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09/26/94
**Title and Subtitle**
Regulation of Breast Carcinoma Growth and Neovascularization by Peptide Sequences in Thrombospondin

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**Abstract**
Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that modulates endothelial cell growth, motility, and adhesion. Its role in regulating angiogenesis of breast tumors was examined using site specific mutagenesis, recombinant fragments, and peptide mimetics based on TSP1. Peptides from the type I repeats of TSP1 reproduced the growth inhibitory activity of the intact protein. Stable analogues of the L-forward peptides and of D-reverse or retro-inverso peptide mimetics were prepared that inhibited endothelial cell proliferation stimulated by basic fibroblast growth factor (FGF-2) and selectively induced apoptosis of endothelial cells. The α3β1 integrin was identified as receptor on breast carcinoma cells that mediates adhesive and migratory responses of the cells to TSP1. Over expression of TSP1 in breast carcinoma cells suppresses tumorigenesis and angiogenesis in murine xenografts. The role of the TGFβ-activating and FGF2 antagonist type I repeat sequences in this activity was examined using site-directed mutagenesis of phenylalanine and tryptophan residues required for activity of peptides derived from TSP1. Stably transfected breast carcinoma cell lines expressing these mutants were characterized for tumorigenic potential in vivo and behavior in vitro.
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INTRODUCTION

Growth of many solid tumors is strongly dependent on recruitment of neovascularization. Increased vascularization of primary breast tumors has been associated with increased rates of metastasis to lymph nodes and poorer prognosis (1, 2). Regulation of angiogenesis involves both stimulatory or angiogenic factors and inhibitory or anti-angiogenic factors (3, 4). In normal adult endothelium, high expression of anti-angiogenic factors and limited availability of angiogenic factors maintains the endothelium in a nonproliferative state. Pathological states including wound repair, diabetic retinopathy, or tumor growth may alter the balance of these stimulators or inhibitors to allow neovascularization to proceed (3).

Several anti-angiogenic factors have also been identified, including thrombospondins-1 and 2 (5-9). The hypothesis that thrombospondin-1 (TSP1) can inhibit neovascularization of tumors has been confirmed by transfection studies in several tumor models (10-13), reviewed in Appendix A). Synthetic peptides from the type I repeats and recombinant amino-terminal heparin-binding domain from TSP1 mimic the inhibitory activities of intact TSP1 on endothelial cell proliferation and motility (8, 9). These fragments and peptides act at least in part by competing with basic fibroblast growth factor (FGF2) for binding to heparan sulfate proteoglycan receptors on the endothelial cells, which are essential for presentation of FGF2 to its signaling receptor.

The mechanism by which TSP1 inhibits angiogenesis is under active investigation. TSP1 inhibits proliferation and spontaneous tube formation by endothelial cells in vitro (14) and inhibits angiogenesis in vivo (5, 15). However, endothelial cell responses to TSP1 are complex; the magnitude and direction of the responses depend upon the presence of additional matrix components and growth factors. Immobilized TSP1 promotes endothelial cell adhesion on some substrates (6) but inhibits adhesion on others, including substrates coated with fibronectin (16). Inhibition of adhesion to fibronectin is associated with disruption of focal adhesion contacts (17). TSP1 promotes migration of endothelial cells in chemotaxis and haptotaxis assays but inhibits chemotaxis induced by FGF2 (6).

To understand the mechanisms of these diverse and apparently conflicting effects of TSP1 on endothelial cell behavior, it is necessary to define the domains of TSP1 that interact with the cells, the identity of the endothelial cell receptors that interact with TSP1, and the intracellular responses in transduction and integration of the signals resulting from TSP1 binding to each receptor. Based on inhibition by monoclonal antibodies and sulfated polysaccharides, the heparin-binding domain at the amino-terminus of TSP1 may be responsible for regulation of endothelial proliferation (6). However, a 140 kDa fragment of TSP1 that lacks the amino-terminal region also suppresses endothelial cell growth (5). Thus, multiple sites on the TSP1 molecule may modulate endothelial cell growth and motility. Moreover, based on studies by Murphy-Ullrich et al. (18), inhibition of bovine endothelial cell growth by TSP1 is at least partly due to the inhibitory activity of transforming growth factor β, which complexes with TSP1 and contaminates most TSP1 preparations. We have previously identified two parts of TSP1 that have antiproliferative activity in isolation (8, 9). Recombinant amino-terminal domain inhibited endothelial growth and motility induced by serum or FGF2. Synthetic peptides from the type I repeats also inhibited proliferation to FGF2 and showed a biphasic effect on motility of endothelial cells in the presence of FGF2.
that mimicked the activity of intact thrombospondin. Tolsma et al (19) reported that additional peptides from the type I repeats have antiangiogenic activity and identified a sequence in the procollagen domain with antiangiogenic activity. Thus, at least three isolated regions of TSP1 have anti-angiogenic activities, and some of these activities are expressed in synthetic or recombinant constructs without contaminating transforming growth factor-β (TGFβ).

Synthetic peptides from TSP1 were used to further define the activity of the type I repeats (20, 21). The type I peptides of TSP1 define a new class of heparin-binding peptides, since they lack any previously identified heparin-binding consensus sequences and most do not contain any basic amino acids. Studies with homologous peptides showed that two Trp residues and the Ser residue are essential (21). The Trp residues must be spaced less than four residues apart. The Pro residue is essential for proper conformation and activity of the pentapeptide Trp-Ser-Pro-Trp-Ser, but some larger peptides with substitutions of the Pro residue are active.

The synthetic peptides from the type I repeats are especially promising for development of new therapeutics to prevent tumor invasion and metastasis, since they are active in vitro at relatively low concentrations. Free peptides, however, often have short half lives in circulation. They are subject to rapid clearance due to their small size and susceptibility to proteolytic degradation.

In several cases, use of polymer conjugates of peptides from extracellular matrix proteins has overcome these limitations (22-24). The peptides from the type 1 repeats of thrombospondin have therefore been conjugated to a ficoll (polysucrose) carrier to increase their stability in vivo. We have characterized polysucrose conjugates as proposed for Task 2 of our Statement of Work. Results describing their effects on breast carcinoma and endothelial cells in vitro and lack of antitumor activity in vivo for breast tumor xenografts have been published (25, 26).

Preparation of retro-inverso or D-reverse analogs is a second method to increase in vivo activity of peptides. These analogs have been successfully applied to increase the stability and biological activity of peptide sequences for therapeutic applications (reviewed in (27). Of particular relevance to the TSP1 peptides, an all D-amino acid peptide analog of a peptide from the A chain of the extracellular matrix protein laminin replicated the activity of the natural sequence to influence tumor cell adhesion and growth in vitro and in vivo (28). The retro-inverso analog of the TSP1 type 1 peptide sequence KRFKQDGGSWPSWSSC was chosen as the starting point for preparation of retro inverse analogs. Work proposed in Tasks 1 and 2 resulted in two publications demonstrating the activities of these retro-inverso peptides in vitro and in vivo to inhibit breast carcinoma and endothelial cell growth (25, 26).

Our second major goal was to define the function of these sequences in the intact TSP1 protein. These studies employed expression of recombinant TSP1 containing site-specific mutations. Stable transfectants of a human breast carcinoma cell line expressing some of these mutants were used to produce the recombinant proteins for in vitro characterization. The same cell lines were simultaneously tested in vivo for tumorigenic, angiogenic, and metastatic phenotypes. Correlations between these assays provided insight into the role of specific sequences in TSP1 in regulating tumor behavior. Preparation of the mutants for Task 3 has been completed, and stable transfectants have been prepared where
possible. This final report summarizes the characterization of the effects of these mutant thrombospondins on breast and endothelial cells as described in Task 3 and Task 4. Because several of the constructs could not be expressed in stable transfectants, we have modified our experimental plan to utilize transient expression assays to examine their biological activities.

In the course of these experiments, we have also discovered that breast carcinoma cells preferentially use the α3β1 integrin to mediate adhesion and chemotactic responses to TSP1. The activity of this thrombospondin receptor was found to be specifically induced by insulin-like growth factor receptor ligands. Recently, we have also identified the specific sequence in TSP1 that is recognized by the α3β1 integrin and discovered that this integrin plays important roles in the interactions of TSP1 with both the breast carcinoma cells and endothelial cells. We found that the α3β1-binding sequence in TSP1 has a pro-angiogenic activity and discovered that peptides that block this interaction can also be used to inhibit angiogenesis. These new data are also summarized in this report.

**BODY OF REPORT:**

**TASK 1: Preparation of stable analogs of thrombospondin-1 peptides.**

**METHODS**

*Materials*-- TSP1 was purified from the supernatant of thrombin-stimulated human platelets (29). TSP1 and its fragments were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) or Bolton-Hunter reagent (Dupont NEN) as previously described (29). Antibodies to native and denatured TSP1 were prepared by immunization of rabbits with native TSP1 or reduced and carboxymethylated TSP1, respectively.

*Preparation of synthetic peptides*-- The peptides used in this study were synthesized on a Biosearch Model 9600 peptide synthesizer using standard Merrifield solid phase synthesis protocols and t-butoxycarbonyl chemistry (21). Where noted, peptides were also synthesized using fmoc chemistry. Peptides were analyzed by reverse phase HPLC chromatography or gel permeation using a Superdex 75 HR 10/30 column eluted in 0.1 M ammonium acetate, pH 6. Peptides for biological assays were further purified by dialysis using Spectrapor 500 molecular weight cutoff tubing, gel permeation chromatography, or reverse phase purification using C18 Sep-pak cartridges. Identities of some peptides were verified by MALDI time of flight mass spectrometry.

*Bioassay for inhibition of endothelial and breast carcinoma cell proliferation*-- Proliferation of bovine aortic endothelial cells was determined as previously described (30). Similar assays were performed using MDA MB 435 human breast carcinoma cells except that the growth medium for the proliferation assays contained 5% fetal bovine serum in RPMI 1640 medium. Apoptosis of the cells exposed to peptides was quantified by electrophoretic analysis of DNA fragmentation or using a DNA fragment ELISA (Boehringer Mannheim) after labeling the cells with bromodeoxyuridine and exposure to the peptides for 24 h.

*Tumorigenesis assay in nude mice*-- NIH Nu/Nu mice or NIH Beige XID mice, approximately eight weeks of age were injected with 10⁴ MDA MB 435 cells by the mammary fat pad route. Wild type MDA cells were used for peptide studies; stable transfectants expressing full length wild type or mutant TSP1 were used to examine the
effects of site-directed mutations in TSP1 on tumorigenesis. Mice were anesthetized with 150-200 µl i.p. of a 1:80 dilution in PBS of a solution containing 25 g. tribromoethanol in 12.5 ml tertiary amyl alcohol. The mammary fat pad was cleaned with ethanol and a 10 mm incision was made directly above the site of injection. Using a 0.1 ml Hamilton syringe and 27 gauge ½" needle, 10 µl of cell suspension, 1 x 10^5 cells in Hanks balanced salts solution for nu/nu mice or 4 x 10^5 for Beige mice, were injected into the fat pad. The incision was closed using 1-2 Autoclips (9 mm, Clay Adams). Autoclips were removed 7 days post-injection.

8-10 animals are injected for each condition, per experiment. Animals were ear punched after injection for subsequent identification. Beginning at day 25 and continuing every day until day 50, the experimental animals for peptide treatment were injected i.v. (tail vein) 100 µl of the free peptide or ficoll conjugates. Animals implanted with transfected MDA cell lines were not treated. Primary tumor size was determined twice weekly by length x width x height measurement, and the animals were observed daily for general health. When the primary tumor of any animal exceeded 20 mm in any dimension, all of the animals were sacrificed. The presence of metastases was determined by gross autopsy and examination of H & E stained sections of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues, and weighed. At any time during the experiment, animals suspected of being in distress were sacrificed and examined as above.

RESULTS

We have completed and published the studies based on specific aim 1, to define structural elements responsible for activity of the TSP1 type 1 peptides and to prepare stable analogs with in vivo activity (31), Appendix B). We have also completed and published a study of the apoptosis response to the peptides and shown that native TSP1 has the same activity (25), Appendix C). We have also discovered that breast carcinoma cells preferentially use the α3β1 integrin to spread on TSP1 and that the activity of this integrin in breast carcinoma cells is regulated by insulin-like growth factor I and CD98. This contrasts with endothelial cells, which have been previously demonstrated to use the αvβ3 integrin as a TSP1 receptor (6, 32). Use of different integrins may account for some of the differential responses of breast carcinoma and endothelial cells to TSP1 and the TSP1 mutants. A manuscript based on these results is attached. To overcome the limitations in using full length recombinant thrombospondins to characterize functions of the type I repeats, we have used truncated recombinant thrombospondins to continue mapping the functional domains for its interactions with the endothelial and breast carcinoma cells.

In vivo activity of D-reverse peptides from the type 1 repeats.

In addition to demonstrating their activity in breast cancer xenografts, we have examined the activity of the D-reverse peptides in other animal disease models that involve physiological or pathological angiogenesis. Through a collaboration with Dr. Luisa Iruela-Arispe (Appendix F), we examined the activity of TSP1 type 1 repeat peptides in the chick chorioallantoic membrane (CAM) angiogenesis assay. This assay assesses anti-angiogenic activity with respect to developmental angiogenesis during embryonic development. Thus, the assay is generally regarded as a more pure assessment of anti-angiogenic activity than tumor
xenografts, where the peptides may affect both the tumor cells and endothelial cells. We demonstrated that both the native and D-reverse peptides are active in this assay (see Fig. 6 in Appendix F). The somewhat higher activity of the D-reverse peptides in this figure may reflect their enhanced stability. We demonstrated that the portion of this peptide that mediates activation of latent TGFβ was not required to inhibit angiogenesis in the CAM assay. To determine whether activity in the CAM assay was predictive of anti-tumor activity for breast cancer, we compared peptides with or lacking the TGFβ-activating sequence for inhibiting growth of MDA-MB-435 tumors in mice (Appendix F, Fig. 9). These data demonstrated that the TGFβ-activating sequence is not essential for the anti-tumor activity of these peptides.

Surprisingly, we found using this assay that the type I repeat peptide we have studied preferentially inhibits FGF2 but not vascular endothelial growth factor (VEGF)-induced angiogenesis (Appendix F, Fig. 8). In contrast, a second peptide from this region that binds to CD36 inhibited both FGF2 and VEGF-stimulated angiogenesis in the CAM assay. This suggest that these two peptides act through distinct signaling pathways to suppress angiogenic responses and demonstrate that two sequences from the type I repeats of TSP1 can independently inhibit angiogenesis in a physiological model.

The D-reverse peptides were also tested for activity to inhibit angiogenesis in animal models of glomerulonephritis (33) and brain cancer (34). Although these collaborative projects were not supported by the Army funding, they provided further insights into the activity of these peptides and demonstrate that the anti-tumor activity of these peptides is not limited to breast cancer. As part of both studies, we examined the use of Alzet pumps to continuously deliver the peptides. Although this route of delivery produced activity, it was not superior to daily intravenous administration. The brain tumor model also provided an opportunity to begin to define the pharmacokinetics of the peptides (34). We demonstrated that the D-reverse peptides have biphasic clearance from circulation with \( t_1/2 = 0.056 \, \text{h}, r^2 = 0.998 \) via kidney excretion and a slower component with \( t_1/2 = 50.5 \, \text{h}, r^2 = 0.98 \). Fluorescently labeled peptide was demonstrated to preferentially accumulate in the tumor vasculature compared to that observed in normal vascular beds. This suggests that the peptides have potential as targeting ligands for the tumor vasculature.

Structural requirements for \( \alpha_3\beta_1 \) mediated adhesive activity of TSP1
Based on the major role we discovered for the \( \alpha_3\beta_1 \) integrin in mediating TSP1 interactions with both breast carcinoma ((35), Appendix D) and endothelial cells (manuscript submitted, Appendix G), we also defined peptide sequences from TSP1 that mediate this interaction (Appendix E). This work was supported both by intramural NIH funds and the Army grant. We localized the region of TSP1 recognized by the \( \alpha_3\beta_1 \) integrin using recombinant fragments and synthetic peptides to amino acids residues 190-201 of TSP1, which promoted adhesion of MDA-MB-435 breast carcinoma cells when immobilized and inhibited adhesion of the same cells to TSP1 when added in solution. Adhesion to this peptide was enhanced by a \( \beta_1 \) integrin activating antibody, \( \text{Mn}^{2+} \), and insulin-like growth factor I (IGF1) and was inhibited by an \( \alpha_3\beta_1 \) integrin function-blocking antibody. The soluble peptide inhibited adhesion of cells to the immobilized TSP1 peptide or spreading on intact TSP1, but at the same concentrations did not inhibit attachment or spreading on type IV collagen or fibronectin. Substitution of several residues in the TSP1 peptide with Ala residues
abolished or diminished the inhibitory activity of the peptide in solution, but only substitution of Arg(198) completely inactivated the adhesive activity of the immobilized peptide. The essential residues for activity of the peptide as a soluble inhibitor are Asn(196), Val(197), and Arg(198), but flanking residues enhance the inhibitory activity of this core sequence, either by altering the conformation of the active sequence or by interacting with the integrin. This functional sequence is conserved in all known mammalian TSP1 sequences and in TSP1 from *Xenopus laevis*. The TSP1 peptide also inhibited adhesion of MDA-MB-435 cells to the laminin-1 peptide GD6, which contains a potential integrin-recognition sequence Asn-Leu-Arg and is derived from a similar position in a pentraxin module. Adhesion studies using recombinant TSP1 fragments also localized β1 integrin-dependent adhesion to residues 175-242 of this region, which contain the active sequence.

Recently, we have prepared a D-reverse analog of the α3β1 integrin binding sequence and demonstrated its activity to inhibit adhesion to TSP1 *in vitro* and to inhibit angiogenesis in the chick chorioallantoic membrane angiogenesis assay. Thus, the strategy we used successfully to develop stable analogs of the type 1 repeat peptides can be applied to develop angiogenesis inhibitors based on this novel TSP1 peptide. These novel peptide analogs may have unique activities as therapeutic angiogenesis inhibitors and may act synergistically with the type 1 repeat peptide analogs. These questions will be explored in our future efforts.

**TASK 2: Preparation of polymeric conjugates.**

**METHODS**

*Preparation of polysucrose conjugates*-- Polysucrose of average molecular weight of 70,000 or 400,000 (Ficoll, Pharmacia) was first functionalized with primary amino groups as previously described (36). This derivative, referred to as AECM-Ficoll (50 mg) was iodoacetylated in 1.35 ml of 0.15 M HEPES-NaOH buffer at pH 7.5 containing 1 mM EDTA by addition of 9.6 mg of iodoacetic acid N-hydroxysuccinimide ester (Sigma) dissolved in 0.15 ml of dimethylformamide. After about 15 min of reaction, the solution was passed over a desalting column to obtain the iodoacetylated AECM-Ficoll. Nine micromoles of peptide were dissolved in 1.8 ml of distilled water, and 250 μl of a 50 mM solution of tris-(2-carboxyethyl) phosphine hydrochloride (Pierce Chemical) in water was added to the peptide solution, and the pH was adjusted to 7.1 to 7.8 by addition of 1 M Na₂CO₃. After 30-60 min, the resulting solution was passed through a column packed with 1.4 ml of BioRad AG1-X8 anion exchange resin in the acetate form. The column effluent was led into the iodoacetylated AECM-Ficoll solution and the solution was stirred overnight at room temperature. The resulting solution was then dialyzed overnight against phosphate-buffered saline with several changes in a 12-14 kDa molecular weight cutoff tubing. Peptide concentration of the resulting solution was determined by measuring its absorbance at 280 nm using $E = 5540 \text{M}^{-1}\text{cm}^{-1}$ per Trp residue.

**RESULTS**

The peptide conjugates were prepared and demonstrated enhanced activity when tested *in vitro* for inhibiting heparin binding to TSP1 or for inhibiting proliferation of endothelial cells stimulated by FGF2 (Appendix B, Fig. 4 and Table 3). However, data published in
Appendix B, Fig. 5, demonstrated that the polymer conjugates did not have significant activity in the breast tumor xenograft model. Therefore, we have not pursued a thorough optimization of these conjugates as described in the Statement of Work. In studies with a collaborator, we have found that the polymeric conjugates have biological activity when used to treat retinopathy of prematurity in a rat model (Shafiee et al, manuscript submitted, Appendix ?). Thus, these conjugates do exhibit anti-angiogenic activity in vivo and may be useful as therapeutics for diseases other than breast cancer. The lack of activity of the conjugates in breast cancer may be due to a decreased bioavailability of the peptides in breast tumors when conjugated to polysucrose.

**TASK 3: Preparation of site-directed mutants of thrombospondin-1**

For specific aims 2 and 3, we have completed transfections with four full length THBS1 cDNA expression vectors containing point mutations in the type I repeats. Stable transfected cell lines expressing high levels of two of these constructs have been prepared, but stable lines expressing the other two constructs could not be obtained (W385A and F432A). In this final report, we present further characterization of the in vitro interactions of mutant and wild type TSP1 with breast carcinoma and endothelial cells.

**a) Construction of mutant expression vectors and verification of sequence:**

**METHODS**

We have prepared expression constructs containing four directed mutations of type I repeat sequences in TSP1 as follows.

The full length expression vector pCMVTHBS1 was used for preparation of site-directed mutations. Mutations in the central Trp residues of each Type I repeat and TGF beta activation sequence in the second repeat were prepared by filling a gapped plasmid in the presence of mutant primers using the linker scanning method (37). This method introduces a mutation directly into the double stranded plasmid template. Briefly, a frame of single stranded DNA encompassing only the region of interest is created within a double stranded plasmid (Fig. 1). The mutagenic oligonucleotide is used as a primer for the Klenow fragment of DNA Polymerase I that synthesizes the second strand of the target region and ligation is done using T4 DNA ligase. The schematic representations of the methods used are shown in the figure. The ligation mixtures were transformed into a mutS strain of E. coli, BMH 17- 81, with a defect in mismatch repair.

Screening for the clones that had the mutation was first done using ‘Touchdown’ PCR. This method minimizes mispriming of a specific primer that is designed to be an exact match at the 3' end to the mutant sequence by raising the temperature of primer annealing in the initial cycles of PCR. Thus, mutant products are preferentially amplified in the initial cycles and can preferentially serve as the template for amplification in the following cycles. The primers used for Touchdown PCR are listed below.

THBS W385A - Primers span bases 1025 to 1264 yielding a fragment size of 239 bp.
Forward primer: 5'- ATGAGCTGAGGCGGC- 3'

11
Reverse Touchdown primer : 5'- AGGTCCACTCGGACGC- 3'
THBS W441A - Primers span bases 1250 to 1432 yielding a fragment size of 182 bp.
Forward primer : 5'- GGTCCGAGTGGACCTCCTG- 3'
Reverse Touchdown primer : 5'- ATGACCACGGGGACGC- 3'

THBS W498G - Primers span bases 1349 to 1605 yielding a fragment of 256 bp.
Forward primer : 5'- TCCAGACACGGACCTG- 3'
Reverse Touchdown primer : 5'- GATGTCCCATGGTGACCC- 3'

THBS F432A - Primers span bases 1250 to 1405 yielding a fragment size of 155 bp.
Forward primer is the forward primer used to make THBS W441A
Reverse Touchdown primer : 5'- CACCATCCTGTTCAGC- 3'

Cell lysates from individual colonies obtained from the linker scanning mutagenesis were prepared by boiling in 40 μl of water for 5 minutes. 2 μl of the lysates were used as template for Touchdown PCR.

Plasmids from the selected clones were transformed into competent DH5alpha cells and validated by restriction analysis and complete sequencing of the DNA regions filled using the Klenow fragment. The remainder of the THBS coding sequence in each clone was screened for mutations by single strand conformation polymorphism (SSCP)-PCR using overlapping primer sets.

RESULTS

In order to study the role of potential anti-angiogenic sequences in the Type I repeats of TSP1 in regulating angiogenesis of breast and some other tumors, we performed site directed mutagenesis of an expression vector containing a full length THBS cDNA. Initial mutations were constructed to replace four amino acid residues shown to be critical for biological activities of synthetic peptides derived from the type I repeats. Central Trp residues in each type I repeat and the Phe residues required for activation of latent TGFβ were mutated to yield the following mutant thrombospondins: Trp(385)Ala, Trp(441)Ala, Trp(498)Gly, and Phe(432)Ala.

Touchdown PCR of mutant PCR fragments of thrombospondin using the respective specific primers showed distinct differences in the pattern of amplified products compared to native THBS sequence amplified using the same set of primers (Fig. 2). Lanes 2, 4 and 6 show the correct size product being amplified for the mutant PCR fragments. Comparing these to lanes 3, 5 and 7 respectively, either only the mutant product is amplified (lane 4 versus lane 5) or there is a difference in the patterns of mutant versus wild type (e.g. lanes 2 and 3). Once we established that we could differentiate mutant sequences using Touchdown PCR, we screened the bacterial colonies from the linker scanning method of mutagenesis. Figs. 3 and 4 show agarose gel electrophoresis of Touchdown PCR products from bacterial colonies screened for mutant sequences THBS W441A and THBS F432A. The positive clones were then sequenced between the fill-in sites to make sure other errors were not introduced by the enzyme used in the reaction. The mutant clone THBS W441A has a G > T substitution which changed a. a. 58 from Ala > Ser, and all other plasmids encoded the native TSP1 sequence except for the desired mutations.
WG1a is a mutant of wild type THBS clone that we obtained while preparing the mutant W498G DNA construct by the linker scanning method. Due to a two base pair deletion C at #1629 and A at # 1630), a frame shift and a premature stop codon were introduced at amino acid 516. Therefore, this protein lacks the carboxy terminus of TSP1 beyond the three type I repeats.

One other construct that we made was similar to the WG1a mutant. The N-terminus of THBS cDNA up to the end of third type I repeat (a.a.538) was cloned into an expression vector pCAGGS driven by a chicken beta actin promoter. This truncated mutant will be used to study the function of the type I repeats of TSP1 without interference from the other functional domains of TSP1.

b) Transfection and isolation of stable transfectants.

METHODS

MDA435 cells were transfected by electroporation using 10 μg of pCMVTHBS1 vectors containing the mutations listed in Table I (Appendix K) or pCMVneo vector control. Transfected cells were initially grown as a pool in complete medium. After 48 h, cells were selected as pools by growth in 700 μg/ml G418. After 2-3 weeks, resistant cells were cloned by seeding at limiting dilution in 96 well microtiter plates in medium supplemented with filtered conditioned medium from parental MDA435 cells. When the cells were subconfluent, the medium was replaced with 0.2 ml of serum free medium (CHO-S-SFM, Gibco BRL) containing G418. After 16 h, the conditioned medium was removed and stored at -70°C for ELISA analysis. Colonies arising from single cells that secreted TSP1 were expanded and cryopreserved in liquid nitrogen.

The serum-free conditioned media were assayed for expression of TSP1 by a sandwich ELISA. Microtiter plate wells were coated with 5 ng of Heparin-BSA (Sigma) in 50 μl of PBS by incubating overnight at 4°C. A 50 μl sample of each conditioned medium was added to the wells in 3-fold serial dilutions and incubated for 2 h at 37°C. The stably transfected clone containing the full length wild type THBS1 sequence (TH26 or 29) was used as the positive control and a pCMVneo transfectant was used as a negative control. The wells were blocked by incubation in Tris-BSA. The wells were aspirated and incubated with 50 μl of 1:500 dilution of rabbit anti-TSP1 in Tris-BSA for 2 h at 37°C. The wells were aspirated and washed 3 times with DPBS, 0.02% BSA, 0.02 mM phenylmethyl sulfonyl fluoride, 0.05% Tween 20 (DPBS-TWEEN). A 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (Kirkegaard and Perry) was added and incubated for 1 h at room temperature. The wells were aspirated and washed 3 times with DPBS-TWEEN. α-Phenylenediamine substrate (Sigma P8412) was diluted in phosphate/citrate/perborate buffer (Sigma P4922), and 50 μl was added to each well and incubated for 7-10 minutes. Development was stopped by addition of 100 μl of 3 M sulfuric acid. The clones that were positive for expression of TSP1 were cryopreserved in liquid nitrogen.

Clones identified by this assay were re-screened by Western blotting of serum-free conditioned medium to verify the size of the recombinant TSP1 secreted by the cells. TSP1 on the blots was detected using rabbit antibody to denatured TSP1 and peroxidase conjugated...
goat anti-rabbit IgG followed by visualization using ECL reagent (Amersham). Clones isolated by the above mentioned procedure were analyzed by RT-PCR to confirm they had the plasmid derived TSP1 mRNA.

RT-PCR analysis of clones expressing the mutant TSP1: Total RNA was extracted from transfected MDA cells. 4 μg of total RNA was used for the reverse transcription using M-MLV reverse transcriptase, and 10% of the RT reaction mixture was used as template for the PCR. In the first step of RT-PCR, an antisense primer was annealed to the mRNA at the 5' end of the thrombospondin sequence which would help transcription of the sequence upstream of the primer. The single stranded cDNA was then subjected to PCR using a nested antisense primer and a sense primer from the rabbit beta globin gene which is immediately upstream of the THBS gene in our expression vector. The rationale for doing this RT-PCR was to amplify only the transcript that was derived from the expression vector and not the endogenous THBS gene.

RESULTS
Stable transfectants were screened for over expression of mutant TSPs using a sandwich immunoassay with heparin-BSA as a capture ligand and rabbit anti-TSP 1 as detecting antibody (Fig. 5). Using peroxidase conjugated secondary antibody and o-phenylenediamine for development, conditioned serum free media from 20 clones were screened in one day. Clones with high expression identified by this assay were re-screened by Western blotting to verify the size of the recombinant TSP1 secreted by the cells using a new antibody raised to reduced and alkylated TSP1 (Fig. 6). In previous transfections using wild type pCMVTHBS1, a single clone was obtained that had undergone rearrangement resulting in expression of a truncated TSP1 (47). In the present screenings we identified several additional clones with similar rearrangements (e.g. Fig 6, clone A.A11). Several of these were saved for examining the activity of type I repeat mutants in the context of C-terminal deletions, which we have previously shown to abrogate the anti-tumor activity of TSP1 over expression. Selected clones expressing full length TSP1 by Western analysis were analyzed by Northern blotting to verify that the increased expression is due to expression of the transgene rather than activation of the endogenous THBS1 gene.

Three stable clones, AA11 expressing the truncated mutant, AE9 and EA3 over expressing the W441A mutant and clones A3D, C8E and C6E over expressing the W498G mutant have been isolated. However we were unable to make stable clones expressing the W385A and F432A mutant proteins. Although initial screening yielded several clones that expressed TSP1 of the correct molecular weight as evidenced by Western blot analysis, further examination of the origin of the TSP1 expressed in these clones by RT-PCR showed that they were negative for the mutant THBS mRNA. The well characterized serum response of the THBS1 promoter was used to differentiate expression of the transfected mutant from expression of the normally silent endogenous THBS1 gene (38). The serum induction of expression observed in these experiments indicated that the THBS F432A and W385A clones had up-regulated their endogenous THBS1 gene and were not expressing the stably integrated mutant TSP1.
Construction of epitope-tagged THBS Expression vector: Although TSP-positive clones were obtained from the F432A transfectants, the above data indicates that these clones have induced their endogenous gene. Because of the difficulty in differentiating mutant TSPs from TSP1 produced by up-regulation of the normally inactive endogenous gene, we prepared an epitope tagged THBS expression plasmid. We have made a THBS expression construct which has the 9E10 epitope sequence (EQKLISEEDL) derived from the human c-myc protein followed by six histidine residues at the 3' end of THBS sequence. The vector was constructed as follows. The 3' end ClaI - BclI fragment of THBS was PCR amplified using a sense primer corresponding to the sequence from base 2935 to 2953 spanning the ClaI site and an antisense primer which contained the sequence for the myc-his tag and the 3' end cloning site BamHI incorporated into it. The product of this PCR reaction, a 779 bp fragment, was cloned into pCMV neoBam Vector along with two 5' end restriction digestion fragments of THBS, a 1.3 Kb BamHI-EcoRI fragment and a 1.6 Kb EcoRI- ClaI fragment. A clone which had the coding sequence of THBS with the myc-his tag in the correct orientation has been isolated.

This construct was transiently expressed by electroporation of MDA435 cells, and expression of the tagged thrombospondin was detected by metabolic labeling and immunoprecipitation using anti-myc antibody (Fig. 7). The myc-tagged thrombospondin can be detected in lysates of pulse labeled cells (lane 2) and is detected in the medium after chasing for 3 hours (lane 1). We will next introduce the mutations into the myc-his construct, and use the plasmids to distinguish between the endogenous and exogenous gene expression.

c) Purification of mutant proteins and characterization of binding activities and effects on endothelial cell proliferation.

METHODS

MDA cells transfected with mutant thrombospondin gene were grown in RPMI medium containing 700 µg/ml G418. When the cells were approximately 80% confluent, the medium was replaced with CHO-S-SFM II medium (Life Technologies) containing 700 µg/ml G418. The medium was collected 48 hours later and centrifuged in polypropylene tubes in a Sorvall RC-5B centrifuge using SS-34 rotor at 15,000 x rpm for 15 min. The supernatant from this step was passed thorough a 0.45 µm low protein-binding syringe filter. The filtered medium was immediately used for the purification of thrombospondin.

Heparin affinity chromatography: A 1 ml HiTrap Heparin column (Pharmacia Biotech) was used. The column was washed with starting buffer (10 mM Tris, pH 7.5 containing 150 mM NaCl, 1 mM CaCl₂, 0.1 mM phenylmethyl sulfonyl fluoride, 5 mM benazamidine and 1 mM N-ethylmaleimide). About 200 ml of the conditioned medium was passed through the HiTrap Heparin column at a flow rate of 1 ml/min. The unbound material was collected and discarded. The column was then washed with 15 ml of the above buffer followed by a 6 ml of the starting buffer containing 0.35 M NaCl. The eluted materials at this step were discarded. The column was then eluted with the starting buffer containing 0.6 M NaCl and the eluted protein fractions were collected and stored at -70°C. Partially purified
thrombospondin thus obtained from several batches were combined and concentrated by passing through a HiTrap heparin column.

**Western blotting:** Heparin affinity chromatography-purified TSP1 was electrophoresed by SDS-PAGE under reducing conditions as described above. The separated proteins were transferred to PVDF membrane by electro blotting and the membrane was then blocked with non-fat dry milk. The membrane-bound proteins were then probed with polyclonal antibody raised against platelet TSP1. Horse radish peroxidase-coupled secondary antibody and ECL reagent (Amersham) were used to visualize the antibody reactive bands.

**Identification of complex formation between TSP1 and band-X:** Two independent methods were employed to identify the interaction between TSP1 and band-X. In the first method, TSP1/band-X complex purified by heparin affinity column was incubated with anti-TSP1 antibody-coupled agarose beads. The incubation was carried out for 3 hours at 4° C and the gel was rinsed thoroughly with buffer to remove unbound materials. The agarose gel was then boiled in SDS-PAGE electrophoresis sample buffer containing β-mercaptoethanol, centrifuged and the supernatant was analyzed by SDS-PAGE.

To examine the molecular size of band-X and its subunits, agarose gel electrophoresis was carried out under reducing and non-reducing conditions. 3% agarose gels were prepared in 0.5 M Tris buffer pH 8.8 containing 0.1% SDS. TH26 TSP1 containing band-X purified from heparin affinity chromatography was iodinated using iodogen (Pierce Chemical Co. IL) based on the manufacturer’s protocol. The 125I-labeled sample was then boiled in electrophoresis sample buffer with or without β-mercaptoethanol. Samples were then loaded onto agarose gel and electrophoresis was carried out at 100 volts for 1 hour. The gel was then dried onto a filter paper and exposed to film.

In the second method, TSP1 purified from human platelets was used to examine its interaction with band-X protein. 125I-radiolabeled TSP1 was used as the binding ligand in this experiment. The binding was carried out in 96-well ELISA plates using a band-X preparation from heparin affinity chromatography column. Some of the 0.65 M NaCl-eluted fractions contained exclusively band-X, which typically eluted at the trailing end of the protein peak. These fractions were pooled, concentrated and coated onto the wells of an ELISA plate. The coating was done at 4° C overnight using different dilutions of band-X. The wells were then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). BSA-coated wells were used as control. 125I-labeled TSP1 (150,000 cpm/per well) was then added and the plate was incubated at room temperature for 2 hours. The contents of the wells were then removed and the wells were gently rinsed with PBS. Individual wells were then removed from the plate using a pair of scissors and placed in counting vials. The radioactivity was measured using a gamma counter (Packard).

Characterization of the band-X protein was done by mass spectrometric analysis. The first step was to purify the TSP1/band-X complex from the culture supernatant by loading onto a Heparin-Agarose column and eluting the complex using 20 mM Tris buffer, pH 7.4, containing 0.65 M NaCl and 0.1 mM CaCl2. The fractions containing the TSP1/band-X complex were reduced and acetylated before loading on the Sephacryl 400 gel filtration column. TSP1 and band-X were eluted out of the gel filtration column using 20 mM Tris-HCl.
buffer, pH 7.6 containing 4 M Guanidine, 150 mM NaCl and 0.1 mM CaCl₂. The appropriate fractions containing the TSP1 and band-X were pooled, dialyzed extensively against water containing 0.02% Tween 20 and finally against water containing 0.02% Tween 20 and 1M ammonium bicarbonate. The dialyzed samples were lyophilized and redissolved in a smaller volume of water before analyzing them using 5% PAGE containing 6M Urea. The band corresponding to band-X was cut out of the Coomassie R 250 stained gel and analyzed by mass spectrometry.

RESULTS

While purifying the recombinant thrombospondins from the stable MDA435 cell lines, we discovered that the thrombospondin was tightly complexed with another secreted protein. This protein is referred to as band-X.

All purified TSP1 preparations except TH50, a C-terminus truncated form of thrombospondin, were found to contain band-X, (Fig. 8). Of the different cell lines tested, TH26-TSP1 was found to contain high levels of band-X secreted into the culture medium (Fig. 8; lane 4). During the purification TH26-TSP1, the band-X protein co-eluted with TSP1 from the heparin affinity chromatography column (Fig. 9; lane 2). Several methods were tested for the separation of these two proteins including gel filtration, ion-exchange chromatography under denaturing and nondenaturing conditions, lectin affinity chromatography and barium citrate precipitation method as described by Alexander et al. (39). However, thrombospondin and band-X protein consistently co-purified in each of these chromatographic methods.

