AD NUMBER

ADB233109

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Oct 97. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St., Fort Detrick, MD 21702-5012.

AUTHORITY

USAMRMC ltr, 1 Jun 2001.

AD_____

GRANT NUMBER DAMD17-94-J-4499

TITLE: Regulation of Breast Carcinoma Growth and Neovascularization by Novel Peptide Sequences in Thromospondin

PRINCIPAL INVESTIGATOR: David D. Roberts, Ph.D.

CONTRACTING ORGANIZATION: National Institutes of Health
Bethesda, Maryland 20892

REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1997	Annual (30 Sep 96 - 29 Sep 97)

4. TITLE AND SUBTITLE
Regulation of Breast Carcinoma Growth and Neurovascularization by Novel Peptide Sequences in Thromospondin

5. FUNDING NUMBERS
DAMD17-94-J-4499

6. AUTHOR(S)
David D. Roberts, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
National Institutes of Health
Bethesda, Maryland 20892

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Oct 97). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Thrombospondin-1 (TSP) 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 TSP. Peptides from the type I repeats of TSP reproduced the growth inhibitory activity of the intact protein and specifically interact with heparin, heparan sulfate proteoglycans, fibronectin, and TGFβ. 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 FGF-2 and selectively induced apoptosis of endothelial cells. Over expression of TSP 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 TSP. Stably transfected breast carcinoma cell lines expressing these mutants were characterized for tumorigenic potential *in vivo* and behavior *in vitro*.

14. SUBJECT TERMS
Breast Cancer extracellular matrix, thrombospondin, angiogenesis, site-directed mutagenesis, peptidomimetics, growth factor antagonists, apoptosis

15. NUMBER OF PAGES
74

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

DL Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

DL In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

DL In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

DL In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

DL In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Daniel C. Roberts 10-28-97

PI - Signature Date

TABLE OF CONTENTS

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). Since normal endothelium is non-proliferating, neovascularization of tumors requires specific induction of endothelial cell growth and migration. The factors that are produced by tumors and their mechanism for regulating angiogenesis are poorly understood. The current model for regulation of angiogenesis incorporates 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 simulators or inhibitors to allow neovascularization to proceed (3).

The major identified stimulators of angiogenesis produced by tumors are basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial cell growth factor (VEGF). These are potent stimulators of endothelial cell growth and motility in vitro. Several anti-angiogenic factors have also been identified, including thrombospondin (5, 6), interferon-alpha (7), platelet factor 4 (8), SPARC (9), apolipoprotein E (10), angiostatin (11), and a proteolytic fragment of fibronectin (12). The mechanism for action of angiogenesis inhibitors is less clear. Some of these proteins bind to heparin, and this binding activity may be responsible for some of the anti-angiogenic activities. We have recently shown that apolipoprotein E and heparin-binding recombinant fragments and synthetic peptides from thrombospondin can compete for binding of bFGF to endothelial cells or heparin and inhibit proliferative and migratory responses of endothelial cells to bFGF (10, 13).

Thrombospondin is a major component of the α-granules of platelets and is a member of a gene family synthesized by many cell types in tissue culture (reviewed in 14, 15). Thrombospondin-1 (TSP) is the product of the *THBS1* gene (16) and is the major form of thrombospondin in human platelets, which are the source of TSP for all studies of the purified protein. In examining the role of thrombospondins in tumor metastasis, we have used TSP from platelets. Metastasis is a complex process involving escape of tumor cells from a primary tumor, local invasion of surrounding tissue, invasion through capillaries, arrest in specific target organs, extravasation, and colonization of the target organ (17). Expression of oncogenes or loss of tumor suppressor genes presumably lead to expression of the many matrix degrading enzymes, adhesion molecules, motility factors, and growth factors that regulate tumor metastasis.

