, but ligation of the α3β1 integrin modulates endothelial cell proliferation. In solution, both intact TSP1 and the α3β1 integrin–binding peptide from TSP1 inhibit proliferation of sparse endothelial cell cultures independent of their CD36 expression. However, TSP1 or the same peptide immobilized on the substratum promotes their proliferation. The TSP1 peptide, when added in solution, specifically inhibits endothelial cell migration and inhibits angiogenesis in the chick chorioallantoic membrane, whereas a fragment of TSP1 containing this sequence stimulates angiogenesis. Therefore, recognition of immobilized TSP1 by α3β1 integrin may stimulate endothelial cell proliferation and angiogenesis. Peptides that inhibit this interaction are a novel class of angiogenesis inhibitors.

INTRODUCTION

Angiogenesis under normal and pathological conditions is regulated by both positive and negative signals received from soluble growth factors and components of the extracellular matrix (reviewed by Folkman, 1995; Polverini, 1995; Hanahan and Folkman, 1996). Thrombospondins are a family of extracellular matrix proteins that have diverse effects on cell adhesion, motility, proliferation, and survival (reviewed by Bornstein, 1992, 1995; Roberts, 1996). Two members of this family, thrombospondin-1 (TSP1) and thrombospondin-2, are inhibitors of angiogenesis (Good *et al.*, 1990; Volpert *et al.*, 1995). TSP1 inhibits growth, sprouting, and motility responses of endothelial cells in vitro (Good *et al.*, 1990; Taraboletti *et al.*, 1990; Iruela Arispe *et al.*, 1991; Canfield and Schor, 1995; Tolsma *et al.*, 1997) and, under defined conditions, induces programmed cell death in endothelial cells (Guo *et al.*, 1997b). TSP1 inhibits angiogenesis in vivo in the rat corneal pocket and chick chorioallantoic membrane (CAM) angiogenesis assays (Good *et al.*, 1990; Iruela-Arispe *et al.*, 1999). The ability of TSP1 overexpression to suppress tumor growth and neovascularization in several tumor xenograft models provides further evidence for an antiangiogenic activity of TSP1 (Dameron *et al.*, 1994; Weinstat-Saslow *et al.*, 1994; Sheibani and Frazier, 1995; Hsu *et al.*, 1996). Circulating TSP1 may also inhibit neovascularization of micrometastases in some cancers (Morelli *et al.*, 1998; Volpert *et al.*, 1998). A few studies, however, have concluded that TSP1 also has proangiogenic activities under specific conditions (BenEzra *et al.*, 1993; Nicosia and Tuszynski, 1994). Observations of increased TSP1 expression during endothelial injury and wound repair are also difficult to explain with a purely antiangiogenic activity for TSP1 (Vischer *et al.*, 1988; Munjal *et al.*, 1990; Reed *et al.*, 1995). These

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Abbreviations used: BAE, bovine aortic endothelial; CAM, chorioallantoic membrane; HDME, human dermal microvascular endothelial; HUVE, human umbilical vein endothelial; peptide 678, FQGVLQNVRFVF; peptide 686, FQGVLQAVRFVF; peptide 690, FQGVLQNVAFVF; TSP1, thrombospondin-1; VE-cadherin, vascular endothelial cadherin.

apparently contradictory reports have led to confusion about the physiological role of TSP1 as an angiogenesis regulator.

To understand the factors that control the complex responses of endothelium to TSP1, we must define the receptors and signaling pathways that mediate its actions. TSP1 interacts with several receptors on endothelial cells, including the $\alpha v\beta 3$ integrin (Lawler *et al.*, 1988), heparan sulfate proteoglycans (Vischer *et al.*, 1997), CD36 (Dawson *et al.*, 1997), the low-density lipoprotein receptor–related protein (Godyna *et al.*, 1995), and CD47 (Gao *et al.*, 1996). TSP1 peptides that bind to CD36, CD47, or heparan sulfate proteoglycans inhibit endothelial responses to growth factors in vitro and angiogenesis in vivo (Tolsma *et al.*, 1993; Vogel *et al.*, 1993; Iruela-Arispe *et al.*, 1999; Kanda *et al.*, 1999). CD36 expression is required for TSP1 to inhibit the motility response of bovine and human endothelial cells stimulated by FGF2 (Dawson *et al.*, 1997). However, proliferation of several cell types that do not express CD36, including large vessel endothelial cells, is also inhibited by TSP1 and heparin-binding peptides from TSP1 (Guo *et al.*, 1997a, 1998). Based on activities in the CAM angiogenesis assay, both of these TSP1 sequences can inhibit angiogenesis in vivo (Iruela-Arispe *et al.*, 1999). Finally, a sequence from the N-terminal domain of TSP1 can disrupt focal adhesions in endothelial cells, but the effects of this response on angiogenesis have not been defined (Murphy-Ullrich *et al.*, 1993).

TSP1 may also influence angiogenesis indirectly through activation of latent TGFβ (Schultz-Cherry and Murphy-Ullrich, 1993), which in turn can either stimulate or inhibit angiogenesis (Roberts *et al.*, 1986; Passaniti *et al.*, 1992). Based on differences in the phenotypes of *thbs1* and *tgfβ1* null mice and the inability of TGFβ antagonists to block many activities of TSP1 in vitro, activation of latent TGFβ probably mediates only a subset of endothelial responses to TSP1 (Crawford *et al.*, 1998).

Integrins are also known to regulate angiogenesis (Brooks *et al.*, 1994). Antagonists of the $\alpha v\beta 3$ integrin are potent inhibitors of neovascularization induced by growth factors or in tumors (Brooks *et al.*, 1995). Although $\alpha v\beta 3$ is a known TSP1 receptor on endothelial cells (Lawler *et al.*, 1988), its role in the modulation of angiogenesis by TSP1 has not been defined. The CD47-binding sequence in TSP1 may increase binding of $\alpha v\beta 3$ integrin ligands, including TSP1 itself (Gao *et al.*, 1996; Sipes *et al.*, 1999). However, a recombinant fragment of TSP1 containing the type 3 repeats that bind to $\alpha v\beta 3$ did not inhibit angiogenesis (Iruela-Arispe *et al.*, 1999), suggesting that the RGD sequence in TSP1 is not involved in its effects on angiogenesis.

TSP1 interacts with several $\beta 1$ integrins, including $\alpha 4\beta 1$ and $\alpha 5\beta 1$ on T lymphocytes (Yabkowitz *et al.*, 1993), $\alpha 3\beta 1$ on neurons (DeFreitas *et al.*, 1995), and $\alpha 3\beta 1$ and $\alpha 4\beta 1$ on breast carcinoma cells (Chandrasekaran *et al.*, 1999; Krutzsch *et al.*, 1999). The $\alpha 3\beta 1$ integrin is localized in cell–cell junctions of endothelial cells in a complex with some tetraspan family proteins (Yanez-Mo *et al.*, 1998). Antibodies to several components of this complex, including the $\alpha 3\beta 1$ integrin, inhibited endothelial cell motility in wound repair assays (Yanez-Mo *et al.*, 1998). Based on this observation and our recent finding that recognition of TSP1 by the $\alpha 3\beta 1$ integrin is tightly regulated in breast carcinoma cells (Chandrasekaran *et al.*, 1999) and small cell lung carcinoma cells (Guo *et al.*, 2000), we have examined the role of this integrin in the responses of endothelial cells to TSP1 and the regulation of angiogenesis. We demonstrate here that recognition of TSP1 by endothelial cell $\alpha 3\beta 1$ integrin is selectively induced after loss of cell–cell contact. These cells efficiently spread on immobilized TSP1, and this interaction stimulates endothelial cell proliferation. An $\alpha 3\beta 1$ integrin–binding peptide from the N-terminal domain of TSP1 (Krutzsch *et al.*, 1999) also modulates endothelial cell proliferation and is a potent inhibitor of endothelial wound repair in vitro and angiogenesis in vivo.

MATERIALS AND METHODS

Proteins and Peptides

TSP1 and plasma fibronectin were purified from human platelets and plasma, respectively, obtained from the National Institutes of Health Blood Bank (Bethesda, MD) (Akiyama and Yamada, 1985; Roberts *et al.*, 1994). Human vitronectin was obtained from Sigma Chemical (St. Louis, MO), and bovine type I collagen was obtained from Becton Dickinson Labware Division (Franklin Lakes, NJ). Human placental laminin was obtained from GIBCO–Life Technologies (Gaithersburg, MD). Recombinant N-terminal fragments of TSP1 were described previously (Vogel *et al.*, 1993). Synthetic peptides from TSP1 and laminin-1 that are recognized by the $\alpha 3\beta 1$ integrin and structural analogues defective in $\alpha 3\beta 1$ integrin binding were prepared as described previously (Guo *et al.*, 1992; Krutzsch *et al.*, 1999), and the peptide GRGDSP was obtained from Life Technologies–BRL (Grand Island, NY). Nonpeptide antagonist of $\alpha v\beta 3$ (SB223245) was provided by Dr. William H. Miller (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (Keenan *et al.*, 1997).

Cells and Culture

Bovine aortic endothelial (BAE) cells were isolated from fresh bovine aortae and were used at passages 3–10. BAE cells were maintained at 37°C in 5% CO_2 in DMEM (low-glucose) medium containing 10% FCS, 4 mM glutamine, 25 μg/ml ascorbic acid, and 500 U/ml each of penicillin G, potassium, and streptomycin sulfate. Media components were obtained from Biofluids (Rockville, MD). Primary human umbilical vein endothelial (HUVE) cells were provided by Dr. Derrick Grant (National Institute of Dental and Craniofacial Research, Bethesda, MD; NIDCR), and human dermal microvascular endothelial (HDME) cells were purchased from Clonetics (San Diego, CA). HUVE cells were maintained in medium 199 supplemented with 20% FCS, 10 μg/ml heparin, 80 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA), glutamine, penicillin, and streptomycin sulfate. HDME cells were maintained in MCDB medium containing glutamine, 5% FCS, 10 ng/ml EGF, 1 μg/ml hydrocortisone, 50 μg/ml ascorbic acid, 30 μg/ml heparin, 4 ng/ml FGF2, 4 ng/ml VEGF, 5 ng/ml insulin-like growth factor-1, and 50 μg/ml gentamicin.