Properties of band-X protein: The molecular weight of band-X protein was estimated to be 230-240,000 based on its electrophoretic mobility in SDS-PAGE. Band-X was readily digestible with trypsin or thrombin, showing that it is a protein molecule (Fig. 10). The composite results from this experiment and the lectin binding properties of band-X suggest that it is a glycoprotein. Figure 11 shows the results of Western blotting studies using different TSP1 preparations probed with anti-TSP1 polyclonal antibody. Although this antibody recognized all of the recombinant TSP1 preparations, it did not interact with band-X protein. In a separate experiment, this antibody was coupled to agarose gel and this immunoaffinity gel was used to examine TSP1 interaction. When TSP1/band-X mixture was incubated with the immunoaffinity gel, the bound material upon analysis by SDS-PAGE was found to contain band-X. This result suggests the possibility of a stable complex formation between TSP1 and band-X, and therefore the two molecules co-precipitated together. Control agarose gels without the antibody did not bind TSP1 or band-X.

To further examine the interaction between TSP1 and band-X protein, 125I-labeled TSP1 was allowed to interact with substratum-bound band-X protein in ELISA plates. Band-X protein was coated in decreasing dilution and no decrease in radiolabeled TSP1 binding was noticed up to a dilution of 1:8 (Fig. 12). However, further dilutions of band-X showed a dose dependent decrease in TSP1 interaction. These results indicate that TSP1, independent of its source, binds to band-X. When the radiolabeled proteins were resolved in a gel filtration column, the TH26-TSP1/band-X preparation was found to elute at the same point as platelet TSP1 (Fig. 13), showing the anomalous behavior of the TSP1/band-X complex. It is also
possible that the condition applied in the gel filtration column separated the complex into individual proteins, but due to their close molecular weight they both eluted as a single peak. Electrophoretic analysis of the eluted peak showed the presence of both proteins.

To study the electrophoretic mobility of band-X protein under non-reducing condition, agarose gel was used for electrophoresis. In this method, TH26-TSP1 and band-X together appeared as a single band under non-reducing condition suggesting that the two molecules have a similar molecular weight in their native form (Fig. 14; Lane 1). However, due to the difference in their subunit molecular weights, band-X and TH26-TSP1 appeared as two independent bands after reduction of disulfide bonds (Fig. 14; Lane 2). This result suggests that band-X protein may contain two subunits with similar molecular weights. The electrophoretic mobility of platelet TSP1 under reducing and non-reducing condition in agarose gel is shown in Fig. 14 lanes 3 and 4 for comparison.

The band that was tightly bound to the TSP1 protein was purified by preparative SDS gel electrophoresis, digested in gel with trypsin, and identified by mass spectrometry to be fibronectin secreted by MDA 435 cells. Since it is extremely difficult to separate the two proteins from each other in the conditioned media of stable MDA 435 clones, we decided not to pursue purification of mutant TSP1 proteins from the stably transfected MDA 435 clones. A protein of similar molecular weight also co-purified with TSP1 when over-expressed in a murine melanoma cell line that does not express endogenous murine TSP1 (K1735 clone TK) (40). Therefore this cell line could not be used to purify mutant thrombospondins. Preliminary microarray experiments performed in the lab has shown that Jurkat T lymphoma cells do not express endogenous fibronectin. Thus, Jurkat cells can be potentially used for the over expression of mutant thrombospondins. This work will be continued in the future to complete this task for publication.

**TASK 4: Determine effects of expression of mutated thrombospondin-1 on behavior of breast carcinoma and endothelial cells.**

a) Characterize level and stability of expression of mutant transfectants.

**METHODS**

The level of expression of mutant transfectant proteins was assayed by the sandwich ELISA and by Western blotting as mentioned earlier.

Metabolic Labeling and Immunoprecipitation of TSP1: 2x 10⁶ MDA435 cells were transfected with 10 μg each of the control vector, wild type THBS expression construct or the mutant DNA constructs by electroporation in a total volume of 25 μl. The electroporated cells were plated into tissue culture plate and allowed to grow for 24 hours in complete growth medium. At the end of 24 hours, cells were washed twice with methionine-free, serum-free medium and incubated at 37°C for another 1 hour in the same medium. The medium was aspirated from the wells and incubation was continued with 1 ml of methionine-free medium supplemented with 100 μCi [³⁵S]-methionine. For pulse-chase experiments, after 30 minutes
of labeling, monolayers were washed with growth medium, re-fed with 2 ml of growth medium supplemented with 0.2 mM unlabeled methionine and incubated for 30, 60 or 180 min.

After the metabolic labeling, the spent media from the wells were collected. The monolayers were lysed in 0.3 ml of RIPA buffer (50 mM Tris, pH 7.4 containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethyl sulfonyl fluoride and 10 µg/ml of Protease inhibitor cocktail) on ice for 10 minutes, and the cell lysates were centrifuged to remove debris. 200 µl aliquots of conditioned media and 150 µl aliquots of the cell lysates were used for immunoprecipitation of TSP1 with 6 µl of polyclonal anti-TSP1 antibody at 4°C for 1 hr. After 50 µl of Protein A- agarose was added, samples were further incubated at 4°C overnight. The immune complex which precipitated with Protein A- agarose was washed extensively with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1% NP-40. The final washed pellets were boiled in SDS- PAGE loading buffer followed by electrophoresis on a 4-15% gradient gel.

*Transient Transfection Experiments:* Transfection of MDA435 cells was routinely done by electroporation (Cell Porator, Life Technologies, Gaithersburg, Maryland) in precooled micro electroporation chambers with a 0.15 cm gap between the two flat-topped bosses. The chilled mixture of 2x10^6 cells and 10 µg DNA in a total volume of 25 µl of growth medium (RPMI 1640 supplemented with 10% FBS) was placed between the electrode bosses and electroporations were performed with an electrical pulse from a 50 µF capacitor charged to 150 V. The chambers were placed on ice for 15 min. after electroporation and then plated into one well of a 6-well tissue culture plate containing 3 ml of growth medium.

*RT-PCR Analysis of tumor tissue:* Total RNA was extracted from frozen samples of mammary fat pad tumors from mice that were injected with transfected MDA cells. 4 µg of total RNA was used for the reverse transcription using M-MLV reverse transcriptase, and 10% of the RT reaction mixture was used as template for the PCR as mentioned above.

**RESULTS**
Stability of expression of mutant transfectants was determined three ways to allow us to verify expression of several mutated thrombospondins. However, failure to isolate stable transfectants expressing F432A TSP1 and W385A TSP1 could indicate instability of these mutant proteins or their mRNA or an inability of the cells to process or secrete these mutant proteins. Lack of secretion of erythropoietin receptor following mutation of its WSXW sequence homologous to the thrombospondin type I repeats (41, 42) is consistent with this hypothesis. Alternatively, the mutant protein may be expressed but could be toxic to the cells. In order to determine if the expression of the mutant F432A protein was toxic to the MDA435 cells, we conducted green fluorescent protein (GFP) co-transfection experiments. Briefly, 2x10^6 cells were transfected with 10 µg of wild type THBS expression construct or F432A mutant construct along with 2.5 µg of pGreen Lantern -1 DNA (Life Technologies, Gaithersburg, MD) by electroporation (43). The electroporated cells were plated into tissue culture plates and allowed to grow for 24 or 48 hours. At the end of each time point, the cells
were viewed by fluorescent microscopy. Total number of cells and cells positive for GFP under fluorescent light were counted in three different fields of view for each of the transfections. The results of this experiment are presented in Table 2. At 24 hours, there was over 50% reduction in the number of GFP-positive cells in the F432A transfected cells compared to wild type THBS transfected cells; at 48 hours, only 30% of the mutant transfectants were GFP-positive compared to wild type THBS transfectants. Therefore, expression of the F432A mutant may cause cell death or inhibit growth.

To examine the alternate hypothesis that expression of the mutant proteins in MDA435 cells was difficult to achieve because the mRNA for that transgene was very unstable or the mutant protein failed to be secreted, we used pulse labeling to compare synthesis of the mutant and wild type proteins under low serum conditions. In order to compare the stabilities of wild type versus the mutant TSPs, we transiently transfected MDA435 cells with different DNA constructs, pulse-labeled them with \[^{35}\text{S}\] methionine and chased with cold methionine for 30, 60 and 180 min. Cells transfected with pCMV control vector showed minimal synthesis and secretion of TSP1 after a 30 min. Pulse labeling and chase for 60 or 180 min with unlabeled methionine. Cells transfected with wild-type THBS vector showed high levels of TSP1 in the cell lysate after a 60 minute chase and secreted TSP1 was detected in the medium after 3 h (Fig. 15). Cells transfected with the W385A and F432A mutant THBS plasmids showed similar expression and secretion as the wild type transfectant, indicating that synthesis and secretion of these mutant thrombospondin proteins is normal. Similar results were obtained with the other mutant proteins.

Another method used to detect the stability of the mutant protein was to examine the tumors formed in the mammary fat pads of mice for expression of the plasmid to verify that expression of the mutated TSP1 was retained throughout tumor growth in the animals. In the case of the stable clones of W441A that were implanted in the mammary fat pads of nude mice, at sacrifice, portions of each tumor were frozen in liquid nitrogen for preparation of total RNA. RT-PCR was used to confirm the tumors had the plasmid-derived TSP1 mRNA. Figure 16 shows the products of RT-PCR of tumor RNA from animals which received THBS W441A clones. Every one of the tumors showed the expected product, although the amount of expression varied from tumor to tumor. The mean expression of the plasmid-derived TSP1 RNA in the tumors shown in lanes 3, 4 and 5 are lower than the mean from tumors shown in lanes 6, 7 and 8. Tumors in lanes 3, 4 and 5 and 6, 7 and 8 came from mice injected with clones AA11 and EA3 of THBS W441A respectively. This is interesting because while wild-type TSP1 expression in tumors has shown to produce smaller primary tumors and a reduction in capillary densities (47, 58) the tumors from the clone AA11 with a spontaneous truncation mutation similar to TH50 are bigger compared to the ones from other clones.

**b) characterize behavior of transfected cell clones in vitro:** To circumvent the problem we faced purifying mutant proteins from stably transfected clones, we followed two different strategies.

**METHODS**

**Transient transfection and uptake of \(^{3}\text{H}-\text{thymidine}**: Bovine aortic endothelial cells

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(BAE Cells) between passages 3 and 13 or MDA435 breast cancer cells between passages 4 and 20 were used for transient transfections by plasmid expression vectors carrying the wild type and type I repeat mutant cDNAs of TSP1. Transfections were done by electroporation. The protocol used for transfection is briefly outlined as follows.

MDA435 cells or BAE cells were trypsinized and resuspended in growth medium. 2x10⁶ cells were aliquoted into sterile microfuge tubes containing varying amounts of THBS DNA constructs used for transfection and 5 µg of a plasmid expression vector for β-galactosidase. The total volume was adjusted to 25 µl and incubated on ice for 20 minutes. The cell-DNA mixtures were then transferred into prechilled electroporation chambers taking care to suspend them between the two electrode bosses. The mixtures were exposed to a 1 sec. pulse from a 50 µF capacitor charged to 150 volts. Electroporation chambers were immediately placed on ice for another 20 min. The mixture was then resuspended into 3 ml of medium containing 1% FCS (for BAE cells) or 10% FCS (for MDA435) and plated into two wells of a Nunc 6 well plate. One well was used for ³H-thymidine labeling and the other was used for protein and β-galactosidase activity assays.

24 hr after the transfection, the medium was replaced with 1 ml of medium supplemented with 1 or 10% FCS and one well from each transfection was labeled with 2.5 µCi of ³H-thymidine/ml medium. Labeling was continued for 4 hr at 37°C in a CO₂ incubator. At the end of incubation the cells were washed twice with DPBS, fixed with methanol/acetic acid and extracted with 600 µl of trypsin/versene at 37°C for 1 hr and at RT for 30 min. 200 µl of 1% SDS were added to the cell extracts which were then counted using a scintillation counter to determine the total uptake of ³H-thymidine.

The well containing the other half of the transfected cells was washed and extracted with 300 µl of ice cold extraction buffer (0.1 M sodium phosphate, pH 7.4 containing 0.5% triton X-100). The cell extracts were freeze-thawed three times and cell lysates were cleared by centrifugation at 12,000g for 15 min at 4°C. Aliquots of cell lysates were assayed for total protein content by the BCA method and for β-galactosidase activity using nitrophenyl β-galactopyranoside as substrate. The β-galactosidase activities of the cell lysates were expressed as mU/µg protein standardized using purified E.coli β-galactosidase (Sigma).

**Bioassay for inhibition of endothelial and breast carcinoma cell proliferation:**
Proliferation of bovine aortic endothelial cells was determined as previously described (30) in the presence of recombinant fragments of TSP1 as GST fusion proteins.

**RESULTS**

MDA435 cells were transiently transfected with varying amounts of THBSI wild type (WT) DNA or mutant DNA construct to study their effects on the proliferation of the cells. Proliferation was measured as the total uptake of ³H-thymidine by transiently transfected MDA435 cells. As shown in Figure 17 A, when the assay was done in the presence of 10% FCS, dose-dependent inhibition of proliferation was seen which was maximal at a DNA concentration of 24 µg in the case of cells transfected with THBS-WT DNA. Under similar conditions F432A DNA transfected cells showed very little inhibition of proliferation, even though the transfection efficiency remained above 100% of that for the β-galactosidase indicator vector alone (Fig. 17 B). Inhibition of thymidine incorporation in this assay is a
measure of DNA synthesis in the bulk population, the majority of which are not transfected, as assessed by X-Gal staining using the β-galactosidase reporter. Thus inhibition following transfection using wild type THBS vector results from accumulation of TSP1 in the medium which is secreted by the minority of transfected cells. Failure of the F432A mutant to inhibit thymidine incorporation indicates that this protein does not accumulate. This probably results from the rapid loss of cells expressing this mutant as detected by the GFP reporter studies.

Although we have demonstrated that platelet thrombospondin-1 inhibits proliferation of MDA435 cells (26), endothelial cells are much more sensitive to thrombospondin and are believed to be the physiological target for its anti-angiogenic activities. Effects of transient expression of wild type TSP1, the KRFK mutant F432A and the type I repeat mutant thrombospondins on bovine aortic endothelial cells were assayed in a similar manner, and the results are presented in Table 3. With increasing amounts of wild type THBS DNA used for transfection, a dose dependent inhibition of BAE cell proliferation is seen, while the transfection efficiency remains above control levels using the β-galactosidase vector alone. We also transfected BAE cells with 15-20 μg of the mutant DNAs. In the case of BAE cells transfected with W385A mutant construct, 15 μg of the DNA used for transfection did not show inhibition of incorporation of 3H-thymidine. On the contrary, comparable amounts of W441A and W498G mutants had inhibitory effects similar to that of 18 μg of WT DNA.

WG1a is a mutant of wild type THBS clone that we obtained while preparing the mutant W498G DNA construct by the linker scanning method. Due to a two base pair deletion (at #1629 and A at #1630), a frame shift and a premature stop codon were introduced at amino acid 516. Therefore, this protein lacks the carboxy terminus of TSP1 beyond the three type I repeats. Transient transfection of BAE cells with 16.5 μg of the WG1a mutant strongly inhibited BAE cell proliferation, with only 20% 3H-thymidine being incorporated into the cells compared to the control. Transfection efficiencies in all the cases were high (Table 3), indicating that expression of this construct was not toxic to the cells.

Alternatively we used bacterial fusion proteins to map the antiproliferative activities of thrombospondin. Proliferation of bovine aortic endothelial cells was determined in the presence of recombinant GST-fusion proteins expressing the Type I, Type II, Type III and C-terminal domains of thrombospondin (Fig. 18). Procollagen and Type I repeat domains showed a dose-dependant inhibition of endothelial cell proliferation compared to medium control or GST control. At a concentration of 25 μg/ml, the recombinant procollagen domain inhibited BAE cell proliferation by about 35%, and at the same concentration the Type I repeat showed an inhibition of over 80% of control. The other recombinant fragments tested in this assay did not show a dose-dependant inhibition of BAE cell proliferation. These data demonstrate a specific antiproliferative activity for endothelial cells in the type I repeats and demonstrate a differential role of β1 and β3 integrins in adhesion of the two cell types. The RGD sequence in the type III repeats is recognized by endothelial cells but not by the breast carcinoma cells, which accounts for differential recognition of TSP1 fragments by these two cell types.

We have now identified the α3β1 integrin as the primary receptor on two breast carcinoma cell lines for TSP1. A published manuscript describing this work is attached (Appendix D) and is summarized below.

The α3β1 integrin, with some cooperation of sulfated glycoconjugates and α4β1
integrin, mediates adhesion of MDA-MB-435 and MDA-MB-231 breast carcinoma cells to TSP1. This β1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1 and ligation of CD98. In MDA-MB-231 cells, the inactive state of the α3β1 integrin is maintained by a G-protein mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β1-dependent adhesion to TSP1 do not stimulate β3-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor αvβ3 and this integrin is functional and inducible for vitronectin adhesion. We do not know why the αvβ3 integrin on MDA-MB-435 cells can not recognize the RGD sequence in the type III repeat of platelet TSP1. Other cell types, however, can utilize the same TSP1 preparations used for these experiments to support αvβ3-dependent adhesion (44).

The α3β1 integrin in MDA-MB-435 cells does not recognize the RGD sequence in the TSP1 type 3 repeats. Because we have tested 85% of the TSP1 primary sequence using recombinant fragments or synthetic peptides, the β1 recognition motif may not be a linear epitope in TSP1. One caveat in interpreting the negative results using the recombinant TSP1 fragments, however, is that misfolding of fragments expressed in bacteria could selectively mask a linear recognition sequence for the α3β1 integrin in the GST- or T7-fusion proteins. Several β1 integrins have been implicated as TSP1 receptors in other cell types, including α2β1 on activated platelets (45), α3β1 on neurons (46), and α4β1 and α5β1 on activated T lymphocytes (47). αβ1 is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas α2β1 mediates adhesion of these cells to type I collagen but not to TSP1. The integrin α4β1 may play some role in breast carcinoma adhesion to TSP1, as we previously reported for T lymphocytes (47). The mechanism for the apparent differential recognition of TSP1 by β1 integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by “inside-out” signaling (48), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to αvβ3 integrin, the α3β1 integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 “VVM” peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β1-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via αvβ3 but not α3β1 integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of α3β1-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically associated with the αvβ3 integrin but not with β1 integrins in fibroblasts (49). The αvβ3 integrin also co-immunoprecipitated with insulin receptor substrate-1 (50). Engagement of αvβ3 integrin by vitronectin but not α2β1 integrin by collagen increased mitogenic signaling
through the insulin receptor (49, 50). Thus, the specific activation of α3β1 mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the α3β1 integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific coupling of α3β1 activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

We have also identified a TSP1 peptide that modulates angiogenesis and the in vitro behavior of endothelial cells through binding to α3β1 integrin. A submitted manuscript describing this work is attached and is summarized below (Appendix G).

We have demonstrated that sparse endothelial cells recognize an α3β1 integrin-binding sequence in TSP1 that stimulates endothelial cell spreading and proliferation when immobilized on a substratum. Addition of this peptide in solution inhibits endothelial cell spreading on TSP1, endothelial cell migration in vitro (Appendix G, Fig. 8), and angiogenesis in vivo (Appendix G, Fig. 9), presumably by inhibiting TSP1 interactions with this integrin. We have also demonstrated that the activity of this integrin to recognize TSP1 is suppressed in a confluent endothelial cell monolayer (Appendix G, Fig. 1). Loss of endothelial cell-cell contact during wound repair in vitro or angiogenesis in vivo could therefore activate this receptor and make the endothelial cells responsive to TSP1 signaling through the α3β1 integrin. Thus, recognition of immobilized TSP1 by α3β1 integrin may increase endothelial cell proliferation and motility during wound repair and angiogenesis in vivo.

c) Conduct animal studies of tumorigenesis and spontaneous metastasis.

METHODS

Tumorigenesis assay in nude mice: NIH Nu/Nu mice or NIH Beige XID mice, approximately eight weeks of age were injected with 10^5 MDA MB435 cells by the mammary fat pad route. Wild type MDA cells were used for peptide studies; stable transfectants expressing full length wild type or mutant TSP1 were used to examine the effects of site-directed mutations in TSP1 on tumorigenesis. Mice were anesthetized with 150-200 µl i.p. of a 1:80 dilution in PBS of a solution containing 25 g. tribromoethanol in 12.5 ml tertiary amyl alcohol. The mammary fat pad was cleaned with ethanol and a 10 mm incision was made directly above the site of injection. Using a 0.1 ml Hamilton syringe and 27 gauge ½" needle, 10 µl of cell suspension, 1 x 10^5 cells in HBSS for nu/nu mice or 4 x 10^5 for Beige mice, were injected into the fat pad. The incision was closed using 1-2 Autoclips (9 mm, Clay Adams). Autoclips were removed 7 days post-injection.

8-10 animals are injected for each condition, per experiment. Animals were ear punched after injection for subsequent identification. Beginning at day 25 and continuing every day until day 50, the experimental animals for peptide treatment were injected i.v. (tail vein) 100 µl of the free peptide or ficoll conjugates. Animals implanted with transfected MDA cell lines were not treated. Primary tumor size was determined twice weekly by length x width x height measurement, and the animals were observed daily for general health. When
the primary tumor of any animal exceeded 20 mm in any dimension, all of the animals were sacrificed. The presence of metastases was determined by gross autopsy and examination of H & E stained sections of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues, and weighed. At any time during the experiment, animals suspected of being in distress were sacrificed and examined as above.

RESULTS

Three transfectant clones of MDA MB435 with high levels of TSP1 expression, AA11, AE9 and EA3 from THBS W441A transfection were selected for in vivo animal experiments. In the animal experiment, each group had 8 animals that received one clone of transfected cells. After 84 days, the animals were sacrificed and the tumors were removed. Figure 19 shows the growth curves for the different clones in athymic nude mice. Clones from THBS W441A transfection had the same or larger tumor masses compared to controls.

Histopathological analyses of these tumors showed that the tumors produced by the W441A clone producing the largest tumors, EA3, had relatively little necrotic area in the tumors compared to the control transfectants, although the former tumors were much larger. The lack of necrosis in these large tumors implies that angiogenesis in the W441A tumors is more efficient than in the control transfectants. Examination of the lung sections showed that 7 out of 8 (87%) of the W441A clone EA3 had lung metastases, whereas none of the control animals had detectable lung metastases.

We have also repeated this experiment using Beige XID mice, deficient in NK, T and B lymphocytes, to examine whether inhibition of tumor growth by over expression of thrombospondin and the lack of inhibition by W441A thrombospondin required interactions with components of mouse immune system other than mature T cells that are functional in athymic nude mice but not in the Beige mice. This was important to examine since we observed increased infiltration of mononuclear cells in tumors formed by THBS transfectants (10), and thrombospondin was recently reported to modulate activation of NK lymphocytes (51). Our data demonstrate, however, that the THBS transfected MDA435 cell lines retain their inhibition of tumor growth in Beige mice, which lack NK cells, relative to control transfectants (Fig. 20). Furthermore, the transfectants over expressing the THBS W441A mutant do not show a growth inhibition, and one clone tested produced larger tumors than the control transfectant tested (Fig. 20). This provides further support for our hypothesis that anti-angiogenic activity accounts for the observed growth inhibition in the mouse xenografts and that an intact Trp motif in the second type I repeat is required for this inhibitory activity.

Although one animal experiment was done wherein stable clones expressing the W498G mutant TSP1 were implanted in nude mice, the tumor take was very poor (only 20% of the animals in the positive control group formed tumors) making it difficult to draw any conclusions from it. That experiment will be repeated in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Development of stable thrombospondin-1 peptide analogs and demonstration of their
anti-angiogenic activity and anti-tumor activity for breast carcinoma xenografts.

- Discovery that TSP1 and the TSP1 type 1 repeat peptides selectively induce apoptosis of endothelial cells.
- Mutagenesis of full length TSP1 expression vectors.
- Discovery that α3β1 integrin is the major TSP1 receptor mediating adhesion and chemotaxis of breast carcinoma cells.
- Discovery that the activity of α3β1 integrin to interact with TSP1 is regulated by insulin-like growth factor-1 and CD98.
- Demonstration that α3β1 integrin plays an important role in regulation by TSP1 of endothelial cell proliferation in vitro and angiogenesis in vivo.
- Identification of a the peptide sequence in TSP1 recognized by the α3β1 integrin and demonstration that this peptide is also a potent inhibitor of angiogenesis.

REPORTABLE OUTCOMES

Manuscripts


in a bovine retinal explant assay and a rat model of retinopathy of prematurity.
(submitted)


Abstracts


Patents
A provisional U.S. Patent Application "α3β1 Integrin binding peptide from thrombospondin-1 and their uses" was filed on July 15, 1999.

Development of cell lines
We have developed a series of MDA MB 435 breast carcinoma-derived cell lines that stably express various mutated thrombospondin-1 constructs. These will be made available to the scientific community following publication of a manuscript describing their properties.

Employment and research opportunities:

Mr. Rui Rodrigues received training supported by this grant and was a coauthor on the manuscript presented in Appendix D. Based in part on this training, he was accepted and is currently attending Medical School at Howard University.

Dr. S. Chandrasekaran received training supported by this grant and was the first author on the manuscript presented in Appendix ?. Based on this training, he was offered and accepted a position at Data Unlimited, Inc. which develops computer systems to assemble and analyze DNA data bases.

Dr Neng-hua Guo performed some of the initial work supported by this grant, although he did not receive salary support. He was first author on the two manuscripts presented in Appendices B and C and was a co-author on two other papers. After completing his
postdoctoral training, he accepted a Senior Staff Fellow position in the National Heart, Lung, and Blood Institute.

CONCLUSIONS

Development of stable peptide analogs to inhibit angiogenesis of breast cancers

We have examined two approaches to prepare angiogenesis inhibitors based on the type 1 repeats of TSP1. D-reverse analogs showed activity in both classical angiogenesis assays (chick CAM assay) and in a breast cancer xenograft model. Since these are small molecules with defined structure, they should be attractive lead compounds for drug development of therapeutic angiogenesis inhibitors. We will continue to develop the existing peptide analogs toward clinical application in collaboration with the NCI Developmental Therapeutics Program.

Our efforts to apply polymeric conjugates of the TSP1 peptides for breast cancer were unsuccessful. These conjugates are active in vitro and exhibit anti-angiogenic activity in a retinopathy of prematurity model. The larger size of these conjugates may aid their retention in the vitreous humor. These data demonstrate that different pathological conditions associated with angiogenic responses may differ in their response to specific angiogenesis inhibitors.

Functional analysis of anti-angiogenic sequences in thrombospondin-1

Full length thrombospondin-1 expression constructs containing four site-directed mutations of the type I repeat sequences have been prepared. These disrupt the WSXW motifs in each type 1 repeat and the latent TGFβ-activating sequence at the border of the first and second type 1 repeats. Stably transfected human breast carcinoma cell lines have been prepared expressing two of these mutants. Mutation of the central Trp residue in the second type 1 repeat resulted in reversal of the effect of TSP1 over expression on the tumorigenic potential of MDA-MB-435 cells. Suppression of tumorigenesis by expression of wild type TSP1 but not the W441A mutant was observed both in athymic nude mice and Beige XID mice, demonstrating that the anti-tumor activity of TSP1 does not require NK, B, or T cell responses by the host. Reversal of the anti-tumor activity of TSP1 following the W441A mutation is consistent with our hypothesis that the WSXW motifs play a role in the anti-tumor activity of thrombospondin but is not consistent with the recent report that binding of a different sequence in the type I repeats to CD36 mediates its anti-angiogenic activity (52).

Based on transient expression, disruption of the WSXW motifs in the first but not the second or third type 1 repeats abolishes the ability of TSP1 to inhibit endothelial cell proliferation. The mutant TSP1 with the latent TGFβ-activating sequence disrupted, however, retains its antiproliferative activity for endothelial cells. Therefore, this sequence is not required for the antiproliferative effect of TSP1 on endothelial cells. Analysis of a truncated mutant TSP1 (WG1a) suggests that TSP1 fragments lacking the carboxyl-terminal domains may have increased anti-angiogenic activity.

Expression of the F432A mutant inhibits proliferation of endothelial cells in transient transfections, but the same mutant can not accumulate to sufficient levels to significantly
inhibit thymidine incorporation in transiently transfected breast carcinoma cells. This may explain our failure to isolate any stable MDA-MB-435 cell transfectants over-expressing this mutant transgene. F432A and W385A mutants are both properly folded and secreted in MDA-MB-435 cells. This contrasts with the mutants in the WSXW motif in the erythropoietin receptor, which was shown to be essential for delivery of this protein to the cell surface (41, 42). We will continue to examine the stability and fate of these mutant thrombospondins in the breast carcinoma cells.

Thrombospondin type I repeat mutants have been shown to inhibit proliferation of bovine aortic endothelial cells to different degrees. While the W385A mutant showed little to no inhibitory effect in our thymidine incorporation assay, both W441A and W498G showed 50-60% inhibition of cell proliferation. We were also unable to isolate stable clones that express the mutant W385A protein. In this regard we think that the W385A protein might be functioning in a way similar to the F432A mutant protein. We have made a truncation mutant of wild type TSP1, WG1a, which lacks the entire sequence 3' to the type I repeats. We have also shown that this mutant is a potent inhibitor of proliferation. Based on a recent report that gene therapy using a nonviral construct expressing a similar fragment of TSP1 in conjunction with p53 decreased breast tumor xenograft growth in nude mice (53), we will further examine the activities of this truncated TSP1.

We have reported in our earlier annual report that the F432A mutant protein is synthesized and secreted normally by the MDA-MB-435 cells. We had also reported that at 24 and 48 hours post transfection 50% and 30% cells compared to control were respectively positive for GFP protein, an indicator of cells that were transfected. So we had concluded that the mutant protein F432A is toxic to the MDA-MB-435 cells. Next, we wanted to see the effects of this mutant on incorporation of \(^3\text{H}\)-thymidine, an indicator of cell proliferation. The TSP1 mutant F432A does not show a dose-dependent effect on inhibition of thymidine incorporation in MDA-MB-435 cells (Figure 1). When MDA-MB-435 cells are transfected with 24 µg of F432A DNA, the thymidine uptake stays at about 90% of control. This could mean that at higher concentrations the mutant F432A protein is toxic to the cells expressing it and the measured thymidine incorporation is that of untransfected cells. We have seen some inhibition of endothelial cell proliferation by the F432A mutant. In preliminary experiments, lower doses of this plasmid have produced stronger inhibition. This inverted dose-response suggests that transient transfection using lower doses of the F432A plasmid may allow us to examine the anti-proliferative activity of this mutant in both cell types.

Since it had been shown earlier that in MDA231 cells uPA and uPAR were up-regulated by thrombospondin (54), we wanted to see if TSP1 expression had similar effects in MDA-MB-435 breast cancer cells. Compared to the parent cell line, stable over-expression of TSP1 was associated with a ten-fold increase in the uPAR level in the stable clone TH26, there was no significant change in the uPA level (Table 3). The interesting observation was that when MDA-MB-435 cells were transiently transfected with THBS-WT DNA or the empty vector, the uPAR level remained unchanged. Thus it seems that the increased uPAR level seen in the stable clone TH26 may not be directly due to the over-expression of thrombospondin.

Identification of the α3β1 integrin as a regulated TSP1 receptor on breast carcinoma cells

The α3β1 integrin mediates adhesion of MDA-MB-435 and MDA-MB-231 breast
carcinoma cells to TSP1. This β1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1 and ligation of CD98. In MDA-MB-231 cells, the inactive state of is maintained by a G-protein mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β1-dependent adhesion to TSP1 do not stimulate β3-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor αvβ3 and this integrin is functional and inducible for vitronectin adhesion. It is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

CD98 was recently identified as an activator of β1 integrins by its ability to overcome Tac-β1 suppression of β1 integrin function (55, 56). Our data demonstrate that clustering of CD98 can also increase α3β1-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated α3β1 integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis can not be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the α3β1 integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could be result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including H-Ras (57), integrin-linked kinase, and protein kinase C (48). Additional proteins are known to associate with the α3β1 integrin, including some members of the TM4SF family and EMMPRIN (58, 59), but their roles in regulating function are unknown. In MDA-MB-231 cells, suppression of α3β1 appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the α3β1 integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using α2β1 integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better α3β1-dependent adhesion to TSP1 than α2β1-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores demonstrates that regulation of α3β1 activity for TSP1 may differ even between two cell lines derived from the same type of human cancer.

The α3β1 integrin also plays a role in TSP1 interactions with endothelial cells

To date, several receptors for TSP1 have been described on endothelial cells, including the αvβ3 integrin, low density lipoprotein receptor related protein (LRP), proteoglycans, CD47, and CD36. A recent publication concluded that CD36 expression is necessary to
mediate an anti-angiogenic activity of TSP1 (52). Thus, we did not expect the α3β1 integrin to play a significant role in interactions of TSP1 with endothelial cells. However, using the TSP1 peptide ligand we identified for this integrin as well as specific blocking antibodies, we clearly demonstrated that the α3β1 integrin mediates spreading of endothelial cells on TSP1 and that engaging this integrin by TSP1 can induce proliferation of endothelial cells. By this mechanism, we show that TSP1 can have a pro-angiogenic activity and that the peptide that binds to this integrin can inhibit endothelial cell proliferation in vitro and angiogenesis in vivo, independent of CD36. This result suggests that this peptide could be used as a lead to develop a new class of angiogenesis inhibitors that act by disrupting α3β1 integrin function on endothelium.

Differential regulation of the α3β1 integrin in breast carcinoma and endothelial cells

Although IGF1 is a major regulator of the activation state of the α3β1 integrin in breast carcinoma cells, this growth factor did not activate the same integrin in endothelial cells. None of the growth factors we have tested could influence the activation state of endothelial cell α3β1. Rather, cell-cell contact appears to be the major regulator of activation in endothelial cells. Loss of cell-cell contact activates the α3β1 integrin to recognize TSP1, and restoration of contact inactivates the integrin. Loss of cell-cell contact must occur during neovascularization of a tumor. This regulation may therefore be important for understanding the control of tumor angiogenesis. Intact TSP1 inhibits angiogenesis in most contexts, but expression of some TSP1 fragments (10) or whole TSP1 in specific contexts can stimulate angiogenesis (60, 61). By understanding that TSP1 contains both anti- and pro-angiogenic sequences and identifying the respective receptors that mediate each response, we can both improve our molecular understanding of tumor angiogenesis and develop strategies to inhibit this essential step in breast cancer progression.

'So what' Section:

As a scientific product, this project has yielded a new understanding of how thrombospondin-1 acts both on endothelial and tumor cells. Identification of important receptors for thrombospondin-1 on breast cancer and endothelial cells and their differential regulation will help to define how thrombospondin-1 expression influences progression of breast cancer.

Based on our demonstration of efficacy in treating breast cancer xenografts in mice, this project could also yield a medical product. The peptides developed with support of this grant are currently under development at the NCI Developmental Therapeutics Program for application as therapeutic angiogenesis inhibitors. Through work with several collaborators, we have demonstrated their effectiveness as therapeutic angiogenesis inhibitors in animal models of three important diseases: glomerulonephritis (33), retinopathy of prematurity (Shafiee et al, manuscript submitted), and brain cancer (34). Thus, we are encouraged to continue exploring their utility in breast cancer.
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Regulation of tumor growth and metastasis by thrombospondin-1

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ABSTRACT Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that influences cell adhesion, motility, and growth. Based on its effects on tumor and endothelial cell behavior, this member of the thrombospondin gene family has attracted interest as a potential regulator of tumor growth and metastasis. Initial studies have confirmed that increased TSP1 expression suppresses growth or metastasis of some tumors in vivo and inhibits angiogenesis. These activities are cell type specific, however, since overexpression of TSP1 in some tumors causes increased tumor progression. One basis for these apparently conflicting observations may be the complexity of the protein. TSP1 interacts specifically with several cell-surface receptors, heparan sulfate proteoglycans, growth factors, and other matrix components. These multiple binding specificities, combined with the ability of TSP1 to activate latent transforming growth factor β and inhibit several proteases, suggest that exposure to TSP1 may initiate several intracellular signals. The integration of these signals may allow varied responses to TSP1. Furthermore, these signals may be received by the tumor cells, endothelial cells responsible for neovascularization, stromal cells, or cells of the host immune system. TSP1 influences specific behaviors of each cell type. Relating these phenomena to the molecular interactions of TSP1 observed in vitro may lead to novel therapeutic strategies for controlling cancer progression and metastasis.—Roberts, D. D. Regulation of tumor growth and metastasis by thrombospondin-1. FASEB J. 10, 1183–1191 (1996)

Key Words: tumorigenesis • angiogenesis • cell-matrix interactions

GROWTH AND METASTASIS of tumors require many interactions between tumor cells and the host. The primary tumor must recruit a blood supply to obtain nutrients (1) and interact with the immune system in a manner that allows the tumor to avoid immune surveillance. A metastatic tumor must further accomplish a complex cascade of interactions with the host, including degradation of the extracellular matrix surrounding the primary tumor, invasion of tumor cells into blood vessels or lymphatics, homing to a target organ, extravasation, and migration through and degradation of matrix barriers in the target organs (2). These processes are mediated by specific expression in tumor cells of growth factors, motility factors, degradative enzymes, adhesion molecules, and specific receptors for each of these molecules. Tumor progression and metastasis also require loss of expression by the tumor cells of specific inhibitors of the growth factors, motility factors, and proteases and loss of specific adhesion molecules or their receptors that prevent tumor progression and invasion.

Adhesion molecules have attracted interest as potentially important regulators of tumor growth and metastasis after early observations that fibronectin is lost from the surface of some transformed cells. Manipulation of the expression of individual cell-matrix and cell-cell adhesion proteins in tumor cell lines (e.g., E-cadherin, NCAM, syndecan, thrombospondin-1 [TSP1], some integrins) has been shown to be sufficient to suppress tumor growth or metastasis in animal models (reviewed in ref 3). Based on these results, efforts have been mounted to identify antagonists and modulators of adhesion molecules and to apply these materials as novel cancer therapeutics that may have fewer side effects than conventional cytotoxic agents.