The role of TSP in development or progression of breast cancer is not known. Thrombospondin is synthesized by normal breast stromal cells in tissue culture (18) and is a normal component of human milk (19). Immunohistochemical analyses of TSP expression in malignant breast tissues demonstrated strong staining in desmoplastic stroma and in the basement membrane associated with malignant ductal epithelium (20). However, TSP is also

expressed in the basement membrane of normal myoepithelial cells, and most invasive ductal carcinoma cells do not express TSP (21). High expression of TSP in breast carcinoma is restricted to invasive lobular carcinoma (21). Thus, expression of TSP may be lost in some types of invasive breast carcinoma. This finding correlates with the *in vitro* observation that expression of TSP in hybrids of normal mammary epithelial cells with MCF-7 breast cancer cells was inversely related to their invasive behavior (22).

TSP may play a role in several steps in the metastatic cascade. We have shown that TSP promotes tumor cell adhesion and motility (23, 24), which are important in several steps of metastasis. Thrombospondin enhances melanoma cell interactions with platelets (25), which is critical to arrest and extravasation of circulating tumor cells during hematogenous metastasis. TSP is a tight binding inhibitor of several neutral proteases including plasmin, neutrophil elastase and cathepsin G (26, 27), and so could regulate tumor invasion through matrix. Finally, we and others have recently identified TSP as an inhibitor of angiogenesis (5, 6, 28), which is critical for recruitment of blood vessels needed to support growth of the primary tumor and for development of hematogenous metastases. Because the ability to induce angiogenesis is associated with increased frequency of lymph node metastasis in breast cancer (1, 2), we are focusing on the effects of TSP both on tumor cells and on endothelial cells.

TSP is present at very low levels in plasma, but its concentration is elevated at sites of platelet activation. TSP is found in intracellular granules of endothelial cells and is enriched in the subendothelial matrix *in vivo* (29, 30). Thus, endothelial cells are probably exposed to significant concentrations of TSP *in vivo*. Endothelial cell responses to TSP are complex; the magnitude and direction of the responses depend upon the presence of additional matrix components and growth factors. Immobilized TSP promotes endothelial cell adhesion on some substrates (6) but inhibits adhesion on others, including substrates coated with fibronectin (31). Inhibition of adhesion to fibronectin is associated with disruption of focal adhesion contacts (32). TSP promotes migration of endothelial cells in chemotaxis and haptotaxis assays but inhibits chemotaxis induced by bFGF (6). TSP inhibits proliferation and spontaneous tube formation by endothelial cells *in vitro* (28) and inhibits angiogenesis *in vivo* (5). A 140 kD fragment of TSP has been identified as the anti-angiogenic factor in conditioned medium of hamster kidney cells (5).

To understand the mechanisms of these diverse and apparently conflicting effects of TSP on endothelial cell behavior, it is necessary to define the domains of TSP that interact with the cells, the identity of the endothelial cell receptors that interact with TSP, and the intracellular responses in transduction and integration of the signals resulting from TSP binding to each receptor. Based on inhibition by monoclonal antibodies and sulfated polysaccharides, the heparin-binding domain at the amino-terminus of TSP may be responsible for regulation of endothelial proliferation (6). However, a 140 kDa fragment of TSP that lacks the amino-terminal region also suppresses endothelial cell growth (5). Thus, multiple sites on the TSP molecule may modulate endothelial cell growth and motility.

Moreover, based on recent studies by Murphy-Ullrich et al. (33), inhibition of bovine endothelial cell growth by TSP is at least partly due to the inhibitory activity of transforming growth factor β, which complexes with TSP and contaminates most TSP preparations. Recently we have identified two parts of TSP that have antiproliferative activity in isolation (10, 13). Recombinant amino-terminal domain inhibited endothelial growth and motility induced by serum or bFGF. Synthetic peptides from the type I repeats also inhibited proliferation to bFGF and showed a biphasic effect on motility of endothelial cells in the presence of bFGF that mimicked the activity of intact thrombospondin. Recently Tolsma et al (34) 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 thrombospondin have antiangiogenic activities, and some of these activities are expressed in synthetic or recombinant constructs without contaminating TGFβ.