Cell proliferation was measured with the use of the Cell-Titer colorimetric assay (Promega, Madison, WI) as described previously (Vogel *et al.*, 1993). A 100-μl volume of BAE cell suspension at 50,000 cells/ml in DMEM containing 1% FBS and supplemented with 10 ng/ml FGF2 was plated in triplicate in 96-well tissue culture plates either in the presence of peptides in solution or in wells that were precoated with 100 μl of the peptides at 4°C overnight and blocked with 1% BSA before cells were added. Cells were grown for 72 h at 37°C in a humidified incubator with 5% CO_2. HUVE cell proliferation was measured by the same protocol except that medium 199 containing 5% FCS without heparin was used. HDME cell proliferation was measured in MCDB growth medium containing 5% FCS but without heparin, VEGF, or FGF2.

Immunoprecipitation and Western Blotting

Cells grown under sparse and confluent conditions were surface labeled with a 1 mg/ml solution of sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) at 4°C for 1.5 h. After lysis in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM NaF, supplemented with 10 μg/ml each of the following protease inhibitors: antipain, pepstatin A, chymostatin, leupeptin, aprotinin, soybean trypsin inhibitor, and 1 mM PMSF), the lysate was precleared by centrifugation and the protein concentration was determined by bicinchoninic acid assay (Pierce). Equal volumes containing equal protein concentrations were immunoprecipitated with the use of the α3β1 integrin antibody P1B5 prebound to anti-mouse immunoglobulin G agarose (Sigma). The immune complexes were washed three times with Tris-buffered saline (140 mM NaCl, 20 mM Tris, pH 7.5, 1% Tween 20), eluted with sample buffer containing 10% 2-mercaptoethanol, heated, and fractionated on precast SDS gels (Bio-Rad, Richmond, CA). After transfer to polyvinylidene difluoride membrane, the proteins were detected with the use of HRP-streptavidin (Pierce) and visualized with the use of chemiluminescent substrate (Pierce).

For Western analysis, proteins on membranes were incubated with anti-VE-cadherin (Transduction Laboratories, Lexington, KY). After repeated washes, bound antibody was detected with the use of HRP-conjugated anti-mouse antibody, followed by chemiluminescent substrate.

Adhesion

TSP1 and TSP1 peptides in Dulbecco's PBS were adsorbed on bacteriological polystyrene dishes by overnight incubation at 4°C. After blocking with 1% BSA in Dulbecco's PBS, adhesion assays were performed by adding cells suspended in DMEM (BAE cells) or medium 199 (human cells) containing 1 mg/ml BSA. Cell attachment and spreading was quantified microscopically. For some experiments, cell spreading was quantified morphometrically with the use of Image-Pro Plus version 4 software (Media Cybernetics, Silver Spring, MD).

Inhibition assays were performed with the use of the following function-blocking antibodies: P1B5 (Life Technologies-BRL; α3β1), P4C2 (Life Technologies-BRL; α4β1), and mAb13 (Dr. Ken Yamada, NIDCR; antiβ1). The β1 integrin–activating antibody TS2/16 (Hemler *et al.*, 1984) and the CD98 antibody 4F2 were prepared from hybridomas obtained from the American Type Culture Collection (Rockville, MD). The function-blocking vascular endothelial (VE)-cadherin (cadherin-5) antibody, clone 75, was obtained from Transduction Laboratories, and the function-blocking PECAM-1 (CD31) antibody, HEC7, was from Endogen (Woburn, MA).

To examine the regulation of endothelial cell adhesion by cell–cell contact, cells were grown to confluent monolayers in tissue culture dishes. The confluent cells were pretreated with a function-blocking anti-VE-cadherin antibody (Hordijk *et al.*, 1999), anti-PECAM-1 antibody, histamine, or lipopolysaccharide or dissociated with the use of EDTA and replated as indicated at low density to prevent cell–cell contact.

In some experiments, the cells were treated with 5-fluorouracil to prevent proliferation. BAE cells were grown to confluence in 100-mm tissue culture dishes with complete growth medium. Twenty-four hours before the adhesion assay was performed, the confluent cells were treated with a sterile solution of 5-fluorouracil to a final concentration of 10 μg/ml. In parallel, another 100-mm dish of confluent endothelial cells was split into several 100-mm dishes such that after 24 h the cells would not contact each other. The sparse cells were treated with the same concentration of 5-fluorouracil as the confluent cells. Appropriate controls were treated in the same manner without 5-fluorouracil. After incubation at 37°C for 24 h, the cells were harvested by dissociation with EDTA and used in the adhesion assay. Complete inhibition of DNA synthesis was verified by [^{3}H]thymidine incorporation.

Scratch Wound Repair

The in vitro wound-healing assay used was a slight modification of that described by Joyce *et al.* (1989). A confluent monolayer of BAE cells pretreated with 10 μg/ml 5-fluorouracil for 24 h was used in this assay. A straight wound ~2.0 mm wide was made in the monolayers with the use of the flat edge of a sterile cell scraper (Costar 3010, Corning, NY), and the cells were allowed to migrate back into the wound site in the presence of TSP1 peptides. Mitosis of the BAE cells in the monolayers was inhibited by the addition of 5-fluorouracil, so that the rate of wound closure was due solely to the migration of cells into the wound sites. The distances between the wound margins were measured as soon as the wound was made and 24 h later with the use of a grid incorporated into the eyepiece of the microscope. All data represent the results obtained from three independent scratch wounds for each peptide tested.

CAM Angiogenesis Assay

Fertilized Leghorn chicken eggs were obtained from Ramona Duck Farm (Westminster, CA). At d 3 of development, the embryos were placed on 100-mm Petri dishes. Assays were performed as described previously (Iruela-Arispe *et al.*, 1999). Briefly, vitrogen gels containing growth factors FGF-2 (50 ng/gel) and VEGF (250 ng/gel) were allowed to polymerize in the presence or absence of TSP1 peptides. Peptides were filtered on Centricon P100 (Amicon, Inc, Beverly, MA) before their analysis on the CAM assays to eliminate traces of endotoxin. Pellets were applied to the outer one-third of the CAM, and the assay was performed for 24 h. Detection of capillary growth was done by injection of FITC-dextran in the bloodstream and observation of the pellets under a fluorescent inverted microscope. Positive controls (growth factors and vehicle) as well as negative controls (vehicle alone) were placed in the same CAM and used as reference of 100% stimulation or baseline inhibition (0%), and the response to the peptides was determined according to these internal controls. Assays were performed in duplicate in each CAM and in four independent CAMs (total of eight pellets). Statistical evaluation of the data was performed to determine whether groups differed significantly from random by analysis of contingency with Yates' correction.

RESULTS

α3β1 Integrin Is a TSP1 Receptor on Endothelial Cells

Based on previous publications, αvβ3 is regarded as the major integrin receptor for TSP1 on endothelial cells (Lawler *et al.*, 1988; Gupta *et al.*, 1999). However, an α3β1 integrin–binding sequence from residues 190–201 of TSP1 (peptide 678 [FQGVLQNVRFVF]) (Krutzsch *et al.*, 1999) also promoted endothelial cell adhesion (Figure 1A). Endothelial cells attached specifically on immobilized TSP1 peptide 678 but not on the control peptide 690 (FQGVLQNVAFVF), in which the essential Arg residue was substituted with an Ala residue. Two related peptides with amino acid substitutions that diminished their activity for mediating α3β1-dependent adhesion of breast carcinoma cells (Krutzsch *et al.*, 1999) only weakly supported endothelial cell adhesion (Figure 1A). All of the peptides had similar capacities for adsorption on the polystyrene substrate used for these assays (2.5–3.8 pmol/mm^2), so the differences in activities of these peptides did not result from differences in their adsorption.

Recognition of TSP1 by the α3β1 Integrin Is Regulated by Cell–Cell Contact

Although some investigators have reported that TSP1 promotes spreading of endothelial cells (Taraboletti *et al.*, 1990;