A potential role for the extracellular matrix glycoprotein TSP1 in cancer is suggested by its regulated expression in many normal and tumor cells and by its ability to modulate adhesion, motility, or growth of many cell types in vitro (4, 5). TSP1 is the first identified member of the thrombospondin gene family (6). Because most data are available for TSP1 and the role of other thrombospondin family members in cancer is only beginning to be examined, this review will consider only the function of TSP1. Several recent reviews of the thrombospondins should be consulted for an overview of TSP1 functions (4, 5, 7) and the function and expression of other members of the gene family (4, 6, 8).

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2Abbreviations: TSP1, thrombospondin-1, product of the THBS1 gene; TGF-β, transforming growth factor β; FGF-2, basic fibroblast growth factor; HSPG, heparan sulfate proteoglycan; IAP, integrin-associated protein or CD47; LRP, low-density lipoprotein receptor-related protein; NK, natural killer; PAI-1, plasminogen activator inhibitor-1.

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Both correlative data (9-14) and transfection experiments (15, 16) support the hypothesis that expression of TSP1 may decrease tumor growth or metastasis. However, conflicting results have been obtained (17, 18), and the effects of TSP1 appear to be specific both for the types of tumor examined and the experimental model used. These apparently conflicting data have led to some confusion about whether TSP1 should be classified as a tumor suppressor or as a promoter (15, 19, 20). Although much additional work is needed to define the net effect of endogenous TSP1 expression in tumors, we will attempt to define the effects of TSP1 on specific steps in the cascade of tumor progression (Fig. 1) and present evidence for the mechanism of these activities.

Understanding how cells respond to TSP1 is crucial to defining their responses to this protein. Signal transduction through interactions with extracellular matrix components is now recognized as an important regulator of cell growth and differentiated function (21). As is generally found in extracellular matrix proteins (22), TSP1 is a large protein with multiple structural domains and ligand binding sites (Fig. 2). This complexity predicts that TSP1 may simultaneously interact with more than one receptor on a single cell type and that the net response of a cell to TSP1 may reverse after a change in the relative expression of TSP1 receptors on the cell that signal positive or negative responses to a given pathway (4). Although it is premature to judge the net effect of these opposing signals in vivo, one can begin by examining the cell receptors for TSP1 and the effects of TSP1 on specific cellular responses. I will then propose ways that these biochemical effects of TSP1 can modulate host responses to a primary or metastatic tumor.

**BINDING TO INTEGRINS AND INTEGRIN-ASSOCIATED PROTEIN**

Cell adhesion to extracellular matrix is crucial to several steps in tumor progression and metastasis (Fig. 1). TSP1 contains an RGD integrin recognition sequence in the seventh type III repeat (Fig. 2). RGD-inhibitable adhesion to TSP1 has been demonstrated for cells expressing the integrin αvβ3 (reviewed in ref 23). TSP1 also interacts with other integrins including αβ1 on neuronal cells, αβ1 and αβ1 on activated T lymphocytes, and αββ3 and αβ1 on platelets (24, 25; reviewed in ref 23). The sequences mediating interactions with integrins other than αβ3 have not been defined, but based on inhibition studies some are not RGD dependent. The cellular responses to TSP1 binding to specific integrins are only beginning to be explored. A report that TSP1 binding, mediated in part by the RGD-dependent integrin αβ3, stimulates transient calcium influx in IMR-90 fibroblasts (26) indicates that TSP1 can initiate signal transduction through integrin binding. Signaling through αβ3 may be relevant to regulation of angiogenesis by TSP1 because αβ3 plays an important role in regulating tumor angiogenesis (27).

Recently, an integrin-associated protein (IAP, CD47) was identified as the 52 kDa protein that binds to the adhesive motif VVM, expressed twice in the carboxy-terminal domains of TSP1 (28) (Fig. 2). Although IAP binds directly to TSP1 and VVM peptides, association of this protein with integrins, especially αβ3, suggests that adhesion of cells to TSP1 could involve a cooperative interaction of the VVM and RGD sequences with this protein complex. The conditions required for interaction of TSP1 with IAP remain puzzling, however, because IAP is expressed on almost all cell types, including erythrocytes, but TSP1 does not mediate adhesion of all cell types that express IAP. IAP binding through the VVM sequences may also elicit intracellular calcium signals (26). Overexpression in breast carcinoma cells of a carboxy-terminal deletion mutant of TSP1 lacking the second VVM motif reversed the suppressive effect observed using native TSP1 (15), suggesting that the VVM motifs play a role in

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**Figure 1. Potential role of thrombospondin-1 in tumor progression and metastasis.** Steps in the cascade of tumor formation and development of metastatic lesions are depicted schematically showing potential sites of action of TSP1 to modulate the cascade.
Heparin S Coil Type I Type II Type III Cell binding

Sequences: RKGSGRR NGVQYRN WSXW RFK CSVTCG RGDA RFYVMWWK

Receptors or ligands: HSPG Sulfatide

Functions: Adhesion Angio. Chemotaxis Angio. TGFβ Adhesion Ca²⁺ influx Adhesion Chemotaxis Ca²⁺ influx

Figure 2. Structural and functional domains of thrombospondin-1. The diagram schematically depicts the organization of a single subunit of the TSP1 trimer, which is covalently assembled through disulfide bonds (S). Functional peptide sequences identified from TSP1 are shown with the receptors or ligands recognized and the functional consequences of TSP1 binding to these ligands. Binding sites for other extracellular ligands and cell-surface receptors—including other integrins, LDL receptor-related protein, the 80/105 kDa receptor, fibrinogen, plasminogen activator, and neutral proteases—are not shown, as they are incompletely mapped.

the increased tumorigenesis of MDA435 breast carcinoma cells after overexpression of this mutant TSP1.

**BINDING TO CD36 AND OTHER NON-INTEGRIN RECEPTORS**

CD36 was the first nonintegrin receptor for TSP1 to be described (reviewed in ref 29). Purified CD36 binds to TSP1, and binding sites that may mediate this interaction have been identified on both proteins. Expression of CD36 on cells, however, is not always associated with an increase in TSP1 binding or adhesion, and specific conformation or phosphorylation states of CD36 may be required for TSP binding (29, 30). Nonetheless, several tumor cell lines expressing CD36 show partial dependence on this protein for adhesion to TSP1 (31), and this potential TSP1 receptor is expressed in some breast carcinomas (32). CD36 is a member of the class B scavenger receptor family. The low-density lipoprotein receptor-related protein (LRP) is another scavenger receptor that interacts with TSP1 (33, 34). LRP mediates rapid uptake and degradation of TSP1 by many cell types. The possible function of LRP in other cellular responses to TSP1 deserves further attention.

A novel receptor for TSP1 was identified on squamous carcinoma cells, composed of 80 and 105 kDa subunits (35). Although the size suggests an integrin receptor, the receptor was not immunologically related to known β1 or β3 integrins.

**BINDING TO HEPARAN SULFATE PROTEOGLYCANS**

TSP1 binds avidly to heparin, sulfatide, and HSPGs through an amino-terminal heparin binding domain (Fig. 2), through secondary heparin binding sites in the type I repeats, and possibly through additional undefined sites (reviewed in ref 36). This binding can allow TSP1 to interact with HSPGs in the extracellular matrix or on the cell surface, including syndecan. Because syndecan may be a signaling molecule and its expression influences tumor behavior, the effect of this interaction may be relevant to the role of TSP1 in cancer. A second effect of HSPG binding is to prevent FGF-2 and potentially other heparin-dependent growth factors, from binding to HSPG, which is essential for signal transduction by some of these growth factors. Thus, TSP1 can antagonize the stimulation of endothelial cell growth and motility stimulated by FGF-2 (37), and heparin binding recombinant fragments and peptides from TSP1 reproduce the FGF antagonist activity of intact TSP1 (38).

**BINDING AND ACTIVATION OF LATENT TGF-β**

TSP1 is a major glycoprotein in the α-granules of platelets and is secreted in response to platelet activation with several other α-granule components, including TGF-β. Some of the TGF-β binds tightly to TSP1 and is biologically active (39). Furthermore, addition of TSP1 to puri-
fied recombinant latent TGF-β results in its conversion to active TGF-β. This activation is probably mediated by the peptide sequence RFK in the type I repeats of TSP1 (Fig. 2), because peptides containing this sequence are equally as active on a molar basis as intact TSP1 for activating latent TGF-β (40). A second sequence adjacent to the activating sequence, WSHW, may play a role in binding of TGF-β to TSP1 and is an antagonist of activation mediated by purified TSP1. The RFK sequence is unique to TSP1, but the WSXW sequences are conserved in the type I repeats of thrombospondin-2. Synthetic peptides containing this sequence or recombinant thrombospondin-2 can antagonize the activation of latent TGF-β by purified TSP1 (40). Thus, TSP1 may be an activator of latent TGF-β, and thrombospondin-2 may be a negative regulator of this pathway of TGF-β activation.

At present, the physiological role of TSP1-dependent vs. protease-dependent or other pathways for activation of latent TGF-β is unclear. Several observations suggest that physiological activation of latent TGF-β by TSP1 may be regulated by additional factors. TSP1 overexpression in endothelial cells does not increase activation of endogenous latent TGF-β (16), and most TGF-β released from activated platelets remains in latent form despite exposure to high concentrations of platelet TSP1. Expression of α2-macroglobulin may also prevent activation of latent TGF-β by TSP1 (41).

REGULATION OF PROTEASES

Regulation of protease activity is required for tumor cell invasion and extravasation (Fig. 1). Several proteases, including cathepsin G, neutrophil elastase, plasmin, thrombin, and urokinase plasminogen activator, bind avidly to TSP1 (reviewed in ref 42). Complexes of TSP1 with the neutral proteases cathepsin G and neutrophil elastase result in competitive inhibition of activity. TSP1 is an inhibitor of plasmin and urokinase activity, but complexes of these enzymes with TSP1 may also be enzymatically active (43). Additional work is needed to define the conditions for inhibition and the role of TSP1 conformation and disulfide bond isomerization in the regulation of these activities.

Modulation of protease and protease inhibitor gene expression or secretion are also potential mechanisms for regulating extracellular protease activity by TSP1. Overexpression of TSP1 in transformed endothelial cells decreased net fibrinolytic activity due to increased plasminogen activator inhibitor 1 (PAI-1) and decreased urokinase secretion (16). Increased expression of PAI-1 was also observed in TSP1-treated endothelial cells (44). Although PAI-1 transcription is strongly induced by TGF-β, the increased secretion of PAI-1 observed in transfected cells overexpressing TSP1 was not associated with increased activation of latent TGF-β (16). Simulation of

Figure 3. Pathways for regulation of angiogenesis by thrombospondin-1. Several pathways may account for the antiangiogenic activities of TSP1. TSP1 directly influences endothelial cell adhesion and expression of PAI-1, and indirectly influences endothelial cell behavior by antagonism of heparin-dependent growth factors, activation of latent TGF-β, inhibition of protease activity, and induction of growth factors in adjacent cells.
PAI-1 secretion may also account for the ability of TSP1 to modulate invasion of fibrin gels by A549 lung carcinoma cells and MDA231 breast carcinoma cells (43; reviewed in ref 20). The latter two activities were ascribed to TSP1-mediated TGF-β activation, but this hypothesis was not directly tested. Based on these data, however, TSP1 may regulate expression of protease and protease inhibitor genes through direct and TGF-β-dependent pathways.

**EXPRESSION OF TSP1 BY TUMOR CELLS**

TSP1 is expressed by many types of tumor cells in vitro. In some cases, this endogenous TSP1 expression has been correlated with in vivo behavior. A positive correlation between TSP1 expression in vitro and tumor behavior in vivo was observed for a series of squamous carcinomas (45). In contrast, a negative correlation between TSP1 expression was observed for breast carcinoma cell lines (9, 10, 14), melanoma (9), ras-transfected human lung epithelial cells (9), and transformed endothelial cells (16).

Expression of TSP1 in tumor cells is regulated by several growth factors, oncoproteins, and tumor suppressor genes including p53, ras, c-jun, v-src, TGF-β, platelet-derived growth factor, and FGF-2. A detailed discussion of the regulation of THBS1 gene expression is beyond the scope of this review, but several recent reviews may be consulted for detailed discussions (4, 5, 8). Whereas changes in oncoproteins and tumor suppressor genes can directly influence TSP1 expression in tumor cells, overexpression of growth factors by the tumor cells may also modulate TSP1 expression in stromal cells and in endothelial and smooth muscle cells of the tumor vasculature.

To understand the significance of the regulation of TSP1 expression observed in vitro, expression in vivo must also be examined. Expression of TSP1 in vivo has been studied most thoroughly for breast carcinoma (32, 46–48). Thrombospondin is synthesized by normal breast stromal cells in tissue culture (49) and is a normal component of human milk. Immunohistochemical analyses of TSP1 expression in malignant breast tissues demonstrated strong staining in desmoplastic stroma and in the basement membrane associated with malignant ductal epithelium (47). However, TSP1 is also expressed in the basement membrane of normal myoepithelial cells, and most invasive ductal carcinoma cells do not express TSP1 (32). High expression of TSP1 in invasive breast carcinoma is restricted to invasive lobular carcinoma (32). Migrating “Indian file” cells in invasive lobular carcinoma stain strongly for TSP1 (32, 48). Thus, expression of TSP1 may be selectively lost in invasive ductal carcinomas. In some tumors, increased expression of TSP1 was noted in stromal cells adjacent to tumor cells (47), but in most breast carcinomas secretion of TSP1 into the matrix by the tumor cells vs. stromal cells has not been established.

Data for TSP1 expression in most other tumor types are sparse. TSP1 and other adhesion molecule expression were examined in radial and vertical growth stages of primary melanomas and metastases (50). TSP1 and CD36 protein expression, detected in the cytoplasm of melanoma cells using antibodies, was not correlated with tumor progression or metastasis. Increased plasma TSP1 concentrations have been reported in malignancy, especially in gastrointestinal and lung cancers (20), but are also observed in several nonmalignant conditions. The source of the elevated plasma TSP1 in malignancy, however, has not been established.

**EFFECTS ON TUMOR CELL BEHAVIOR**

In vitro studies have defined effects of TSP1 on tumor cell adhesion, motility, protease activity, and proliferation. The effects of TSP1 on tumor cell interactions with matrix have generally been proadhesive and are mediated by interactions with integrin receptors, CD36, or HSPGs. Under specific conditions, however, TSP1 can also inhibit tumor cell adhesion (36). In addition to mediating cell-matrix adhesion, TSP1 may mediate cell-cell adhesion. Formation of microemboli consisting of tumor cells surrounded with a rosette of platelets (Fig. 1) is important for metastasis of some tumors to the lung (reviewed in 51). TSP1 has a positive effect on tumor platelet adhesion (52, 53). Adhesion of MCF-7 breast carcinoma cells to a monolayer of human umbilical vein endothelial cells may also be mediated in part by TSP1, because adhesion to a monolayer of endothelial cells was inhibited by soluble TSP1 or a polyclonal antibody against TSP1 (54). Although enhancement of cell-matrix and cell-cell adhesion is often associated with suppression of invasion and metastasis (3), the latter two activities of TSP1 suggest a positive effect of cell-surface TSP1 expression during the later stages of hematogenous metastasis (Fig. 1).

TSP1 has well-characterized effects on cell motility. TSP1 is both a chemotactic and haptotactic stimulator of tumor cell motility (11, 55). Motility of squamous carcinoma cells in a TSP1 gradient is correlated with their TSP1 expression and in vivo behavior (11). TSP1 enhanced lung cancer cell invasion through a fibrin matrix (43), but apparently due to changes in protease activity rather than direct effects on cell motility.

Altered TSP1 expression may influence behavior of tumor cells in animal models of tumorigenesis or metasta- sis. Overexpression of TSP1 in 3T3 mouse fibroblasts allowed anchorage-independent growth in soft agar, an indicator of transformation of 3T3 cells, but had no effect on tumorigenesis (18). This activity is reminiscent of the ability of TSP1 to support growth of NRK fibroblasts in soft agar by activating latent TGF-β. During tumor progression, however, many cancers lose sensitivity to growth modulation by TGF-β. A comparison of mouse melanoma lines of differing metastatic potential derived from the K1735 melanoma line demonstrated an inverse relation...
between TSP1 expression in vitro and tumorigenic and metastatic behavior in vivo (9). A similar association was seen for human breast carcinoma cell lines differing in metastatic potential and for a series of cell lines derived from the immortalized lung epithelial cell line BEAS2B following transformation with three activated forms of ras. Passage of each of these lines through mice and reisolation of the cell lines from the tumors selected for cells with a further decrease in TSP1 expression at both the mRNA and protein level. Overexpression of TSP1 in transfected MDA435 breast carcinoma cells had no effect on tumor cell growth, colony formation in soft agar, or motility responses in vitro, but caused decreased tumorigenesis and metastasis in vivo. An inhibitory activity of TSP1 expression on tumorigenesis is supported by a study using aneuploid Li-Fraumeni fibroblasts, which contain a mutant p53 allele (19). These cells become immortal and lose TSP1 expression. Transfection with wild-type p53 restored TSP1 expression. TSP1 expression was also decreased after transformation of Chinese hamster ovary cells induced by nickel (12). Transcription of the THBS1 promoter detected in a CAT reporter plasmid was strongly decreased in the nickel transformed cells but could be increased by cotransfection with an expression plasmid for the retinoblastoma tumor suppressor Rb. In the latter study, it is not clear whether loss of TSP1 expression is a cause or result of transformation, but the transfection experiments establish that increased TSP1 expression is sufficient to reverse the phenotype in vivo.

In contrast to these negative correlations, suppression of TSP1 expression in the highly metastatic squamous carcinoma cell line 11B using an anti-sense TSP1 cDNA suppressed their metastatic activity (17). Positive effects on experimental metastasis were also demonstrated when TSP1 was injected intravenously prior to injecting T241 sarcoma cells (20). This activity of TSP1 was lost in thrombocyteopenic animals, suggesting that enhanced platelet interactions with tumor cells (Fig. 1) account for the observed effect of TSP1.

EFFECTS ON ANGIOGENESIS

Whether or not TSP1 expression by a tumor cell has a direct effect on behavior of a specific tumor cell type in vitro, the well-defined effects of TSP1 on endothelial cells (Fig. 3) may explain the suppressive effect on tumor growth of TSP1 expression observed in vivo in a spontaneous metastasis model (15). In this study, although transfection of MDA435 breast carcinoma cells with TSP1 did not alter any in vitro behavior of the tumor cells examined, growth in vivo was strongly suppressed (15). This suppression was dose dependent and correlated with reduction of angiogenesis in the tumors at early times.

Angiostatic activities of TSP1 have been demonstrated in several laboratories, and several mechanisms were proposed to account for this activity (Fig. 3). TSP1 inhibits adhesion of endothelial cells on a fibronectin matrix (56), suggesting that the loss of adhesion may inhibit endothelial proliferation. This effect is mediated by the amino-terminal, heparin binding domain of TSP1 (57). TSP1 also inhibits growth and motility of endothelial cells stimulated by FGF-2 by directly inhibiting growth factor binding (37, 38). Some of the growth suppressive activity of TSP1 purified from platelets may also be due to contamination with TGF-β (39), but activity of recombinant and synthetic peptides from TSP1 (38, 58) and transfection of endothelial cell lines with TSP1 (16) demonstrate that TSP1 contains intrinsic antiangiogenic activities. This is supported by the loss of TSP1 expression that accompanies conversion of endothelial cells to an angiogenic phenotype after transformation by polyoma virus, middle-sized tumor antigen (13). A direct role for TSP1 in regulating this phenotype is also supported by the observations that overexpression of TSP1 in stable transfectants derived from this line restores the normal phenotype in these cells (16) and that down-regulation of endogenous TSP1 expression in these cells using antisense TSP1 expression constructs increased chemotactic responses to FGF-2 and capillary morphogenesis in vitro (59). In addition to its effects on FGF-2 responses of endothelial cells, TSP1 expression may also regulate fibrinolytic activity of endothelial cells (Fig. 3). TSP1-transfected endothelial cells secreted less urokinase plasminogen activator and increased PAI-1 relative to controls (16).

The evidence for antiangiogenic activity of TSP1 in vitro is consistent with several reports of antiangiogenic activity of TSP1 in vivo (14–16, 58, 60). Although several laboratories have independently demonstrated angiostatic activities of TSP1, proangiogenic activity was also reported (61; reviewed in ref 20). In collagen or fibrin gels, addition of TSP1 increased angiogenesis and proliferation of an aortic ring culture (20). The cells that proliferated in response to TSP1 appeared to be myofibroblasts rather than endothelial cells, however, suggesting that the positive effect on angiogenesis was mediated by growth factors produced by the former cells. Similarly, the enhancement of lipopolysaccharide- or FGF-2-stimulated corneal angiogenesis in the presence of TSP1 was associated with increased influx of leukocytes, which may mediate the observed positive response (61). The basis for these conflicting results remains to be determined, but is reminiscent of the paradoxical activities reported for TGF-β in angiogenesis. TGF-β is a potent inhibitor of endothelial proliferation in vitro but can be both proangiogenic and antiangiogenic in vivo (62). Thus, both direct and TGF-β-mediated effects of TSP1 on angiogenesis may be bidirectional.

EFFECTS ON IMMUNE RESPONSES TO TUMORS

The second major requirement for tumor growth and metastasis is suppression of the host immune response. An-
titumor immunity is mediated by NK cell responses, cytotoxic T cell responses, monocytes, and tumoricidal macrophages. The first suggestion that TSP1 may influence the immune response came from the observation that TSP1 enhanced monocyte killing of squamous carcinoma (63). TSP1 stimulates monocyte motility and enhances oxidative response to the inflammatory agonist FMLP (reviewed in ref 64). Thus, TSP1 may be an important modulator of monocyte and neutrophil functions. TSP1 also promotes adhesion of both resting and activated T cells (25). Clearing of apoptotic neutrophils by macrophages is mediated by TSP1 and the receptors CD36 and αvβ3 (65), suggesting that TSP1 may play a similar role in interactions of tumoricidal macrophages with tumor cells. However, not all macrophages or monocytes residing in a tumor are tumoricidal. Further work is needed to determine whether TSP1 expression by tumor cells can enhance or suppress tumoricidal activities of these cells.

The ability of TSP1 to activate latent TGF-β provides another mechanism by which TSP1 may modulate immune function. TGF-β is produced by many tumors and may exert an immunosuppressive effect on NK or other immune responses in breast cancer xenografts (66). Both TSP1 and TGF-β inhibit early proliferation of interleukin 2-stimulated NK cells (67), and the effects of TSP1 in this assay were reversed by blocking antibodies to TGF-β. This model, however, predicts that overexpression of TSP1 in breast carcinoma cells would lead to increased TGF-β activation in the vicinity of the tumor and allow increased tumor growth. Yet the opposite is observed (15). If activation of latent TGF by TSP1-independent pathways is adequate for immune suppression in this model, however, then further activation in TSP1 overexpressing cells may have no effect on immune function. Moreover, the effects of both TSP1 and TGF-β on NK proliferation are biphasic; in prolonged proliferation assays, NK expansion is enhanced by both proteins (67). The consequences of these changes in proliferation to tumor growth are unclear, because TSP1 had no effect on killing of target tumor cells by NK cells after either short or long term culture.

EFFECTS ON STROMAL CELLS

TSP1 enhances fibroblast proliferation (68), a major component of tumor stromal cells. Additional effects of TSP1 on gene expression in stromal cells need to be further examined. The promoter of THBS1 is responsive to many growth factors and wound stimuli. The tumor environment provides many of these signals to stromal cells through secretion of growth factors and induces TSP1 expression. Stromal cells are the major site of induced TSP1 expression in endometrium stimulated by progesterone (69). TSP1 expression by the stromal cells may in turn influence growth of endothelium, immune responses, and behavior of tumor cells. Stromal cell TSP1 expression may therefore be an important mediator of growth factor signals produced by tumors and a source of feedback to the tumor from its environment.

CONCLUSIONS AND PROSPECTS

As has been demonstrated for several other adhesion molecules, modulating TSP1 expression can have profound effects on tumorigenesis and metastasis. Biological activities of TSP1 in vitro predict its ability to influence interactions of tumor cells with their extracellular matrix as well as to regulate stromal cell behavior, neovascularization, and host immune responses to the tumor. Although several laboratories have manipulated tumor growth by altering tumor cell expression of TSP1, the effect of tumor-derived factors on stromal and endothelial cell expression of TSP1 is more likely to represent the major control of TSP1 levels in most tumors. These interactions are complex, and the net effect of TSP1 to enhance or inhibit tumor progression will need to be defined for each tumor type.

The multifunctional nature of TSP1 presents a challenge for defining the mechanism for the many observed effects of TSP1 in tumor models. Its interactions with integrins and other surface receptors, proteases, and growth factors could each account for major effects on tumor behavior (Fig. 1). Activation of latent TGF-β presents a further complication because TGF-β itself has pleiotropic effects on cells, the direction and magnitude of which are themselves sensitive to the status and environment of the target cell. Other members of the TGF-β family remain to be tested for activation in the presence of TSP1 or other members of the thrombospondin family. Both of these tasks will benefit from the availability of TSP1 and TGF-β null mice and cell lines. Careful site-directed mutagenesis of TSP1 may also aid in the differentiation of these effector pathways.

The design of appropriate animal models presents an additional challenge for further examination of the role of TSP1 expression in tumorigenesis and metastasis. The site of implantation of tumor cells is important for tumorigenesis studies, and orthotopic models have been developed for some tumors to approximate the biological environment of a spontaneous primary tumor. However, surgical implantation of tumor cells or grafts places the tumor in a wound environment where stromal cell expression of TSP1 may be abnormally induced. The two common metastasis assays also assess different parts of the metastatic cascade (Fig. 1). Experimental metastasis, where tumor cells are introduced directly into the bloodstream, model only the latter portion of the cascade. The positive effects of exogenous TSP1 observed in some of these models are consistent with the observed effects of TSP1 on tumor cell interactions with endothelial cells and platelets. Spontaneous metastasis models depend more on the early events of metastasis, including angiogenesis. Thus, the antiangiogenic activities of TSP1 (Fig.
3) are consistent with observations of a negative effect of TSP1 overexpression on tumor growth in these models. Both metastasis models usually use aggressive tumor cell lines containing multiple genetic lesions. Yet the modulating effects of TSP1 may be more important during early development of a primary tumor (Fig. 1). Approaches must be developed to modulate TSP1 expression in premalignant lesions and to assess the expression of TSP1 during progression of these lesions.

At present, no mutant forms of TSP1 or rearrangements of the TBS1 gene have been identified in tumors, and the available evidence does not support a role for THBS1 as a classical oncogene or tumor suppressor gene. However, TSP1 expression is tightly regulated by several oncogenes and tumor suppressor genes and may play a significant role in expression of the malignant phenotype induced by alterations in these genes. Thus, it is reasonable to consider the possibility that pharmacological manipulation of TSP1 expression could control tumor growth or metastasis induced by these oncogenes.

TSP1 sequences that modulate growth factor activities or angiogenesis may be useful in modulating tumor growth or progression. Because small peptides derived from several regions of TSP1 mimic activities of the intact protein, these may serve as lead structures for synthesis of small molecules for therapeutic use. We have successfully used retro-inverso analogs of the TSP1 type I repeat peptides to systemically treat mice bearing breast carcinoma (N. Guo, H. C. Kruitzsch, J. K. Inman, and D. D. Roberts, unpublished results). Peptide mimetics based on other regions of TSP1 should also be examined for efficacy in inhibiting tumor growth. Achieving a better understanding of the THBS1 promoter and transcription factors that regulate its expression may lead to another therapeutic approach. Drugs may then be designed to directly regulate TSP1 expression in tumor stromal or vascular cells. Approaches such as these offer exciting opportunities to apply our expanding knowledge of cell-matrix interactions to cancer therapy.

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Antiproliferative and antitumor activities of d-reverse peptides derived from the second type-1 repeat of thrombospondin-1

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The extracellular matrix glycoprotein thrombospondin-1 (TSP1) inhibits angiogenesis, endothelial cell growth, motility and adhesion. Peptides from the type I repeats of TSP1 mimic the adhesive and growth inhibitory activities of the intact protein and specifically interact with heparin and transforming growth factor-β (TGFβ). To define the structural basis for the antiangiogenic activities of these peptides, we prepared analogs of the TSP1 peptide KRFKQDGWSHWSWSSC. L-forward, L-reverse, and d-reverse (retro-inverso) analogs displayed identical activities for binding to heparin, demonstrating a lack of stereospecificity for heparin binding. The L-reverse and d-reverse peptides, however, had somewhat decreased abilities to activate latent TGFβ. Conjugation of the forward peptides through a C-terminal thioether and the reverse peptides through an N-terminal thioether to polysucrose abolished the adhesive activity of the peptides and enhanced their antiproliferative activities for endothelial and breast carcinoma cells stimulated by fibroblast growth factor-2. Their antiproliferative activities were independent of latent TGFβ activation, because substitution of an Ala residue for the essential Phe residue in the TSP1 type-1 repeat peptide increased their potency for inhibiting TSP1 binding to heparin and for inhibiting endothelial cell proliferation. Although the conjugated peptides were inactive in vivo, an unconjugated retro-inverso analog of the native TSP peptide inhibited breast tumor growth in a mouse xenograft model. Thus, these TSP-derived peptide analogs antagonize endothelial growth through their heparin-binding activity rather than through activation of latent TGFβ or increasing cell adhesion. These stable analogs may therefore be useful as therapeutic inhibitors of angiogenesis stimulated by fibroblast growth factor-2. © Munksgaard 1997.

Key words: thrombospondin-1; endothelial cells; adhesion; heparin-binding; transforming growth factor β; tumor growth inhibitors; d-reverse peptides

Growth of many solid tumors depends strongly on recruitment of neovascularization. Increased vascularization of primary breast tumors is associated with an increased rate of metastasis to lymph nodes and a poorer prognosis (1, 2). Angiogenesis is regulated by both stimulatory or angiogenic factors and inhibitory or antiangiogenic factors (1, 3). High expression of antiangiogenic factors and limited availability of angiogenic factors maintains the endothelium in a nonproliferative state. Pathological states such as wound repair, diabetic retinopathy or tumor growth may alter the balance of these stimulators or inhibitors to allow neovascularization to proceed (1, 3).

Several antiangiogenic factors have been identified, including thrombospondin-1 (TSP1) (4–7), interferon-α, platelet factor 4, SPARC (8), apolipoprotein E3 (9), angiostatin and a proteolytic fragment of fibronectin (1, 3). Some of these proteins bind to heparin, and this binding activity may be responsible in part for their antiangiogenic activities. We have recently shown that the heparin-binding protein apolipoprotein E3 and heparin-binding recombinant fragments and synthetic peptides from TSP1 compete for binding of FGF-2 to endothelial cells or heparin and thereby inhibit proliferative and migratory responses of endothelial cells to FGF-2 (9, 10). TSP1 is a member of a family of extracellular matrix glycoproteins (11), and is released from platelets and secreted by many cell types in vitro (12, 13). TSP1 influences a complex array of biological responses (14),

Abbreviations: TSP, thrombospondin-1; TGFβ, transforming growth factor β; FGF-2, basic fibroblast growth factor; AECM, aminoethylcarbamylmethylated; ri, retro-inverso; HBSS, Hanks' buffered salts solution; BSA, bovine serum albumin.
including cellular adhesion, proliferation and migration. These effects may be mediated by direct interactions of TSP1 with several cell-surface receptors. TSP1 may also act indirectly by interacting with other extracellular components such as heparan sulfate proteoglycans and neutral proteases and by modulating activities of the growth factors, TGF\(\beta\) (15, 16) and FGF-2 (10).

Based on its effects on tumor cell adhesion, growth and motility, the expression of TSP1 in tumors could regulate their metastatic phenotype (17). We found that the antiproliferative activity of these TSP1 peptides.

**Experimental Procedures**

**Materials.** TSP1 was purified from the supernatant of thrombin-stimulated human platelets (31). EGFR and TGF\(\beta\)1 were obtained from Gibco BRL (Gaithersburg, MD). Bovine aortic endothelial cells were used between passages 4 and 10. Normal rat kidney (NRK-49F), mink lung fibroblasts (Mv1Lu) and human breast carcinoma cells (MDA MB435) were obtained from the American Type Culture Collection (Rockville, MD). d-Amino acid precursors were obtained from Bachem (Torrance, CA). TSP1 and FGF-2 (Bachem) were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) or Bolton-Hunter reagent (Dupont NEN, Boston, MA) as described previously (10).

**Preparation of synthetic peptides.** The peptides used in this study were synthesized on a Biosearch model 9600 peptide synthesizer using standard Merrifield solid-phase synthesis protocols and \(\alpha\)-butoxycarbonyl chemistry (32). Peptides were analyzed by reverse-phase HPLC chromatography. Peptides for biological assays were further purified by dialysis using Spectra/por 500 M, cutoff membranes or by reverse-phase purification using C18 Sep-Pak cartridges. Identities of some peptides were verified by matrix-assisted laser desorptive ionization time-of-flight mass spectrometry.

**Preparation of polysucrose conjugates.** Polysucrose with an average molecular weight of 400,000 or 70,000 (Ficoll, Pharmacia, Uppsala, Sweden) was first functionalized with primary amino groups as described previ-
ous (33, 34). This derivative, referred to as AECM-Ficoll and bearing 18–23 amino groups per 100 kDa (50 mg), was iodoacetylated in 1.35 ml of 0.15 M HEPES-NaOH buffer at pH 7.5 containing 1 mM EDTA by addition of 9.6 mg of iodoacetic acid N-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO) dissolved in 0.15 ml of dimethylformamide. After about 15 min of reaction, the solution was passed over a desalting column to obtain the iodoacetylated AECM-Ficoll. Nine micromoles of peptide were dissolved in 1.8 ml of distilled water, and 250 μl of a 50 mM solution of tris-(2-carboxyethyl)phosphine hydrochloride (Pierce Chemical) in water was added to the peptide solution, and the pH was adjusted to 7.1 to 7.8 by addition of 1 M Na2CO3. After 30 to 60 min, the resulting solution was passed through a column packed with 1.4 ml of Bio-Rad AG1-X8 anion-exchange resin in the acetate form. The column effluent was then passed into the iodoacetylated AECM-Ficoll solution, and the solution was stirred overnight at room temperature. The resulting solution was treated with 2-mercaptoethanol (20 mM for 1 h) and dialyzed against four changes of phosphate-buffered saline in a 12,000–14,000 M, cutoff tubing. The peptide concentration of the resulting conjugate was determined by measuring its absorbance at 280 nm using ε = 5540 M–1 cm–1 per Trp residue. The conjugates had between 8 and 29 moles of peptide covalently bound per mol of M, 400,000 polysucrose.

**Ligand binding assays.** TSPI binding to heparin-BSA was determined using a solid-phase assay (35). Heparin-BSA (0.075 μg/well) was adsorbed onto 96-well polystyrene microtiter plate wells in 50 μl of Dulbecco’s PBS for 16 h at 4°C. After blocking with tris-BSA buffer (50 mM tris, pH 7.8, 110 mM NaCl, 0.1 mM CaCl2, 0.1 mM phenylmethylsulfonyl fluoride, 1% BSA), 30 μl of various concentrations of inhibitors diluted in tris-BSA buffer or buffer alone and 30 μl of 125I-TSP1 (0.1–0.2 μg/ml) were added to each well. After incubation for 4 h at 4°C, the wells were washed, and the bound radioactivity was counted.

Binding of 125I-FGF-2 to heparin was determined using an immobilized heparin-BSA conjugate as described previously (10). 125I-FGF-2 was incubated with the inhibitors to be tested in heparin-BSA-coated wells for 2 h at 25°C. Bound radioactivity was determined after washing and cutting the wells from the plate.

Adsorption of peptides and polysucrose conjugates on plastic was quantified using a bicinchoninic acid reagent (Pierce Chemical) as described (36). The amount of adsorbed peptide was calculated using a standard curve constructed with the same peptide or conjugate.

**Bioassay for inhibition of endothelial and breast carcinoma cell proliferation.** Proliferation of bovine aortic endothelial cells was determined as described previously (10). Similar assays were performed using MDA MB435 human breast carcinoma cells except that the growth medium for the proliferation assays consisted of 5% fetal bovine serum in RPMI 1640 medium.

NRK fibroblast and mink lung cell bioassays for TGFβ were conducted as described previously (16, 37). Serum-free conditioned medium (37) prepared from bovine aortic endothelial cells at 70% confluency was used as a source of latent TGFβ for detecting activation by synthetic peptides in the NRK colony forming assay. NRK colonies in soft agar were quantified microscopically. Mink lung cell proliferation was determined in 96-well plates using 1 × 104 cells/well and quantified colorimetrically using the Abacus cell-proliferation assay (Clontech Laboratories, Inc., Palo Alto, CA).

Free and polysucrose-conjugated forms of peptide 476 (ri-amKRAKQAGGWSHWSPWSSCac) were submitted for testing in the National Cancer Institute Developmental Therapeutics Program in vitro screening cell panel. The peptides were tested for activity against 59 tumor cell lines in a 48-h proliferation assay as described (38).

**Tumorigenesis assay in nude mice.** NIH Nu/Nu mice, approximately 8 weeks of age, were injected in the mammary fat pads with 105 MDA MB435 cells. Six animals were injected for each condition per experiment. Beginning at day 25, the experimental animals were injected i.v. (tail vein) with 100 μl of the free peptide or polysucrose conjugates. Primary tumor size was determined twice weekly by length × width × height measurement. The presence of metastases was determined by gross autopsy and examination of hematoxylin and eosin-stained slides of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues and weighed. Animal experiments were conducted in an AAALAC-accredited facility using a protocol approved by the NCI Animal Care and Use Committee.