Interactions of thrombospondin with tumor cells are also complex. Two regions of the TSP molecule have been identified that mediate adhesive and migratory responses of cultured human melanoma cells to TSP (23, 24). The carboxyl-terminal domain mediates attachment and haptotaxis, and the amino-terminal domain mediates cell spreading and chemotaxis (23, 24). The cell receptors recognizing these two regions of TSP are under investigation. Sulfated glycoconjugates, including heparan sulfate proteoglycans and sulfated glycolipids, interact with the amino-terminal domain of TSP. An unusual sulfated glycolipid, present only in melanoma cell lines that spread on TSP, binds to TSP and participates in melanoma cell spreading on TSP but not on fibronectin (35). Integrin and non-integrin receptors for the carboxyl-terminus of TSP have been characterized in several types of tumor and normal cells (36-38).

At least two regions of thrombospondin interact with sulfated glycoconjugates. Proteolytic or recombinant fragments from the amino terminus of thrombospondin bind specifically to heparin or sulfatide (35, 39). Basic consensus sequences occur in the amino terminal domain of TSP (40, 41) and were shown to be active using recombinant fragments containing these sequences. A second putative heparin binding site was identified in the type I repeats of TSP (42). Synthetic peptides from TSP were used to further define this heparin binding site in the type I repeats. These studies led to the discovery of a novel heparin binding sequence (43, 44). The TSP peptides which inhibit heparin binding, but not adjacent peptides from the TSP sequence containing the previously identified adhesive motif Val-Thr-Cys-Gly (45), promote melanoma cell adhesion when immobilized on plastic. The peptides also inhibit heparin-dependent binding of TSP or laminin to human melanoma cells. The active peptides 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 (44). 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. Adjacent basic residues in the second type I repeat enhance binding to

heparin but not to sulfatide. Using defined oligosaccharides from heparin, the two heparin binding sequences from TSP were shown to have different binding specificities (Yu et al, manuscript submitted). The type I peptides of TSP thus define a new class of heparin-binding peptides.

Based on its effects on tumor cell adhesion, growth, and motility, expression of TSP by tumor cells may regulate their metastatic phenotype. We found that TSP mRNA and protein expression were decreased in subclones of K1735 melanoma cells selected for high metastatic potential in mice and in human lung epithelial cell lines transfected with activated forms of ras and selected for tumor formation by growth in nude mice (46). We have recently shown that over expression of thrombospondin-1 in breast carcinoma cells suppresses tumor growth in nude mice (47), identifying *THBS1* as a potential tumor and metastasis suppressor gene.

These data, combined with recent data from this and other laboratories demonstrating that TSP inhibits endothelial cell growth *in vitro* and angiogenesis *in vivo* (1, 5, 6), suggest that TSP may inhibit neovascularization of tumors. The synthetic peptides from the type I repeats and recombinant amino-terminal heparin-binding domain from TSP mimic the inhibitory activities of intact TSP on endothelial cell proliferation and motility (10, 13). These fragments and peptides act at least in part by competing with bFGF for binding to heparan sulfate proteoglycan receptors on the endothelial cells, which are essential for presentation of bFGF to its signaling receptor. This may be a general mechanism for inhibition of angiogenesis by heparin-binding proteins, as we have recently shown that the heparin-binding protein apolipoprotein E is a potent inhibitor of endothelial cell proliferation and motility *in vitro* (12) and *in vivo* (48).

Establishing the molecular mechanisms involved in adhesion and metastatic migration of tumor cells may also lead to development of inhibitory agents to prevent tumor invasion and metastasis. The synthetic peptides from the type I repeats are especially promising in this regard, since they are active *in vitro* at relatively low concentrations. The strong antiproliferative activity of the TSP peptides suggested that these may also be useful for inhibition of pathological angiogenesis *in vivo*. Free peptides, however, often have short half lives in circulation. They are subject to rapid clearance due to their small size and susceptible to proteolytic degradation. In several cases, use of polymer conjugates of peptides from extracellular matrix proteins has overcome these limitations (49-51). The peptides from the type 1 repeats of thrombospondin have therefore been conjugated to a ficoll carrier to increase their stability *in vivo*.