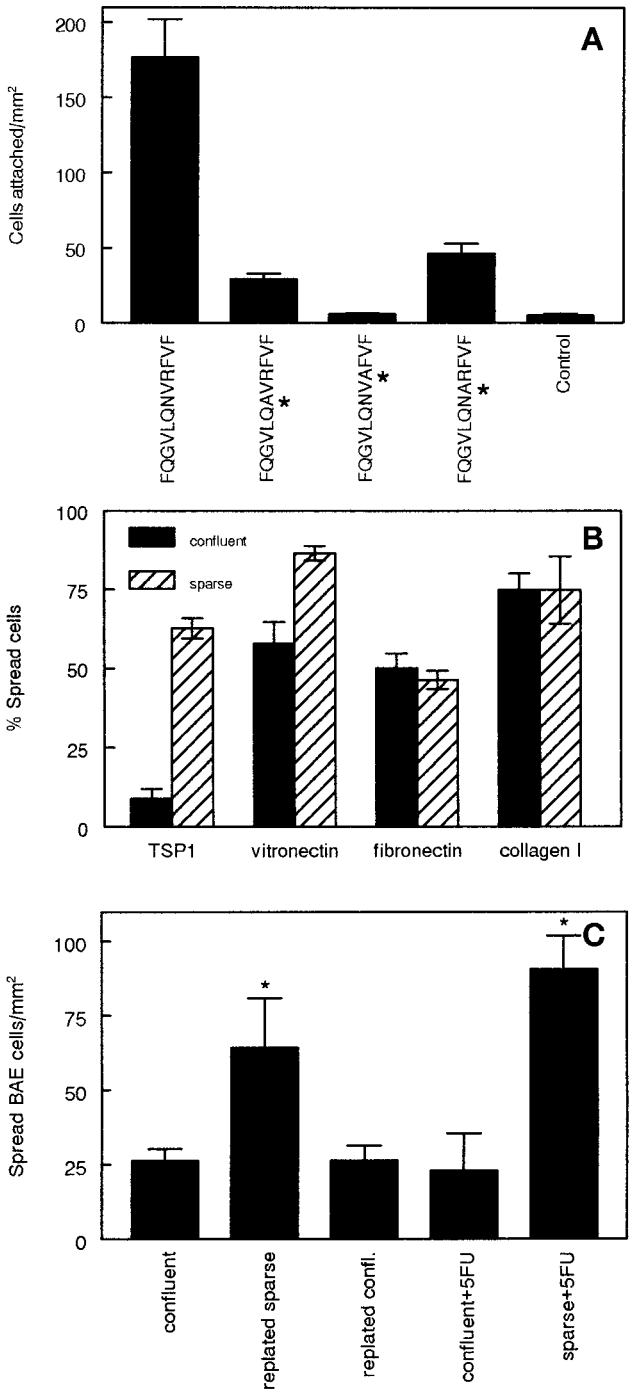

Figure 1. Spreading of BAE cells on TSP1 is regulated by confluence but not by proliferation. (A) Adhesion of endothelial cells on an $\alpha 3\beta 1$ integrin–binding peptide from TSP1. TSP1 peptide 678 (FQGVLQNVRFVF) or analogues of this peptide with the indicated Ala substitutions (*) were adsorbed on bacteriological polystyrene substrates at 10 μM in PBS. Direct adhesion of BAE cells to the adsorbed peptides or uncoated substrate (control) is presented as means ± SD (n = 3). (B) Loss of cell–cell contact stimulates endothelial cell spreading on TSP1. Two flasks of BAE cells were grown to confluence. One flask was harvested and replated in fresh medium at 25% confluence. Fresh medium was added at the same time to the second flask. After 16 h, cells from both flasks were dissociated with the use of EDTA, and adhesion was measured on substrates coated with 40 μg/ml TSP1, 10 μg/ml vitronectin, 20 μg/ml plasma fibronectin, or 5 μg/ml type I collagen. The percent spread of cells from confluent (closed bars) or sparse cultures (striped bars) after 60 min is presented as means ± SD (n = 3) for a representative experiment. (C) Cell contact–dependent regulation of $\alpha 3\beta 1$ integrin is independent of proliferation. Confluent BAE cell cultures and duplicate cultures treated with 5-fluorouracil were fed (confluent and confluent+5FU), dissociated and replated at low density in fresh medium 24 h before use (replated sparse and sparse+5FU), or dissociated and replated at their original density in fresh medium for 24 h (replated confl.). After 24 h, the cells were dissociated and tested for spreading on TSP1. Results are presented as means ± SD (n = 3). Significance was assessed with the use of a two-tailed *t* test, and values of $p < 0.05$ compared with the confluent control are indicated by asterisks above the bars.

Morandi *et al.*, 1993), others have concluded that TSP1 cannot promote endothelial cell spreading and disrupts spreading of endothelial cells attached on other matrix proteins (Lahav, 1988; Lawler *et al.*, 1988; Murphy-Ullrich and Höök, 1989; Chen *et al.*, 1996). In agreement with the latter reports, BAE cells harvested from a confluent cobblestone did not spread on TSP1 (Figures 1B and 2, a and g). However, when a duplicate culture of the same cells was replated at low density to minimize cell–cell contact and harvested at the same time after feeding, they did spread on TSP1 (Figures 1B and 2, c and g). Up-regulation of spreading on TSP1 after loss of cell–cell contact was highly significant ($p < 0.0001$) and specific for TSP1, because spreading on fibronectin and collagen were not induced under the same conditions (Figures 1B and 2, b and d). Sparse cells also displayed a significant increase in spreading on vitronectin ($p = 0.001$), although ~60% of the cells harvested from a confluent monolayer also spread on vitronectin, compared with <10% on TSP1 (Figure 1B).

Density-dependent spreading on intact TSP1 was inhibited by the $\alpha 3\beta 1$ integrin–binding peptide 678 added in solution but was not significantly inhibited by the control peptide 690 (Figure 2e and our unpublished results). Inhibition by the active peptide was specific for endothelial cell spreading on TSP1, because peptide 678 did not inhibit spreading on fibronectin (Figure 2f).

The increase in spreading observed in the sparse culture was not observed in a parallel culture replated at confluent density (Figure 1C). To determine whether this response was triggered by cell contact signals or proliferation induced by loss of cell–cell contact, BAE cells were pretreated with 5-fluorouracil to block proliferation. Treatment with 5-fluorouracil had no effect on the spreading on TSP1 of cells harvested at confluence ($p > 0.6$), and the stimulation of spreading induced by replating without cell–cell contact was not inhibited by 5-fluorouracil (Figure 1C). Therefore, activation of $\alpha 3\beta 1$ integrin after loss of cell–cell contact is independent of proliferation.

Similar density dependence for spreading on TSP1 and the TSP1 peptide 678 was observed with microvascular and large vessel human endothelial cells (Figure 3). HUVE cells harvested from a confluent monolayer spread less on immobilized TSP1 than those from a duplicate sparse culture

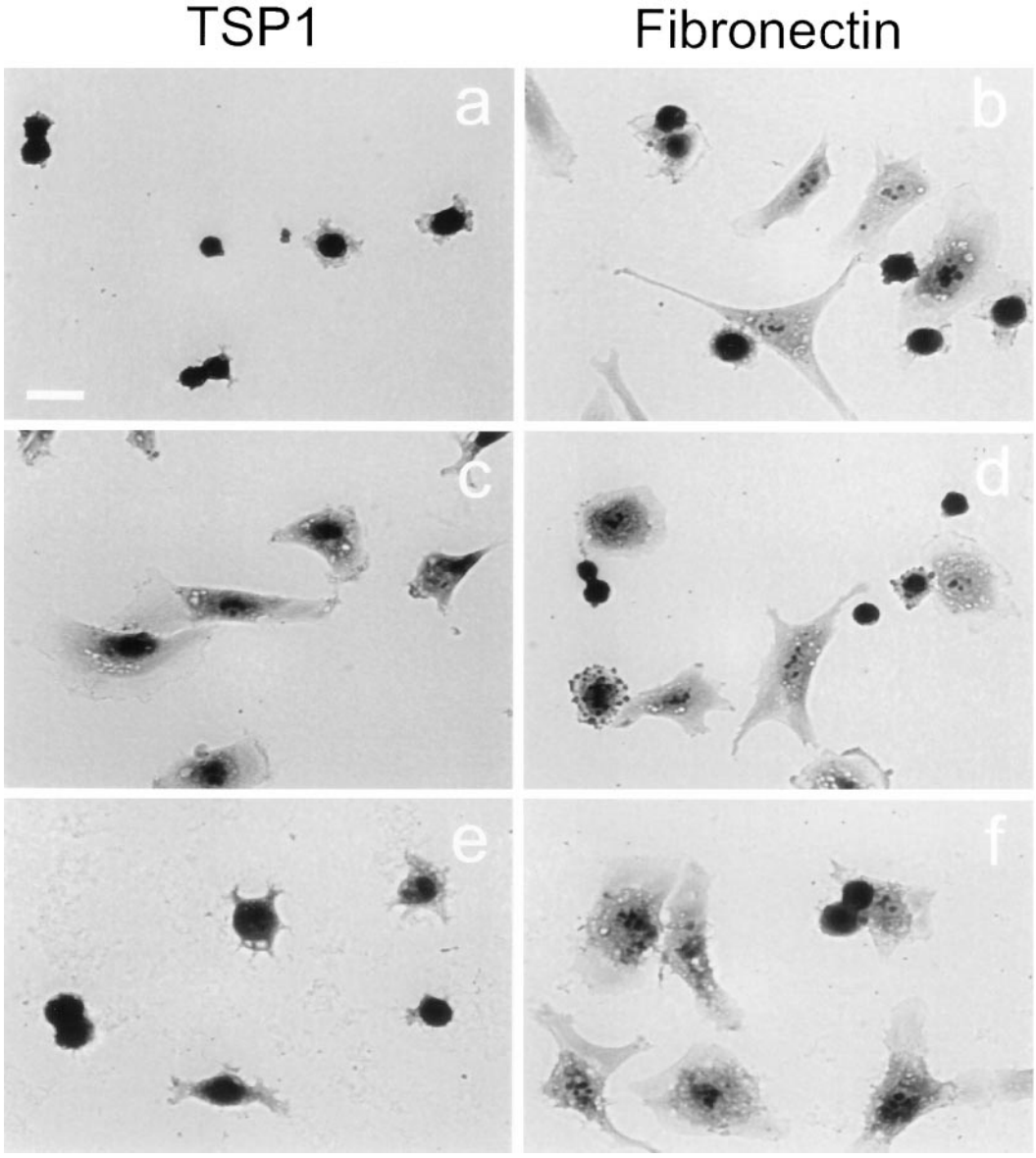

Figure 2. Spreading on TSP1 induced by loss of cell–cell contact is inhibited by the α3β1 integrin–binding peptide from TSP1. BAE cells dissociated with the use of EDTA from confluent (a and b) or 16-h sparse cultures (c–f) were incubated for 60 min on substrates coated with 40 μg/ml TSP1 (a, c, and e) or 20 μg/ml fibronectin (b, d, and f). Adhesion was performed in the presence of 30 μM TSP1 peptide 678 (e and f). Cells were fixed with 1% glutaraldehyde and stained with the use of Diff-quik (Dade, Miami, FL). Bar in a, 25 μm. (g) Spread of cell areas for 100 cells each from the experiments presented in a (○) and c (●) were quantified from digitized images with the use of Image-Pro Plus software.