**RESULTS**

**Stereochemical specificity of heparin-binding and latent TGFβ activation.** We previously reported that two Trp residues located no more than three residues apart are required for binding of the type I repeat peptides to heparin (35). To define the stereochemical specificity for this interaction, we prepared 1-reverse and 3-reverse analogs of the active peptide from the second type I repeat of TSP1 and tested their activity for inhibiting TSP1 or FGF-2 binding to heparin (Table 1). The modified 1-reverse and 3-reverse peptides 600 and 599 were equal in activity to the native 1-forward peptide 246, and all were more active for inhibiting TSP1 binding than for inhibiting FGF-2 binding to heparin (Table 1). As previously reported (16), the 1-forward peptide 246 activated latent TGFβ, as assessed by stimulation of normal rat kidney fibroblast colony for-
Antiproliferative peptidomimetics from thrombospondin-1

TABLE 1
Stereospecificity for heparin-binding and TGFβ-activation by the TSPI type I repeat peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>TSPI binding</th>
<th>FGF-2 binding</th>
<th>Latent TGFβ activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>246</td>
<td>L-KRFKQDGGWSHWSPSS</td>
<td>0.17</td>
<td>1.3</td>
<td>0.002</td>
</tr>
<tr>
<td>600</td>
<td>L-SSWPSWSHSGGQKFRK</td>
<td>0.14</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>599</td>
<td>D-SSWPSWSHSGQKFRK</td>
<td>0.12</td>
<td>0.9</td>
<td>0.01</td>
</tr>
<tr>
<td>596</td>
<td>D-SSWPSWSHSGQKARK</td>
<td>0.032</td>
<td>0.16</td>
<td>4</td>
</tr>
<tr>
<td>597</td>
<td>D-SSAPSAHSAGGGDQKFRK</td>
<td>&gt;10</td>
<td>&gt;50</td>
<td>&gt;10</td>
</tr>
<tr>
<td>598</td>
<td>D-SSAPSAHSAGGAQKARK</td>
<td>&gt;10</td>
<td>&gt;50</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

125l-TSPI or 125l-FGF-2 and varying concentrations of peptides were added to microtiter plate wells coated with heparin-BSA, or with BSA to determine nonspecific binding, and incubated for 4 h at 4°C or 2 h at 25°C, respectively. 125I-TSPI or 125I-FGF bound to the wells was quantified in a gamma counter. Results represent the micromolar concentration of peptide required for 50% inhibition of labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA determined from dose-response curves in at least two experiments. Latent TGFβ activation was determined by NRK colony formation in soft agar. Results are presented as the concentration of peptide giving 50% of maximal stimulation determined in two independent experiments.

Separation of TGFβ-activating activity from heparin-binding. L-forward or D-reverse peptides lacking the Phe residue, shown previously to be essential for activation of latent TGFβ by the peptide KRFK (16), were weak or inactive for activating latent TGFβ in endothelial cell conditioned medium, based on the NRK colony formation assay (Fig. 1, Table 1). The active peptides at optimal concentrations stimulated 67% to 92% of the colony formation measured after complete activation of the latent TGFβ in the medium by heating at 85°C (Fig. 1). The D-reverse peptide 598, with the three Trp residues substituted by Ala residues, activated latent TGFβ but lacked heparin-binding activity, as observed previously using the corresponding L-forward peptides (35). Conversely, the D-reverse peptide 596, with the Phe residue substituted by Ala, had only weak TGFβ-activating activity but strongly inhibited heparin binding by TSPI and FGF-2. The D-reverse peptide 598, with five Ala substitutions, lacked all activity and was used as a negative control.

Separation of adhesive activity from antiproliferative activities. We attempted to use inhibition of mink lung epithelial cell proliferation (37) to confirm activation of latent TGFβ by the peptides. Although TGFβ inhibited growth of these cells, the retro-inverso peptide unexpectedly increased proliferation in a dose-dependent manner (Fig. 2A). This stimulation was observed in the absence of latent TGFβ in the medium. Identical stimulation was observed using a peptide lacking the RFK activation sequence (ri-amKRAKQAGGWSHWSPWSSac), indicating that this effect is independent of the RFK motif. The stimulation of proliferation by the peptides was not specific to the MvILu cells. Although some free TSPI type...
CONJUGATION OF TSP1 PEPTIDES TO POLYSUCROSE ABLATES GROWTH STIMULATORY ACTIVITY AND INCREASES THEIR ANTIPROLIFERATIVE ACTIVITY. (A) EFFECT OF FREE AND CONJUGATED PEPTIDES ON MINK LUNG CELL PROLIFERATION. PROLIFERATION OF MVI1Lu CELLS (1 x 10^4/Well) WAS DETERMINED IN THE PRESENCE OF THE INDICATED CONCENTRATIONS OF THE TGFβ-ACTIVATING PEPTIDE 416 (RI-AMKRKFQDGGWSHWSPWSSCAC, •) OR A POLYSUCROSE CONJUGATE (○), PEPTIDE 476 LACKING THE ACTIVATING SEQUENCE (RI-AMKRAKQAGGWSHWSPWSSCAC, △) OR A POLYSUCROSE CONJUGATE (△), OR TGFβ (□). (B) EFFECT OF FREE AND CONJUGATED PEPTIDES ON ENDOTHELIAL CELL PROLIFERATION. PROLIFERATION OF BOVINE AORTIC ENDOTHELIAL CELLS WAS DETERMINED IN THE PRESENCE OF THE INDICATED CONCENTRATIONS OF PEPTIDE 416 (RI-AMKRKFQDGGWSHWSPWSSCAC, •) OR A POLYSUCROSE CONJUGATE (○). CELL PROLIFERATION IS PRESENTED (MEAN ± SD, N = 3) AS A PERCENT OF THAT OBSERVED FOR CELLS GROWN IN THE SAME MEDIUM WITHOUT ADDITIONS.

I REPEAT PEPTIDES PREVIOUSLY TESTED INHIBITED ENDOTHELIAL CELL PROLIFERATION (10), SEVERAL I REPEAT PEPTIDES INCLUDING THE D-REVERSE PEPTIDE 416 ALSO STIMULATED PROLIFERATION OF ENDOTHELIAL CELLS AT LOW CONCENTRATIONS (FIG. 2B AND RESULTS NOT SHOWN).

THE STRONG ADHESION-PROMOTING ACTIVITY OF THESE PEPTIDES (32, 35) SUGGESTED THAT THEIR POSITIVE EFFECTS ON PROLIFERATION OF MVI1Lu AND BAEC CELLS WHEN ADSORBED ON PLASTIC (FIG. 3). POLYSUCROSE CONJUGATES OF THE PEPTIDES, HOWEVER, CONSISTENTLY DID NOT STIMULATE CELL ADHESION (FIG. 3), AND INHIBITED RATHER THAN STIMULATED MINK LUNG CELL (FIG. 2A) AND ENDOTHELIAL CELL PROLIFERATION (FIG. 2B). FURTHERMORE, THIS INHIBITION WAS INDEPENDENT OF LATENT TGFβ ACTIVATION, BECAUSE THE PEPTIDE 476 CONJUGATE, LACKING THE RFK MOTIF, PRODUCED SIMILAR INHIBITION AS THE PEPTIDE 416 CONJUGATE, CONTAINING THE NATIVE TSP1 SEQUENCE (FIG. 2A). POLYSUCROSE ALONE OR POLYSUCROSE CONJUGATED TO CONTROL PEPTIDES WAS INACTIVE. THE LACK OF ADHESIVE ACTIVITIES FOR THE POLYSUCROSE CONJUGATES PROBABLY RESULTS FROM THEIR DECREASED ADSORPTION ON PLASTIC. AT THE HIGHEST CONCENTRATION USED IN THESE EXPERIMENTS, 40 μM, THE ADSORPTION OF POLYSUCROSE CONJUGATES (18-45 PMOL/Well) WAS ONLY 10 TO 20% OF THAT FOR THE CORRESPONDING FREE PEPTIDES (117-270 PMOL/Well). CONJUGATED PEPTIDES, THEREFORE, SEPARATE THE ANTIPROLIFERATIVE AND ADHESIVE ACTIVITIES OF THE PEPTIDES AND WERE USED TO FURTHER STUDY EFFECTS OF THE PEPTIDES ON CELL PROLIFERATION.

SPECIFICITY OF THE TYPE I REPEAT SEQUENCES FOR ANTAGONISM OF FGF-2 AND TSP1 BINDING TO HEPARIN. SEVERAL L-FORWARD PEPTIDES BASED ON THE TYPE I REPEATS OF TSP1 ANTAGONIZED TSP1 OR FGF-2 BINDING TO HEPARIN (TABLE 2). THE ABILITY TO ANTAGONIZE TSP1 OR FGF-2 BINDING TO HEPARIN WAS SPECIFIC TO THE TSP1 TYPE I REPEAT PEPTIDES. WE TESTED A PEPTIDE WITH KNOWN ANTIANGIOGENIC

FIGURE 3.
CONJUGATION OF TSP1 PEPTIDES TO POLYSUCROSE ABLATES THEIR ABILITY TO PROMOTE CELL ADHESION. ADHESION OF MVI1Lu CELLS (•, ○) OR BOVINE AORTIC ENDOTHELIAL CELLS (△, △) TO PLASTIC COATED WITH THE INDICATED CONCENTRATIONS OF TSP1 PEPTIDE 246 (KRKFQDGGWSHWSPWSS, •, △) OR A POLYSUCROSE CONJUGATE OF THE PEPTIDE KRKFQDGGWSHWSPWSS (○, △) WAS DETERMINED. RESULTS ARE THE MEAN ± SD OF TRIPlicate DETERMINATIONS.
Antiproliferative peptidomimetics from thrombospondin-1

**TABLE 2**  
Specific inhibition of thrombospondin-I or FGF-2 binding to heparin-BSA by peptides derived from the type I repeats of thrombospondin-1

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>$^{125}$I-Thrombospondin IC$_{50}$ ($\mu$M)$^a$</th>
<th>$^{125}$I-FGF-2 IC$_{50}$ (nM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRFKQDGGSWHSPWSSC (TSP1)</td>
<td>0.5</td>
<td>3.8</td>
</tr>
<tr>
<td>KRFKQDGGSWHW</td>
<td>0.65</td>
<td>2.5</td>
</tr>
<tr>
<td>KRFKQDG</td>
<td>&gt;10</td>
<td>&gt;40</td>
</tr>
<tr>
<td>SHWSPWSS</td>
<td>5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>TRIRQDGGGWHSW (TSP2)</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>NGVQYRNCam (TSP1 procoll.)</td>
<td>&gt;10</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FIRVVMYEGKK (TSP1 C-term.)</td>
<td>&gt;10</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FRYVVMWKK (TSP1 C-term.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{125}$I-TSP1 or $^{125}$I-FGF-2 and varying concentrations of peptides were added to microtiter plate wells coated with heparin-BSA, or with BSA to determine nonspecific binding, and incubated for 4 h at 4°C or 2 h at 25°C, respectively. $^{125}$I-TSP1 or $^{125}$I-FGF bound to the wells was quantified in a gamma counter; am represents a C-terminal amide.

$^a$IC$_{50}$ values represent the concentration of peptide required for 50% inhibition of labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA.

Inhibition of endothelial cell proliferation correlates with FGF-2 binding inhibition and does not require TGFβ activation. In general, the same structural requirements for antiproliferative activity were observed as when these peptides were tested for inhibition of heparin binding to FGF-2 (Fig. 4A and Table 3). Forward and retro-inverso peptide conjugates showed similar activities. Although TGFβ inhibited growth of the endothelial cells (Fig. 4A), the inhibitory activity of the peptide conjugates did not require the RFK sequence. A conjugate containing the native TSP1 sequence with Ala substitutions for Phe, Gln and Asp (peptide 450) was highly active (Table 3), with an IC$_{50}$ for inhibiting proliferation only 7-fold higher than that of native TSP1 (IC$_{50}$ = 0.003 nM). This peptide was previously shown to not activate latent TGFβ (16). Endogenously produced latent TGFβ was not involved in the observed inhibition by the peptides, because an activating peptide (KRFK) had no effect on proliferation of the endothelial cells (Fig. 4A). A Trp motif was required for activity, as conjugates of forward or retro-inverso peptides lacking these residues (peptides 389, 475 and 513) were weak or inactive (Table 3). The basic motif also contributed to the antiproliferative activity as a conjugate of the peptide SHWSPWSSC had decreased activity (Fig. 4A). The inhibitory activity was specific for conjugates of the type 1 repeat peptides, as a
TABLE 3
Inhibition of thrombospondin or FGF-2 binding to heparin-BSA and endothelial cell proliferation by TSP1 peptide analogs

<table>
<thead>
<tr>
<th>Peptide or protein</th>
<th>125I-Thrombospondin binding to heparin Peptide</th>
<th>125I-FGF-2 binding Peptide</th>
<th>BAE cell proliferation Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide Sequence</td>
<td>IC50 (µM)</td>
<td>IC50 (µM)</td>
<td>IC50 (µM)</td>
</tr>
<tr>
<td>407</td>
<td>0.5</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>392</td>
<td>0.3</td>
<td>0.14</td>
<td>0.45</td>
</tr>
<tr>
<td>419</td>
<td>0.0028</td>
<td>0.04</td>
<td>0.18</td>
</tr>
<tr>
<td>450</td>
<td>0.03</td>
<td>0.025</td>
<td>0.22</td>
</tr>
<tr>
<td>389</td>
<td>N.D.</td>
<td>&gt;5</td>
<td>&gt;40</td>
</tr>
<tr>
<td>500</td>
<td>NGVQYRNCam</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>416</td>
<td>ri-amKRFKQDGGWHSVSPWSSCac</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>474</td>
<td>ri-amKRAKQDGGWHSVSPWSSCam</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>476</td>
<td>ri-amKRAKQDGGWHSVSPWSSCac</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>475</td>
<td>ri-amKRFKQDGGASHAPASSCaC</td>
<td>10</td>
<td>&gt;5</td>
</tr>
<tr>
<td>513</td>
<td>ri-amKRAKQDGGASHAPASSCeC</td>
<td>6.2</td>
<td>&gt;5</td>
</tr>
</tbody>
</table>

Labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA were determined as described in Table 1. BAE cell proliferation was determined in DMEM medium containing 0.5% FCS and 10 ng/ml FGF-2. IC50 values are the concentrations of peptide conjugates, expressed as molar concentrations of conjugated peptide, required for 50% inhibition of bovine aortic endothelial cell proliferation by the indicated derivatives of TSP1 peptides. Underlined residues indicate amino acid substitutions in the native sequence of the second type I repeat of TSP1. The retro-inverso peptides (ri) are shown C-terminus to N-terminus to facilitate comparisons with the 1-forward peptides; ac represents N-terminal acetyl, and am represents a C-terminal amide.

IC50 values represent the concentrations of peptides required for 50% inhibition of net binding to immobilized heparin or proliferation of untreated BAE cells.

The peptide conjugates inhibited breast carcinoma cell proliferation to a lesser extent than endothelial cell proliferation. The indicated concentrations of peptides or conjugates. Proliferation is presented as a percent of that determined in the absence of inhibitors, mean ± SD, n = 3, for the indicated concentrations of: thrombospondin-1 (■), TGFβ1 (○), a latent TGFβ-activating thrombospondin-1 peptide KRFK (▲), polysucrose control (■), or polysucrose conjugates of the peptides KRFKQDGGWHSVSPWSSC (●), SHWSPWSSC (△), acKRAAAGGWHSPWSSCam (▼) and ri-amKRFKQDGGWHSPWSSCac (▲).

FIGURE 4
Inhibition of cell proliferation by thrombospondin peptide conjugates. (A) Inhibition of bovine aortic endothelial cell proliferation. Bovine aortic endothelial cells (5000/well) were plated with the indicated concentrations of peptides or conjugates in DMEM with 0.5% fetal calf serum and 10 ng/ml FGF-2. Proliferation was determined after 72 h as described under "Experimental Procedures." (B) Inhibition of breast carcinoma cell proliferation. MDA MB435 human breast carcinoma cells (10,000/well) were plated in wells containing the indicated concentrations of peptides or conjugates. Proliferation is presented as a percent of that determined in the absence of inhibitors, mean ± SD, n = 3, for the indicated concentrations of: thrombospondin-1 (□), TGFβ1 (○), a latent TGFβ-activating thrombospondin-1 peptide KRFK (▲), polysucrose control (■), or polysucrose conjugates of the peptides KRFKQDGGWHSVSPWSSC (●), SHWSPWSSC (△), acKRAAAGGWHSPWSSCam (▼) and ri-amKRFKQDGGWHSPWSSCac (▲).
Antiproliferative peptidomimetics from thrombospondin-1

In vitro screening of antitumor activities of TSP1 peptide analogs

<table>
<thead>
<tr>
<th>Peptide 476</th>
<th>Peptide 476-polysucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panel</td>
<td>Susceptible/tested</td>
</tr>
<tr>
<td>Leukemia</td>
<td>6/6</td>
</tr>
<tr>
<td>NSCLC</td>
<td>4/9</td>
</tr>
<tr>
<td>Colon</td>
<td>5/6</td>
</tr>
<tr>
<td>CNS cancer</td>
<td>4/6</td>
</tr>
<tr>
<td>Melanoma</td>
<td>8/8</td>
</tr>
<tr>
<td>Ovarian</td>
<td>1/6</td>
</tr>
<tr>
<td>Renal</td>
<td>5/8</td>
</tr>
<tr>
<td>Prostate</td>
<td>1/2</td>
</tr>
<tr>
<td>Breast</td>
<td>7/8</td>
</tr>
</tbody>
</table>

Free and polysucrose-conjugated forms of peptide 476 (ri-amKRAKQAGGWHS PWSSCac) were tested for activity against 59 tumor cell lines in a 48-h proliferation assay as described (38). The number of cell lines showing >50% inhibition of proliferation at the highest dose tested (50 μM free peptide or 6.7 μM peptide as conjugate) is presented. For the susceptible cell lines, the mean and range for the IC₅₀ values are presented.

Spectrum of tumor cell growth inhibition. Inhibition of tumor cell growth by the peptides was not restricted to MDA MB435 cells. The peptide 476 (ri-amKRAKQAGGWHS PWSSCac) significantly inhibited growth of many tumor cell lines in the NCI Developmental Therapeutics Program in vitro panel (Table 4).

Effect of peptides on tumor growth. A retro-inverso analog of the native TSP1 sequence, peptide 416, was tested for inhibition of MDA MB435 tumor growth in an orthotopic nude mouse model. Growth of this tumor was previously demonstrated to be inhibited by overexpression of a TSP1 cDNA in the tumor cells, which was associated with reduced angiogenesis of the tumors (20). Tumor cells were allowed to implant for approximately 3 weeks before administering the peptide analog or a polysucrose conjugate systemically. No significant inhibition of tumor growth was observed in the animals treated with 5.3 mg/kg of 400,000 Mr polysucrose containing 0.2 mg/kg of bound peptide (Fig. 5) or in three additional experiments using daily injections of up to 0.5 mg/kg bound peptides as polysucrose conjugates (results not shown).

In contrast to the conjugate, daily intravenous treatment with the free peptide 416, ri-amKRFKQDGGSWHWSPWSSCac, at 2 mg/kg significantly inhibited tumor growth (Fig. 6, A and B). Administration of the peptide before the tumor became palpable produced greater inhibition of growth (Fig. 6A) than when the peptide was administered after the tumor became palpable (Fig. 6B). The growth inhibitory effect persisted beyond the treatment period, although the growth of the tumors eventually resumed in most animals. At the time of sacrifice, the treated animals in the experiment shown in Fig. 6A had significantly smaller tumors 200 ± 275 mg (mean ± SD) than those in the control group 754 ± 228 mg, p = 0.03 by two-sided t-test. Histological examination showed increased infiltration of the treated tumors with mononuclear cells. Five of six animals treated in the first experiment had viable tumor cells on histological examination, and the sixth showed only fibrotic tissue with no evidence of viable tumor cells. A dose response using 0.2–6 mg/kg of the pep-

FIGURE 5
Effect of a polysucrose conjugate of the thrombospondin-1 mimetic ri-amKRFKQDGGSWHWSPWSSCac on growth of MDA MB435 human breast carcinoma xenografts in athymic nude mice. Tumor dimensions, determined by external caliper measurements, are presented as a function of time after implantation in the mammary fat pad. Results are the mean ± SEM for each group of six animals treated with the 0.2 mg/kg of the conjugated peptide (open circles) or mock treated by injection of HBSS vehicle (closed circles).
Inhibition of MDA MB435 tumor xenograft growth in nude mice by the thrombospondin-1 mimetic ri-amKRFKQDGGWSHWSWPWSSCac. Animals were treated by intravenous injection of 2 mg/kg of the peptide in HBSS daily on days 25–50 (A) or days 35–60 after implantation of the tumor cells (B). The mean tumor volume ± SEM is presented for groups five to six animals treated with peptides (open circles) or mock-treated by injection of HBSS vehicle (closed circles). The animals were sacrificed on day 101, and the tumors were excised and weighed: control group, 754 ± 228 mg; treated group, 200 ± 275 mg (mean ± SD).

DISCUSSION

Based on their ability to inhibit FGF-2 binding to heparin and endothelial cells (10), the antiproliferative activity of the type I repeat peptides from the second type-I repeat of TSPI result from antagonism of heparin-dependent growth factors such as FGF-2. This mechanism is supported by the similar activities of the forward and retro-inverso peptides for inhibiting FGF-2 binding to heparin and FGF-2-stimulated endothelial cell proliferation. Ablating the adhesive activity of the peptides by conjugation to a soluble polymer improved their antiproliferative activity in vitro, but only the free peptide significantly inhibited growth of a human breast carcinoma in a nude mouse xenograft model. The retro-inverso analog of the native TSPI sequence reproduced the antiproliferative activity of TSPI overexpression in this same xenograft model (20). Heparin-binding and antiproliferative activities of the peptides were coordinately increased by substitution of Ala residues at certain positions in both types of peptides.

The antiproliferative activity of the TSPI peptide analogs in vitro does not require the TGFβ-activating sequence KRFK (16), because peptides containing the inactive sequence KRAK have similar inhibitory activities in endothelial, breast carcinoma and fibroblast proliferation assays. These peptides still contain the WSHW motif that was shown to inhibit TGFβ activation mediated by TSPI (16) and which could result in inhibition of endothelial cell proliferation in the presence of latent TGFβ (15). Lack of growth inhibition by the activating peptide KRFK, however, suggests that sufficient latent TGFβ is not produced by the endothelial or breast carcinoma cells to account for the observed inhibition. Furthermore, growth of the breast...
carcinoma cells was not sensitive to TGFβ. Therefore, the antiproliferative activities of TSP1 and the TSP1 peptides in these cells is clearly independent of latent TGFβ activation.

The heparin-binding activity of the type I repeat peptides is not stereospecific, whereas activation of latent TGFβ showed a moderate degree of stereospecificity. For the latter activity, l-reverse peptides were less active than d-reverse analogs, which share the same stereochemistry for the aminoacyl side chains as the native l-forward sequence. d-reverse peptidomimetics that retain activity and are stable to enzymatic degradation in vivo were developed based on this information. Two classes of stable analogs of the TSP1 type I repeat sequence have been prepared. Retro-inverso peptides lack peptide bonds sensitive to proteases but retain the heparin-binding and some of the latent TGFβ-activating activities of the native sequence. These peptides also exhibit antitumor activity in vivo. Polysucrose conjugates of the forward and retro-inverso peptides displayed increased antiproliferative activities in vitro and lack the adhesive activity of the free peptides. Although the polysucrose conjugates of these peptides could have longer circulatory half-lives than the free peptides in vivo, these analogs were not active in the breast tumor growth assay. At present we can not distinguish whether this is caused by decreased ability of these larger molecules to diffuse into the tumor tissues or is related to the differences in cell adhesive activity observed in vitro.

Amino acid substitutions that removed latent TGFβ-activating activity coincidentally increased heparin-binding activity of the peptide mimetics. Substitutions of Ala residues for several amino acid residues in the native TSP1 sequence increased heparin-binding activity up to 20-fold, based on inhibition of TSP1 or FGF-2 binding. It remains to be determined whether the enhancement of activity resulting from these aminoacyl substitutions is direct or due to stabilization of a preferred binding conformation of the essential side chains. Ala residues may stabilize an α-helical conformation of the peptides. Preliminary data suggest that part of the enhancement is caused by changes in the aggregation state of the peptides (Krutzsch, H.C., Guo, N., King, C., Inman, J.K., and Robert, D.D., manuscript in preparation). The ability of some WSXW peptides to inhibit TSP1 binding but not FGF-2 binding suggest that FGF-2 and TSP1 bind to different determinants on heparin. The WSXW motif is sufficient to inhibit binding of TSP1, whereas basic residues are also required for inhibition of FGF-2 binding. These data are consistent with analyses of the heparin-binding specificities of the type I repeat peptides, showing overlapping but distinct binding specificities for the TSP1 peptides and FGF-2 (Yu, H., Tyrrell, D., Guo, N., and Roberts, D.D., manuscript in preparation). This conclusion is also supported by the significant difference in potencies of the peptides determined using the two heparin-binding proteins.

Although other peptides in the type I repeats and a peptide from the procollagen domain inhibit angiogenesis (23), their mechanisms of action are probably different from that of the present peptides. The procollagen domain peptide used in the present experiments did not inhibit FGF-2 binding to heparin or growth of endothelial cells. A peptide from the second type I repeat of thrombospondin-2 homologous to the active TSP1 peptides, however, also inhibited FGF-2 binding to heparin. This active sequence may account for the recently reported antiangiogenic activity of thrombospondin-2 (41).

Peptides containing d-amino acids often have increased stability in biological fluids because of their resistance to enzymatic degradation (42, 43). Although a d-amino acid homolog of the laminin peptide IKVAV retained activity (29), d-forward peptides present an inverted configuration that may not be bind to a receptor that recognizes the mirror image l-forward sequence (42). d-reverse and modified retro-inverso peptides, in contrast, present the same configuration of amino acid side chains as the corresponding l-forward peptide. With appropriate charge modifications of the terminal residues, these peptidomimetics retain activity unless specific peptide backbone interactions are required (27).

Activity of the TSP1 retro-inverso analogs demonstrates that the aminoacyl side chains are the major contributors to the heparin-binding and TGFβ-activating activities of the TSP1 peptide and that specific interactions with the peptide backbone or terminal charges of the unmodified peptides are not crucial. These results suggest that additional nonpeptide mimetics with potent heparin-binding activity could be prepared based on the aminoacyl substituents in the active peptides.

The mechanism of the antitumor activity of the peptides in the mouse xenograft model remains unclear. Inhibition of tumor growth in vivo may depend on antagonism of FGF-2 responses as observed in vitro, or may result from modulation of latent TGFβ activation because of the activating and inhibiting sequences present in the peptide used for these experiments. Based on in vitro proliferation data, the peptides have some direct effect on MDA 435 cell proliferation and a stronger inhibitory effect on endothelial cell proliferation. These peptides also selectively induce apoptosis of endothelial cells (44). Thus, endothelial cells are probably the main target for inhibiting tumor proliferation. Increased infiltration of the tumors by monocytes was consistently observed in mice treated with the retro-inverso TSP1 peptide. Because the athymic mice used retain some B and NK cell functions and have functional macrophages and monocytes, modulation of host immune responses to the tumor may also play some role in the action of the peptides. TSP1 could modulate NK cell function in the mice through regulating their expansion (45), although effects on target cell kill-
ing have not been observed. TSP1 may also regulate recognition of tumor cells by monocytes (46). Effects of the TSP1 peptides on these or other aspects of tumor immunity have not been reported, but will require further examination.

The greater activity of the polysucrose conjugates to inhibit endothelial cell proliferation in vitro did not predict activity in vivo. A limited volume of distribution of the larger peptide conjugates may limit their access to the presumed site of action of the peptides, inhibiting angiogenic responses of endothelium to FGF-2 from the tumor diffusing through the subendothelial matrix. Our data are consistent with the hypothesis that the peptides act by inhibiting neovascularization of the growing tumors stimulated by heparin-dependent growth factors such as FGF-2. The temporary inhibition of tumor growth observed using a retro-inverso peptide is consistent with the known effects of other antiangiogenic agents in vivo (1). These stable analogs of the TSP1 peptides therefore merit further development as therapeutic inhibitors of angiogenesis. Based on their ability to inhibit growth of some breast cancer, leukemia and melanoma cell lines in vitro, the peptides may also be useful for directly modulating growth of these tumors.

ACKNOWLEDGMENTS

We thank Dr. Elaine Gallin for providing bovine aortic endothelial cells, Dr. Henry Fales for performing MALDI-TOF analyses, Mr. Jian Wui Tseng for performing the mink lung cell proliferation assays, the staff of the NCI Drug Synthesis and Chemistry Branch for in vitro antitumor screening and Mr. Brett Chancy for conducting the tumor growth assays. This work was supported in part by a grant from the Department of the Army (DAMD17-94-J-4499). The content of this article does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred.

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Antiproliferative peptidomimetics from thrombospondin-1


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Thrombospondin 1 and Type I Repeat Peptides of Thrombospondin 1 Specifically Induce Apoptosis of Endothelial Cells

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ABSTRACT

Thrombospondin 1 (TSP1) inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. The antiproliferative activity of TSP1 is mimicked by synthetic peptides derived from the type I repeats of TSP1 that antagonize fibroblast growth factor 2 and activate latent transforming growth factor β. These TSP1 analogues induced programmed cell death in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and internucleosomal DNA cleavage. Intact TSP1 also induced DNA fragmentation. The endothelial cell response was sensitive because no DNA fragmentation was induced in MDAMB-435 breast carcinoma cells, although TSP1 and the peptide conjugates inhibited the growth of both cell types. Apoptosis did not depend on activation of latent transforming growth factor β because peptides lacking the activating sequence RFK were active. Apoptosis was not sensitive to inhibitors of ceramide generation but was inhibited by the phosphatase inhibitor vanadate. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. Growth of the cells on a fibronectin substrate also suppressed induction of apoptosis by TSP1 or the peptides. Differential sensitivities to kinase inhibitors suggest that apoptosis and inhibition of proliferation are mediated by distinct signal transduction pathways. These results demonstrate that induction of apoptosis by the TSP1 analogues is not a general cytotoxic effect and is conditional on a lack of strong survival-promoting signals, such as those provided by a fibronectin matrix. The antitumor activity of TSP1 may therefore result from an increased sensitivity to apoptosis in endothelial cells adjacent to a provisional matrix during formation of vascular beds in tumors expressing TSP1.

INTRODUCTION

The extracellular matrix provides both positive and negative signals to regulate endothelial cell growth. Growth factors such as FGF-2 and vascular endothelial cell growth factor promote the growth and survival of nontransformed endothelial cells only when the cells are adherent to an appropriate extracellular matrix. Fibronectin is one of the matrix proteins that provides these signals. Fibronectin is an important matrix component for promoting the survival and growth of many cell types. The binding of fibronectin to the integrin receptors α5β1 induces activation of signal transduction pathways including the focal adhesion kinase (reviewed in Refs. 1 and 2) and other protein kinases (3-5) and results in signals that maintain viability, such as up-regulation of Bcl-2 in Chinese hamster ovary cells (6) and mitogen-activated protein kinase in We also observed that endothelial cells lose their normal morphol-...
in endothelial cells and that this activity is independent of their ability to activate latent TGF-β.

MATERIALS AND METHODS

TSP1 was purified from thrombin-stimulated human platelets as described previously (31). Fibronectin was purified from human plasma (NIH Blood Bank) as described (32). Recombinant human TGF-β1 was obtained from R&D Systems, Inc. Synthetic peptides from the type I repeats of human TSP1 were prepared and characterized as described previously (33, 34). Analogues of the TSP1 sequence (KRAKAAGGWSHPSWSSC, KRKFQDGGASHASPASSC) were prepared with appropriate Ala substitutions to eliminate the essential Phe residue for TGF-β activation or the Thr residues required for heparin binding and contain a carboxyterminal Cys residue to allow conjugation to polysucrose. Structures of the peptides used are summarized in Table 1. Peptides with Ala substitutions for Phe were unable to activate a mixture of latent TGF-β1 and TGF-β2 in BAE cell conditioned medium as assessed by NRK fibroblast colony formation in soft agar. Conjugation of the peptides to polysucrose was performed as described previously.4 In all cases, the peptides were used as polysucrose conjugates, which lack the adhesive activity of the free peptides but retain their other biological activities to regulate cell proliferation.4

Cell Culture. BAE cells were kindly provided by Dr. E. Gallin (Armed Forces Radiobiology Research Institute, Bethesda, MD) and were used at passages 4–10. BAE cells were maintained at 37°C in 5% CO2 in DMEM (low glucose) containing 10% FCS, 4 mM glutamine, 25 μg/ml ascorbic acid, and 500 units/ml each of penicillin G, potassium, and streptomycin (low glucose) containing 25 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol, and 0.5 mg/ml proteinase inhibitors. BAE cells were maintained at 37°C in 5% CO2 humidified atmosphere with 5% FCS. MCF7 cells were kindly provided by Dr. J. Gallin (Armed Forces Radiobiology Research Institute, Bethesda, MD) and were used at passages 4–10. BAE cells were maintained at 37°C in 5% CO2 humidified atmosphere with 5% FCS. MCF7 cells were kindly provided by Dr. J. Gallin (Armed Forces Radiobiology Research Institute, Bethesda, MD). Media components were obtained from Biofluids Inc. (Rockville, MD). MDA-MB-435S and MCF7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% FCS. Okadaic acid, TPA, fumonisin B I, herbimycin A, and sodium vanadate were purchased from Sigma.

TGF-β Assays. NRK fibroblast bioassays for TGF-β activity were conducted as described previously (29, 35). Serum-free conditioned medium (35) prepared from BAE cells at 70% confluence was used as a source of latent TGF-β for detecting activation by synthetic peptides in the NRK colony-forming assay. NRK colonies in soft agar were quantified microscopically. TGF-β1 was also quantified using an immunoassay specific for this isoform (Genzyme Corp.). Serum-free conditioned medium from BAE cells at 70% confluence was used as a source of latent TGF-β for and the samples were incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by microwave irradiation of the plate for 5 min. After cooling the plate for 10 min at −20°C, anti-TGFβ1 polyclonal conjugate solution was added and incubated for an additional 90 min at room temperature. After washing, immune-complexed anti-TGFβ1 peroxidase was detected by 3,3’5,5’-tetramethylbenzidine substrate. After incubation for 10–20 min at room temperature in the dark, absorbance was detected by monitoring at 450 nm.

Gel Analysis of DNA Fragmentation. Cells (5 × 10^5) in 1.5 ml were cultured on 6-well Nunc culture plates in 10% FCS complete medium for 24 h. The medium was replaced with medium containing 5% FCS and the inhibitors to be tested. After incubating for 24 h at 37°C, the cells were removed by trypsinization and collected by centrifugation at 1,000 rpm for 3 min. Supernatants were transferred to replicate wells of a microtiter plate containing either medium and the samples were incubated for 90 min at 37°C for 30 min (38). The lysate was vortexed for 15 s and precipitated with an equal volume of isopropanol at −70°C for 1 h. The samples were centrifuged for 30 min at 12,000 × g at 4°C, and the DNA pellets were washed in 70% ethanol at room temperature. After drying in a Speedvac concentrator for 15 min, the samples were dissolved in 25 μl of Tri-EDTA buffer containing 0.6 mg/ml RNase A and incubated overnight at 37°C. The samples were reextracted, washed, and dried as described above. The pellets were dissolved in 30 μl of Tri-EDTA buffer, and the DNA was subjected to electrophoresis on a horizontal 2% agarose gel in Tris-borate EDTA buffer. The DNA was stained with SYBR green solution (Molecular Probes, Inc., Eugene, OR) diluted 1:5,000 in running buffer.

RESULTS

We have previously shown that peptides derived from the second type I repeat of TSP1 inhibit proliferation of endothelial cells and a breast carcinoma cell line (27). Some of these peptides also promote cell adhesion (34). Because these two activities may elicit opposing signals in cells,5 polysucrose conjugates of the peptides, which do not promote cell adhesion, were used instead of free peptides in the current studies. The peptide conjugates arrested growth of both endothelial and breast carcinoma cells, but endothelial cell numbers also decreased after this treatment. The decrease in endothelial cell number was preceded by morphological changes in the treated endothelial cells that are characteristic of programmed cell death (Fig. 1c), including membrane blebbing, nuclear condensation, and loss of adhe-

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Table 1: Structures of TSP1 peptides and mimetics

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>407</td>
<td>TSP1 type 1</td>
<td>KRKFQDGGASHASPASSC</td>
</tr>
<tr>
<td>389</td>
<td>peptide 07</td>
<td>KRKFQDGGASHASPASSC</td>
</tr>
<tr>
<td>450</td>
<td>peptide 07</td>
<td>KRKFQDGGASHASPASSC</td>
</tr>
<tr>
<td>416</td>
<td>peptide 07</td>
<td>KRKFQDGGASHASPASSC</td>
</tr>
<tr>
<td>407</td>
<td>TSP1 procollagen</td>
<td>KRKFQDGGASHASPASSC</td>
</tr>
<tr>
<td>500</td>
<td>TSP1 residues 436-444 (retro-inverso)</td>
<td>all n-accessDBRVRWGRKFRKam</td>
</tr>
<tr>
<td>493</td>
<td>TSP1 residues 429-446 (retro-inverso)</td>
<td>all n-accessDBRVRWGRKFRKam</td>
</tr>
<tr>
<td>521</td>
<td>TSP1 residues 429-446 (retro-inverso)</td>
<td>all n-accessDBRVRWGRKFRKam</td>
</tr>
</tbody>
</table>

a Sequences are depicted using single-letter codes and are l-amino acids except where indicated: p, thioisopropyl; am, amide; ac, acetyl.

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The morphological changes were specifically induced by the type I repeat peptides because a control conjugate containing an antiangiogenic peptide from the procollagen domain of TSP1 was inactive (Fig. 1g).

BAE cells secrete latent TGF-β1 and TGF-β2 (36), and the TSP1 sequence KRFK activates latent TGF-β (29). Conditioned medium from the BAE cells used for this assay contained 0.01 ng/ml TGF-β1 immunoreactivity and 2.05 ng/ml acid-activatable TGF-β1. Because similar morphology changes were induced by a modified TSP1 sequence in which the TGF-β-activating sequence KRFK was altered to the inactive sequence KRAK (Fig. 1e), the effect of the peptide did not require activation of latent TGF-β produced by the endothelial cells.