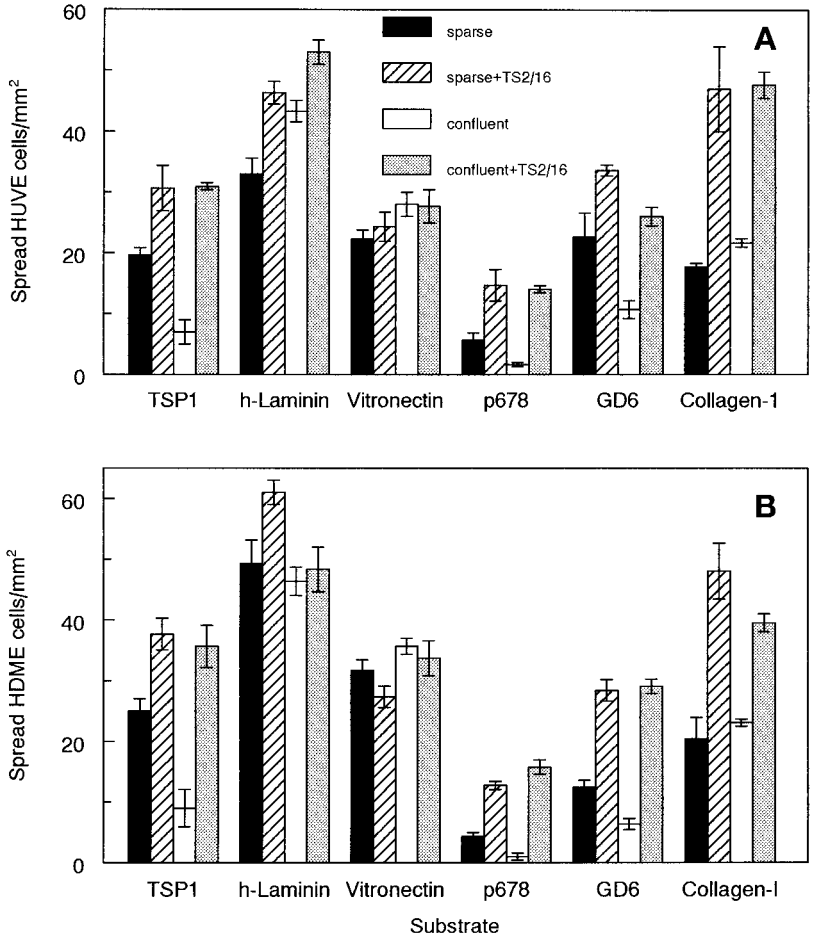

Figure 3. Cell contact specifically regulates spreading of human endothelial cells on $\alpha 3\beta 1$ integrin ligands. (A) HUVE cells harvested from sparse (closed and striped bars) or confluent cultures as in Figure 1 (open and gray bars) were plated on substrates coated with 10 μg/ml TSP1, 10 μg/ml human placental laminin, 5 μg/ml vitronectin, 5 μM TSP1 peptide 678, 5 μM laminin-1 peptide GD6 (KQNCLSSRASFRGCVRNLRLSR), or 5 μg/ml type I collagen. The cells were suspended in medium 199/0.1% BSA (closed and open bars) or the same medium containing 5 μg/ml $\beta 1$ integrin–activating antibody TS2/16 (striped and gray bars). The number of spread cells was determined at 60 min and is presented as means ± SD (n = 3). (B) HDME cells were treated as in A.

(Figure 3A; $p < 0.001$). For both umbilical vein and microvascular cells, addition of the $\beta 1$ integrin–activating antibody TS2/16 increased spreading on TSP1 or the $\alpha 3\beta 1$-binding sequence from TSP1 to the same extent (Figure 3), suggesting that the activation state of $\alpha 3\beta 1$ rather than its level of expression was induced by loss of cell–cell contact. When HUVE cells were replated at their original density for 24 h, spreading on TSP1 ($p = 0.2$) or the peptide ($p = 0.3$) was not induced significantly, replicating the behavior of bovine endothelial cells shown in Figure 1.

Regulation of integrin activation by cell–cell contact was specific for $\alpha 3\beta 1$ in the human endothelial cells. In contrast to the BAE cells, sparse cultures of both HUVE and HDME cells spread slightly less on the $\alpha v\beta 3$ ligand vitronectin than did cells from confluent cultures (Figure 3). The $\alpha 2\beta 1$ integrin was also not activated by loss of cell–cell contact, as assessed by spreading on a type I collagen substrate. The dependence on $\alpha 2\beta 1$ for adhesion on type I collagen was verified with the use of an $\alpha 2\beta 1$-blocking antibody (our unpublished results). However, in both sparse and confluent cultures, the $\alpha 2\beta 1$ integrin was only partially active based on stimulation of spreading on collagen by the activating antibody TS2/16. Although human placental laminin was reported to be an $\alpha 3\beta 1$ integrin ligand (Delwel *et al.*, 1994), cells from sparse endothelial cultures showed similar or decreased spreading on placental laminin compared with cells from confluent cultures, and their spreading was only slightly stimulated by TS2/16 (Figure 3). Adhesion of the endothelial cells on laminin may be mediated primarily by $\alpha 6\beta 1$ integrin (Defilippi *et al.*, 1992), which could mask the regulation of $\alpha 3\beta 1$ binding. To detect whether $\alpha 3\beta 1$-dependent laminin recognition was regulated, we tested an $\alpha 3\beta 1$ integrin–binding sequence from laminin-1, peptide GD6 (Gehlsen *et al.*, 1992). Sparse endothelial cultures showed the expected increase in spreading on the laminin-1 peptide and comparable activation by the antibody TS2/16 in both umbilical vein and microvascular cells (Figure 3). Therefore, regulation of integrin activation under these conditions is specific for $\alpha 3\beta 1$ and can be detected with the use of $\alpha 3\beta 1$-binding sequences from both TSP1 and laminin-1.

Relative Roles of $\alpha v\beta 3$ and $\alpha 3\beta 1$ Integrins and CD36 in Endothelial Cell Adhesion on TSP1

The increased spreading of sparse BAE cells on TSP1 is mediated at least in part by $\alpha 3\beta 1$ integrin, because a TSP1 peptide that binds to this integrin (Krutzsch *et al.*, 1999) inhibited spreading on TSP1 by 55% but did not inhibit spreading on fibronectin or vitronectin substrates (Figure 4A). The $\alpha v\beta 3$ integrin also plays some role in BAE cell

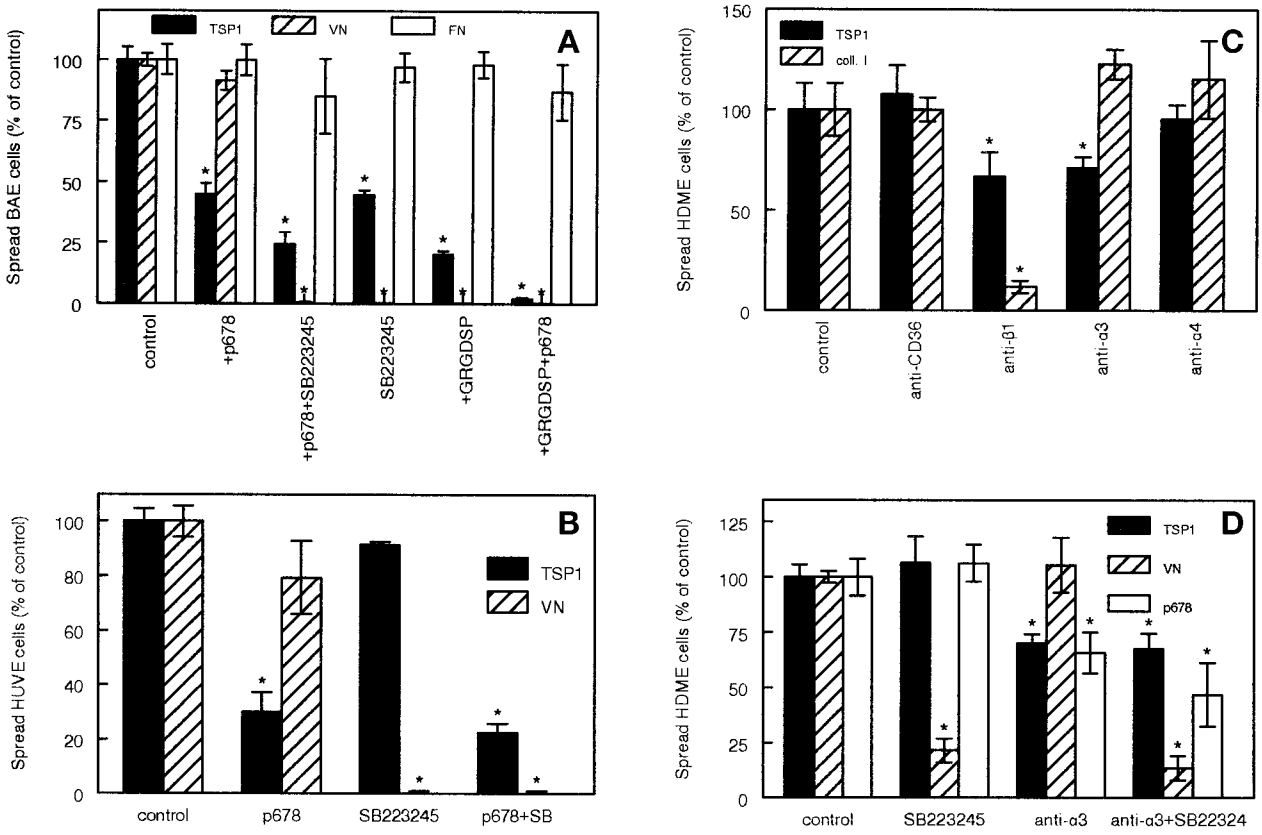

Figure 4. αvβ3 integrin contributes to spreading of bovine but not human endothelial cells on TSP-1. (A) Spreading of BAE cells from sparse cultures on TSP1 (closed bars), vitronectin (striped bars), or plasma fibronectin (open bars) was measured in the presence of 30 μM TSP1 peptide 678, 1 μM αvβ3 integrin antagonist SB223245, 300 μM integrin antagonist peptide GRGDSP, or the indicated combinations. Results are expressed as percent of the response for untreated cells (mean ± SD, n = 3). Inhibition with $p < 0.05$ relative to the control is indicated by asterisks. (B) HUVE cell spreading on substrates coated with TSP1 (closed bars) or vitronectin (striped bars) was determined in the presence of 20 μM peptide 678, 1 μM αvβ3 antagonist SB223245, or 20 μM peptide 678 plus 1 μM SB223245. Spreading is presented as a percent of the respective controls without inhibitors (31 cells/mm^2 for TSP1 and 10 cells/mm^2 for vitronectin). (C) Inhibition of HDME cell spreading on TSP1 (closed bars) or type I collagen (striped bars) was determined in the presence of the indicated function-blocking antibodies at 5 μg/ml: anti-CD36 (OKM5), anti-integrin β1 (mAb13), anti-integrin α3 (P1B5), and anti-integrin α4 (P4C2). (D) HDME cell spreading on substrates coated with TSP1 (closed bars), vitronectin (striped bars), or TSP1 peptide 678 (open bars) was determined in the presence of 1 μM αvβ3 antagonist SB223245, 5 μg/ml P1B5 (anti-α3), or both inhibitors. In all panels, results that differ significantly from their respective controls ($p < 0.05$) are marked with asterisks.

spreading on TSP1, because the αv integrin antagonist SB223245 partially inhibited spreading on TSP1. The effect of these two inhibitors was additive, producing a 76% inhibition of spreading when combined ($p = 0.006$ compared with peptide 678 alone). Similar results were obtained with the use of the αvβ3 peptide antagonist GRGDSP alone and in combination with peptide 678. Approximately 20% of the spreading response on TSP1 was resistant to the GRGDSP peptide, but combining this peptide with the α3β1 integrin–binding peptide completely inhibited spreading on TSP1 ($p = 0.003$ compared with peptide 678 alone).

In contrast, sparse culture of human endothelial cells used the α3β1 integrin exclusively to mediate spreading on TSP1 (Figure 4, B–D). Umbilical vein (HUVE) cell spreading on TSP1 was inhibited 70 ± 7% by peptide 678 ($p < 0.001$), whereas spreading on vitronectin was only marginally inhibited (Figure 5B; $p = 0.06$). Conversely, the αvβ3 antagonist SB223245 completely inhibited spreading on vitronectin but did not significantly inhibit spreading on TSP1. Combining the two antagonists produced no significant increase in inhibition relative to peptide 678 alone ($p = 0.2$), indicating that αvβ3 plays no significant role in spreading of HUVE cells on TSP1. HUVE cell spreading on TSP1 and TSP1 peptide 678 was also specifically inhibited by an α3β1-specific function-blocking antibody (Figure 5B; see also Figure 7B).

Microvascular (HDME) cell spreading on TSP1 was partially inhibited by the function-blocking integrin antibodies specific for the β1 subunit (mAb13) or α3β1 integrin (P1B5; $p = 0.02$) and by TSP1 peptide 678 but not by the α4β1-blocking antibody P4C2 ($p = 0.6$) (Figure 4, C and D), verifying that spreading of these microvascular cells on TSP1 is also mediated by the α3β1 integrin. Inhibition of spreading on TSP1 by the α3β1-blocking antibody was specific, because it did not inhibit spreading of the same cells on type I collagen (Figure 4C).

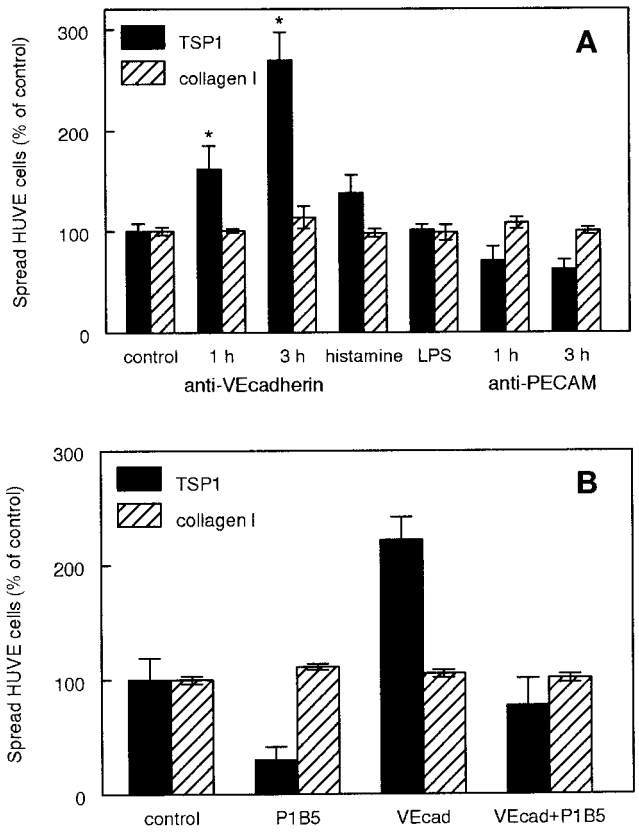

Figure 5. Activation of endothelial cell $\alpha3\beta1$ integrin is regulated by disruption of VE-cadherin. (A) Inhibition of VE-cadherin binding specifically induces spreading on TSP1 of confluent HUVE cell cultures. Confluent HUVE cell cultures were treated for 1 or 3 h with 5 μg/ml function-blocking VE-cadherin antibody clone 75, for 3 h with 3 μM histamine, 10 ng/ml lipopolysaccharide (LPS), or for 1 or 3 h with 5 μg/ml function-blocking PECAM-1 antibody HEC7. The cells were then dissociated with the use of EDTA and spreading was determined on substrates coated with 40 μg/ml TSP1 (closed bars) or 5 μg/ml type I collagen (striped bars). Results for both proteins are presented as percent of the spreading determined for control confluent cultures treated for 3 h with the medium alone (mean $\pm$ SD, n = 3). (B) $\alpha3\beta1$ integrin mediates endothelial cell spreading stimulated by disrupting VE-cadherin. Confluent HUVE cells were mock treated or treated for 3 h with 5 μg/ml function-blocking VE-cadherin antibody clone 75. The cells were harvested, and spreading on TSP1 or type I collagen was determined in the presence or absence of the $\alpha3\beta1$ integrin-blocking antibody P1B5.

The $\alpha v\beta3$ integrin did not contribute significantly to spreading of microvascular cells on TSP1, because the antagonist SB223245 did not inhibit spreading on TSP1 and did not increase the inhibition when combined with the $\alpha3\beta1$-blocking antibody ($p = 0.6$; Figure 4D). A function-blocking antibody recognizing the TSP1 receptor CD36 also did not block adhesion of HDME cells (Figure 4C). Of the human endothelial cells used, only HDME cells expressed CD36 as measured by reverse transcription–PCR. Therefore, expression of CD36 is not required for endothelial cell spreading on TSP1. These data are consistent with the previous report that HDME cell adhesion on TSP1 is independent of CD36 and the $\alpha v\beta3$ integrin (Chen *et al.*, 1996). Heparin also had no effect on spreading of HDME cells on a TSP1 substrate (our unpublished results). These results demonstrate that the $\alpha3\beta1$ integrin mediates spreading of several types of endothelial cells on TSP1. The $\alpha v\beta3$ integrin also plays a role in bovine endothelial cells, but the human endothelial cells display some $\alpha3\beta1$-independent spreading activity for which no known TSP1 receptor could be assigned.

Disrupting VE-Cadherin Specifically Activates Endothelial Cell $\alpha3\beta1$ Integrin

To further differentiate cell contact signals from signals that may result from replating the cells, we used several agents to directly perturb endothelial cell–cell contacts in a confluent monolayer. VE-cadherin is a major mediator of cell–cell contact signaling in endothelial cells (Dejana *et al.*, 1999). Pretreatment of a confluent HUVE cell monolayer with a function-blocking VE-cadherin antibody (Hordijk *et al.*, 1999) produced a time-dependent increase in spreading of the cells when subsequently plated on TSP1 but not when plated on the $\alpha2\beta1$ integrin ligand type I collagen (Figure 5A). Thus, blocking VE-cadherin function specifically induces $\alpha3\beta1$ but not $\alpha2\beta1$ integrin activity on endothelial cells. After 3 h, the enhancement of spreading observed on TSP1 was 62% of the maximal response induced with the use of the $\beta1$ integrin–activating antibody TS2/16. The spreading stimulated by treatment with the VE-cadherin antibody was verified to be mediated by $\alpha3\beta1$ integrin with the use of the function-blocking antibody P1B5, which reversed the spreading induced in anti-VE-cadherin–treated cells (Figure 5B).

Two other agents that disrupt endothelial cell contacts, histamine (Andriopoulou *et al.*, 1999) and lipopolysaccharide (Bannerman *et al.*, 1998), were less effective (Figure 5A). Histamine disrupts endothelial cell contacts in part through disrupting VE-cadherin (Andriopoulou *et al.*, 1999) and somewhat stimulated spreading on TSP1 ($p = 0.03$). Lipopolysaccharide, however, was inactive.

To confirm the specificity of the integrin response induced by disrupting VE-cadherin, we also examined PECAM-1, another homotypic adhesion protein on endothelial cells that mediates cell–cell adhesion but is not present in adherens junctions. PECAM-1 and VE-cadherin play distinct roles as adhesion molecules to mediate signaling from cell–cell contacts (Bach *et al.*, 1998; Halama *et al.*, 1999). We used a function-blocking PECAM-1 antibody, HEC7, to examine the role of PECAM-1 in regulating $\alpha3\beta1$ integrin activity. Confluent HUVE cells treated for 1 or 3 h with this antibody showed a decrease in spreading on TSP1 but no change in spreading on type I collagen (Figure 5A). Thus, the suppression of $\alpha3\beta1$ integrin activity in confluent endothelial cells can be reversed by disrupting VE-cadherin– but not PECAM-1–mediated endothelial cell interactions.

Although the maximal spreading response on TSP1 that could be induced by the integrin-activating antibody TS2/16 was not increased by depriving endothelial cells of cell–cell contact (Figure 3), we wanted to verify that the increase in adhesion on TSP1 after replating was not due to changes in $\alpha3\beta1$ integrin expression. Analysis of HUVE cell surface $\alpha3\beta1$ expression by immunoprecipitation and Western blotting demonstrated that surface expression of the integrin was similar in sparse and confluent cultures (Figure 6).

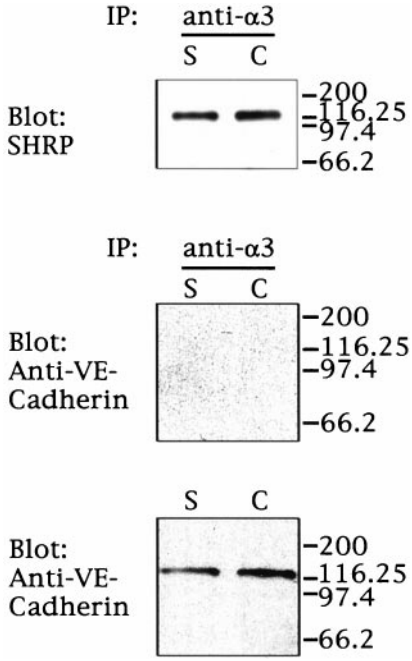

Figure 6. α3β1 integrin and VE-cadherin expression in endothelial cell cultures. HUVE cells grown under sparse (S) or confluent (C) conditions were biotinylated, immunoprecipitated with the use of anti-α3 integrin antibody (P1B5), and fractionated on 10% SDS-polyacrylamide gels (upper and middle panels) along with equal amounts of proteins from total cell lysates (10 μg; lower panel). Surface proteins were detected with the use of HRP–streptavidin (SHRP) and enhanced chemiluminescence. VE-cadherin in the α3 immunoprecipitate (middle panel) or total lysate (lower panel) was detected by blotting. Migration of molecular weight markers is indicated on the right.