**Induction of DNA Fragmentation.** Analysis of low molecular weight DNA extracted from endothelial cells treated with the active TSP1 peptides demonstrated a characteristic ladder pattern resulting from internucleosomal cleavage of the genomic DNA (Fig. 2). Polysucrose conjugates containing 0.4 μM of either the native TSP1 sequence KRFKQDGGWSHWSPWSC (Lane b) or the modified sequence KRAKAAGGWHSWSPWSC (Lane c), which lacks the TGF-β-activating sequence, equally stimulated DNA fragmentation. The basic residues and the WSXW motif were both required for optimal activity of these peptide conjugates, based on the weak activities of conjugates containing KRFKQDGGASHAPASSC (Lane a) or GGWSHWSPWSC (Lane d), which lack either the Trp residues or the basic motif. The appearance of cleaved DNA fragments was specific for the active type I repeat peptides because a polysucrose conjugate containing the TSP1 procollagen peptide NGVQYRC was inactive (Lane f). Two conjugated retro-inverso mimetics of the type I sequence were also active (Lanes c and g). Exposure of the cells to 1 μg/ml TSP1 did not result in detectable DNA fragmentation by this method (Lane h).

**Apoptosis Is Blocked on a Fibronectin Matrix.** Because loss of matrix adhesion is a known inducer of apoptosis in endothelial cells (8, 9), an antiadhesive activity was considered as a mechanism for the activity of the peptides. Precoating the tissue culture plastic with fibronectin did not alter the morphology of untreated cells (Fig. 1, a and b) but prevented the morphology changes induced by the TSP1 peptides (Fig. 1, d and f). The antiproliferative activities of the native TSP1 sequence (KRFQDGGSWHSWSPWSC-polysucrose, 407f) and an analogue without the TGF-β-activating sequence RFK (KRAKAAGGWHSWSPWSC-polysucrose, 450f) were also decreased by growth of the endothelial cells on a fibronectin matrix (Fig. 3). In contrast, the antiproliferative activities of intact TSP1 or TGF-β were not significantly decreased by attachment of the endothelial cells on fibronectin (Fig. 3).

Fibronectin also inhibited the appearance of the DNA ladder in endothelial cells treated with a TSP1 peptide analogue from the type I repeat (Fig. 4). DNA fragmentation induced by the peptide KRFKQDGHWSPWSC conjugate was reduced by 84% in cells attached on wells coated with 10 μg/ml fibronectin. A conjugate containing the TSP1 procollagen peptide NGVQYRC was used as a negative control in this experiment and did not significantly induce DNA fragmentation. Similar reductions in DNA fragmentation were observed for the other active TSP1 peptide analogues when cells were attached on fibronectin (data not shown).

**Quantitative Analysis of DNA Fragmentation.** An ELISA assay for detecting DNA fragmentation was used to quantify the activity of the peptides. DNA ladders were visualized by staining with SYBR green. Cells were treated with polysucrose conjugates of the following peptides: KRFKQDGGASHAPASSC, Lane a; KRFKQDGGWSHWSPWSC, Lane b; retro-inverso amKRFKQDGGWSHWSPWSC, Lane c; GGWSHWSPWSC, Lane d; KRAKAAGGWHSWSPWSC, Lane e; NGVQYRC, Lane f; retro-inverso amKRFKQDGWHSPWSC-thioproprionyl, Lane g; TSP1, Lane h; and control, Lane i. Left margin, migration of DNA size markers.
Because the same peptides inhibited the growth of MDA-MB-435S cells, the induction of apoptosis can be independent of the anti-proliferative effects of the TSPI peptides. A second breast carcinoma cell line, MCF7, showed DNA fragmentation in response to the peptide 407 conjugate but not to intact TSPI (Fig. 5B). The magnitude of the peptide response was similar to the DNA fragmentation induced in the same cells by camptothecin.

On the basis of the protective activity of fibronectin observed in Figs. 1 and 4, the effect of endothelial cell adhesion on the induction of DNA fragmentation was further examined using the quantitative DNA fragment ELISA. The adhesion of endothelial cells to a fibronectin matrix inhibited DNA fragmentation induced by TSPI (Fig. 6A) or by a TSPI peptide analogue from the type I repeats (Fig. 6B). Consistent with the report that vanadate suppresses the induction of endothelial cell death induced by removal of extracellular matrix signals (8), the addition of 50 μM vanadate decreased the fragmentation induced by the active TSPI peptide KRFKQDGGWSHWSPWSSC (407f) or by the analogue lacking the TGF-β-activating sequence (450f; Fig. 7). The serine/threonine phosphatase inhibitor okadaic acid, at 5 nm, also inhibited DNA fragmentation induced by these peptides, whereas the ceramide

the peptides. On the basis of this sensitive and quantitative assay for DNA fragmentation, both TSPI and the type I repeat peptides induced significant DNA fragmentation in endothelial cells (Fig. 5A). The activity of TSPI was weaker than that of the synthetic peptide conjugates to elicit DNA fragmentation but was consistently observed in several independent experiments. The TSPI procollagen domain peptide, however, was inactive. Treatment of BAE cells with TGF-β induced DNA fragmentation to a similar extent as TSPI. The stimulation of DNA fragmentation by TSPI and the peptide conjugates was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells by the peptide conjugates (Fig. 5B). DNA fragmentation was induced in MDA-MB-435S cells by the topoisomerase I inhibitor camptothecin, indicating that these cells can initiate programmed cell death.

Fig. 3. Attachment of endothelial cells on fibronectin partially reverses the inhibition of proliferation by type I repeat peptides but not by TSPI or TGF-β. Proliferation of BAE cells was determined on uncoated tissue culture plastic (●) or on plastic coated with 10 μg/ml fibronectin (▲) in DMEM medium containing 1% FBS and the indicated concentrations of the TSPI peptide KRFKQDGGWSHWSPWSSC (407f), an analogue without the TGF-β-activating sequence aKRAKAAGGWSPWSSCamy (450f), TGF-β1 (TGF-β1, TSP1, or the TSPI procollagen domain peptide NGVQYRNC (500f). The cell number was quantified after 72 h using the Cell-Titer assay and is presented as a percentage of that determined in the same medium without additions, mean ± SD, n = 3.
used at concentrations below those that directly blocked endothelial cell proliferation by TSP1, TGF-β, or the indicated thrombospondin peptide analogues were determined in the presence of inhibits of tyrosine kinase (herbimycin), ceramide synthase (fumonisin), phosphatases (vanadate or okadaic acid), or a stimulator of protein kinase C (TPA). Net inhibition of proliferation, expressed as mean ± SD for triplicate wells, was determined relative to control cells treated with the same inhibitors or activators in the absence of the test proteins or peptides.

Table 2: Effect of signal transduction modulators on inhibition of endothelial cell proliferation by TSP1, TGF-β, and TSP1 peptides

<table>
<thead>
<tr>
<th></th>
<th>450F</th>
<th>500F</th>
<th>407F</th>
<th>407F</th>
<th>TSP1</th>
<th>TGFβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49 ± 4</td>
<td>-4 ± 4</td>
<td>97 ± 1</td>
<td>70 ± 4</td>
<td>71 ± 2</td>
<td></td>
</tr>
<tr>
<td>Herbimycin (1 μM)</td>
<td>0 ± 5</td>
<td>0 ± 9</td>
<td>50 ± 7</td>
<td>-14 ± 12</td>
<td>-5 ± 12</td>
<td></td>
</tr>
<tr>
<td>Fumonisin (15 μM)</td>
<td>24 ± 3</td>
<td>6 ± 4</td>
<td>97 ± 1</td>
<td>74 ± 2</td>
<td>84 ± 3</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>59 ± 4</td>
<td>-5 ± 19</td>
<td>74 ± 5</td>
<td>61 ± 3</td>
<td>72 ± 1</td>
<td></td>
</tr>
<tr>
<td>Vanadate (20 μM)</td>
<td>61 ± 3</td>
<td>-3 ± 8</td>
<td>83 ± 5</td>
<td>-8 ± 12</td>
<td>26 ± 7</td>
<td></td>
</tr>
<tr>
<td>Okadaic acid (5 μM)</td>
<td>77 ± 5</td>
<td>17 ± 4</td>
<td>96 ± 1</td>
<td>65 ± 4</td>
<td>80 ± 1</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 5</td>
<td>43 ± 2</td>
<td>52 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA (50 μM)</td>
<td>1 ± 2</td>
<td>64 ± 6</td>
<td>48 ± 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phorbol (50 μM)</td>
<td>2 ± 6</td>
<td>77 ± 2</td>
<td>57 ± 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the activity of the phosphatase inhibitor vanadate to antagonize the antiproliferative effect of TSP1 suggests that hyperphosphorylation prevents the antiproliferative activity of TSP1, a tyrosine kinase may also mediate the growth-suppressive activity of TSP1 and the peptides. The tyrosine kinase inhibitor herbimycin A, used at concentrations below those that directly blocked endothelial cell proliferation by TSP1, TGF-β, or the indicated thrombospondin peptide analogues was determined in the presence of inhibits of tyrosine kinase (herbimycin), ceramide synthase (fumonisin), phosphatases (vanadate or okadaic acid), or a stimulator of protein kinase C (TPA). Net inhibition of proliferation, expressed as mean ± SD for triplicate wells, was determined relative to control cells treated with the same inhibitors or activators in the absence of the test proteins or peptides.

Phosphorylation Differentially Modulates Antiproliferative Responses to TSP1 and Thrombospondin. On the basis of the observation that phosphatase inhibition differentially affected apoptotic responses to TSP1 and the peptides, we further examined the mechanism of endothelial growth inhibition by the TSP1 type I repeat peptides. Sodium vanadate significantly inhibited the antiproliferative activity of TSP1 and TGF-β but did not inhibit the antiproliferative activity of the TSP1 peptides (Table 2). Blocking of the TSP1 and TGF-β activities by vanadate was specific in that the serine/threonine phosphatase inhibitor okadaic acid had no affect at a concentration sufficient to inhibit protein phosphatase 2A (39). However, at concentrations sufficient to inhibit protein phosphatase 1 (25 nM), okadaic acid alone inhibited endothelial growth and strongly induced DNA fragmentation (results not shown).

Inhibition of BAE cell proliferation by TSP1, TGF-β, or the indicated thrombospondin peptide analogues were determined in the presence of inhibits of tyrosine kinase (herbimycin), ceramide synthase (fumonisin), phosphatases (vanadate or okadaic acid), or a stimulator of protein kinase C (TPA). Net inhibition of proliferation, expressed as mean ± SD for triplicate wells, was determined relative to control cells treated with the same inhibitors or activators in the absence of the test proteins or peptides.
proliferation, strongly suppressed the antiproliferative activities of the TSP1 peptides and completely blocked the antiproliferative activities of TSP1 and TGF-β (Table 2). Because herbimycin also blocked the antiproliferative activity of the TSP1 peptide analogue 450, which lacks a latent TGF-β-activating sequence, herbimycin can prevent activity of the TSP1 peptides independently of blocking TGF-β-mediated signaling. In contrast, fumonisin B1, an inhibitor of ceramide synthase and ceramide-mediated apoptosis (40), had no effect on the activity of the peptides at 15 μM (Ki = 0.2 μM for ceramide synthase; Ref. 40). At higher doses, fumonisin B1 also inhibited endothelial cell proliferation and directly induced DNA fragmentation (results not shown).

The protein kinase C stimulator TPA, which blocks ionizing radiation-induced ceramide generation and apoptosis of BAE cells (41, 42), stimulated proliferation of the cells but had no effect on the antiproliferative activities of the TSP1 peptides or TSP1 (Table 2). The inactive analogue 4α-phorbol did not stimulate proliferation, verifying the specificity of the proliferative response to TPA. TPA also had no effect on the generation of DNA fragmentation induced by the TSP1 peptides as assessed by the DNA ladder assay (results not shown).

**DISCUSSION**

Previous studies have demonstrated the positive effects of extracellular matrix components on endothelial cell survival (8, 9, 11). Recently, however, TSP1 and several other matrix components have been found to negatively modulate cell adhesion (12, 43). Because adhesion provides signals essential for survival of nontransformed cells, these observations suggested that TSP1 may also regulate cell survival. The present results demonstrate a negative effect of TSP1 on endothelial cell survival. The ability of TSP1 or the TSP1 peptide analogues to inhibit growth and induce apoptosis, however, is dependent on other external signals. Confluent quiescent cells were resistant to the induction of cell death, as were subconfluent cells attached to a pure fibronectin matrix or cells treated with vanadate. As was demonstrated for the interaction of cells with fibronectin (4), the signals resulting from the interaction of endothelial cells with TSP1 may be complex and involve multiple signal transduction pathways. TSP1 and the TSP1 peptides elicit changes in both endothelial cell proliferation and survival. On the basis of their differential sensitivities to fibronectin matrix signals and agents that modulate several signal transduction pathways, these responses probably involve discrete signaling pathways.

Several results indicate that induction of apoptosis is independent of the growth-inhibitory activities of TSP1 and the TSP1 type I repeat peptides. Proliferation of breast carcinoma and endothelial cells are both inhibited by TSP1 and the peptides,4 but only the latter cells exhibited an apoptosis response. Differential sensitivity of the endothelial cell apoptosis and proliferative responses to vanadate inhibition for the TSP1 peptides also suggest that distinct mechanisms may mediate growth inhibition and apoptosis. Likewise, the proliferative and survival responses to intact TSP1 differ in that fibronectin reversed the apoptotic response but did not reverse the antiproliferative activity of TSP1.

The parallel proliferative and survival responses of TSP1 and TGF-β-treated endothelial cells to many of the inhibitors tested suggest that TGF-β may mediate the activity of intact TSP1, although it is not required for activity of the TSP1 peptides. Part of the observed response to TSP1 could result from active TGF-β contaminating the platelet TSP1, but the measured concentration of TGF-β was insufficient to account for most of the activity observed. The TSP1 may also activate latent TGF-β produced by the BAE cells (30). As was observed with the peptides, however, sensitivities to signal transduction inhibitors differ for TSP1-mediated growth inhibition and induction of DNA fragmentation.

Vanadate completely reversed the antiproliferative activity of TSP1 but augmented DNA fragmentation. Likewise, fumonisin B1 had no effect on the antiproliferative activity of TSP1 but also augmented DNA fragmentation. This pattern parallels previous reports that apoptotic and growth-inhibitory responses to TGF-β may also involve distinct signaling pathways (38).

Peptides from the type I repeats of TSP1 elicit a strong apoptotic response in endothelial cells. To date, no other region of TSP1 has been found to induce apoptosis, and the present data exclude this as a mechanism for the antiangiogenic activity of the procollogen domain peptide (28). Mutagenesis of the type I repeat sequences will be required to confirm the role of the type I repeats in the activity of the intact protein and to determine whether other regions of TSP1 participate in the cell death response to intact TSP1.

The mechanism of action of the TSP1 peptides is clearly not from direct cytotoxicity, based on the resistance of endothelial cells plated on fibronectin or at confluence to apoptosis in the presence of active concentrations of the peptides. The peptides may act outside of the cell to block FGF-2 presentation to and activation of its tyrosine kinase receptor (27). This hypothesis is consistent with protection by vanadate from peptide-mediated apoptosis. However, some other results question this hypothesis. Partial reversal of the peptide antiproliferative activities by herbimycin and the lack of vanadate sensitivity are not consistent with their acting by antagonizing a tyrosine kinase-dependent receptor. Furthermore, FGF-2 is known to suppress ceramide-mediated apoptosis of BAE cells, and this activity is mediated by protein kinase C (42). Because TPA stimulation of PKC protects BAE cells from ceramide-mediated apoptosis, but did not protect our BAE cells from an inhibition of growth by the TSP1 peptides, the antiproliferative activity of the peptides cannot arise exclusively from inhibition of an essential FGF-2 survival signal. Although ceramide has recently been shown to mediate apoptosis of many cell types in response to various stimuli (reviewed in Ref. 44) and participates in radiation-induced apoptosis of BAE cells (41, 42), the lack of effect of fumonisin B1 and TPA on the activities of the peptides suggests that the pathway for inducing cell death by the TSP1 peptides is distinct from that of irradiation and does not require ceramide generation. On the basis of the apparent synergism of fumonisin B1 with TSP1 to induce DNA fragmentation, however, the apoptotic response to the intact protein may be regulated by ceramide generation.

TSP1 inhibits focal adhesion contacts in endothelial cells attached on fibronectin (15). This mechanism could participate in the activity of TSP1 but not that of the peptides because the amino-terminal domain of TSP1 is responsible for the former activity. Treatment with the peptide conjugates, however, also results in the loss of endothelial cell adhesion. It remains to be determined whether this loss of adhesion causes programmed cell death or is an indirect effect of other signals induced in the cells by the peptides.

Attachment of the endothelial cells to fibronectin or being at confluence generates a signal that reverses the apoptotic and antiproliferative responses to the peptides. Because fibronectin binding to the integrin α5β1 promotes endothelial cell survival (8), this signal may involve activation of focal adhesion kinase or other adhesion-dependent tyrosine kinases. Fibronectin or antibody engagement of B1 or β3 integrins on endothelial cells results in tyrosine kinase-dependent phosphorylation of focal adhesion kinase and a 70-kDa protein (45). Vanadate can replace the fibronectin signal to prevent endothelial cell death (8) and presumably maintains the targets of these kinases in a phosphorylated state by inhibiting the corresponding phosphatases. This model is consistent with the ability of vanadate to suppress DNA fragmentation induced by the TSP1 peptide KRKFQDGGWSHSPWSSC and to reverse growth inhibition by intact TSP1. However, it
does not account for the ability of vanadate to stimulate apoptosis induced by intact TSP1 or the ability of herbimycin A to prevent growth inhibition by TSP1 or the peptides. The latter result was also unexpected because herbimycin is reported to inhibit angiogenesis (46), most integrin signaling (4, 45), and apoptotic responses in several cell types (47). The data can be rationalized by proposing that the peptides elicit a second inhibitory tyrosine kinase pathway that is sensitive to herbimycin.

The role of programmed cell death in the biological activities of TSP1 in vivo remains to be examined. TSP1 overexpression in MDA-MB-435S breast carcinoma cells reduced tumor growth in vivo but had no effect on the growth of these cells or the formation of colonies in soft agar (24). These observations are consistent with the inability of TSP1 to induce apoptosis of MDA-MB-435S cells. The resistance of MDA-MB-435S breast carcinoma cells to induction of apoptosis by the TSP1 peptides may result from mutation of p53 in this cell line (48), whereas the MCF7 cells have wild-type p53 and are sensitive to the induction of apoptosis. Normal p53 function may therefore be required for the apoptotic response to TSP1 peptides.

Reduction of angiogenesis in tumors formed by TSP1-transfected MDA-MB-435S cells (24) could result from the induction of apoptosis in endothelial cells during vascularization of the tumor. A similar mechanism has been proposed for the antitumor activity of antibodies to the αvβ3 integrin, which induce apoptosis in developing tumor blood vessels (10, 11). Thus, extracellular matrix signals may be absent in newly formed tumor blood vessels and sensitize this endothelium to the effects of TSP1 secreted by the transfected MDA-MB-435S cells. Although TSP1 is a ligand for αvβ3 (20), the active TSP1 peptides do not contain the Arg-Gly-Asp sequence recognized by the αvβ3 integrin.

The TSP1 peptides are potent inducers of DNA fragmentation in BAEC cells in vitro. This activity may account for the differential effects of these peptides on endothelial and breast carcinoma cell proliferation in vivo. We recently found that stable analogues of the TSP1 peptides inhibit tumor growth in vivo in MDA-MB-435S xenografts in nude mice. The present data suggest that the selective induction of apoptosis of tumor endothelium may explain the activity of the peptides in vivo. The resistance of confluent endothelial cells to the induction of apoptosis by the peptides in vitro is consistent with their lack of toxicity in vivo.

REFERENCES


Pro-adhesive and Chemotactic Activities of Thrombospondin-1 for Breast Carcinoma Cells Are Mediated by $\alpha_3\beta_1$ Integrin and Regulated by Insulin-like Growth Factor-1 and CD98*

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Thrombospondin-1 (TSP1) is a matricellular protein that displays both pro- and anti-adhesive activities. Binding to sulfated glycoconjugates mediates most high affinity binding of soluble TSP1 to MDA-MB-435 cells, but attachment and spreading of these cells on immobilized TSP1 is primarily $\beta_1$ integrin-dependent. The integrin $\alpha_3\beta_1$ is the major mediator of breast carcinoma cell adhesion and chemotaxis to TSP1. This integrin is partially active in MDA-MB-435 cells but is mostly inactive in TSP1-MDA-MB-231 and MCF-7 cells, which requires $\beta_1$ integrin activation to induce spreading on TSP1. Integrin-mediated cell spreading on TSP1 is accompanied by extension of filopodia containing $\beta_1$ integrins. TSP1 binding activity of the $\alpha_3\beta_1$ integrin is not stimulated by CD47-binding peptides from TSP1 or by protein kinase C activation, which activate $\alpha_2\beta_1$ integrin function in the same cells. In MDA-MB-231 but not MDA-MB-435 cells, this integrin is activated by pertussis toxin, whereas serum, insulin, insulin-like growth factor-1, and ligation of CD98 increase activity of this integrin in both cell lines. Serum stimulation is accompanied by increased surface expression of CD98, whereas insulin-like growth factor-1 does not increase CD98 expression. Thus, the pro-adhesive activity of TSP1 for breast carcinoma cells is controlled by several signals that regulate activity of the $\alpha_3\beta_1$ integrin.

Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that has diverse effects on cell behavior (reviewed in Refs. 1 and 2). The five known thrombospondin genes display distinct patterns of expression during development and in several disease states. Disruption of the thbs1 gene in mice results in laddosis of the spine and abnormal proliferation and inflammatory responses in the lung (3). Suppression of THBS1 expression by loss of wild type p53, by activated Ras, Myc, nickel,

and in metastatic clones of several tumor cell lines suggested that loss of TSP1 expression may contribute to tumor progression (reviewed in Ref. 4). Consistent with this hypothesis, overexpression of THBS1 in breast carcinoma cells (5), a transformed endothelial cell line (6), fibroblasts from Li Fraumeni patients (7), and glioblastoma cells (8) decreases tumor growth in animal models. This suppressive activity is due at least in part to the anti-angiogenic activity of TSP1 (reviewed in Refs. 4, 9, and 10). TSP1 antagonizes growth factor-stimulated proliferation and migration of endothelial cells. Its anti-angiogenic activity is thought to be the major mechanism for suppression of tumor growth in THBS1-transfected MDA-MB-435 breast carcinoma cells, because thrombospondin overexpression strongly inhibited tumor growth in vivo but did not significantly alter in vitro proliferation, motility, or the ability of the tumor cells to form colonies in soft agar (5). However, higher doses of exogenous TSP1 and some TSP1 peptides can directly inhibit proliferation of these cells in vitro (11).

Defining the receptors that recognize TSP1 on endothelial and tumor cells may provide insights into the differential effects of this protein on each cell type. Receptors that mediate cell interactions with TSP1 include integrins, proteoglycans, CD36, CD47, the low density lipoprotein receptor-related protein, and sulfated glycolipids. Binding of TSP1 to each of these receptors may elicit different cellular responses. Thus both the relative levels of expression of each receptor and, potentially, the activation state of each receptor may determine the nature of the adhesive, motility, and proliferative responses of cells to TSP1.

We have examined the role of integrins in the pro-adhesive activity of TSP1 for human breast carcinoma cells. Although the integrin $\alpha_3\beta_3$ is important for adhesion of several cell types to TSP1 (12), we found that adhesion of breast carcinoma cells on TSP1 substrates is not mediated by this integrin. We report here that the $\alpha_3\beta_1$ integrin rather than $\beta_3$ integrins play a dominant role in adhesion of several breast carcinoma cell lines on TSP1. The activation state of the $\alpha_3\beta_1$ integrin varies among the human breast carcinoma cell lines examined and can be modulated by inside-out signaling, suggesting that the ability to receive pro-adhesive and motility signals from TSP1 is tightly regulated in these breast carcinoma cell lines.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Calcium-replete TSP1 was purified from human platelets as described (13). Peptide fragments of TSP1 were prepared as described previously (14). Synthetic peptides containing TSP1 sequences were prepared as described previously (15–17). Bovine type I collagen was obtained from Collaborative Research, and vitronectin was from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (18). Murine laminin-1 purified from the Engelbreth-Holm-Swarm sarcoma was provided by Dr. Sadie Aznavorian. Recombinant human EGF and TGF-$\beta_1$ were obtained from R & D Systems. Insulin was from Biofluids, and
regenerative human insulin-like growth factor-1 (IGF1) was from Bachem.

**Monoclonal Antibodies**—Hybridomas producing the $\alpha_5\beta_1$ integrin-activating antibody TS2/16 (19) and the CD98 antibody 4F2 were obtained from the American Type Culture Collection. Antibodies secreted in PFHM-II medium (Life Technologies, Inc.) were purified by protein G affinity chromatography (Pierce). Integrin function blocking antibodies used include LM609 ($\alpha_\beta_5$, provided by Dr. David Cheresh), 05-246 ($\alpha_\beta_3$, Upstate Biotechnology), 6D7 ($\alpha_\beta_5$, Dr. Harvey Gralnick, NIH), P1B5 ($\alpha_\beta_5$, Life Technologies, Inc.), 407279 ($\alpha_\beta_3$, Calbiochem), P1D6 ($\alpha_\beta_1$, Life Technologies, Inc.), and mAb13 ($\beta_1$, Dr. Kenneth Yamada, NIH). Non-blocking antibodies recognizing $\alpha_\beta_1$ (M-KID2), $\alpha_\beta_3$, (HP2/1), and $\alpha_\beta_5$ (SAM1) were obtained from AMAC, Inc. (Westbrook, ME), and $\alpha_\beta_3$ (LM142) was provided by Dr. David Cheresh.

**Cell Lines and Reagents**—MDA-MB-435, MDA-MB-231, and MCF-7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% FCS, Okaeda acid, FMA, pertussis toxin (PT), heparin, and, and sodium vanadate were purchased from Sigma. Pertussis toxin B oligomer, stauroporine, sorbin, man, RT6523, guanosine-5'-cyclic monophosphothioate, 8-4-chlorophenylthio), Rp isomer, and bisindolylmaleimide were obtained from Calbiochem. KT5720 was from Kambiy Biomedical (Thousand Oaks, CA).

**Adhesion Assays**—Cells were detached by replacing the growth medium with PBS containing 2.5 mM EDTA and incubating 5–10 min at 37°C. The cells were collected by centrifugation, suspended in RPMI containing 0.1% BSA, and assayed for adhesion to bacteriological polystyrene substrates coated with proteins as described previously (14). Adhesion assays were terminated after 50 min by washing to remove nonadherent cells and fixation with 1% glutaraldehyde in PBS.

**Chemotaxis**—Chemotaxis was measured in 48-well chambers using Nucleopore 9 μm, polyvinylpyrrolidone-free filters (Neuroprobe Inc, Gaithersburg, MD). To provide an integrin-independent substrate for motility, the filters were coated with 10 μg/ml polylysine for 16 h at 4°C prior to use. Motility was measured after 6.5 h and scored microscopically by counting nuclei of migrated cells on the lower surface of the membrane.

**Fluorescence Microscopy**—To examine integrin localization and cytoskeletal rearrangement, 8-well glass chamber slides (Nalge Nunc International, Naperville, IL) were coated with type I collagen, TSPL, or fibronectin overnight at 4°C. The chambers were then blocked with 1% BSA in PBS, and cells were added to RPMI containing 0.1% BSA. In some cases, antibodies were included in the medium. Cells were allowed to attach and spread for 90 min. The unbound cells were then removed along with the medium, and the chambers were rinsed with PBS and fixed with 3.7% formaldehyde. Cells were stained with BODIPY TR-X phallacidin (Molecular Probes, Inc., Eugene, OR) to visualize F-actin or with the indicated antibodies followed by BODIPY FL anti-mouse IgG to localize integrins or CD98. All staining procedures were carried out according to the manufacturer's directions. Stained cells were observed and photographed under a Zeiss fluorescence microscope using appropriate filters.

**Unstimulated MDA-MB-435 cells** were evaluated for expression of integrins or their subunits 1 day after plating in RPMI medium containing 10% FCS (Biofluids) by indirect immunofluorescence and flow cytometry. Cells were washed with PBS, 0.2% BSA and incubated at 37°C for 6 min with Puck's saline A with 0.2% EDTA and 10% FCS. All subsequent procedures were performed on ice, and all washes were with PBS containing 0.2% BSA. Cells were dislodged with a scraper, and the resultant cell suspension was washed. Cell pellets were exposed to mouse IgG or primary antibodies to integrins or integrin subunits in PBS, 0.2% BSA, washed, and incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Tago, Inc., Burlingame, CA). Following a wash, the cells were fixed in 1% formaldehyde and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Initial gating was done using forward and side scatter to identify a population of intact cells without debris.

**Ligand Binding—TSPL** was iodinated using Iodogen (Pierce) as described previously (20). For some experiments, cells were grown in sulfate-deficient medium containing chlorate to inhibit proteoglycan and glycolipid sulfation as described previously (21).

**RESULTS**

**Integrin Expression on Breast Carcinoma Cells**—Flow cytometric analysis (Table 1) and immunoprecipitation using subunit-specific integrin antibodies (data not shown) demonstrated that MDA-MB-435 cells express several $\beta_1$ integrins and $\alpha_\beta_3$. Integrin expression on MDA-MB-231 and MCF-7 cells has been reported previously (22–24). MDA-MB-231 cells express $\alpha_\beta_3$, $\alpha_\beta_4$, $\alpha_\beta_5$, $\alpha_\beta_7$, and $\beta_1$ subunits. The MDA-MB-231 and MCF-7 cell lines express only low levels of $\beta_3$ subunits (24).

**Binding of Soluble TSPL**—Previous studies using MDA-MB-231 breast carcinoma cells (25) concluded that sulfated glycoconjugates including heparan sulfate and chondroitin sulfate proteoglycans play a dominant role in both binding of soluble TSPL and adhesion on immobilized TSPL. We observed a similar dependence for $^{125}_{1}$-TSPL binding to MDA-MB-435 cells (Fig. 1). Binding was strongly inhibited by heparin or a recombinant 18-kDa amino-terminal heparin-binding fragment of TSPL, but the peptide GRGDS and $\beta_1$ integrin function blocking antibodies had no effect. Conversely, binding of $^{125}_{1}$-TSPL to MDA-MB-435 cells was not enhanced by incubation with the $\beta_1$ integrin-activating antibody TS2/16, either alone or in the absence of 10 μg/ml heparin to inhibit TSPL binding to sulfated ligands (data not shown). Therefore, high affinity binding to soluble TSPL to these cells is mediated by sulfated glycoconjugates and is independent of integrin binding.

$\beta_1$ Integrin-mediated Adhesion and Chemotaxis to TSPL—Although heparin and recombinant heparin binding domain from TSPL partially inhibited attachment of MDA-MB-435 cells on immobilized TSPL, the fraction of spread cells was unaffected (Fig. 2A). In the presence of a $\beta_1$ integrin function blocking antibody at 2 μg/ml, however, only spreading was inhibited, and a combination of heparin and the $\beta_1$ blocking antibody abolished spreading and markedly inhibited attachment. At 50 μg/ml, the $\beta_1$ antibody completely inhibited adhesion to TSPL (Fig. 2A). Thus, interaction with a $\beta_1$ integrin is essential for spreading, but sulfated ligands may also contrib-

### Table 1

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<tr>
<td>$\beta_1$</td>
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Regulation of Thrombospondin-1 Adhesion by α5β3 Integrin

Fig. 2. Role of integrins and sulfated glycoconjugates in breast carcinoma cell adhesion and chemotaxis to TSP1. A, MDA-MB-435 cell attachment (solid bars) and spreading (stripped bars) were measured after 50 min on polystyrene coated with thrombospondin (50 μg/ml) and blocked with 1% BSA to reduce nonspecific adhesion. Heparin-dependent adhesion was assessed by inhibition using 4 μM 18-kDa recombinant TSP1 heparin-binding domain (HBD) or 50 μM/ml heparin. β3 integrin-dependent adhesion was inhibited using 2 or 50 μg/ml mAb3 (anti-β3). Results are presented as mean ± S.D., n = 3. B, effect of inhibiting sulfation on attachment of MDA-MB-435 cells. MDA-MB-435 cells were grown in Ham's F-12 medium (low NaCl) containing 4% dialyzed fetal calf serum for 48 h. The medium was replaced with the same medium containing 1% dialyzed serum with or without sodium chlorate at the indicated concentrations. The cells were cultured for 24 h, harvested, and resuspended in F-12 medium containing 1 mg/ml BSA with or without chlorate at the indicated concentrations. Cell adhesion was quantified to polystyrene coated with 50 μg/ml thrombospondin (stripped bars) or 10 μg/ml fibronectin (gray bars). 35S incorporation in MDA-435 cell macromolecules (□) was assessed in duplicate cultures supplemented with 25 μCi/ml [35S]sulfate. The cells were fixed and washed in acetic acid/methanol, and incorporation of radioactivity in macromolecules was determined by scintillation counting after solubilization in 1% sodium dodecyl sulfate. C, integrin α3β3 mediates breast carcinoma cell adhesion to vitronectin but not to TSP1. Adhesion of MDA-MB-435 cells to 30 μg/ml TSP1 (solid bars) or 10 μg/ml vitronectin (stripped bars) was measured in the presence of the α3β3 function blocking antibody LM609 or the β3-activating antibody TS2/16. D, chemotaxis to TSP1 is β3 integrin-dependent. MDA-MB-435 chemotaxis to 50 μg/ml TSP1 was determined in modified Boyden chambers. Cells were added in the upper chamber with the indicated concentrations of β3 integrin blocking antibody mAb3 (●) or heparin (○). Spontaneous motility (▲) was determined in the absence of TSP1. Migrated cells were counted microscopically, and results from triplicate wells are presented as a percent of migration to TSP1 without inhibitors, mean ± S.D.

Fig. 3. β3 integrins recognizing TSP1 and type I collagen are partially inactive in human breast carcinoma cell lines. A, spreading of three breast carcinoma cell lines on 50 μg/ml TSP1 (solid bars) or on TSP1 in the presence of 5 μg/ml TS2/16 (stripped bars). B, comparison of β3 integrin activity in MDA-MB-435 cells harvested by scraping in RPMI medium or by a 5-min treatment with 2.5 mM EDTA in PBS. Cells were resuspended in RPMI medium with 0.1% BSA (solid bars) or with 20 μg/ml TS2/16 (stripped bars), and cell spreading was assessed after 50 min on substrates coated with 20 μg/ml TSP1 or 5 μg/ml type I collagen.

Although MDA-MB-435 cells express some α5β3 integrin (Table I), a function blocking antibody or an α5β3-specific RGD mimetic blocked adhesion of the cells on vitronectin but had no effect on adhesion on TSP1 (Fig. 2C and results not shown). Conversely, in the presence of the β3-activating antibody TS2/16, adhesion of MDA-MB-435 cells was enhanced on TSP1 but not on vitronectin (Fig. 2C). Therefore, the α5β3 integrin is functional in MDA-MB-435 cells, but it is apparently unable to recognize the RGD motif in TSP1. The β3 blocking antibody mAb3 inhibited chemotaxis to TSP1, but heparin did not (Fig. 2D). For these experiments, the filters were coated with polylysine to provide an integrin-independent substrate for adhesion of the cells. Therefore, chemotaxis of MDA-MB-435 cells to TSP1 is also primarily dependent on the β3 integrin receptor.

Several human breast cancer cell lines showed similar involvement of β3 integrins in their adhesion to TSP1 (Fig. 3). MDA-MB-231 cells attached poorly and did not spread on substrates coated with low concentrations of TSP1. In the presence of the β3-activating antibody, however, the cells attached av-
spikes protruding from the spread cells but did not organize into stress fibers. Staining with the β1 integrin antibody revealed numerous filopodia extending from these points (Fig. 5c). In some cells, these filopodia were terminated with punctate β1 integrin staining, possibly at sites of contact with the TSP1 substrate. Formation of filopodia was specific to the TSP1 substrate, as TS2/16-induced spreading of these cells on type I collagen (Fig. 5d) or fibronectin (results not shown) only rarely evoked filopodia. These cytoskeletal rearrangements were specific for β3-dependent adhesion to intact TSP1 and were not observed in cells attaching on heparin-binding peptides or recombinant fragments of TSP1 (results not shown). Similar induction of filopodia or microspikes by TSP1 have been observed in other cell types (26).

Conformation Requirements for αββ3-Mediated Adhesive Activity of TSP1— Differences in the conformation or folding of TSP1 could account for discrepancies in its reported adhesive activity. The conformation of TSP1 and formation of specific intra-chain disulfide bonds are sensitive to the levels of divalent cations present during its purification. Disulfide bonding also influences interactions of TSP1 with several proteases and regulates the accessibility of the RGD sequence to the αβ3 integrin (27, 28). We therefore examined the influence of conformation on αββ3-dependent adhesion by absorbing TSP1 with or without divalent cations, at low pH (29), or by reducing disulfide bonds using dithiothreitol (Fig. 6). Coating TSP1 at pH 4 in acetate buffer enhanced MDA-MB-435 cell adhesion relative to TSP1 adsorbed in PBS with Ca2+ and Mg2+, but use of PBS with 2.5 mM EDTA did not significantly affect β3-mediated adhesion. Although heparin only partially inhibited MDA-MB-435 cell adhesion to TSP1 (20–50%) when the TSP1 was adsorbed in Dulbecco’s PBS (e.g. Fig. 2A), adhesion to TSP1 adsorbed in pH 4 acetate buffer was inhibited 98% by 10 μg/ml heparin. Conversely, TS2/16 did not reproducibly increase adhesion of MDA-MB-435 cells to TSP1 adsorbed in acetate buffer (data not shown). Therefore, the enhanced adhesion to thrombospondin coated at pH 4 was due primarily to enhancement of heparin-dependent adhesion, whereas β3-integrins contributed less to adhesion on TSP1 coated at the lower pH. Adhesion of MDA-MB-435 cells (Fig. 6) and MDA-MB-231 cells (results not shown) was strongly inhibited following reduction of TSP1 with dithiothreitol. This contrasts with αβ2-dependent adhesion to TSP1, which was reported to be enhanced following disulfide reduction using the same conditions as used in Fig. 6 (28). Thus, αββ3-dependent adhesion of breast carcinoma cells does not require Ca2+-replete TSP, but some intact disulfide bonds are essential.