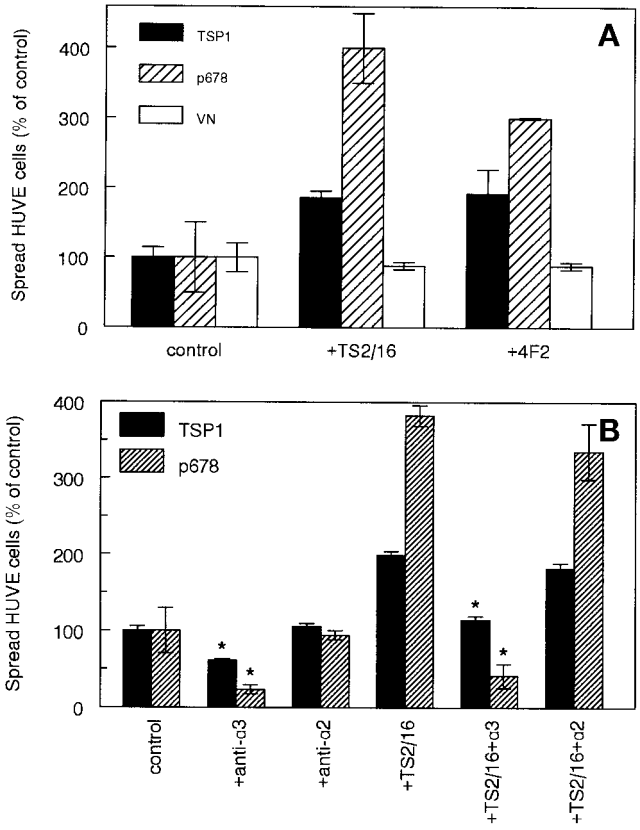

Figure 7. β1 integrin– and CD98-activating antibodies induce HUVE cell spreading on TSP1 and TSP1 peptide 678. (A) Untreated HUVE cells harvested at 80% confluence were suspended in medium 199/0.1% BSA alone (control) or in the presence of 5 μg/ml β1 integrin–activating antibody (TS2/16) or the CD98 antibody (4F2) and incubated on substrates coated with 40 μg/ml TSP1 (closed bars), 5 μM peptide 678 (striped bars), or 5 μg/ml vitronectin (open bars). Cell spreading is expressed as a percent of the response for untreated cells (means ± SD, n = 3). (B) Cells treated as in A were incubated on substrates coated with TSP1 or peptide 678 in the presence of the integrin function-blocking antibodies P1B5 (α3) or 6D7 (α2) at 5 μg/ml. Inhibition with $p < 0.05$ is indicated by asterisks.

Similar α3β1 integrin expression in both cultures was verified by flow cytometry (our unpublished results). Therefore, endothelial cells deprived of cell–cell contact show increased α3β1 functional activity without a corresponding increase in their expression of this integrin.

Surface expression of VE-cadherin was also similar in sparse and confluent cells (Figure 6). No VE-cadherin could be detected in α3β1 integrin immunoprecipitated from either culture under mild conditions, suggesting that regulation of the activation of α3β1 is mediated by intracellular signaling rather than by a direct association between these membrane proteins.

CD98 Ligation Stimulates α3β1 Integrin Recognition of TSP1

Based on the localization of CD98 in endothelial cells spreading on TSP1 (our unpublished results) and its ability to activate α1 integrins (Fenczik *et al.*, 1997; Chandrasekaran *et al.*, 1999), we examined the effect of the CD98 antibody 4F2 on HUVE cell spreading on TSP1 (Figure 7A). The CD98 antibody enhanced spreading on TSP1 and peptide 678 to a similar degree as the β1 integrin–activating antibody TS2/16. Stimulation of spreading by both antibodies was specific in that spreading of the treated cells on vitronectin, an αvβ3 integrin ligand, was not affected (Figure 7A). Spreading stimulated by the β1-activating antibody remained α3β1 dependent, based on complete reversal by the α3β1-blocking antibody but not by an α2β1-blocking antibody (Figure 7B).

TSP1 Modulates Endothelial Cell Proliferation through α3β1 Integrin

Interaction of the α3β1 integrin with its ligands can regulate epithelial cell proliferation (Gonzales *et al.*, 1999). Therefore, we examined the effect of the α3β1 integrin–binding sequence from TSP1 on endothelial cell proliferation. Peptide 678 inhibited BAE cell proliferation in a dose-dependent manner when added in solution (Figure 8A). Of two control peptides with amino acid substitutions that diminish integrin binding (Krutzsch *et al.*, 1999), peptide 686 (FQGV-

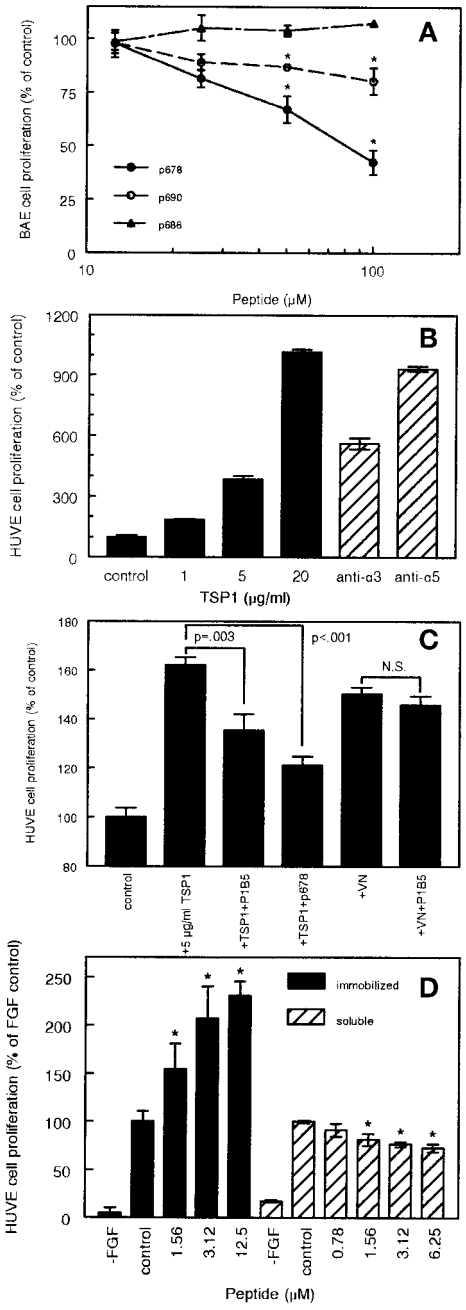

Figure 8. Modulation of endothelial cell proliferation by an $\alpha3\beta1$ integrin–binding peptide from TSP1. (A) Proliferation of BAE cells was assayed in the presence of the indicated concentrations of TSP1 peptide 678 (FQGVLQNVRFVF; ●) or the control peptides 686 (FQGVLQAVRFVF; ▲) or 690 (FQGVLQNVAFVF; ○). Briefly, 100 μl of a 5×10^4 cell/ml suspension of BAE cells was seeded in triplicate into a 96-well tissue culture plate in DMEM containing 1% FCS, 10 ng/ml FGF2, and peptides at 1–40 μM concentrations. Cells were incubated for 72 h, and proliferation was measured with the use of the Cell-Titer tetrazolium assay (Promega). (B) HUVE cell proliferation was measured at 72 h for cells plated on wells coated with the indicated concentrations of TSP1 (closed bars) or 1 μg/ml antibody P1B5 (anti-$\alpha3\beta1$ integrin) or P1D6 (anti-$\alpha5\beta1$ integrin) in medium 199 containing 5% FCS. (C) $\alpha3\beta1$ integrin mediates the proliferative response to immobilized TSP1. HUVE cells were plated in medium 199 containing 20% FCS on wells coated with 5 μg/ml TSP1, 5 μg/ml vitronectin, or BSA (control) alone or in the presence of 5 μg/ml $\alpha3\beta1$-blocking antibody P1B5 or 20 μM TSP1 peptide 678. Proliferation was determined at 72 h and is presented as a percent of the control (means ± SD, n = 3 for experimental points and n = 6 for control). (D) HUVE cell proliferation was determined in the presence of the indicated concentrations of TSP1 peptide 678 immobilized on the substrate (closed bars) or added in solution (striped bars). Conditions that significantly differed from their respective controls based on a two-tailed *t* test with $p < 0.05$ are marked with asterisks.

LQAVRFVF) was inactive and peptide 690 inhibited proliferation of BAE cells by only 19% at the highest dose tested (100 μM).

Previous publications have consistently reported that soluble TSP1 inhibits proliferation of endothelial cells (Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990; Sheibani and Frazier, 1995; Panetti *et al.*, 1997). In contrast, TSP1 immobilized on the growth substrate stimulated dose-dependent proliferation of HUVE cells (Figure 8B). Ligation of the $\alpha3\beta1$ integrin was sufficient to stimulate this proliferative response, because immobilized $\alpha3\beta1$ integrin antibody also stimulated proliferation (Figure 8B). In this experiment, an $\alpha5\beta1$ integrin antibody was used as a positive control, because ligation of this integrin is known to promote endothelial cell proliferation and survival. Stimulation of proliferation by immobilized TSP1 was $\alpha3\beta1$ dependent, based on significant reversal of the growth stimulation in the presence of either the function-blocking $\alpha3\beta1$ antibody or TSP1 peptide 678 in solution (Figure 8C). Specificity of the antibody inhibition was verified by its lack of a significant effect on endothelial cell proliferation stimulated by immobilized vitronectin (Figure 8C). Consistent with the activity of the immobilized $\alpha3\beta1$ antibody, plating of HUVE cells on immobilized TSP1 peptide 678 increased their proliferation (Figure 8D). However, adding the same peptide in solution significantly inhibited HUVE cell proliferation (Figure 8D).

Similar enhancement of microvascular (HDME) cell proliferation was observed after plating on immobilized TSP1 or TSP1 peptide 678 (Figure 9). As reported previously for several types of endothelial cells, however, soluble TSP1 inhibited proliferation of HDME cells stimulated by FGF2 (Figure 9). Therefore, even microvascular endothelial cells that express the antiangiogenic TSP1 receptor CD36 (Dawson *et al.*, 1997) can proliferate in response to TSP1 when it is immobilized.

Inhibiting $\alpha3\beta1$ Integrin Prevents Endothelial Wound Repair

To examine the role of the $\alpha3\beta1$ integrin–binding sequence of TSP1 in endothelial cell motility, we determined the effect of peptide 678 on endothelial scratch wound repair (Figure 10). Cells were arrested with the use of 5-fluorouracil to measure the effects on endothelial cell motility in the absence of proliferation. Peptide 678 was a dose-dependent inhibitor of BAE cell migration into the wound. At 30 μM, peptide 678 significantly inhibited endothelial cell migration relative to the control ($p = 0.016$; two-tailed *t* test), and this inhibition was specific in that the inactive analogue peptide

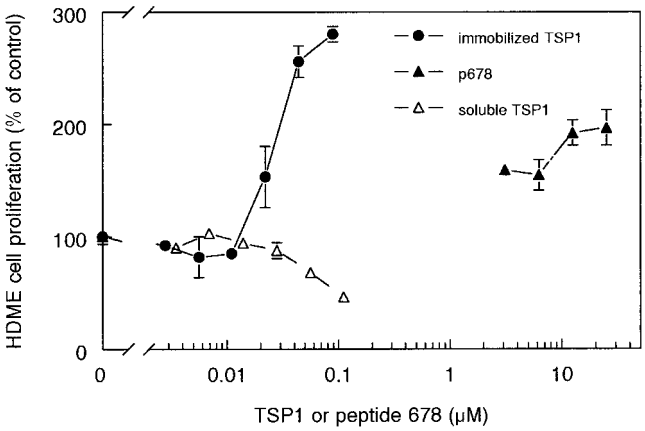

Figure 9. HDME cell proliferation in MCDB growth medium with 5% FCS was determined in the presence of 10 ng/ml FGF2 and the indicated concentrations of TSP1 added in the medium (△) or immobilized on the substrate (●) or in wells coated with the indicated concentrations of peptide 678 (▲). Results are presented as means ± SD and are normalized to controls without TSP1 or peptide.

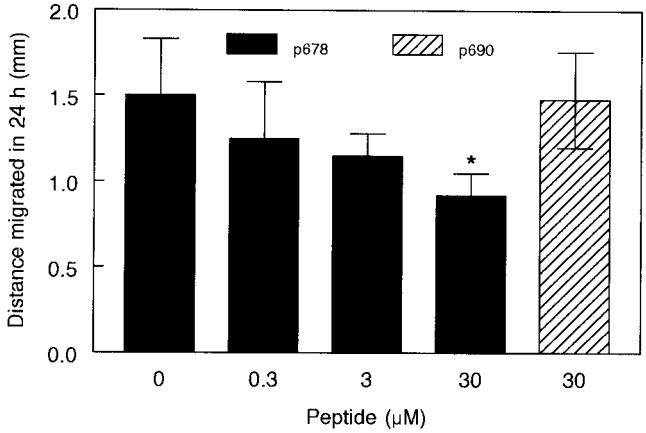

Figure 10. TSP1 peptide 678 inhibits wound healing of BAE cells. BAE cells were seeded at a density of 2×10^5 cells/well of six-well tissue culture plates in complete growth medium supplemented with 10% FBS. After the cells formed a confluent cobblestone, cells were arrested with the use of 10 μg/ml 5-fluorouracil for 48 h. Scrape wounds of 2 mm width were made in the wells, and the cells were further incubated with medium containing 10% FBS, 10 μg/ml 5 fluorouracil, and peptides 686 (closed bars) or 690 (striped bars). Measurements of the distance between the wound margins were taken at 0 and 24 h, and the net migrations for a representative experiment of three performed are presented as means ± SEM (n = 3). Significant inhibition relative to the control ($p < 0.05$) is indicated by an asterisk.

690 did not inhibit cell motility in this assay ($p > 0.5$). Inhibition by peptide 678 was not significant at the lower concentrations ($p = 0.08$ at 3 μM) but was consistently observed in multiple experiments.

The α3β1-binding Sequence from TSP1 Inhibits Angiogenesis

The α3β1 integrin also contributes to angiogenesis in vivo, because peptide 678 inhibited angiogenesis in the chick CAM assay ($p < 0.005$ at 20 μM; Figure 11). The dose dependence for inhibition (Figure 11A) was consistent with the reported IC_{50} of this peptide for blocking α3β1 integrin–dependent adhesion (Krutzsch *et al.*, 1999) and for inhibiting endothelial cell proliferation in vitro. Inhibition of angiogenesis by TSP1 peptide 678 was specific in that substitution of the essential Arg residue with Ala (peptide 690) abolished inhibitory activity in the CAM assay (Figure 11A). The extent of angiogenesis inhibition by peptide 678 was comparable to that for the previously described inhibitor from the type 1 repeats, peptide 246, and for intact TSP1 (Figure 11B). In contrast to the type 1 repeat peptide, however, which inhibited responses to FGF2 but not VEGF (Iruela-Arispe *et al.*, 1999), the integrin-binding peptide 678 comparably inhibited angiogenesis stimulated by both growth factors (Figure 11B).

The α3β1-binding Sequence Promotes Angiogenesis When Expressed with the Heparin-binding Domain of TSP1

Because intact TSP1 contains at least two sequences that inhibit angiogenesis (Tolsma *et al.*, 1993; Iruela-Arispe *et al.*, 1999), we used recombinant fragments from the N-terminal heparin-binding domain of TSP1 that lack these known inhibitory sequences to examine the angiogenic activity of the α3β1 integrin–binding sequence. Addition of a recombinant heparin-binding fragment (residues 1–174) that lacks the α3β1 integrin–binding sequence at residues 190–201 had no effect on growth factor–stimulated angiogenic responses, but a longer fragment (residues 1–242) that includes this integrin-binding sequence significantly augmented angiogenic responses stimulated by FGF2 or a combination of FGF2 and VEGF (Figure 11B). A similar stimulation of angiogenesis, which was also specific for the longer TSP1 fragment, was observed in the absence of growth factors (Figure 11C). Thus, residues 175–242 of TSP1, which contain the α3β1 integrin–binding sequence, exhibit proangiogenic activity in the CAM assay. Intact TSP1 did not significantly stimulate angiogenesis in the absence of growth factors, presumably because of the presence of the known inhibitory sequences. Peptide 678 was also inactive in this assay, suggesting that the heparin-binding domain of the recombinant fragment plays a role by immobilizing the fragment.

DISCUSSION

Although TSP1 is generally recognized as an inhibitor of angiogenesis (Good *et al.*, 1990; Iruela-Arispe *et al.*, 1999), conflicting reports about the effects of TSP1 on endothelial cell adhesion, motility, and proliferation have precluded a clear understanding of the mechanism for its antiangiogenic activity (Good *et al.*, 1990; Taraboletti *et al.*, 1990; Iruela Arispe *et al.*, 1991; BenEzra *et al.*, 1993; Nicosia and Tuszynski, 1994; Canfield and Schor, 1995). Recognizing that endothelial cells can modulate the expression or activation state of specific TSP1 receptors that transduce opposing signals may lead to a resolution of this conflict (Figure 12). We have demonstrated that endothelial cells deprived of cell–cell

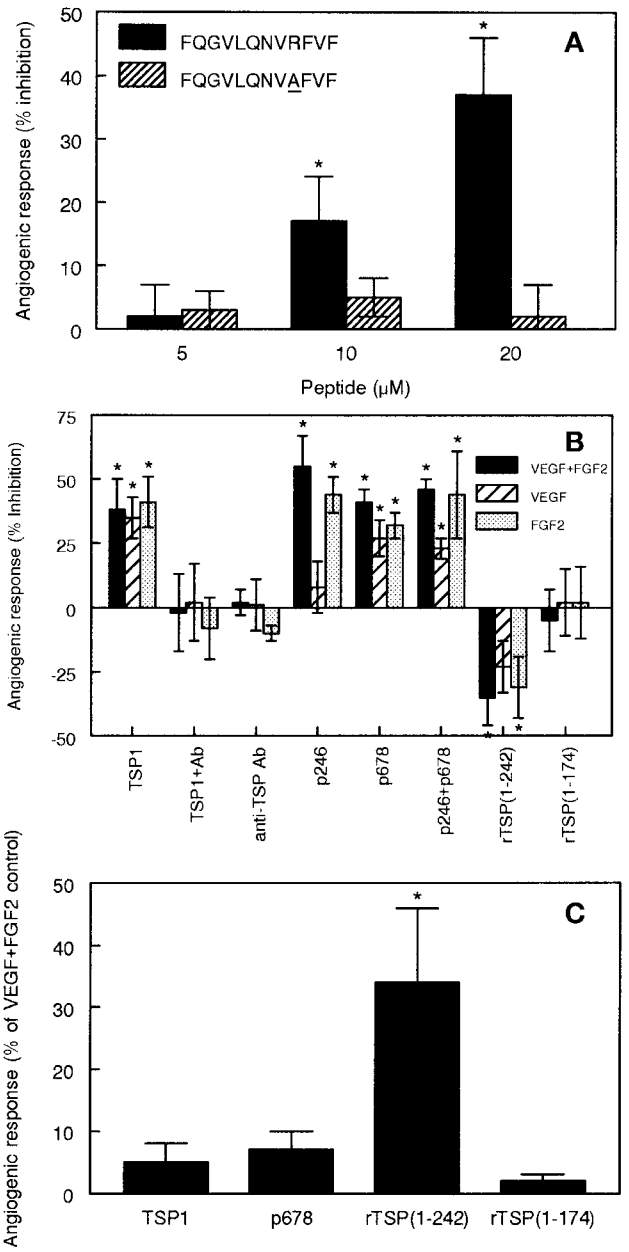

Figure 11. Modulation of chick CAM angiogenesis by the $\alpha3\beta1$-binding sequence in TSP1. (A) The TSP1 $\alpha3\beta1$ integrin–binding peptide inhibits angiogenesis. Polymerized collagen gels containing the angiogenic growth factors VEGF and FGF2 in the presence or absence of the indicated concentrations of the TSP1 peptide FQGVLQNVRFVF (peptide 678; closed bars) or the control peptide FQGVLQNVAFVF (peptide 690; striped bars) were placed on the outer one-third of 10-d chick CAMs for 24 h. Each CAM contained two pellets for each peptide concentration as well as positive and negative controls. The ability of the peptides to modulate growth factor–driven angiogenesis was assessed by injection of FITC-dextran and digital image analysis. The percent inhibition relative to controls is presented as means ± SD for each group (n = 8). Conditions that significantly differed from their respective controls based on a two-tailed *t* test with $p < 0.05$ are marked with asterisks. (B) Inhibition of CAM angiogenesis stimulated by VEGF and FGF2 (closed bars) or by VEGF (striped bars) or FGF2 alone (shaded bars) was assessed as in A in the presence of the indicated effectors: 10 μg of TSP1, 10 μg of TSP1 plus 25 μg of anti-TSP1, 25 μg of anti-TSP1 (Ab), 20 μM peptide 246 (KRFKQDGGWSHWSPWSS from the type 1 repeats of TSP1), 20 μM peptide 678, 20 μM peptide 246 plus peptide 678, or 20 μg of recombinant TSP1 fragments containing residues 1–242 or 1–174 of the mature protein. Results are presented as means ± SD (n = 3–9). (C) Direct stimulation of angiogenesis by recombinant TSP1 fragments. Angiogenesis in the absence of growth factors was determined in the presence of 10 μg of TSP1, 20 μM peptide 678, or 20 μg of recombinant TSP1 (residues 1–242 or 1–174). Results are presented as percent of the positive control stimulated by VEGF plus FGF2 (means ± SD, n = 4–9).