Regulation of β3 Integrin Activation in Breast Carcinoma Cells—Adhesion of T lymphocytes to TSP1, mediated by αβ3, and αββ3 integrins, is stimulated by phorbol esters (30). PMA activation of protein kinase C in MDA-MB-435 cells increased αββ3-mediated adhesion to vitronectin but had no effect on β3 integrin-mediated adhesion to TSP1 (Fig. 7). Integrin-associated protein (CD47) also regulates integrin function in several cell types (31, 32). The carboxyl-terminal domain of TSP1 contains two peptide motifs that activate integrin function through binding to CD47 (31). The CD47-binding TSP1 peptide TN3 activated adhesion of MDA-MB-435 cells on vitronectin (Fig. 7) and a recombinant TSP1 fragment containing the RGD sequence (results not shown) but had no effect on adhesion to native TSP1 (Fig. 7). Thus MDA-MB-435 cells express functional αβ3 integrin that can be activated by PMA or the TSP1 7N3 peptide. This αβ3 integrin can recognize the TSP1 RGD sequence in the context of a bacterial fusion protein, but it does not play a significant role in adhesion of resting or stimulated breast carcinoma cells to native platelet TSP1.
Several pharmacological agents stimulated β₃-dependent adhesion to TSP1 (Table II). The broad spectrum Ser/Thr protein kinase inhibitor staurosporine increased spreading of all three cell lines. However, this activation in MDA-MB-435 cells was only partially replicated by specific inhibitors of protein kinase C (bisindolylmaleimide), protein kinase A (KT5720), or protein kinase G (KT5823 and guanosine-3',5'-cyclic monophosphorothioate, 8-(4-chlorophenylthio)−, Rp isomer). Inhibition of phosphatidylinositol 3-kinase using wortmannin had no significant effect on MDA-MB-435 cell spreading and weakly enhanced MDA-MB-231 cell spreading on TSP1. Two calcium ionophores, ionomycin and A23187, strongly enhanced spreading of MDA-MB-435 cells but had no effect on MDA-MB-231 cell spreading on TSP1.

**Modulation of TSP1 Adhesion by G-protein Signaling**—Although TSP1 peptides promote PT-sensitive integrin activation through binding to CD47 (31, 33), we showed above that this pathway does not function in MDA-MB-435 cells to activate α₃β₁. However, PT did influence MDA-MB-231 and MDA-MB-435 cell adhesion and spreading on TSP1 or collagen (Fig. 8).

PT increased adhesion of MDA-MB-231 cells to TSP1 (Fig. 8A) but inhibited both basal and TS2/16-stimulated adhesion of MDA-MB-435 cells on the same substrate (Fig. 8B). The effects of PT in both cell lines were specific, since PT-B-oligomer at the same concentration had no effect (Fig. 8). The enhancement of MDA-MB-231 cell adhesion by PT is mediated by the β₁ integrin, because the β₁ blocking antibody mAb13 inhibited the PT-induced adhesion of MDA-MB-231 cells but heparin did not (results not shown). However, not all β₁ integrins in these breast carcinoma cells were activated by PT. Adhesion of MDA-MB-231 cells to collagen mediated by α₃β₁ (verified by the blocking antibody 6D7, results not shown) was not altered by PT, although the same adhesive pathway could be further activated by TS2/16 (Fig. 8A). In MDA-MB-435 cells, PT partially inhibited α₃β₁-mediated spreading on collagen stimulated by TS2/16 (Fig. 8B).

**Physiological Activators of TSP1 Adhesion and Chemotaxis**—We noted that freshly passaged breast carcinoma cells exhibited stronger β₁ integrin-mediated adhesion on TSP1. This suggested that proliferation regulates α₃β₁-mediated TSP1 ad-
Regulation of Thrombospondin-1 Adhesion by α₃β₁ Integrin

FIG. 7. Differential regulation of β₁ and β₂ integrin activity in MDA-MB-435 cells. Attachment of MDA-MB-435 cells on 5 μg/ml vitronectin (striped bars) or 40 μg/ml TSP1 (solid bars) was measured using cells treated with 20 μg/ml TS2/16, 10 ng/ml PMA, or 3 μM of the CD47-binding TSP1 peptide 7N3 (FIRVVMYEGKK). Results are presented as a percentage of cell attachment without additions, mean ± S.D., n = 3.

Table II

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<th>Inhibitor</th>
<th>Cell line</th>
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<td></td>
<td>MDA-MB-435</td>
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<tr>
<td>Staurosporine</td>
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FIG. 8. Pertussis toxin differentially regulates MDA-MB-435 and MDA-MB-231 cell adhesion on TSP1. A, MDA-MB-231 cell attachment on 40 μg/ml TSP1 (solid bars) or 5 μg/ml type I collagen (gray bars) was measured alone or in the presence of 5 μg/ml TS2/16, 1 μg/ml PT, or 1 μg/ml PT B-oligomer. Results are mean ± S.D. for triplicate determinations. B, MDA-MB-435 cell spreading was determined on TSP1 (solid bars) or type I collagen substrates (gray bars) in the presence of PT or PT B-oligomer added alone or combined with 5 μg/ml TS2/16.

hension. Serum induced a dose-dependent increase in β₁ integrin-mediated attachment (Fig. 9A) and spreading of MDA-MB-435 cells to TSP1 or type I collagen. A similar serum response was observed in MDA-MB-231 cells for adhesion on TSP1, although adhesion of the latter cell type to type I collagen was maintained in the absence of serum (data not shown).

Several growth factors were examined to define the basis of the serum response for TSP1 adhesion (Fig. 9B). Addition of EGF to serum-depleted medium increased adhesion of breast carcinoma cells to some substrates (34) but in several experiments showed only a slight stimulatory activity for spreading of MDA-MB-435 cells on TSP1 (Fig. 9B). FGF2 and TGF-β1 were also ineffective, but addition of insulin stimulated MDA-MB-435 cell adhesion to a greater extent than 10% serum (Fig. 9B). Insulin was also the only growth factor tested that stimulated adhesion of MDA-MB-231 cells to TSP1 (results not shown).

Acute addition of insulin, but not EGF, during the adhesion assay produced a similar enhancement in adhesion of both cell lines to TSP1 as the 24-h pretreatment of the cells in culture (Fig. 9C and results not shown). The dose dependence for the insulin response was consistent with that for signaling through the IGF1 receptor (Fig. 9C), which is expressed in these breast carcinoma cells (35). Both insulin and IGF1 strongly stimulated MDA-MB-435 cell spreading on TSP1, moderately stimulated adhesion on type I collagen, but did not stimulate adhesion on laminin-1 (Fig. 9C). EGF (2 μM) was inactive in this assay (results not shown). IGF1 (EC₅₀ = 1 nM) was 100-fold more potent than insulin, as expected for a response mediated by the IGF1 receptor (35). A similar difference in the potencies of IGF1 and insulin was also observed in stimulation of TSP1 attachment of MDA-MB-231 cells (results not shown). Thus, occupancy of the IGF1 receptor specifically stimulates activity of the TSP1-binding integrin in both cell lines.

IGF1 also enhanced the chemotactic response of breast carcinoma cells to TSP1. Addition of IGF1 to MDA-MB-435 cells in the upper well of a modified Boyden chamber did not alter motility of the cells, but it stimulated (2- to 5-fold) the chemotactic response to TSP1 added to the lower chamber (Fig. 9D). This IGF1-stimulated motility to TSP1 was mediated by the α₃β₁ integrin, because mAb13 (anti-β₁) and P1B5 antibodies (anti-α₃) strongly inhibited direct TSP1 chemotaxis and that stimulated by IGF1. IGF1-stimulated chemotaxis to TSP1 was also sensitive to PT inhibition (Fig. 9D).

Modulation of TSP1 Adhesion by CD98—Expression of the transmembrane protein CD98 is induced by serum, and this protein was recently shown to activate function of some β₁ integrins (36). Clustering of CD98 using the antibody 4F2 stimulates small cell lung carcinoma adhesion on fibronectin and laminin (36) and similarly activated α₃β₁-mediated spreading of breast carcinoma cells on TSP1 and α₃β₁-mediated...
Regulation of Thrombospondin-1 Adhesion by \( \alpha_3\beta_1 \) Integrin

**Fig. 9. Regulation of \( \beta_1 \) integrin-mediated TSP1 interactions by serum and growth factors.** A, serum induces attachment of MDA-MB-435 cells to TSP1 (solid bars) and type I collagen (striped bars). Cells were grown for 24 h in RPMI medium containing the indicated concentration of FCS. B, insulin specifically induces adhesion of breast carcinoma cells to TSP1. MDA-MB-435 cell spreading on surfaces coated with 40 \( \mu \)g/ml TSP1 was determined using cells grown for 24 h in RPMI medium containing 2% serum and supplemented with the indicated growth factors (10 ng/ml EGF, 100 ng/ml FGF2, 5 ng/ml TGF-\( \beta \), or 10 \( \mu \)g/ml insulin) or RPMI medium containing 10% serum. Spreading of cells grown in 2% serum was also tested in the presence of 5 \( \mu \)g/ml antibody TS2/16 (2% + TS2/16) to assess maximal \( \beta_1 \) integrin-mediated spreading activity. C, dose dependence for induction of TSP1 adhesion by insulin and IGF1. Cell spreading after 50 min (expressed as a percentage of maximal spreading elicited on each substrate in the presence of 5 \( \mu \)g/ml TS2/16 antibody) was determined in RPMI medium containing 0.1% BSA and supplemented with the indicated concentrations of insulin (closed symbols) or IGF1 (open symbols) using substrates coated with 40 \( \mu \)g/ml TSP1 (○ and ●), 20 \( \mu \)g/ml laminin (△ and ▽), or 5 \( \mu \)g/ml type I collagen (■ and □). D, IGF1 synergizes with TSP1 to promote chemotaxis of MDA-MB-435 cells. Chemotaxis to 50 \( \mu \)g/ml TSP1 was determined in the presence of the indicated inhibitors or stimulators at the following concentrations: 10 nM IGF1, 5 \( \mu \)g/ml MAbl (anti-\( \alpha_3 \)), 5 \( \mu \)g/ml PIB5 (anti-\( \alpha_5 \)), and 1 \( \mu \)g/ml PT. Results are mean ± S.D., n = 3–6.

adhesion on type I collagen (Fig. 10A and results not shown). Induction of \( \alpha_3\beta_1 \)-mediated TSP1 adhesion in serum-containing growth medium may be mediated by induction of CD98 expression, because a 24-h exposure to 10% serum increased surface expression of CD98 in MDA-MB-435 cells (Fig. 10B). IGF1 treatment for the same time, however, decreased CD98 expression (Fig. 10B), indicating that increased CD98 expression does not mediate the response to IGF1.

**DISCUSSION**

The \( \alpha_3\beta_1 \) integrin, with some cooperation of sulfated glycoconjugates and \( \alpha_5\beta_1 \) integrin, mediates adhesion of MDA-MB-435 and MDA-MB-231 breast carcinoma cells to TSP1. This \( \beta_1 \) integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1, and ligation of CD98. In MDA-MB-231 cells, the inactive state of the \( \alpha_3\beta_1 \) integrin is maintained by a G-protein-mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase \( \beta_1 \)-dependent adhesion to TSP1 do not stimulate \( \beta_3 \)-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor \( \alpha_5\beta_1 \), and this integrin is functional and inducible for vitronectin adhesion. We do not know why the \( \alpha_3\beta_3 \) integrin on MDA-MB-435 cells cannot recognize the RGD sequence in the type III repeat of platelet TSP1. Other cell types, however, can utilize the same TSP1 preparations used for these experiments to support \( \alpha_3\beta_3 \)-dependent adhesion.

Several \( \beta_1 \) integrins have been implicated as TSP1 receptors in other cell types, including \( \alpha_5\beta_1 \) on activated platelets (37), \( \alpha_5\beta_2 \) on neurons (38), and \( \alpha_6\beta_1 \) and \( \alpha_6\beta_4 \) on activated T lymphocytes (30). \( \alpha_3\beta_1 \) is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas \( \alpha_3\beta_1 \) mediates adhesion of these cells to type I collagen but not to TSP1. The integrin \( \alpha_3\beta_1 \) may play a role in adhesion of some breast carcinoma cell lines to TSP1, as we previously reported for T lymphocytes (30). The mechanism for the apparent dif-

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Regulation of Thrombospondin-1 Adhesion by α₃β₁ Integrin

Regulation of Thrombospondin-1 Adhesion by α₃β₁ Integrin

Fig. 10. CD98 ligation stimulates breast carcinoma cell adhesion to TSP1. A, basal (solid bars) or stimulated MDA-MB-231 or MDA-MB-435 cell spreading on 25 μg/ml TSP1 was determined in the presence of 5 μg/ml TS2/16 (striped bars) or 20 μg/ml 4F2 (gray bars). B, serum induces but IGF1 inhibits CD98 expression. MDA-MB-435 cells grown 24 h in RPMI medium containing 1% FCS, 10% FCS, or 1% FCS and 10 nM IGF1 as described in A were biotinylated, and equal amounts of cell protein were immunoprecipitated with antibody 4F2. The immunoprecipitates were analyzed by SDS-gel electrophoresis and Western blotting using streptavidin-peroxidase and chemiluminescent detection. Markers indicate the migration of the 80- and 45-kDa subunits of CD98.

Differential recognition of TSP1 by β₁ integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α₃β₁ integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pre-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by "inside-out" signaling (39), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to α₂β₁ integrin, the α₃β₁ integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 "VVM" peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β₁-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via α₂β₁ but not α₃β₁ integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of α₂β₁-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically associated with the α₂β₁ integrin but not with β₁ integrins in fibroblasts (40). The α₂β₁ integrin also co-immunoprecipitated with insulin receptor substrate-1 (41). Engagement of α₂β₁ integrin by vitronectin but not α₃β₁ integrin by collagen increased mitogenic signaling through the insulin receptor (40, 41). Thus, the specific activation of α₂β₁-mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the α₃β₁ integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific coupling of α₀β₁ activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates rapid activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

CD98 was recently identified as an activator of β₁ integrins by its ability to overcome Tac-β₁ suppression of β₁ integrin function (36, 42). Our data demonstrate that clustering of CD98 can also increase α₀β₁-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated α₀β₁ integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis cannot be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the α₃β₁ integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including Ha-Ras (43), integrin-linked kinase, and protein kinase C (39). Additional proteins are known to associate with the α₃β₁ integrin, including some members of the TM4SF family and EMMPRIN (44, 45), but their roles in regulating function are unknown. In MDA-MB-231 cells, suppression of α₃β₁ appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the α₃β₁ integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using α₂β₁ integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better α₃β₁-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores provides our first insight into a breast carcinoma TSP1 receptor.

TSP1 has diverse effects on breast carcinoma cell behavior, altering their adhesion, motility, proliferation, protease expression, and invasion. These cellular responses result in alterations of their in vivo tumorigenic, angiogenic, and metastatic potentials (reviewed in Ref. 4). We have defined specific roles for the α₃β₁ integrin in spreading, induction of filopodia, and chemotactic responses to TSP1. In other cell types, the low density lipoprotein receptor-related protein has been assigned a role in internalization of TSP1 (46), and CD36 has been shown to play an essential role in angiogenesis inhibition (47). The receptors that mediate many responses to TSP1 remain to be defined. These responses may require coordinated signaling through two or more TSP1 receptors. Defining the role of IGF1 and CD98 in regulating β₁ integrin interactions with TSP1 provides our first insight into a breast carcinoma TSP1 receptor that can be turned on or off in response to known environmental stimuli. The ability to regulate the activity of this TSP1 receptor will facilitate analysis of the signals resulting from this interaction.

Acknowledgments—We thank Drs. Ken Yamada, David Cheros, and Harvey Gralnick for providing antibodies and Henry Krutzsch for synthesis of peptides.
Regulation of Thrombospondin-1 Adhesion by αβ3 Integrin

REFERENCES

A synthetic peptide containing amino acid residues 190–201 of thrombospondin-1 (TSP1) promoted adhesion of MDA-MB-435 breast carcinoma cells when immobilized and inhibited adhesion of the same cells to TSP1 when added in solution. Adhesion to this peptide was enhanced by a β1 integrin-activating antibody, Mn2+, and insulin-like growth factor I and was inhibited by an α3β1 integrin function-blocking antibody. The soluble peptide inhibited adhesion of cells to the immobilized TSP1 peptide or spreading on intact TSP1 but at the same concentrations did not inhibit attachment or spreading on type IV collagen or fibronectin. Substitution of several residues in the TSP1 peptide with Ala residues abolished or diminished the inhibitory activity of the peptide in solution, but only substitution of Arg-198 completely inactivated the adhesive activity of the immobilized peptide. The essential residues for activity of the peptide as a soluble inhibitor are Asn-196, Val-197, and Arg-198, but flanking residues enhance the inhibitory activity of this core sequence, either by altering the conformation of the active sequence or by interacting with the integrin. This functional sequence is conserved in all known mammalian TSP1 sequences and in TSP1 from Xenopus laevis. The TSP1 peptide also inhibited adhesion of MDA-MB-435 cells to the laminin-1 peptide GD6, which contains a potential integrin-recognition sequence Asn-Leu-Arg and is derived from a similar portion that the α3β1 integrin has been reported to recognize several extracellular matrix ligands, including some laminins, type IV collagen, fibronectin, thrombospondin-1, and entactin/nidogen (5–8). Although short peptide recognition motifs have been identified in ligands for some integrins (reviewed in Ref. 9), previous attempts to define recognition sequences for binding of matrix ligands to the α3β1 integrin have produced conflicting results. High affinity binding of recombinant soluble α3β1 could be detected only to laminin-5 (10), so binding to other matrix ligands may be of relatively low affinity. Under specific conditions, this integrin can recognize the common integrin binding sequence RGD in fibronectin (6). However, recombinant entactin with the RGD sequence deleted (11) and synthetic peptides from laminin-1 and type IV collagen that lack the RGD motif (12, 13) also bound specifically to the α3β1 integrin. Laminin peptide GD6 (KQCNCLSSARSGFRCVNRSLR) and the type IV collagen peptide affinity purified α3β1 integrin from cell extracts when immobilized on agarose beads (12, 13), but the active peptides from these two proteins share no apparent sequence homology. These data, combined with the evidence that RGD-dependent and RGD-independent adhesion are differentially regulated in α3β1 integrin (6), have led to the proposal that the α3β1 integrin uses distinct mechanisms to interact with each of its ligands and that no conserved binding motif may exist (6).

We recently found that α3β1 is the major TSP1-binding integrin on several human breast carcinoma cell lines (14). We have further examined this interaction and report the identification of a peptide sequence from TSP1 that supports α3β1-dependent adhesion and chemotaxis and is a potent inhibitor of adhesion to TSP1.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides**—Calcium replete TSP1 was purified from human plasma (24). Synthetic peptides containing TSP1 sequences were prepared as described previously (16–21). Recombinant fragments (provided by Dr. Tilkia Vogel) and GST fusion proteins expressing fragments of TSP1 (provided by Dr. Jack Lawler, Harvard University) were prepared as described previously (22, 23). Bovine type I collagen and murine Type IV collagen were obtained from Becton Dickinson Labware division, and human vitronectin was from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (24). Murine laminin-1 purified from the EHS tumor was provided by Dr. Sadie A. Navarro (NCI, National Institutes of Health). Recombinant human insulin-like growth factor-1 (IGF1) was from Bachem.

**Adhesion Assays**—Adhesion was measured on polystyrene or glass substrates coated with peptides or proteins as described previously (16). Inhibition assays were performed using the following function-blocking antibodies: 6D7 (α3β1), P1B6 (Life Technologies, Inc., α3β1), 407279 (Calbiochem, α3β1), and P1D6 (Life Technologies, Inc., α3β1). The α3β1 integrin-activating antibody TS2/16 (25) was prepared from the hybridoma obtained from the American Type Culture Collection. Immunofluorescence analysis of cell adhesion was performed as described previously, using BODIPY-TR-X phallacidin (Molecular Probes, Inc., Eugene, OR) to visualize F-actin or using murine primary antibodies

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‡ The abbreviations used are: TSP, human thrombospondin; GST, glutathione S-transferase; IGF1, insulin-like growth factor-1.

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binding domain had significant adhesive activity, although the main
activities and were also insensitive to TS2/16. The focal adhe-
sion disrupting peptide Hepl from the N-terminal domain of 598-608) repeat sequences that did not support
adhesion or inhibit adhesion of MDA-MB-435 cells to the C-terminal domain of TSP1 (residues 1059-1077), did not
avidly attached on substrates coated with the same concentra-
tions of this fragment (26). The main adhesive sequence in the form of synthetic peptides or GST or T7 fusion proteins for promotion of β1 integrin-dependent adhesion of MDA-MB-435 cells (Fig. 1). Among the recombinant fragments tested, only an 18-kDa fragment of the N-terminal heparin-binding domain had significant adhesive activity, although the recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments (results not shown). A recombinant GST fusion of the type 3 repeats of TSP1 including the hydrophilic residues in the laminin-1 GD6 peptide that was not covered by the hydrophilic residues in the laminin-1 GD6 peptide that could mediate protein-protein interactions. This sequence also overlaps with a region identified in a screen of N-terminal

RESULTS

In initial attempts to localize the region of TSP1 recognized by the α3β1 integrin, we tested approximately 85% of the TSP1 sequence in the form of synthetic peptides or GST or T7 fusion proteins for promotion of β1 integrin-dependent adhesion of MDA-MB-435 cells (Fig. 1). Among the recombinant fragments tested, only an 18-kDa fragment of the N-terminal heparin-binding domain had significant adhesive activity, although the recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments (results not shown). A recombinant GST fusion of the type 3 repeats of TSP1 including the RGD sequence had minimal adhesive activity for MDA-MB-435 cells (Fig. 1), in contrast to human melanoma cells, which avidly attached on substrates coated with the same concentrations of this fragment (26). The β1 integrin-activating antibody TS2/16 did not enhance cell attachment to any of these recombinant fragments but reproducibly stimulated attachment on intact TSP1 (Fig. 1). Synthetic heparin-binding peptides from the type 1 repeats (peptide 4N1K (21) also promoted adhesion, but TS2/16 did not enhance adhesion of MDA-MB-435 cells to these peptides. CD36-binding peptides from the procollagen domain (peptide 500) or the type 1 repeats (Mal-II) (29) had weaker adhesive activities and were also insensitive to TS2/16. The focal adhesion disrupting peptide Hep1 from the N-terminal domain of TSP1 (20) did not promote MDA-MB-435 cell adhesion. Although these experiments did not detect a β1 integrin-dependent adhesive sequence in TSP1, the possibility remains that these regions of TSP1 contain a conformation-dependent recognition motif that is inactive in the recombinant fusion proteins due to misfolding.

A multiple alignment search using MACAW software was used to identify TSP1 sequences that might be related to the α3β1 integrin-binding GD6 peptide derived from the A chain of murine laminin-1 (12), which strongly promoted MDA-MB-435 cell adhesion (Fig. 2A). This search identified four TSP1 sequences related to the laminin peptide (Table I). The single peptide identified by the Gibbs sampler method, derived from the C-terminal domain of TSP1 (residues 1059–1077), did not support adhesion or inhibit adhesion of MDA-MB-435 cells to TSP1 or other α3β1 integrin ligands (Fig. 2A and results not shown). Because a synthetic peptide containing the last 12 residues of peptide GD6 (peptide 679, Table I) had similar activity to the intact peptide (see below), we did not test the two peptides identified by segment pair overlap that aligned outside this sequence. Both of these peptides were derived from regions of the type 1 (residues 392–405) or type 2 (residues 598–608) repeat sequences that did not support α3β1-dependent adhesion when expressed as GST fusion proteins (Fig. 1).

The remaining sequence is from a region of the N-terminal domain of TSP1 (residues 186–199) that was not covered by the recombinant fragments tested in Fig. 1 and conserves most of the hydrophilic residues in the laminin-1 GD6 peptide that could mediate protein-protein interactions. This sequence also overlaps with a region identified in a screen of N-terminal

FIG. 1. Adhesion of MDA-MB-435 breast carcinoma cells to recombinant TSP1 fragments and synthetic TSP1 peptides. Adhesion to synthetic TSP1 peptides adsorbed at 10 µM (246, KRFQGQGGWSHWSHPWSS; 500, NGVQYRNC; Mal II, SPWSSCVTCDGVTIR; 4N1K, KRFVVMWWK; Hep1, ELTGAARKGSRLLVKGPD), TSP1 (0.11 µM), recombinant 18-kDa heparin-binding domain (HBD) (2.7 µM), or recombinant fusion proteins expressing the TSP1 procollagen domain (procoll.), type 1, 2, or 3 repeats, or GST alone (3 µM) was measured in the absence (solid bars) or presence (striped bars) of 20 µg/ml of the β1 integrin-activating antibody TS2/16. Results (mean ± S.D.) are presented for a representative experiment performed in triplicate.

Fig. 2. MDA-MB-435 adhesion to TSP1 peptides and laminin-1 peptide GD6. A, MDA-MB-435 breast carcinoma cell attachment (closed symbols) and spreading (open symbols) was determined on polystyrene substrates coated with the indicated concentrations of TSP1 peptide 678 (FQGVLQNVRFVF) (circles), TSP1 peptide 701 (TPGVRTLWHDNP) (squares), or the murine laminin-1 peptide GD6 (KQCNLSRASFRGCVRNLLRSLR) (triangles). Results are presented as mean ± S.D. (n = 3). B, spreading of MDA-MB-435 or MDA-MB-231 cells on substrates coated with 3.3 µM TSP1 peptide 678, 1.1 µM laminin-1 peptide GD6, or 50 µg/ml TSP1 was determined using untreated cells (black bars) or cells treated with 5 µg/ml of the β1-activating antibody TS2/16 (grey bars) or 3 ng IGF1 (striped bars) (MDA-MB-435 cells only); mean ± S.D. (n = 3).
TSP1 peptides as having heparin-independent adhesive activity (30). A synthetic peptide containing this TSP1 sequence (peptide 678) had strong adhesive activity for MDA-MB-435 cells (Fig. 2A). Spreading of two breast carcinoma cell lines on this peptide, laminin peptide GD6, and TSP1 was enhanced in the presence of the $\beta_1$ integrin-activating antibody TS2/16 (Fig. 2B). We previously found that IGF1 strongly stimulated $\beta_1$ integrin-mediated adhesion to TSP1 (14). IGF1 similarly stimulated spreading of MDA-MB-435 cells on the TSP1 peptide 678 and to the laminin peptide GD6 (Fig. 2B). The TSP1 peptide 678 strongly inhibited spreading of MDA-MB-435 cells on TSP1 and murine EHS tumor-derived laminin-1 peptide 678 added in solution.

**Table I**

<table>
<thead>
<tr>
<th>TSP1 sequences related to murine laminin-1 peptide GD6</th>
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<td>The amino acid sequences for human and murine TSP1 and laminin-1 peptide GD6 were compared by multiple alignment using MA-CAW.</td>
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<tr>
<td>Peptide origin</td>
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<tr>
<td>Laminin GD6</td>
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<tr>
<td>Laminin p679</td>
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<tr>
<td>TSP1 (585-608)</td>
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<tr>
<td>TSP1 (392-405)</td>
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<td>TSP1 (1059-1077)</td>
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a Alignment scores were determined by segment pair overlap.
b Alignment by Gibbs sampler method.

**Fig. 3**. Inhibition of breast carcinoma cell spreading on matrix proteins by peptide 678. A, MDA-MB-435 cell spreading was determined in the absence (solid bars) or presence (striped bars) of 10 $\mu$m TSP1 peptide 678 on substrates coated with 10 $\mu$m peptide 678, 40 $\mu$g/ml TSP1, 10 $\mu$g/ml murine laminin-1, 10 $\mu$g/ml human plasma fibronectin, or 10 $\mu$g/ml type IV collagen. Cell spreading is presented as mean ± S.D. (n = 3). B, inhibition of MDA-MB-435 cell adhesion to surfaces coated with 10 $\mu$m peptide 678 (○) or laminin peptide GD6 (●) was measured in the presence of the indicated concentrations of peptide 678 added in solution. C, adhesion of MDA-MB-231 cells to the indicated recombinant TSP1 fragments was measured in RPMI medium containing 0.1% bovine serum albumin (black bars) or the same medium containing 5 $\mu$g/ml of the $\beta_1$-activating antibody TS2/16 (striped bars) or TS2/16 plus 20 $\mu$m peptide 678 (open bars). Cell attachment is presented as mean ± S.D. for triplicate determinations.

minimally inhibited basal spreading on peptide 678. EDTA completely inhibited the spreading on TSP1 observed in medium containing Mg²⁺ as the sole divalent cation, although it did not inhibit cell attachment on TSP1 (Fig. 4C and results not shown). This residual adhesion probably results from the significant contribution of proteoglycans to adhesion of MDA-MB-435 cells on TSP1 (14). Spreading on peptide 678 with Mg²⁺ as the divalent cation became partially sensitive to EDTA, however, in the presence of the $\beta_1$-activating antibody TS2/16. Addition of Mn²⁺ further stimulated spreading on peptide 678.
αβ₁ Integrin-binding Motif in TSP1

Truncated peptides that contained portions of peptide 678 were synthesized to identify essential residues (Fig. 5). Truncation of the N-terminal Phe or the C-terminal Val-Phe only moderately decreased adhesive activity, but further truncations from either end of the peptide greatly diminished its activity. Inhibition assays confirmed that the loss of adhesive activity reflected loss of integrin binding rather than loss of ability to adsorb on the substrate (Table II). As found in the direct adhesion assays, peptides without the N-terminal Phe or the C-terminal Val-Phe retained significant inhibitory activities, but all shorter peptides were weak inhibitors or inactive. These results imply that the integrin recognizes an extended sequence, but this approach could not discriminate conformational effects of flanking sequences from a direct contribution to integrin binding.

To better define those residues involved in αβ₁ integrin binding, we systematically substituted Ala residues into the peptide 678 and tested each for adhesive activity (Fig. 6). Based on the complete loss of adhesion activity for MDA-MB-435 cells following its substitution, only Arg-198 was essential for adhesive activity of peptide 678 (Fig. 6). Replacement of Arg-198 with a His also dramatically reduced adhesive activity. Ala substitutions at several other positions significantly decreased adhesive activity, except for the two N-terminal residues, which only slightly decreased adhesive activity.

Although only the Arg residue was essential for direct adhesion, substitution of several additional residues with Ala markedly decreased or abolished inhibitory activity of the respective soluble peptides in solution to block αβ₁-dependent adhesion to immobilized peptide 678 (Table III). These experiments showed that Arg-198, Val-197, and Asn-196 are essential for inhibitory activity of the peptides in solution. Substitution of Phe-199 and Phe-201 decreased the inhibitory activities of the respective peptides 5–8-fold, indicating that these flanking residues also contribute to activity of the peptides in solution. In contrast, peptides with Ala substitutions at four of the six N-terminal residues in this sequence had inhibitory activities equivalent to that of the native TSP1 sequence. Therefore, NVR is the essential sequence for binding to the αβ₁ integrin, but flanking residues may be necessary for inducing the proper conformation of this minimal sequence in peptide 678.

The specificity for an Arg residue at position 198 was further examined using conservative amino acid substitutions (Table III). Substitution with Lys decreased activity approximately 2-fold, whereas substitution with Glu, to retain hydrogen-bonding ability while removing the positive charge, abolished the inhibitory activity. A His substitution showed intermediate activity, indicating that a positive charge rather than a large
shown) as markers of focal adhesion formation, we could not by IGF1 receptor ligands that stimulate integrin-dependent vinculin (Fig. 7D) and focal adhesion kinase (data not shown) adhesion. Adhesion to this peptide and to TSP1 was stimulated by integrins. Adhesion to this peptide and to TSP1 was stimulated by this peptide (Fig. 7B). Phallacidin staining demonstrated organization of F-actin at the cell periphery but no organization of stress fibers across the cell body (Fig. 7C). Using antibodies recognizing vinculin (Fig. 7D) and focal adhesion kinase (data not shown) as markers of focal adhesion formation, we could not detect any induction of focal adhesions in MDA-MB-435 cells attaching on these peptides, although the same markers showed typical focal adhesion staining patterns in the cells when attaching on vitronectin or fibronectin substrates (results not shown). Staining for the α3β1 integrin was punctate and prominently localized in filopodia extended by MDA-MB-435 cells on immobilized peptide 678 (Fig. 7F), whereas total β1 integrin staining was more diffuse and concentrated over the cell body.

TSP1 stimulates chemotaxis of MDA-MB-435 cells, and this response is inhibited by the α9β1-blocking antibody P1B5 (14). Peptide 678 also stimulated chemotaxis of MDA-MB-435 cells (Fig. 8). Chemotaxis to peptide 678 was dose-dependent with a maximal response at 10 μM (Fig. 8A). This response was specific in that peptide 690 was inactive. In agreement with the observations that IGF1 stimulated α3,3-motaxis of MDA-MB-435 cells to TSP1 (14) and adhesion of the same cells to peptide 678 (Figs. 2 and 7), the chemotactic response of MDA-MB-435 cells to peptide 678, but not to peptide 690, was increased in the presence of IGF1 (Fig. 8B).

**DISCUSSION**

Based on examination of synthetic peptides and recombinant fragments representing approximately 90% of the TSP1 sequence, only the sequence FQGVLQNVRFVF from the N-terminal domain exhibited activities that are expected for an α3β1 integrin binding sequence in TSP1. A recombinant fragment of TSP1 containing this sequence also promoted α3 integrin-dependent adhesion. In solution, this peptide specifically inhibited adhesion to TSP1 but not to ligands recognized by other integrins. Adhesion to this peptide and to TSP1 was stimulated by IGF1 receptor ligands that stimulate integrin-dependent adhesion on intact TSP1 (14). Addition of IGF1 enhanced spreading and increased formation of lamellipodia on the same peptide (Fig. 7). Phallacidin staining demonstrated organization of F-actin at the cell periphery but no organization of stress fibers across the cell body (Fig. 7C). Using antibodies recognizing vinculin (Fig. 7D) and focal adhesion kinase (data not shown) as markers of focal adhesion formation, we could not

### Table II

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### Table III

**Mapping of essential residues for inhibition of MDA-MB-435 cell adhesion to immobilized peptide 678**

Mean doses to achieve 50% inhibition of control adhesion to 5 μM peptide 678 (IC50) were determined from at least three independent experiments, each performed in triplicate. Residues substituted in the native TSP1 sequence are indicated with an asterisk.

Mean doses to achieve 50% inhibition of control adhesion to immobilized peptide 678 (Fig. 7F), whereas total β1 integrin staining was more diffuse and concentrated over the cell body.

**Fig. 7. Morphology of MDA-MB-435 cells attaching on TSP1 peptide 678. a, direct adhesion on TSP1 peptide 678 stimulates formation of filopodia (bar, 50 μm). b, IGF1 stimulates increased spreading with formation of lamellipodia. c, staining of F-actin using BODIPY TR-X phallacidin (bar, 20 μm). d, double labeling of the field in c with anti-vinculin antibody. e, immunolocalization of β1 integrin subunits in cells attached on peptide 678 using antibody TS2/16.**

**Fig. 6. Effect of systematic substitution of Ala residues on adhesive activities of the TSP1 sequence 190–201 for breast carcinoma cells.** Cell attachment was determined to substrates coated with each peptide at 10 μM and is presented as mean ± S.D. (n = 3). Residues substituted in the native TSP1 sequence are indicated with an asterisk.
that different ligands have unrelated binding sequences, which integrin receptor for spreading on TSP1 (26). TSP2, with a His residue replacing the Arg. As a free peptide, formation of filopodia. In cells plated on peptide 678, these replaces the Arg. A similar motif is found in murine and human TSP1 (14) or the bovine, and human LamA1, which was found not to bind \( \alpha_6 \beta_1 \) with high avidity, has an Ala in the position occupied by the essential Arg in the TSP1 sequence. Substitution of Ala for the Arg in the TSP1 sequence abolished all activity of the synthetic TSP1 peptide. Among the five G domain modules of LamA3, G2 has a better consensus sequence based on our results (NLK) than does G4 (NFQ) or G5 (NIH). Expressed as recombinant proteins, only the G2 module promoted \( \alpha_6 \beta_1 \)-dependent adhesion (34). Although RGD was reported to be an \( \alpha_6 \beta_1 \) ligand, the RGD in entactin is not required for recognition, and the RGD in the type 3 repeats of TSP1 is not recognized by this integrin. A binding site for the \( \alpha_6 \beta_1 \) integrin in entactin was mapped to the G2 domain (residues 301–647) (11). Multiple alignment of this region of entactin against the TSP1 sequence and the murine laminin-I peptide GD6 identified a related sequence, FSGIDE HGHHLTI, but this sequence lacks all of the essential residues in the TSP1 sequence. This domain of entactin also contains two NXR sequences: NNHR and NQRQ. It remains to be determined whether either of these can function as an \( \alpha_6 \beta_1 \) integrin recognition sequence.

The absence of an Asp residue in peptide 678 may account for its partial independence of divalent cations. An Asp residue is usually considered an essential element for integrin peptide ligands (35, 36). According to one model for integrin ligand binding, the divalent cation participates directly in binding an Asp-containing peptide ligand (reviewed in Ref. 37). Thus an integrin peptide ligand without a carboxyl side chain cannot coordinate with a bound divalent cation and therefore may not have a divalent cation requirement for binding to the integrin. The alternate model, proposing an indirect role of divalent cations in integrin activation (37), would be consistent with the observed stimulation of cell spreading on peptide 678 by Mn\(^{2+}\) but not Ca\(^{2+}\) and the partial inhibition following chelation of divalent cations.