contacts recognize an $\alpha3\beta1$ integrin–binding sequence in TSP1 that stimulates their spreading and proliferation when it is immobilized on a substratum. However, addition of this TSP1 peptide in solution inhibits endothelial cell spreading on TSP1, endothelial cell proliferation, and migration in vitro and angiogenesis in vivo, presumably by inhibiting interactions of this integrin with TSP1 or its other known ligands. The activity of this integrin to recognize TSP1 is suppressed in confluent endothelial cell monolayers. Loss of endothelial cell–cell contact during wound repair in vitro or angiogenesis in vivo, therefore, could activate this receptor and make endothelial cells responsive to TSP1 signaling through the $\alpha3\beta1$ integrin. Proangiogenic activity may also be induced by proteolytic processing of TSP1, which rapidly releases heparin-binding fragments of TSP1 that contain the integrin-binding sequence (Lawler and Slayter, 1981). Heparan sulfate proteoglycan–mediated immobilization of these fragments in the extracellular matrix may account for the proangiogenic activity we observed in the CAM assay with the use of this fragment.

We have identified two endothelial cell proteins, VE-cadherin and CD98, that can regulate the activity of $\alpha3\beta1$ integrin (Figure 12). CD98 is a general activator of $\beta1$ integrins (Fenczik *et al.*, 1997), so it probably is not responsible for selective activation of $\alpha3\beta1$ integrin after loss of cell contact.

VE-cadherin is an endothelial adherens junction component that modulates catenin and Shc signaling pathways (Dejana *et al.*, 1999). Antibody blocking demonstrated that disrupting VE-cadherin in confluent endothelial cells is sufficient to activate $\alpha3\beta1$ integrin. Therefore, signaling from ligated VE-cadherin may maintain $\alpha3\beta1$ integrin in an inactive state. The inactive $\alpha3\beta1$ integrin in confluent endothelial cells is concentrated at the cell–cell junctions (Yanez-Mo *et al.*, 1998). This localization may augment the negative signal from VE-cadherin that suppresses the activity of $\alpha3\beta1$ integrin but, based on our immunoprecipitation data, does not reflect a direct interaction between VE-cadherin and $\alpha3\beta1$ integrin. Insulin-like growth factor-1 receptor signaling in breast carcinoma cells (Chandrasekaran *et al.*, 1999) and EGF receptor signaling in small cell lung carcinoma cells (Guo *et al.*, 2000) play analogous roles to regulate activation of the $\alpha3\beta1$ integrin in those cell types. These growth factors do not activate the $\alpha3\beta1$ integrin in endothelial cells (our unpublished results), suggesting that regulation of the activation state of this integrin is cell type specific.

A second TSP1 receptor on endothelial cells that mediates inhibition of growth factor–stimulated cell migration, CD36,

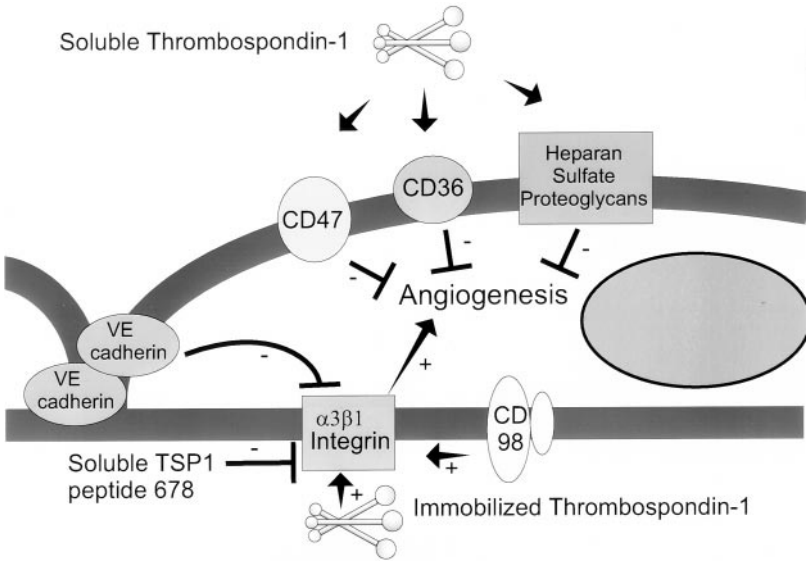

Figure 12. Revised model for modulation of angiogenesis by TSP-1. Inhibition of angiogenesis through binding of TSP1 to CD36, CD47, and heparan sulfate receptors have been documented previously (Dawson *et al.*, 1997; Iruela-Arispe *et al.*, 1999; Kanda *et al.*, 1999).

is differentially expressed in large vessels versus capillaries (Swerlick *et al.*, 1992; Dawson *et al.*, 1997). Thus, CD36-negative endothelial cells with activated α3β1 integrin (represented here by sparse HUVE cells) may recognize TSP1 in the extracellular matrix primarily as an angiogenic signal, whereas CD36-positive endothelial cells with inactive α3β1 integrin (e.g., confluent HDME cells) would receive only an antiangiogenic signal (Dawson *et al.*, 1997). Therefore, endothelial cells receive both proangiogenic and antiangiogenic signals from TSP1, and the net balance of these signals could be controlled by environmental signals that regulate the expression and activity of each TSP1 receptor.

TSP1 expression in endothelial cells is also regulated by cell–cell contact (Mumby *et al.*, 1984; Canfield *et al.*, 1990). Cells without mature cell–cell contacts produce more TSP1 than confluent cells (Mumby *et al.*, 1984). Reports that TSP1 is involved in endothelial cell outgrowth in wound repair assays (Vischer *et al.*, 1988; Munjal *et al.*, 1990), combined with our new data showing that recognition of TSP1 by the α3β1 integrin is activated under the same conditions that stimulate TSP1 production, suggest that coordinate induction of TSP1 expression and activation of its receptor, α3β1 integrin, may stimulate both endothelial cell motility and proliferation during wound repair. This hypothesis is consistent with the pattern of TSP1 expression induced in vascular injury (Reed *et al.*, 1995) and with the observation that function-blocking antibodies recognizing α3β1 integrin inhibited migration of endothelial cells lacking cell–cell contact (Yanez-Mo *et al.*, 1998). Although induction of TSP1 expression during angiogenic responses has been interpreted as a negative feedback pathway to limit angiogenesis (Suzuma *et al.*, 1999), the possibility should be considered that TSP1 immobilized in the extracellular matrix also participates as a positive regulator of neovascularization. This positive signal would be limited, because the α3β1 integrin becomes inactive when endothelial cell–cell contact is established.

The involvement of α3β1 integrin in endothelial cell adhesion on TSP1 is consistent with several recent studies of TSP1–endothelial cell interactions. Binding of soluble TSP1 to HUVE cells was shown to be mediated mostly by heparan sulfate proteoglycans, with some involvement of αvβ3 integrin but not of CD36 (Gupta *et al.*, 1999). However, combinations of these inhibitors could not completely inhibit TSP1 binding to HUVE cells, suggesting that additional TSP1 receptors are present on endothelial cells. More relevant to the present studies, HDME cell adhesion on TSP1 was neither RGD nor CD36 dependent and was concluded to be mediated by an undefined TSP1 receptor (Chen *et al.*, 1996). Based on the present data, the α3β1 integrin mediates this adhesive interaction of HDME cells with TSP1.

Previous publications have identified αvβ3 integrin as a TSP1 receptor on endothelial cells (Lawler *et al.*, 1988; Gupta *et al.*, 1999). We confirmed this result for BAE cells, but we could not detect a significant contribution of the αvβ3 integrin on microvascular and large vessel human endothelial cells to their adhesion on TSP1. Rather, α3β1 seems to be the major TSP1-binding integrin on human endothelial cells.

Other extracellular matrix proteins are known to exert both positive and negative effects on cell proliferation. Altering the architecture of fibronectin (Sechler and Schwarzbauer, 1998) or type I collagen matrices (Koyama *et al.*, 1996) can reverse their effects on cell cycle progression. Differential expression of integrins can reverse the effects of laminins and tenascin on cell proliferation (Yokosaki *et al.*, 1996; Mainiero *et al.*, 1997). TSP1, likewise, expresses both proproliferative and antiproliferative activities for specific cell types, but its activity toward endothelial cells has been generally regarded as antiproliferative (Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990). However, we have now demonstrated that interaction with immobilized intact TSP1 or the TSP1 peptide 678 through the endothelial cell α3β1 integrin stimulates the proliferation of endothelial cells. Binding of laminin-5 to the α3β1 integrin was recently demonstrated to stimulate the proliferation of mammary epithelial cells (Gonzales *et al.*, 1999), suggesting that the growth-promoting activity of immobilized TSP1 for endothelial cells may be a general response to α3β1 ligand binding. Because

addition of a soluble TSP1 peptide that is recognized by this integrin also inhibited endothelial cell motility in the absence of proliferation, $\alpha 3\beta 1$ integrin interaction with intact immobilized TSP1 may stimulate both endothelial cell proliferation and motility. Defining the specific sequences in TSP1 and the respective endothelial cell receptors that are responsible for both its proangiogenic and antiangiogenic activities may allow us to isolate each activity and lead to the development of peptides, gene therapy approaches, or small molecule analogues of TSP1 peptides with more specific antiangiogenic activities.

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