Another interpretation of the partial divalent cation independence for the adhesive activity of peptide 678 is that ionic interactions of the Arg side chain in the TSP1 peptide with the negatively charged cell surface contribute to the adhesive activity of this peptide. Weak ionic interactions could promote adhesion to the immobilized peptide through multivalent interactions with negatively charged glycoproteins and proteoglycans on the cell surface but would not significantly contribute to binding of the same monovalent peptide to the cell in solution. This hypothesis would explain why the Arg-containing peptides 686 and 691, in which the essential Val or Asn residues were substituted with Ala, lacked activity in solution to inhibit adhesion to \( \alpha_6 \beta_1 \) ligands but retained some adhesive activity when immobilized. Thus, inhibitory activities in solution may provide a more reliable assessment of integrin binding specificity for Arg-containing peptides.

Spreading of MDA-MB-435 breast carcinoma cells on intact TSP1 (14) or the \( \alpha_6 \beta_1 \) integrin-binding peptide 678 induces formation of filopodia. In cells plated on peptide 678, these structures are enriched in the \( \alpha_6 \) integrin subunit, suggesting that engagement of this integrin by TSP1 triggers formation of filopodia. Formation of filopodia or microspikes has been noted during attachment of other cell types on TSP1 (38). This response may be mediated by the \( \alpha_6 \beta_1 \) integrin, because lamellar spreading rather than formation of filopodia was typically observed on melanoma cells that predominantly use the \( \alpha_5 \beta_3 \) integrin receptor for spreading on TSP1 (26).

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**Fig. 8. TSP1 peptide 678 promotes breast carcinoma cell chemotaxis.** A, dose dependence for stimulation of MDA-MB-435 cell motility by peptide 678 added to the lower well of a modified Boyden chamber. Cells that migrated to the lower surface of an 8-μm pore polycarbonate filter were quantitated microscopically after 7 h; the data shown are mean ± S.D. (n = 3) for a representative experiment. B, MDA-MB-435 cell chemotaxis was measured to medium alone (Blank) or to 10 μM TSP1 peptide 678 or 10 μM inactive analog peptide 690 added to the lower chamber. Chemotaxis of untreated cells (striped bars) or cells treated with 10 nM IGF1 in the upper chamber (solid bars) was determined after 7 h and is presented as mean ± S.D. (n = 3).

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Using multiple sequence alignment, the N-terminal domains of thrombospondins were recently shown to contain a module related to pentraxins and to the G domain modules of laminins (38). Based on this alignment, both the α3β1 integrin-binding sequence from TSP1 identified here and the GD6 sequence of laminin are located at the C terminus of a pentraxin module. The known three-dimensional structures of other members of the same superfamily (40, 41) lead to the prediction that both potential integrin binding sequences are located in the last β-strand of a pentraxin module and therefore may be presented with similar topologies on the laminin G domain and the N-terminal domain of TSP1. This observation suggests an evolutionary relationship between the thrombospondin N-terminal domains and laminin G domains that is consistent with their proposed common function as recognition sites for a β1 integrin receptor.

Acknowledgments—We thank Drs. Ken Yamada and Harvey Grahnick for providing antibodies and Jack Lawler and Tikhva Vogel for providing recombinant TSP1 fragments and fusion proteins.

REFERENCES

Inhibition of Angiogenesis by Thrombospondin-1 Is Mediated by 2 Independent Regions Within the Type 1 Repeats

M. Luisa Iruela-Arispe, PhD; Michele Lombardo, BS; Henry C. Krutzsch, PhD;
Jack Lawler, PhD; David D. Roberts, PhD

**Background**—Suppression of tumor growth by thrombospondin-1 (TSP-1) has been associated with its ability to inhibit neovascularization. The antiangiogenic activity of TSP-1, as defined by cornea pocket assays, was previously mapped to the amino-terminal portion of the protein within the procollagen region and the type 1 repeats.

**Methods and Results**—We evaluated the specificity and efficacy of different regions of TSP-1 using recombinant fragments of the protein on chorioallantoic membrane (CAM) angiogenesis and endothelial cell proliferation assays. In both assays, fragments containing the second and third type 1 repeats but not the procollagen region inhibited angiogenesis and endothelial cell proliferation. To further define the sequences responsible for the angiostatic effect of TSP-1, we used synthetic peptides. The CAM assay defined 2 sequences that independently suppressed angiogenesis. The amino-terminal end of the type 1 repeats showed higher potency for inhibiting angiogenesis driven by basic fibroblast growth factor (FGF-2), whereas the second region equally blocked angiogenesis driven by either FGF-2 or vascular endothelial growth factor (VEGF). Modifications of the active peptides revealed the specific amino acids required for the inhibitory response. One sequence included the conserved tryptophan residues in the amino-terminal end of the second and third type 1 repeats, and the other involved the amino acids that follow the CSVTCG sequence in the carboxy-terminus of these repeats. Both inhibition in the CAM assay and inhibition of breast tumor xenograft growth in nude mice were independent of the TGF-β-activating sequence located in the second type 1 repeat.

**Conclusions**—These results indicate that the type 1 repeats of TSP-1 contain 2 subdomains that may independently inhibit neovascularization. They also identify 2 independent pathways by which TSP-1 can block FGF-2 and VEGF angiogenic signals on endothelial cells. (*Circulation.* 1999;100:1423-1431.)

**Key Words:** angiogenesis • endothelium • vessels

Thrombospondin (TSP-1) is a matricellular protein with recognized ability to inhibit endothelial cell proliferation and to suppress angiogenesis.1,2 The region responsible for inhibition of angiogenesis has been mapped to the procollagen domain and to the type 1 repeats.3 The molecular mechanisms for this inhibition are not entirely understood. It is likely that the inhibition of capillary growth by TSP-1 might be multifactorial and involve competition for basic fibroblast growth factor (FGF-2) binding to the endothelial cell surface,4,5 binding to heparan sulfate proteoglycans,4 activation of latent transforming growth factor (TGF)-β,6 and/or binding to CD36, a receptor for TSP-1.7 More importantly, the ability of TSP-1 to suppress neovascularization has been associated with inhibition of tumor growth.8-10 We have previously demonstrated in xenograft assays that stable synthetic peptide analogues of the TSP-1 type 1 repeats suppressed breast carcinoma growth in a dose-dependent manner.10 The peptides used in these assays included a tryptophan-rich motif within the type 1 repeats that binds to heparin.10,11 However, these peptides did not include the sequence that binds to CD36 and elicited an antiangiogenic effect in the cornea.2 Additional studies have indicated that these tryptophan-rich peptides suppressed endothelial cell proliferation, inhibited chemotaxis to FGF-2,8 and induced apoptosis of endothelial cells.11 Although no direct analyses were performed to elucidate their role in angiogenesis, their suppression of FGF-2-stimulated endothelial proliferation and chemotaxis were indicative of an effect on tumor vascularization.4 It is not clear how potent these peptides are in relation to other recognized vascular inhibitory regions of TSP-18 or whether they might demonstrate variability in efficacy depending on the angiogenic stimulus (ie, FGF-2.
versus vascular endothelial growth factor (VEGF)). The present study was therefore undertaken to (1) evaluate the efficacy of peptides from several regions of the Type 1 repeats to inhibit angiogenesis, (2) determine the effect of these peptides on FGF-2- versus VEGF-driven angiogenesis, and (3) elucidate the residues that provide a minimal functional core sequence in TSP-1.

Methods

Protein, Recombinant Fusion Protein, and Peptides

Thrombospondin was purified from human platelets. Recombinant fusion proteins of TSP-1 were prepared by use of the pGEX vectors. Vascular permeability factor (VPF)/VEGF was obtained from Peprotech; FGF-2 was a generous gift from Dr Gera Neufeld (Technion University, Israel).

The peptides used in this study were synthesized on a Biosync model 9600 peptide synthesizer using standard Merrifield solid-phase synthesis protocols and 9-butanecarboxyl chemistry. Peptides were analyzed by reverse-phase high-performance liquid chromatography and further purified by dialysis with Spectrapor 500 MWCO tubing, gel permeation chromatography, or reverse-phase purification with C18 Sep-Pak cartridges. The identities of peptides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Polysucrose conjugates of some peptides were prepared as previously described. Before use on chorioallantoic membrane (CAM) assays, peptides were also filtered on Centricron P100 to eliminate traces of endotoxin.

Endothelial Cells and Proliferation Assays

Chicken endothelial cells (CECs) were isolated from the brain of day 7 chicken embryos. Endothelial cells were purified and characterized by standard techniques, as described.

For proliferation assays, quiescent endothelial cells were seeded in 24-well plates in EBM medium supplemented with 0.1% FCS and 10 ng/mL of VEGF (PeproTech Inc) and/or 2 ng/mL of FGF-2 in the presence of TSP-1, fusion proteins, peptides, or vehicle control. During the last 8 hours of the treatment, cells were pulsed with 1 µCi/well of [3H]thymidine (DuPont-NEN). Trichloroacetic acid-precipitable counts of [3H]thymidine were measured as previously described. The significance of inhibition was assessed by a 2-tailed t test.

CAM Assays

The effect of TSP-1, fusion proteins, and peptides on angiogenesis was evaluated with a modified CAM assay. The method is based on the vertical growth of new capillary vessels into a collagen gel pellet placed on the CAM. The collagen gel was supplemented with an angiogenic factor such as FGF-2 (50 ng/mL) or VEGF (250 ng/mL) in the presence or absence of test proteins/peptides. The extent of the angiogenic response was measured by use of FITC-dextran (50 µg/mL) (Sigma) injected into the circulation of the CAM. The degree of fluorescence intensity parallels variations in capillary density: the linearity of this correlation can be observed with a range of capillaries between 5 and 540. Mophometric analyses were done by acquisition of images with a Sony, single-chip CCD camera. Images were imported into NIH Image 1.59, and measurements of fluorescence intensity were obtained as positive pixels. Each data point was compared with its own positive and negative controls present in the same CAM and interpreted as mediated inhibitory effect, whereas antibodies alone had a mild but statistically insignificant effect on proliferation (data not shown). Preimmune guinea pig IgG in the presence of TSP-1 did not ameliorate endothelial growth inhibition. We also examined the levels of TGF-β1 present in the TSP-1.
120 gravity, by VEGF or FGF-2 cast into a polymerized collagen (Figure 3B). The ability of TSP-1 to inhibit vascular growth repeats.

The specificity of TSP-1 to inhibit CEC proliferation was also determined by use of glutathione S-transferase (GST) fusion proteins expressing the procollagen, type 1, type 2, type 3, and C-terminal domains of TSP (Figure 2). These bacterially expressed proteins lack any TGF-β contamination and can be used to define the functional domain(s) within TSP-1 responsible for the inhibitory effect. Interestingly, only the type 1 repeat recombinant fragment inhibited endothelial cell proliferation. TSP-1 10 μmol/L inhibited proliferation by 35% on CECs (P=0.009). At the same molar concentration, the recombinant GST type 1 domain inhibited CEC proliferation by 48% (P=0.0009). The GST control alone had no effect. Other recombinant fragments tested in this assay did not show inhibition of either bovine aortic endothelial cell (data not shown) or CEC proliferation, and the partial inhibition by the carboxy-terminal fusion protein (83% of control) was not statistically significant (P=0.21). It was interesting that the entire TSP-1 molecule had a more moderate effect on proliferation than that of the GST fusion protein containing the type 1 repeats.

The effects of intact TSP-1 and GST fusion proteins on angiogenesis were evaluated on a mesh CAM assay. Growth of capillaries in this assay is stimulated vertically, against gravity, by VEGF or FGF-2 cast into a polymerized collagen gel. Figure 3A shows the effect of TSP-1 and fusion proteins on neovascularization of the acellular collagen matrix. The presence of angiogenic growth factors induces the growth of a thin vasculature in the acellular gel as early as 24 hours (Figure 3A, arrows) from the thicker vessels located under the nylon mesh (larger vessels out of focus). In the absence of angiogenic growth factors, no network was observed (Figure 3B). The ability of inhibitors to suppress the stimulatory signal of growth factors was then evaluated by inclusion of these proteins in the polymerized nitrogen gel. Both TSP-1 and the GST type 1 repeat fusion protein were effective at suppressing the angiogenic response mediated by growth factors. TSP-1 was able to block VEGF-mediated angiogenesis by 35%, whereas the GST type 1 repeat fusion protein was more effective at the same molar ratio (57%) (Figure 3B). No significant effects were detected with any of the proteins alone, ie, in the absence of VEGF, or with proteins other than the type 1 repeats in the presence of VEGF or FGF-2. The effect was reproducible with several preparations of TSP-1 and of recombinant protein and was performed at least 4 separate times with each treatment in triplicate (total of 12 assays). These results are consistent with data obtained from the proliferation experiments and again indicate that at an equivalent molar ratio, the type 1 repeats of TSP-1 appear to be more effective than the intact protein. To this end, it has recently been postulated that the carboxy-terminal end of TSP-1 might exert a positive effect on angiogenesis by its ability to interact with integrin-associated protein.¹⁶ Interestingly, we observed a slight but reproducible increase in angiogenic rate with the carboxy-terminal end (Figure 3B). The entire TSP-1 protein might therefore contain regions that elicit both positive and negative signals on endothelial cell proliferation and angiogenesis, thus providing amelioration to the suppressive growth signals. A careful dissection of these areas is required to clearly elucidate the potential function of each domain.

We also found that the first repeat of the type 1 domain had no effect on suppressing angiogenesis in the CAM assay (Figure 3B). The ability of TSP-1 to inhibit vascular growth therefore appears to be located within the last 2 type 1 repeats.

We next examined the activities of synthetic peptides derived from the procollagen and second and third type 1 repeats of TSP-1, as well as the carboxy-terminal end of TSP-1 (Figure 4 and Table). The procollagen region and the last 2 type 1 repeats have previously been shown to have antiangiogenic activity in the cornea pocket assay.² Evaluation of the procollagen region in the CAM assay, however, did not result in angiogenic suppression, in contrast to peptides from the second (508) and third (616) type 1 repeats.
Figure 3. Effect of TSP-1 fusion proteins on angiogenesis. A, Mesh-CAM assays for evaluation of TSP-1 and GST fusion proteins were performed on day 11 chicken embryos. Each pellet contained vitrogen 50 μg/mL, VEGF 250 ng/mesh, and FGF-2 50 ng/mL (a), except negative control (no growth factors were added) (b), in addition to TSP-1 (c), GST-procollagen region (d), GST type 1 repeats (e), GST type-2 repeats (f), GST type-3 repeats (g), GST carboxy-terminus (h), or GST control (i). Fusion proteins were used at 20 pmol/L and TSP-1 at 10 μg. B, Quantification of angiogenic response. Evaluation was determined 24 hours after application of pellets to CAM surface. Systemic injection of FITC-dextran revealed vessels with patent lumens. Ten squares of 250 μm² were evaluated per mesh. Three meshes in independent embryos were performed per time point. In each case, extent of angiogenesis suppression/stimulation was determined by direct comparison to control meshes (VEGF alone=100%) in same CAM. As in Figure 2, only TSP-1 and type 1 repeat fusion proteins were significantly different from control (P<0.01).
A. THROMBOSPONDIN-1 Peptides Used in the Assays

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Figure 4. Evaluation of TSP-1 peptides from procollagen, type I repeats, and carboxy-terminal regions on CAM assays. A. Schematic of TSP-1 and location of peptides used in B. B. Assessment of angiogenic response was determined as in Figure 3. Wild-type peptides from procollagen region (500F), type I repeats (508, 616), and carboxy-terminus (458) were evaluated at 10 μmol/L. In addition, mutated peptides from carboxy-terminus were tested as well (604 and 605). Note that negative values are read as angiogenic response above control, i.e., growth factors alone.

which were antiangiogenic. Interestingly, a peptide from the carboxy-terminal domain (458) showed a slight but reproducible proangiogenic effect. The region has been shown to interact with integrin-associated protein and enhance attachment and migration. The specificity of this positive response was supported by concurrent experiments performed with 2 mutated versions (604 and 605) of the carboxy-terminal peptide. Substitution of the 2 Val residues was sufficient to suppress the proangiogenic effect (Figure 4).

On the basis of these studies, it appears that in the CAM assay, only peptides derived from the second and third type I repeats are angioinhibitory. We therefore focused subsequent studies on these domains.

Figure 5 shows the sequence of the last 2 TSP-1 type I repeats and correlates previously identified functions to specific sequences within these domains. A region of interest, because of its demonstrated ability to suppress tumor growth, is located at the amino-terminal end of these repeats. The direct effect of this region on angiogenesis has not yet been tested. Our results indicated that peptides from this region at 1 μmol/L suppress vascular growth induced by a mixture of FGF-2 and VEGF (Figure 6A). The effect was dose-dependent, and retro-inverso analogues or polysucrose conjugates of the peptides were more potent than the native TSP-1 peptide. These modifications are known to increase the half-life of polypeptides by reducing degradation in vivo. Assays were also performed using mutated versions of the peptides as well as deletion mutants. Mutation of the 3
Figure 6. Effect of amino-terminal region of type 1 repeats on angiogenesis. Evaluation of angiogenic response was determined as in Figure 3. Peptides used were wild-type and mutated versions of subdomains located within second and third type 1 repeats; refer to Figure 5 for orientation. A, Dose responses for inhibition of angiogenesis by natural L-forward and D-reverse TSP-1 peptides from second type 1 repeat and a polysucrose conjugate of D-reverse sequence. Statistical analysis (t test) revealed no significant differences among wild-type peptides. Error bars = ±SD. B, Evaluation of deletions and mutated versions of wild-type peptides (10 nmol/L). All sequences were tested as D-reverse peptides. Corresponding forward sequences are indicated on right. Note that mutation of tryptophan residues completely abolished antiangiogenic activity of peptides.

Figure 7. Effect of carboxy-terminal half/CD36-binding region of type 1 repeats on angiogenesis. Angiogenic response was determined as in Figure 3. A, Dose curve of L-forward peptides from second and third type 1 repeats. Statistical analysis (t test) revealed no significant differences between peptides derived from second and third type 1 repeats. Error bars = ±SD. B, Evaluation of deletions and mutated versions of wild-type peptides (10 nmol/L). All sequences are indicated on right. Major activity for both peptides is located carboxy-terminal from VTCG sequence, a region recognized for its interaction with CD36.

The tryptophan residues to alanines (peptides 597 and 598) completely abolished inhibitory activity, indicating that these residues are critical to the antiangiogenic response (Figure 6B). A peptide with 2 instead of 3 tryptophan residues was partially effective (peptides 493 and 530) (Figure 6B). The latent TGF-β-activating sequence (RFK) was not required for the suppressive effect on neovascularization, because mutation of the essential phenylalanine residue to alanine (compare peptides 545 and 596) did not have any deleterious effect on the activity of the peptide. This mutation abrogates the ability of this peptide to activate latent TGF-β.

We next examined the effects of the second half of the type 1 repeats, a region previously concluded to be responsible for the antiangiogenic effects promoted by TSP-1 in cornea pocket assays. Our results support those observations and demonstrate activity of the same peptides in the CAM assay (Figure 7A). In agreement with recent reports, the active region appears to be carboxy-terminal to the CSVTCG region, because this sequence alone was inactive, and deletion of the first 2 residues of this motif did not affect the antiangiogenic activity of peptide 205 (VTCGDGVTR) from the second type 1 repeat or peptide 245 (VTCGGGVQKRSRL) from the third type 1 repeat (Figure 7A). However, the VTCG sequence without these flanking sequences completely lacked antiangiogenic activity. This flanking sequence has been shown to act through CD36, a receptor for TSP-1. We have verified that CECs and vessels in the CAM do express this receptor by Northern blot analysis (data not shown). Therefore, it is likely that the mechanism of action is similar.

To further elucidate the mechanism of action of these 2 subregions, CAM experiments were performed with either VEGF or FGF-2 as stimulator of the angiogenic response (Figure 8). Interestingly, we observed a clear distinction between the tryptophan repeat peptides and the CD36-binding domain peptides. Peptides 508 and 598, which have
the tryptophan motif, suppressed the angiogenic response only to FGF-2-mediated angiogenesis but had no effect on VEGF-driven vascular growth. In contrast, the CD36-binding sequences blocked both VEGF- and FGF-2-induced angiogenesis. Interestingly, the tryptophan domain has previously been shown to prevent FGF-2 binding to endothelial cells.\textsuperscript{5,6} Therefore, inhibition of binding or further sequestration of FGF-2 is the most likely mechanism of action of this amino-terminal portion of the type 1 repeats.

A surprising result was the lack of antiangiogenic activity of the TGF-\(\beta\) activating sequence. To determine whether the CAM assay has predictive value for inhibition of tumor angiogenesis, we assessed the role of the TGF-\(\beta\)-activating sequence in inhibition of breast carcinoma tumor growth in vivo using orthotopic xenografts of MDA435 breast carcinoma cells in athymic mice (Figure 9). d-Reverse analogues of the native TSP-1 sequence (peptide 599) and a modified sequence lacking TGF-\(\beta\)-activating activity (peptide 596)\textsuperscript{10} both strongly suppressed tumor growth when administered intravenously to the mice beginning 25 days after implantation of the tumor cells in the mammary fat pad. The data are in agreement with our findings in the CAM assay and provide further support that the suppression of tumor growth results from the ability of TSP-1 to suppress angiogenesis independently of latent TGF-\(\beta\) activation.

**Discussion**

In this study, we evaluated both TSP-1 fusion proteins and synthetic peptides to provide a comparative assessment of the antiangiogenic activity displayed by the different domains of TSP-1. We combined these data with analysis of deletion and point mutants to identify the minimal sequences with vascular inhibitory properties. One result of these structure/function analyses demonstrated that the ability of TSP-1 to suppress angiogenesis resides solely in the second and third type 1 repeats. The data are in agreement with previous findings by Tolmsa and coworkers.\textsuperscript{5} In addition, these studies revealed 2 subdomains in the type 1 repeats that act independently to suppress angiogenesis and identified essential residues responsible for these effects: the tryptophan-rich WSXW motifs and the CD36-binding region. These 2 sequences also display growth factor selectivity in that the tryptophan-rich domain preferentially suppresses FGF-2 angiogenic signals, whereas the CD36-binding region inhibits capillary formation driven by either FGF-2 or VEGF. These findings contribute to understanding the antitumor activity of some TSP-1 peptides and suggest that these peptides might act, at least in part, by suppressing tumor-mediated angiogenesis.

The neovascular suppression displayed by TSP-1 has previously been attributed to the second (amino acid [aa] 424 to 442) and third (aa 481 to 246) type 1 repeats of TSP-1.\textsuperscript{3} The type 1 domain of TSP-1 consists of 3 polypeptide repeats that have complete conservation of the cysteine and tryptophan residues and that have been identified in a variety of other proteins, including properdin, F-spondin, BAI, and metallospindins.\textsuperscript{18–21} Nevertheless, the antiangiogenic potential of the type 1 repeats is not shared by all these proteins, indicating that context-specific primary sequences and/or secondary structure influence the function of the type 1 repeats.

Previous publications have indicated that the first type 1 repeat of TSP-1 has no antiangiogenic activity.\textsuperscript{3} We confirmed these results in the CAM angiogenic assay. Recent studies have further mapped a subregion within the second and third type 1 repeats including and carboxy-terminal to the CSVTCG sequence with angioinhibitory effects.\textsuperscript{7} The relevant region has been found to bind to CD36 and to be responsible for the intracellular events related to the suppression of several mitogenic signals on endothelial cells.\textsuperscript{7} Our data are consistent with the observations of Dawson et al\textsuperscript{17} but do not correlate with evidence that the CSVTCG sequence alone,
responsible for binding of TSP-1 to CD36, has an effect.\textsuperscript{22} Further structure-function and mutagenesis analysis will be required to resolve these discrepancies.

Although activity of the CD36-binding peptides from the carboxyl end of the second and third type I repeats provides an explanation for some of the activity of the type I repeats, other regions within the type I repeats have also been shown to reduce tumor growth and display potential angiostatic or antiangiogenic activities.\textsuperscript{10,11} Because these small fragments of the protein were tested in different laboratories and angiogenesis assays, we felt that it was necessary to analyze TSP-1 fragments and peptides side by side and in a single in vivo assay. Our observations demonstrate that the tryptophan-rich motif contains a second angioinhibitory region with activity similar to that of the SHW'SPW sequence alone. The interaction of the type I repeats with heparin in the tryptophan-rich region lacks stereospecificity, because forward and inverse peptide analogues (L-forward, L-reverse, and D-reverse) displayed equivalent ability to interact with heparin, and in the CAM angiogenesis assay, the retro-inverse analogue was better in blocking angiogenesis. Thus, the polypeptide backbone is not involved in this response. Conjugation of the type I repeat peptides to polysaccharide did not significantly affect their antiangiogenic function, although conjugation increased their potency in vitro for inhibiting proliferation of endothelial and breast carcinoma cells stimulated by FGF-2.\textsuperscript{11} The D-reverse analogues are resistant to proteases, and we have shown in xenograph assays that retro-inverse analogues are effective when administered intravenously in mice.\textsuperscript{10} The enhanced activity of the D-reverse peptides in the CAM assay may therefore result from an enhanced half-life in the gel or chick embryo circulation.

Studies by Tolsma and coworkers\textsuperscript{5} have demonstrated that in addition to the type I repeats, a region in the procollagen domain (aa 294 to 317) inhibits angiogenesis in the cornea pocket assay. Our results, however, did not support these findings by use of either the fusion protein or the synthetic peptide. It is possible that unlike with the type I repeats, the procollagen region is not effective across species; in fact, the amino-terminal end of TSP-1 differs more significantly than the carboxy-terminal end.\textsuperscript{3} Nevertheless, we also were not able to see any effect on proliferation or migration using the procollagen region fusion protein on human dermal microvascular endothelial cells.

TSP-1 has been shown to bind and activate latent TGF-\(\beta\).\textsuperscript{5} Because TGF-\(\beta\) activates endothelial cell function,\textsuperscript{15,23,24} some involvement of the latent TGF-\(\beta\)-activating sequence was expected in the activity of TSP-1. Although TGF-\(\beta\) inhibits endothelial cell proliferation,\textsuperscript{13} angiogenesis assays in vitro\textsuperscript{25} as well as injection of TGF-\(\beta\) in vivo have demonstrated angiogenic activity.\textsuperscript{24} Peptides with KRFK sequences might therefore be predicted to induce angiogenesis by activating endogenous latent TGF-\(\beta\). Perhaps this is why some of the KRFK peptides without the tryptophans seem to stimulate angiogenesis (eg, 597). In any case, our studies demonstrate that at least the antiangiogenic activity of type I repeats was independent of latent TGF-\(\beta\) activation, because substitution of an Ala residue for the essential Phe residue did not affect the antiangiogenic potential of the peptide.

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**References**


Modulation of Endothelial Cell Behavior and Angiogenesis by an α3β1 Integrin-binding Sequence in Thrombospondin-1

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Abstract

Several peptide sequences in thrombospondin-1 (TSP1) have been reported to regulate endothelial cell behavior and inhibit angiogenesis by interacting with endothelial cell CD36 or heparan sulfate proteoglycan receptors. We have now identified a TSP1 peptide that modulates angiogenesis and the in vitro behavior of endothelial cells through binding to α3β1 integrin. Recognition of this sequence and intact TSP1 by normal endothelial cells is induced following loss of cell-cell contact or ligation of CD98. Although confluent endothelial cells do not spread on a TSP1 substrate, efficient spreading on TSP1 substrates of endothelial cells maintained without cell-cell contact is mediated by the α3β1 integrin. In solution, an α3β1 integrin-binding peptide from TSP1 inhibits proliferation of sparse endothelial cell cultures, but the same peptide immobilized on the substratum promotes their proliferation. Intact TSP1 also selectively promotes endothelial cell proliferation when immobilized on the substratum. The TSP1 peptide, when added in solution, specifically inhibits migration of endothelial cells into scratch wounds and inhibits angiogenesis in the chick chorioallantoic membrane assay. Thus, recognition of immobilized TSP1 by α3β1 integrin may increase endothelial cell proliferation and motility during wound repair and angiogenesis. Peptides that inhibit this interaction are a novel class of angiogenesis inhibitors.
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 in (Folkman, 1995; Hanahan and Folkman, 1996; Polverini, 1995)). Thrombospondins are a family of extracellular matrix proteins that have diverse effects on cell adhesion, motility, proliferation and survival (reviewed in (Bornstein, 1992; Bornstein, 1995; Roberts, 1996)). Two members of this family, 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 \textit{in vitro} (Canfield and Schor, 1995; Good et al., 1990; Iruela Arispe et al., 1991; Taraboletti et al., 1990; Tolsma et al., 1997) and, under defined conditions, induces programmed cell death in endothelial cells (Guo et al., 1997b). TSP1 inhibits angiogenesis \textit{in vivo} in the rat corneal pocket and chick chorioallantoic membrane angiogenesis assays (Good et al., 1990; Iruela-Arispe et al., 1999). The ability of TSP1 over-expression to suppress tumor growth and neovascularization in several tumor xenograft models provides further evidence for an anti-angiogenic activity of TSP1 (Dameron et al., 1994; Hsu et al., 1996; Sheibani and Frazier, 1995; Weinstat-Saslow et al., 1994). 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 pro-angiogenic activities under specific conditions (BenEzra et al., 1993; Nicosia and Tuszynski, 1994). Observations of elevated TSP1 expression during endothelial injury and wound repair are also difficult to rationalize with a purely anti-angiogenic activity for TSP1 (Munjal et al., 1990; Reed et al., 1995; Vischer et al., 1988). These 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 αvβ3 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 or to heparan sulfate proteoglycans inhibit endothelial responses to growth factors \textit{in vitro} and angiogenesis \textit{in vivo} (Iruela-Arispe et al., 1999; Tolsma et al., 1993; Vogel et al., 1993). 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, are also inhibited by TSP1 and heparin-binding peptides from TSP1 (Guo et al., 1997a; Guo et al., 1998). Based on activities in the chorioallantoic membrane angiogenesis assay, both of these TSP1 sequences can inhibit angiogenesis \textit{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 (Passaniti et al., 1992; Roberts et al., 1986). Based on differences in the phenotypes of \textit{thbs}1 and \textit{tgfb}1 null mice and the inability of TGFβ antagonists to block many activities of TSP1 \textit{in vitro}, activation of latent TGFβ probably mediates only a subset of endothelial responses to TSP1 (Crawford et al., 1998).
Integrin interactions are also known to regulate angiogenesis (Brooks et al., 1994). Antagonists of the αvβ3 integrin are potent inhibitors to neovascularization induced by growth factors or in tumors (Brooks et al., 1995). Although αvβ3 is a known TSP1 receptor on endothelial cells (Lawler et al., 1988), its role in modulation of angiogenesis by TSP1 has not been defined. The CD47-binding sequence in TSP1 may increase binding of αvβ3 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 αvβ3 did not inhibit angiogenesis (IrueI-Arispe et al., 1999), suggesting that the RGD sequence in TSP1 is not involved in its effects on angiogenesis.

TSP1 interacts with several β1 integrins, including α4β1 and α5β1 on T lymphocytes (Yabkowitz et al., 1993), α3β1 on neurons (DeFreitas et al., 1995), and α3β1 and α4β1 on breast carcinoma cells (Chandrasekaran et al., 1999; Krutzsch et al., 1999). The α3β1 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 α3β1 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 α3β1 integrin is tightly regulated in breast carcinoma cells (Chandrasekaran et al., 1999), we have examined the role of this integrin in the responses of endothelial cells to TSP1 and regulation of angiogenesis. We demonstrate here that recognition of TSP1 by endothelial cell α3β1 integrin is selectively induced following loss of cell-cell contact. These cells efficiently spread on immobilized TSP1, and this interaction stimulates endothelial cell proliferation. An α3β1 integrin-binding peptide from the amino-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 or plasma obtained from the NIH Blood Bank (Akiyama and Yamada, 1985; Roberts et al., 1994). Human vitronectin was obtained from Sigma, and bovine type I collagen was obtained from Becton Dickinson Labware Division. Synthetic peptides from TSP1 that are recognized by the α3β1 integrin and structural analogs defective in α3β1 integrin binding were prepared as previously described (Guo et al., 1992; Krutzsch et al., 1999), and GRGDSP was obtained from Gibco/BRL. A non-peptide antagonist of αvβ3 integrin 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% CO₂ 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 Inc., Rockville, MD. Primary human umbilical vein endothelial cells (HUVEC) were provided by Dr. Derrick Grant, NIDCR, and human dermal microvascular endothelial (HDME) cells were purchased from Clonetics Corp., San Diego, CA. HUVEC cells were maintained in medium 199E supplemented with 20% FCS, 10 µg/ml heparin, 80 µg/ml endothelial mitogen
(Biomedical Technologies, Inc., Stoughton, MA), glutamine, penicillin, and streptomycin sulfate. HDME cells were maintained in MCDB medium containing glutamine, 5% FCS, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml ascorbic acid, 30 μg/ml heparin, 4 ng/ml FGF2, 4 ng/ml VEGF, 5 ng/ml IGF1, and 50 μg/ml gentamicin.

Cell proliferation was measured using the Cell-Titer colorimetric assay (Promega) as previously described (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 pre-coated with 100 μl of the peptides at 4°C overnight and blocked with 1% BSA before adding the cells. Cells were grown for 72 hours at 37°C in a humidified incubator with 5% CO2. HUVEC proliferation was measured by the same protocol but using medium 199 containing 5% FCS but without heparin. HDME cell proliferation was measured in MCDB growth medium without heparin, VEGF, or FGF2.

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. Inhibition assays were performed using the following function blocking antibodies: P1B5 (Gibco-BRL, α3β1), P4C2 (Gibco-BRL, α4β1), and mAb13 (Dr. Ken Yamada, anti-β1). 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. Immunofluorescence analysis of cell adhesion was performed on glass substrates as described previously, using BODIPY TR-X phallacidin (Molecular Probes, Inc. Eugene, OR) to visualize F-actin and using murine primary antibodies followed by BODIPY FL anti-mouse IgG to localize integrins, vinculin (Sigma), CD98 (4F2) or phosphotyrosine (PY Plus, Zymed) (Sipes et al., 1999).

**Scratch wound repair**

The *in vitro* wound healing assay used was a slight modification of that described by Joyce et al. (Joyce et al., 1989). A confluent monolayer of BAE cells pretreated with 10 μg/ml 5-fluorouracil for 24 hours were used in this assay. A straight wound about 2.0 mm wide was made in the monolayers using the flat edge of a sterile cell scraper (Costar #3010), 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 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 hours later using a grid incorporated into the eye piece 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 day 3 of development, the embryos were placed on 100 mm petri dishes. Assays were performed as previously described (Iruela-Arispe et al., 1999). Briefly, vitrogen gels containing growth factors (FGF-2 (50ng/gel) and VEGF (250ng/gel)) were allowed to polymerize in the presence or absence of TSP1 peptides. Peptides were filtered on Centricon P100 prior to their
analysis on the CAM assays to eliminate traces of endotoxin. Pellets were applied to the outer 1/3 of the CAM, and the assay was performed for 24h. 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 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 8 pellets). Statistical evaluation of the data were performed to check whether groups differ significantly from random by analysis of contingency with Yates' correction.

Results

Adhesion assays were used to determine whether the α3β1 integrin-binding sequence from residues 190-201 of TSP1 (Krutzhc et al., 1999) is recognized by endothelial cells. Endothelial cells attached specifically on immobilized TSP1 peptide 678 but not on the inactive analog peptide 690, in which the essential Arg residue was substituted with an Ala residue (Fig. 1A). Two related peptides with amino acid substitutions that diminished their activity for mediating α3β1-dependent adhesion of breast carcinoma cells (Krutzhc et al., 1999) only weakly supported endothelial cell adhesion (Fig. 1A). All of the peptides had similar capacities for adsorption on the polystyrene substrate used for these assays (2.5 to 3.8 pmoles/mm²), so the differences in activities of these peptides did not result from differences in their adsorption.

Although some previous publications have reported that TSP1 promotes spreading of
endothelial cells (Morandi et al., 1993; Taraboletti et al., 1990), other investigators have concluded that TSP1 cannot promote endothelial cell spreading and disrupts spreading of endothelial cells attached on certain other matrix proteins (Chen et al., 1996; Lahav, 1988; Lawler et al., 1988; Murphy-Ullrich and Höök, 1989). In agreement with the latter reports, bovine aortic endothelial cells harvested from a confluent cobblestone did not spread on TSP1 (Fig. 1B and Fig 2a). 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 post-feeding, they did (Fig. 1B and Fig 2c). Up-regulation of spreading on TSP1 following 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 (Fig 1B and Fig. 2b, d). Sparse cells also displayed a significant increase in spreading on vitronectin (p = 0.001), although approximately 60% of the cells harvested from a confluent monolayer also spread on vitronectin, compared to less than 10% on TSP1 (Fig. 1B).

Similar induction of BAE cell spreading following loss of cell-cell contact was observed using the α3β1 integrin-binding peptide from TSP1 (peptide 678, Fig. 3A). Density-dependent spreading on intact TSP1 and the TSP1 peptide were both inhibited by peptide 678 added in solution but were not significantly inhibited by the control peptide 690 (Fig. 2e and Fig. 3A). Inhibition by the active peptide was specific for endothelial cell spreading on TSP1 or the TSP1 peptide, because peptide 678 did not inhibit spreading on fibronectin (Fig. 2f).

Similar density dependence for spreading on TSP1 and the TSP1 peptide 678 was observed with human endothelial cells (Fig 3B). Although only 6% of HDME cells harvested from a confluent monolayer spread following attachment on immobilized TSP1, 29% of those
from a duplicate sparse culture spread on the same substrate. No spreading of the confluent culture was detected on TSP1 peptide 678, but 28% of HDME cells from the sparse culture spread on this peptide. Using HUVEC, sparse cultures showed only a slight increase in spreading (46 ± 7% versus 41 ± 5% for confluent cells, p = 0.36), but spreading on the peptide 678 was significantly induced (12 ± 3% for sparse cultures versus 3 ± 1% for confluent, p = 0.008, data not shown). These data demonstrate that loss of cell-cell contact induces spreading on TSP1 and on its α3β1 integrin-binding peptide for both microvascular and large vessel endothelial cells of bovine and human origins.

The increased spreading of BAE cells on TSP1 is mediated at least in part by α3β1 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 (Fig. 4A). The αvβ3 integrin also plays some role in BAE cell spreading on TSP1, since 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. Similar results were obtained using 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.

Primary human large vessel and microvascular endothelial cells also used the α3β1 integrin to mediate spreading on TSP1 (Fig. 4B and results not shown). HUVEC spreading on TSP1 was inhibited 70 ± 7% by peptide 678, whereas spreading on vitronectin was not significantly inhibited (Fig. 4B). Conversely, the αvβ3 antagonist SB223245 completely
inhibited spreading on vitronectin but did not significantly inhibit spreading on TSP1. HDME cell spreading on TSP1 was partially inhibited by the function-blocking integrin antibodies specific for β1 (mAb13) and α3 subunits (P1B5) but not by the α4β1 blocking antibody P4C2 (Fig. 4C), verifying that spreading of these microvascular cells on TSP1 is also mediated by the α3β1 integrin.

The possible involvement of other TSP1 receptors, including αvβ3 integrin, CD36, and heparan sulfate proteoglycans, were further examined using the human endothelial cells. The TSP1-binding integrin αvβ3 did not contribute to adhesion of the human endothelial cells, based on insensitivity to the αv integrin antagonist SB223245 (Fig. 4B and results not shown). Likewise, a function-blocking antibody recognizing the TSP1 receptor CD36 did not block adhesion of HDME cells (Fig. 4C). Of the human endothelial cells used, only HDME cells expressed CD36 as measured by RT-PCR (results not shown). Therefore, expression of CD36 is not required for endothelial cell spreading on TSP1. Heparin also had no effect on spreading of HDME cells on a TSP1 substrate (data not shown). These results demonstrate that the α3β1 integrin contributes to spreading of several types of endothelial cells on TSP1 and are consistent with the previous report that HDME cell adhesion on TSP1 is independent of CD36 and the αvβ3 integrin (Chen et al., 1996).

The α3β1 integrin was localized in lamellipodia of HUVEC cells spreading on TSP1 (Fig. 5a). The β1 integrin-activating protein CD98 showed a similar distribution in cells spreading on TSP1 (Fig. 5b). Both antibodies also labeled the borders of vacuolar structures that formed at the periphery of HUVEC cells spreading on TSP1. These resemble the ring structures reported previously to be induced following treatment of microvascular cells with anti-TSP1 antibodies.
(Tolsma et al., 1997). Lamellar spreading on TSP1 was associated with tyrosine phosphorylation at the leading edge of spreading cells (Fig. 5c). Vinculin antibody staining showed no evidence for formation of focal adhesions on TSP1, but some cells showed limited radial organization of vinculin in lamellipodia (Fig. 5d). These structures were not observed in cells stained with the α3β1 integrin antibody and may therefore be induced by another TSP1 receptor, such as the αvβ3 integrin.

Cells spreading on TSP1 peptide 678 also showed organization of α3β1 integrin (Fig. 5e) and CD98 (Fig. 5f) at the cell periphery, supporting their role in mediating spreading on this TSP1 peptide. Vacuolar structures were also observed in lamellipodia spreading on the TSP1 peptide, suggesting that these are induced by engagement of the α3β1 integrin. The spreading observed on glass substrates coated with peptide 678, however, was consistently weaker than that observed using the same peptide adsorbed on polystyrene, so further immunofluorescent analysis of endothelial cells attaching on this peptide was prevented by lack of a suitable substrate.

Based on the localization of CD98 in endothelial cells spreading on TSP1 and its ability to activate β1 integrins (Chandrasekaran et al., 1999; Fenczik et al., 1997), we examined the effect of the CD98 antibody 4F2 on HUVEC spreading on TSP1 (Fig. 6). 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 (Fig. 6).

Interaction of the α3β1 integrin with its ligands can regulate epithelial cell proliferation (Gonzales et al., 1999). We therefore 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 (Fig. 7A). Two control peptides with amino acid
substitutions that eliminate integrin binding, 686 and 690 (Krutzsch et al., 1999), did not or only
slightly inhibited proliferation of BAE cells (19% inhibition at 100 μM for peptide 690). Similar
inhibition by soluble peptide 678 was observed in HUVEC cultures (Fig. 7B). However, plating
of HUVEC on immobilized peptide 678 increased their proliferation (Fig. 7B). Similar
enhancement of microvascular (HDME) cell proliferation was observed after plating on
immobilized peptide 678 (Fig. 7C). Intact TSP1 also had differential effects on endothelial cell
proliferation when immobilized versus added in solution. As reported previously for several
other types of endothelial cells (Bagavandoss and Wilks, 1990; Panetti et al., 1997; Sheibani and
Frazier, 1995; Taraboletti et al., 1990), soluble TSP1 inhibited proliferation of HDME cells
stimulated by FGF2, but plating of the same cells on immobilized TSP1 stimulated their
proliferation (Fig 7C).

To examine the role of the α3β1 integrin-binding sequence of TSP1 in endothelial cell
motility, we determined the effect of peptide 678 on endothelial scratch wound repair (Fig. 8).
Cells were arrested using 5-fluorouracil to measure 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, 2-tailed t-test), and this inhibition was specific in that the inactive analog
peptide 690 did not inhibit cell motility in this assay (p > 0.5).

The α3β1 integrin recognition sequence in TSP1 may also contribute to angiogenesis,
because peptide 678 inhibited angiogenesis in the chick CAM assay (p<0.005 at 20 μM, Fig. 9).
The dose dependence for inhibition was consistent with the reported IC₅₀ 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.

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 anti-angiogenic activity (BenEzra et al., 1993; Canfield and Schor, 1995; Good et al., 1990; Iruela Arispe et al., 1991; Nicosia and Tuszynski, 1994; Taraboletti et al., 1990). Recognizing that endothelial cells can modulate their expression or activation state of specific TSP1 receptors that transduce opposing signals may lead to a resolution of this conflict. We have demonstrated that sparse endothelial cells recognize an α3β1 integrin-binding sequence in TSP1 that stimulates endothelial cell spreading and proliferation when immobilized on a substratum. 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 TSP1 interactions with this integrin. We also demonstrated that the activity of this integrin to recognize TSP1 is suppressed in a confluent endothelial cell monolayer. Loss of endothelial cell-cell contact during wound repair in vitro or angiogenesis in vivo could therefore activate this receptor and make endothelial cells responsive to TSP1 signaling through the α3β1 integrin.

The activity of a second TSP1 receptor on endothelial cells that mediates inhibition of
growth factor-stimulated migration, CD36, is regulated by differential expression in endothelial cells from large vessels versus capillaries (Dawson et al., 1997; Swerlick et al., 1992). Thus, CD36-negative endothelial cells with activated α3β1 integrin may recognize TSP1 primarily as an angiogenic signal, whereas CD36-positive endothelial cells with inactive α3β1 integrin would receive only an anti-angiogenic signal (Dawson et al., 1997). Antagonism of FGF2-mediated angiogenic signals by heparin-binding sequences in TSP1 is a second pathway through which TSP1 can inhibit angiogenesis (Iruela-Arispe et al., 1999; Vogel et al., 1993). The responsiveness of this pathway has not been demonstrated to be regulated by endothelial cells. Therefore, endothelial cells receive both pro- and anti-angiogenic signals from TSP1, and the net balance of these signals is 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 (Canfield et al., 1990; Mumby et al., 1984). 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 (Munjal et al., 1990; Vischer et al., 1988), 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). 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.

Involvement of β1 integrins in endothelial cell adhesion on TSP1 is consistent with several recent studies of TSP1-endothelial cell interactions. Binding of soluble TSP1 to HUVEC 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 HUVEC, suggesting that additional TSP1 receptors are present on endothelial cells. More relevant to the present studies, HDME cell adhesion on TSP1 was not RGD- or 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 the αvβ3 integrin as a TSP1 receptor on endothelial cells (Gupta et al., 1999; Lawler et al., 1988). We confirmed this result for BAE cells using a specific nonpeptide antagonist of this integrin, and we found that disrupting cell-cell contact somewhat increases the activity of this integrin for mediating BAE cell spreading on TSP1. In contrast, we could not detect a 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. On BAE cells, α3β1 integrin also plays a major role in mediating spreading on TSP1 and is strongly induced or activated by loss of cell-cell contact. The other known TSP1-binding integrins, α4β1 and α5β1, do not contribute significantly to this response.
The β1 integrin-activating antibody TS2/16 and a CD98 antibody both increased the activity of the α3β1 integrin, demonstrating that this integrin exists on endothelial cells in a partially inactive state. Although sparse cells deprived of cell-cell contact spread better on TSP1 than confluent cells, both showed similar spreading following activation by TS2/16 (unpublished results), suggesting that differential integrin activation rather than differential expression is responsible. Cell-cell contact may therefore transduce a negative signal that suppresses activity of this integrin. We do not know whether exogenous signals could also regulate activation of the α3β1 integrin on endothelial cells. In breast carcinoma cells, IGF1 and insulin acting through the IGF1 receptor were potent activators of the α3β1 integrin for binding to TSP1 (Chandrasekaran et al., 1999). However, IGF1 and insulin had no significant effect on the activity of α3β1 integrin on endothelial cells (Roberts, unpublished results).

The α3β1 integrin is expressed in confluent endothelial cells, but it is concentrated at the cell-cell junctions in association with the TM4 proteins CD9 and CD151/PETA-3 (Yanez-Mo et al., 1998). In the same study, function blocking antibodies recognizing α3β1 integrin inhibited migration of endothelial cells lacking cell-cell contact. In endothelial cells attaching on TSP1, α3β1 integrin localizes to sites of contact with the substrate. Differential association with integrin-binding components in the cytoplasm or membrane accompanying this redistribution of α3β1 integrin may, therefore, activate the integrin to recognize TSP1 and lead to the induction of the spreading response we observed in cells lacking cell-cell contact.

Several other 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 (Mainiero et al., 1997; Yokosaki et al., 1996). TSP1, likewise, expresses both pro- and anti-proliferative activities for specific cell types, but its activity toward endothelial cells has been generally regarded as anti-proliferative (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 α3β1 integrin stimulates proliferation of endothelial cells. Binding of laminin-5 to the α3β1 integrin was recently demonstrated to stimulate 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. Since addition of soluble TSP1 peptide that is recognized by this integrin also inhibits endothelial cell motility in the absence of proliferation, α3β1 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 pro- and anti-angiogenic activities may allow us to isolate each activity and lead to development of peptides, gene therapy approaches, or small molecule analogs of TSP1 with more specific anti-angiogenic activities.

Acknowledgments

This work was supported in part by Department of Defense Grant DAMD17-94-J-4499 (D.D.R.) and NIH Grant CA63356-01 (L. I. A.). The content of this article does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. We thank Dr. James Kaiser for isolation of BAE cells and Drs. William Miller, Ken Yamada, and
Derrick Grant for providing reagents.

Footnotes
Abbreviations: BAE, bovine aortic endothelial; HDME, human dermal microvascular endothelial; HUVEC, human umbilical vein endothelial cells; peptide 678, FQGVLQNVRFVF; TSP1, human thrombospondin-1

References


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Yokosaki, Y., H. Monis, J. Chen, and D. Sheppard. 1996. Differential effects of the integrins $\alpha_9\beta_1$, $\alpha_\nu\beta_3$, and $\alpha_\xi\beta_3$ on cell proliferative responses to tenascin. *J. Biol. Chem.* 271:24144-24150.
Figure legends

Fig. 1. Adhesion of endothelial cells on an α3β1 integrin-binding peptide from TSP1. Panel A: TSP1 peptide 678 (FQGVLQNVRFVF) or analogs 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) are presented as mean ± SD, n = 3. Panel 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% confluency. Fresh medium was added at the same time to the second flask. After 16 h, cells from both flasks were dissociated using 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 cells from confluent (solid bars) or sparse cultures (striped bars) after 60 min is presented as mean ± SD, n = 3 for a representative experiment.

Fig. 2. Spreading on TSP1 induced by loss of cell-cell contact is inhibited by the α3β1 integrin-binding peptide from TSP1. BAE cells from confluent (a, b) or sparse cultures (c-f) were incubated for 60 min on substrates coated with 40 μg/ml TSP1 (a, c, e) or 20 μg/ml fibronectin (b, d, f). Inhibition by 30 μM TSP1 peptide 678 is presented in (e-f). Cells were fixed with 1% glutaraldehyde and stained using Diff-quik. Bar in panel a = 25 μm.

Fig. 3. Loss of cell-cell contact induces endothelial cell spreading on TSP1 peptide 678. Panel A: Adhesion of sparse or confluent BAE cells to substrates coated with 40 μg/ml TSP1 (solid
bars) or 10 μM TSP1 peptide 678 (striped bars) was determined as in Fig. 1B. Spreading was determined microscopically for cells with no additions, in the presence of 10 μM peptide 678, or in the presence of 30 μM of the control peptide 690. Results are presented as mean ± SD, n = 3.

Panel B: HDME cells harvested from confluent or sparse cultures as in Fig. 1 were plated on substrates coated with TSP1 (solid bars), peptide 678 (striped bars), or type I collagen (open bars). The percent spread cells was determined at 60 min.

Fig. 4. Both αβ1 and αvβ3 integrins mediate spreading of endothelial cells on thrombospondin-1. Panel A: BAE cell adhesion to TSP1 (solid bars), vitronectin (striped bars), or plasma fibronectin (open bars) was measured in the presence of 30 μM TSP1 peptide 678, 1 μM of the αvβ3 integrin antagonist SB223245, 300 μM of the integrin antagonist peptide GRGDSP, or the indicated combinations. Results are expressed as percent of the response for untreated cells, mean ± S.D., n = 3. Panel B: HUVEC spreading on substrates coated with TSP1 (solid bars) or vitronectin (striped bars) was determined in the presence of 20 μM peptide 678, 1 μM αIIbβ3 antagonist SB208651, 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/mm² for TSP1 and 10 cells/mm² for vitronectin). Panel C: Inhibition of HDME cell spreading on TSP1 (solid 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).

Fig. 5. Integrin and CD98 localization in endothelial cells spreading on TSP1 or TSP1 peptide
678 substrates. HUVEC attached on TSP1 (panels a-d) or TSP1 peptide 678 (panels e, f) were stained using antibodies to α3β1 integrin (panels a, e), CD98 (panels b, f), phosphotyrosine (panel c), or vinculin (panel d). Bar in panel a = 25 μm.

Fig. 6. β1 Integrin- and CD98-activating antibodies induce HUVEC spreading on TSP1 and TSP1 peptide 678. Untreated HUVEC (control) or cells in the presence of 5 μg/ml of the β1 integrin activating antibody (TS2/16) or CD98 antibody (4F2) were incubated on substrates coated with 40 μg/ml TSP1 (solid 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, mean ± S.D., n = 3.

Fig. 7. Modulation of endothelial cell proliferation by an α3β1 integrin binding peptide from TSP1. Panel A: Proliferation of BAE cells was assayed in the presence of the indicated concentrations of TSP1 peptide 678 (FQGVVLQNVRFVF, ●) or the control peptides 686 (FQGVVLQAVRFVF, ▲), and 690 (FQGVVLQNVAVFVF, ○). Briefly, 100 μl of a 5x10^4 cell/ml suspension of BAE cells were seeded in triplicate into 96 well tissue culture plate in DMEM medium containing 1% FCS, 10 ng/ml of FGF and peptides at 1-40 μM concentrations. Cells were incubated for 72 h, and proliferation was measured using the Celltiter tetrazolium assay (Promega). Panel B: HUVEC proliferation was determined in the presence of the indicated concentrations of TSP1 peptide 678 immobilized on the substrate (solid bars) or added in solution (striped bars). Panel C: HDME cell proliferation 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 mean ± S.D. and are normalized to controls without TSP1 or peptide.

Fig. 8. TSP1 peptide 678 inhibits wound healing of BAE cells. BAE cells were seeded at a density of 2x10⁵ cells/well of 6 well tissue culture plates in complete growth medium supplemented with 10% FBS. After the cells formed a confluent cobblestone, cells were arrested using 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 (solid bars) or 690 (striped bars). Measurements of the distance between the wound margins were taken at 0 and 24 h, and the net migration is expressed as mean ± SEM for triplicates.

Fig. 9. TSP1 peptide 678 inhibits angiogenesis. Polymerized collagen gels containing angiogenic growth factors in the presence or absence of the indicated concentrations of the TSP1 peptide FQGVLQNVRFVF (peptide 678, solid bars) or the inactive analog FQGVLQNVAFVF (peptide 690, striped bars) were placed on the outer 1/3 of 10 d CAMs for 24 h. Each CAM contained two pellets for each peptide concentration, as well as, positive and negative controls. Ability of the peptides to modulate growth-factor driven angiogenesis was assessed by injection of FITC dextran. The percent inhibition relative to controls is presented as mean ± SD for each group (n = 8).
FIGURE 2

TSP1

Fibronectin

(a) (b) (c) (d) (e) (f)
FIGURE 3

A

confluent

sparse

Spread cells/mm²

control
+p678
+p690
control
+p678
+p690

TSP1
p678

B

% spread cells

confluent
sparse

TSP1
p678
Collagen
Spread cells (% of control)

- **TSP1**
- **p678**
- **VN**

- **control**
- +**TS2/16**
- +**4F2**
Figure 8

Distance moved in 24 h (mm)

Peptide (µM)

0  0.3  3  30  30

- p678
- p690

PROPRIETARY DATA
FIGURE 9

The graph shows the angiogenic response (% inhibition) for two peptides, FQGVLQNVRFVF and FQGVLQNVAFVF, across three concentrations: 5, 10, and 20 μM. The data indicates a higher inhibitory effect at 20 μM for both peptides, with FQGVLQNVAFVF showing a more pronounced response.
THROMBOSPONDIN TYPE I REPEAT PEPTIDES SPECIFICALLY INDUCE APOPTOSIS OF ENDOTHELIAL CELLS

Neng-hua Guo, Henry C Krutzsch, John K. Inman, and David D. Roberts, Laboratory of Pathology, NCI, and NIAID, NIH, Bethesda, MD 20892-1500

Thrombospondin-1 (TSP) is an inhibitor of angiogenesis that modulates endothelial cell adhesion, motility, and growth. The anti-proliferative activity of TSP is mimicked by synthetic peptides derived from the type I repeats of TSP that antagonize FGF-2 and activate latent TGFβ. Decreases in cell number and morphological changes induced by these peptides suggested that these TSP analogs may induce programmed cell death. Ficoll conjugates containing peptides from the type I repeats of TSP induced DNA fragmentation and inter-nucleosomal cleavage in bovine aortic endothelial cells. The response was specific to the endothelial cells, as no DNA fragmentation was induced in a breast carcinoma cell line, even though TSP and the peptide conjugates inhibited growth of both cell types. Apoptosis did not depend on activation of latent TGFβ, as peptides lacking the activating sequence KRFK were also active. Induction of programmed endothelial cell death by the peptides was decreased when endothelial cell cultures reached confluence. The activity of the peptides could also be inhibited by growth of the cells on fibronectin substrates or in the presence of the phosphatase inhibitor sodium vanadate. These results demonstrate that induction of apoptosis by the TSP analogs is not a general cytotoxic effect and is dependent on a lack of survival-promoting signals such as those provided by a fibronectin matrix. The anti-tumor activities of TSP and these TSP analogs may therefore result from the selective sensitivity of endothelial cells on provisional matrix in newly formed blood vessels to induction of apoptosis.

Univ. of Wash. Seattle.
Thrombospondin-I (TSP1) is an extracellular matrix protein that modulates endothelial cell responses to growth factors and inhibits angiogenesis. Over-expression of TSP1 in breast carcinoma cells suppresses tumor growth and metastasis in mouse xenografts. To define the role of the type I repeats of TSP1 in these activities, we have examined the activities of synthetic peptide analogs based on these repeats and prepared TSP1 expression vectors with site-directed mutations in these repeats.

Synthetic peptides from the type I repeats inhibit endothelial cell growth. The peptides antagonize endothelial cell responses to basic fibroblast growth factor and activate latent transforming growth factor β (TGFβ). We have now shown that the inhibitory activity of the peptides for endothelial cells is independent of their ability to activate latent transforming growth factor β and can be reproduced by D-reverse peptide analogs.

To define the structural basis for the anti-angiogenic activities of these peptides, we prepared analogs of the TSP1 peptide KRFKQDGWHSHPWSSC. L-forward, L-reverse, and D-reverse (retro-inverso) analogs displayed identical activities for binding to heparin, demonstrating a lack of stereospecificity for heparin binding. The L-reverse, and D-reverse peptides, however, had somewhat decreased abilities to activate latent TGFβ. Conjugation of the forward peptides through a C-terminal thioether and the reverse peptides through an N-terminal thioether to polysucrose abolished the adhesive activity of the peptides and enhanced their anti-proliferative activities for endothelial and breast cells.

**Keywords:** endothelial cells, extracellular matrix, angiogenesis, proliferation, mutagenesis

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carcinoma cells stimulated by basic fibroblast growth factor. Their anti-proliferative activity was independent of latent TGFβ activation, because substitution of an Ala residue for the essential Phe residue in the TSPI type-I repeat peptide increased its potency for inhibiting TSPI binding to heparin and for inhibiting endothelial cell proliferation. Although the conjugated peptides were inactive in vivo, an unconjugated retro-inverso analog of the native TSPI peptide inhibited breast tumor growth in a mouse xenograft model. Thus, these TSPI-derived peptide analogs antagonize endothelial growth through their heparin-binding activity rather than through activation of latent TGFβ or increasing cell adhesion.

These TSPI analogs induced programmed cell death in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and inter-nucleosomal DNA cleavage. Intact TSPI also induced DNA fragmentation. The endothelial cell response was specific, as no DNA fragmentation was induced in MDA435 breast carcinoma cells, even though TSPI and the peptide conjugates inhibited growth of both cell types. Apoptosis did not depend on activation of latent TGFβ by the activating sequence RFK, as peptides lacking this sequence also induced apoptosis. Apoptosis was not sensitive to inhibitors of ceramide generation but was inhibited by the phosphatase inhibitor vanadate. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. Growth of the cells on a fibronectin substrate also suppressed induction of apoptosis by TSPI or the peptides. Differential sensitivities to kinase inhibitors suggest that apoptosis and inhibition of proliferation are mediated by distinct signal transduction pathways. These results demonstrate that induction of apoptosis by the TSPI analogs is not a general cytotoxic effect and is conditional on a lack of strong survival-promoting signals, such as those provided by a fibronectin matrix.

The role of the TGFβ-activating and fibroblast growth factor-antagonist type I repeat sequences in this activity was examined using site-directed mutagenesis of the Phe and Trp residues required for activity of peptides derived from TSPI. Stably transfected breast carcinoma cell lines expressing full length TSPI with a Trp(441)Ala or a Phe(432)Ala mutation in the second type I repeat were characterized for tumorigenic potential in vivo and behavior in vitro. Three transfectant clones of MDA MB435 with high levels of mutant TSP expression were injected orthotopically in the mammary fat pads of athymic mice. Clones from TSPI (W441A) transfection produced tumors with the same or larger tumor masses than controls, whereas those from THBS F432A clones were smaller than controls.

We conclude that stable analogs of active peptides from TSPI may be useful as therapeutic inhibitors of angiogenesis stimulated by basic fibroblast growth factor. These stable peptide analogs inhibit breast cancer growth in mouse xenografts. The anti-tumor activity of TSPI and these peptides may result from an increased sensitivity to apoptosis in endothelial cells adjacent to a provisional matrix during formation of new vascular beds in tumors.
Two Distinct Phases in Integrin-Mediated Raf Activation

Alex Bové and R.L. Juliano. Dept. of Pharmacology and the Linskberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill

We have examined the role of Ras-Raf interaction. Ras membrane localization, and PRC activity in integrin-mediated activation of Raf. Time course experiments involving transfection of epoxygenated wild type Raf (Raf-WT) or a Raf point mutant deficient in Ras binding (Raf/RH), as well as pharmacological inhibition of PRC activity demonstrated that integrin-mediated activation of Raf occurs in two phases. Specifically, efficient early activation of Raf requires Raf-Ras interaction. Raf was not phosphorylated by PRC activity, even when a lower, sustained level of activity was independent of Ras-Raf interaction, but was reduced by PRC inhibitors. The combination of PRC inhibition and lack of Ras binding completely blocked integrin-mediated Raf activation. Interestingly, the activity of a membrane-targeted form of the Raf point mutant (Raf/RH-CAAX) was not regulated by adhesion. Specifically, Raf/RH-CAAX activity was low in non-adherent cells, was rapidly stimulated to wild type levels by adhesion to fibronectin, and remained at near-maximal levels longer than Raf-WT, activity. The activation of Raf by integrin-mediated adhesion was abolished by cycloheximide, demonstrating that cytoskeletal reorganization and organization are required for activation of Raf, even when it is targeted to the membrane. These data suggest that initial and sustained phases of integrin-mediated Raf activation are governed by distinct mechanisms that require Raf membrane localization and possibly PRC activity, respectively, and that integrin-mediated adhesion may regulate a membrane- or cytoskeletal-associated factor responsible for Raf activation.

Extracellular Matrix and Cell Signaling I (1731-1732).

MUTATION OF ANTI-ANGIOGENIC SEQUENCES IN THE TYPE I REPEATS OF THROMBOSPONDIN-1

L. Chambrakar and D. D. Roberts. Laboratory of Pathology, NCI XIII.

We have examined the role of mutations in the anti-angiogenic sequences of the type I repeats of TSP1 as a means of modulating its biological activity. We have previously shown that the type I repeats of TSP1 are necessary for the anti-angiogenic activity of the protein, and that the type I repeats of TSP1 are sufficient to confer anti-angiogenic activity to a non-angiogenic protein. We have also shown that the type I repeats of TSP1 are necessary for the anti-angiogenic activity of the protein in vivo. We have used a series of deletion mutants of TSP1 to study the role of the type I repeats in the anti-angiogenic activity of the protein. We have found that the type I repeats are necessary for the anti-angiogenic activity of the protein in vivo, and that the type I repeats are sufficient to confer anti-angiogenic activity to a non-angiogenic protein. We have also shown that the type I repeats are necessary for the anti-angiogenic activity of the protein in vitro. We have used a series of deletion mutants of TSP1 to study the role of the type I repeats in the anti-angiogenic activity of the protein. We have found that the type I repeats are necessary for the anti-angiogenic activity of the protein in vitro, and that the type I repeats are sufficient to confer anti-angiogenic activity to a non-angiogenic protein.
Appendix K: Tables

Table 1. List of Thrombospondin Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Comments</th>
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<tbody>
<tr>
<td>THBS W385A</td>
<td>Type I repeat 1 mutation of central Trp required for heparin binding to synthetic peptides</td>
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<tr>
<td>THBS W441A</td>
<td>Type I repeat 2 mutation of essential central Trp residue</td>
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<tr>
<td>THBS W498G</td>
<td>Type I repeat 3 mutation of essential central Trp residue</td>
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<tr>
<td>THBS F432A</td>
<td>TGF beta activation sequence mutant</td>
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</table>
Table 2. Analysis of GFP Co-expression In Transient Transfectants
MDA435 cells were transiently transfected with 10 μg of wild type or F432A mutant DNA and 2.5 μg of the pGreen Lantern vector as described in Methods. Transfected cells were plated into 6-well tissue culture plates, and at 24 and 48 hours cells were counted under phase contrast (Total) or fluorescent (GFP +ve) light in three different fields of view.

<table>
<thead>
<tr>
<th>Time</th>
<th>Wild Type</th>
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<tr>
<td></td>
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<tr>
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<td>4</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>9.8</td>
</tr>
</tbody>
</table>
Table 3: Effects of wild type and mutant TSP1s on proliferation of BAE cells.

Effects of the different transiently expressed proteins on proliferation of BAE cells was measured as \(^{3}\text{H}\)-thymidine incorporation. Varying amounts of THBS wild type expression vector (WT) and 15-20 \(\mu\)g of the KRFK mutant (F432A), the first type I repeat mutant (W385A), the second type I repeat mutant (W441A), the third type I repeat mutant (W498G) or the truncation mutant (WG1a) were used for transfection and thymidine incorporation was measured as mentioned in the methods section. The transfection efficiencies are represented as \(\beta\)-galactosidase/\(\mu\)g total protein. The numbers in parentheses indicate number of samples assayed per group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(^{3}\text{H})-thymidine uptake (% Control ± SEM)</th>
<th>Transfection Efficiency as mU (\beta)-gal/(\mu)g protein (% Control ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT - 6 (\mu)g</td>
<td>90.41 ± 14.16</td>
<td>118.20 ± 13.61 (4)</td>
</tr>
<tr>
<td>WT - 12 (\mu)g</td>
<td>72.82 ± 9.01</td>
<td>124.19 ± 18.71 (4)</td>
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<td>WT - 18 (\mu)g</td>
<td>48.34 ± 13.41</td>
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<td>64.30 ± 19.35</td>
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<td>W385A - 15(\mu)g</td>
<td>128.01 ± 40.75</td>
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<td>W498G - 16.5 (\mu)g</td>
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<td>19.06 ± 7.45</td>
<td>259.60 ± 104.35(2)</td>
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Type I repeat # 1, 2 and TGF-β activation sequence mutations

Type I repeat # 3 mutation

FIGURE 1

Linearize with Agel

Digest with BamHI

Denature and anneal primer

A + C + D + B

Fill in with Klenow, ligate and transform.

Linearize with Clal

Digest with NotI/Agel

Denature and anneal primer

A + C + D + B

Fill in with Klenow, ligate and transform.
Screening of transfected MDA clones for TSP secretion
FIGURE 12

Dilution of BandX protein used for coating ELISA plate

- 1:128
- 1:64
- 1:32
- 1:16
- 1:8
- 1:4
- 1:2
- NO DIL
- BSA

Radioactivity, CPM

0 2000 4000 6000 8000
FIGURE 13

Radioactivity, CPM x 10^3

Fraction Volume, ml

- - - Platelet TSP

- - - TH26 TSP/BandX complex
FIGURE 18

Proliferation of BAE Cells in the presence of recombinant TSP proteins

[Graph showing absorbance levels for different inhibitors]
686.V TSP-WA transfected MDA cell lines in nude mice

**Figure 19**

Tumor volume (mm$^3$) vs. Days after implantation

- + - neo
- ▲ - WA A.E9
- ● - WA A.A11
- ▼ - WA E.A3
Tumor Progression in Beige Mice 96-02
MDA-WT and 2WA Mutants

Figure 20

- O - 2WA-AE9
- ▲ - 2WA-EA3
- ▼ - TH26
- □ - TH5
Appendix L: Figures and figure legends

FIGURE LEGENDS

Figure 1: Strategy of construction of mutant DNAs. A, B, C and D represent the different forms of the double stranded expression vector pCMVTHBS containing the full length cDNA formed by reannealing the indicated fragments.

Figure 2: Touchdown PCR of mutant fragments of THBS. Lane 1 is the size marker. Lanes 2, 4 and 6 are Touchdown products of Type I repeat 2, TGF-beta activation sequence and Type I repeat 1 mutant fragments respectively. Lanes 3, 5 and 7 are controls showing lack of products from the corresponding wild type templates.

Figure 3: Screening of bacterial colonies for THBS W441A mutation by Touchdown PCR. Lane 1 is the size marker. Lanes 4 and 5 are mutant and wild type PCR fragments. Lanes 7 - 20 are bacterial colonies amplified by Touchdown PCR. DNA from the colony in lane 12 was positive for the mutation.

Figure 4: Screening of bacterial colonies for THBS F432A mutation. Lane 1 is the size marker. Lane 2 and 3 are mutant and wild type PCR fragments respectively amplified by Touchdown PCR. Lanes 5 - 20 are bacterial colonies screened for the mutation. DNA from the colony in lane 7 was positive for the mutation.

Figure 5: ELISA assay for screening cloned MDA435 transfectants for expression of TSP1 in transfected clones.

Figure 6: Western blot analysis of recombinant TSP1 expression by transfected MDA cell clones.

Figure 7: Immunoprecipitation of myc-his tagged TSP1: Myc-his tagged full-length THBS construct in pCMVneoBam vector was used to transiently transfect MDA435 cells and 24 hr post transfection the cells were labeled with 100 μCi of [35S] methionine for 2 hr. The spent medium (lane 1) and cell lysate (lane 2) were immunoprecipitated with anti-TSP1 antibody as described in the ‘Methods’ section.

Figure 8: Electrophoretic analysis of TSP1 purified by heparin affinity chromatography. Electrophoresis was carried out as described under ‘Methods’ using thrombospondin purified from conditioned medium derived from untransfected MDA cells (lane 1), MDA cells transfected with 2WA-A.E9 mutant TSP1 (lane 2), TH50 mutant TSP1 (lane 3) and TH26 wild type TSP1 (lane 4). The gel was stained using Coomassie blue. The electrophoretic pattern of platelet thrombospondin is shown in lane 5.

Figure 9: Purification of TH26-TSP1 on heparin affinity chromatography. Heparin affinity
chromatography was carried out as described under ‘Methods’. Lane 1 contains the conditioned medium used as the source for purification. Sample eluted using 0.65 M NaCl contained TSP1 band and the high molecular weight protein band-X (Lane 2). A pre-elution step with 0.35 M NaCl did not elute TSP1 or band-X (Lane 3). Platelet TSP1 run in the same gel is also shown (Lane 4).

Figure 10: Proteolytic digestion of band-X protein. Band-X protein eluted from heparin affinity chromatography was digested with trypsin or thrombin. Lanes 1 and 3 contain the starting material for the proteolytic digestion. The protease reaction was carried out as described under ‘Methods’ the proteolytic digests are shown in lane 2 (trypsin digest) and lane 4 (thrombin digest).

Figure 11: Western blotting analysis of thrombospondin. Heparin affinity chromatography purified thrombospondin from untransfected MDA (lane 1), TH26 wild type thrombospondin-transfected MDA (lane 2), 2WA- mutant-transfected MDA (lane 3), and thrombospondin purified from human platelets (lane 4) were run in a 4-15 % gradient gel under reducing condition. The proteins were then transferred to PVDF membrane and probed with anti-thrombospondin polyclonal antibody and the bands were visualized by ECL method.

Figure 12: Assay of thrombospondin interaction with band-X. ELISA plate was coated with different dilutions of band-X protein-purified by heparin affinity chromatography. Fixed amount of $^{125}$I-labeled thrombospondin was used for binding (see Methods). The unbound material was rinsed out and the wells were removed from the plate and counted in a gamma counter. The data presented here represent the mean value of duplicate samples.

Figure 13: Gel filtration profile of TSP1/band-X complex compared to platelet TSP1. $^{125}$I-labeled samples were used for gel filtration on Sephacryl S-500. Fixed volume fractions were collected and the radioactivity was measured in a gamma counter.

Figure 14: Agarose gel electrophoresis. Agarose gel (3%) was used to resolve TSP1 and TSP/band-X complex. TSP1 and band-X mixture under reducing and non-reducing conditions is shown in lane 1 and lane 2 respectively. Lanes 3 and 4 contain platelet TSP1 under reducing and non-reducing conditions respectively.

Figure 15: Synthesis and secretion of TSP1 mutants: MDA435 cells grown in 10 % fetal bovine serum were electroporated with 10 $\mu$g each of pCMVneoBam vector (lanes 1-4), THBS wild type construct (lanes 5-8) or F432A mutant construct (lanes 9-12). 24 hr post transfection cells were pulse-labeled with 100 $\mu$Ci of $^{35}$S methionine for 30 min and subsequently chased for 60 min (lanes 1, 2, 5, 6, 9, and 10) or 180 min (lanes 3, 4, 7, 8, 11 and 12). Spent media (lanes 1, 3, 5, 7, 9 and 11) and cell lysates (lanes 2, 4, 6, 8, 10 and 12) were immunoprecipitated with anti-TSP1 antibody as described in the ‘Methods’ section.

Figure 16: RT-PCR of mammary fat pad tumors from mice injected with THBS W441A transfected clones. Lane 1 is the size marker. Lane 2 is the RT-PCR product of wild-type THBS
cDNA in the expression vector. Lanes 3, 4 and 5 are products from mouse tumors expressing the THBS W441A - A.A11 clone and lanes 6, 7 and 8 are products from tumors expressing the E.A3 clone.

Figure 17: Dose response for inhibiting proliferation of MDA435 breast carcinoma cells by expression of wild type TSP1 or TSP1 with mutation in the TGFβ activation sequence (KRFK). (Panel A) 3H-thymidine incorporation by MDA435 cells transiently transfected with varying amounts of THBS wild type expression vector (WT) and the KRFK mutant (F432A) were measured as described in the methods section. (Panel B) The transfection efficiencies were determined by co-transfection with a constant amount of β-galactosidase vector. Efficiency was calculated as β-galactosidase/μg total protein and is presented as a percent of control transfections without addition of the THBS vectors.

Figure 18: Proliferation Assay using bovine aortic endothelial cells with recombinant fragments of different domains of TSP1. The amounts indicated are μg/ml. Values are averages of triplicate wells. The amino acid residues of TSP1 represented in each of the recombinant fragments used are - Procollagen, 278 - 355; Type I repeat, 385 -522; Type II repeat, 559 - 669; Type III repeat, 784 - 932; COOH-terminal (M3), 877 - 1152; COOH-terminal, 933 -1152.

Figure 19: Growth curves for MDA435(W441A) transfectants in athymic nude mice.

Figure 20: Growth of stably transfected MDA435 breast carcinoma cells implanted in the mammary fat pads of NIH-III Beige nude XID mice. Tumor volumes were determined by external caliper measurements at the indicated times after implantation of MDA435 control transfectant (□), clone TH-26 over expressing wild type thrombospordin (v), clone AE9 expressing W441A thrombospordin (○), or clone EA3 expressing W441A thrombospordin (▲). Results are mean ± SD, n = 10.
Appendix M

BIBLIOGRAPHY

Peer reviewed publications


Abstracts


Appendix N

Personnel

Lakshmi Chandrasekaran, Ph.D.
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Joe Tseng, B.S.
Rui Rodrigues, B.S.
Heba Al-Barazi, Ph.D.
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHILIP M. RINEHART
Deputy Chief of Staff for Information Management
Reports to be changed to "Approved for public release; distribution unlimited"

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