Preparation of retro-inverso 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 52). Of particular relevance to the thrombospondin 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* (53). The

retro-inverso analog of the thrombospondin type 1 peptide sequence KRFKQDGGWSHWSPWSSC was chosen as the starting point for preparation of retro inverso analogs.

Our second major goal is to define the function of these sequences in the intact thrombospondin protein. These studies employ expression of recombinant thrombospondin-1 containing site-specific mutations. Stable transfectants of a human breast carcinoma cell line expressing 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 provide insight into the role of specific sequences in TSP in regulating tumor behavior.

BODY OF REPORT:

MATERIALS AND METHODS:

Materials-- TSP was purified from the supernatant of thrombin-stimulated human platelets (8). TSP and its fragments were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) or Bolton-Hunter reagent (Dupont NEN) as previously described (8). Antibodies to native and denatured TSP were prepared by immunization of rabbits with native TSP or reduced and carboxymethylated TSP, 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 (44). 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 MWCO tubing, gel permeation chromatography, or reverse phase purification using C_{18} Sep-pak cartridges. Identities of some peptides were verified by MALDI time of flight mass spectrometry.

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 (54). 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 Na_2CO_3. 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 kD MWCO tubing. Peptide concentration of the resulting solution was determined by measuring its absorbance at 280 nm using å = 5540 $M^{-1}cm^{-1}$ per Trp residue.

Ligand binding assays -- TSP binding to heparin-BSA was determined using a solid phase assay. Heparin-BSA (0.075 µg/well) was adsorbed onto 96-well polyvinyl chloride microtiter plate wells by incubation in 50 µl of Dulbecco's PBS for 16 h at 4°. The wells were emptied and filled with tris-BSA. After 30 minutes, the wells were emptied and 30 µl of various concentrations of inhibitors diluted in tris-BSA buffer or buffer alone and 30 µl of ^{125}I-TSP (0.1-0.2 µg/ml) were added to each well. After incubation for 4 hours at 4°, the wells were washed 6 times with 0.15 M NaCl, cut from the plate, and the bound radioactivity was counted.

Binding of ^{125}I-bFGF to heparin was determined using an immobilized heparin-bovine serum albumin conjugate as previously described (7). ^{125}I-bFGF, prepared as described (55), was added and incubated for 2 h at 25°. Bound radioactivity was determined after washing and cutting the wells from the plate.

Bioassay for inhibition of endothelial and breast carcinoma cell proliferation-- Proliferation of bovine aortic endothelial cells was determined as previously described (15). Similar assays were performed using MDA MB435 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 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 TSP were used to examine the effects of site-directed mutations in TSP 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.

Mutagenesis of THBS1 cDNA-- The full length expression vector pCMVTHBS1 was used for preparation of site-directed mutations. Site-directed 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 (56). Full length mutants were constructed expressing thrombospondin with the following amino acid substitutions in the type I repeats: W385A, W441A, W498G, and F432A. 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 SSCP-PCR using overlapping primer sets.

Transfection-- MDA435 cells were stably transfected by electroporation using 10 µg of pCMVTHBS1 vectors containing the mutations listed in Table I 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° for ELISA analysis. Colonies arising from single cells that secreted TSP were expanded and cryopreserved in liquid nitrogen.

The serum-free conditioned media were assayed for expression of TSP 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°. 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°. 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-TSP in tris-BSA for 2 h at 37°. The wells were aspirated and washed 3 times with DPBS, 0.02% BSA, 0.02 mM PMSF, 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. o-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 TSP 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 TSP secreted by the cells. TSP on the blots was detected using rabbit antibody to denatured TSP and peroxidase conjugated goat anti-rabbit IgG followed by visualization using ECL reagent (Amersham).

Three transfected cell lines expressing the THBS W441A and three cell lines expressing the THBS F432A mutations were tested for tumorigenic potential in athymic nude mice following orthotopic implantation of $1x\ 10^5$ transfected cells in the mammary fat pads as described above. At sacrifice, portions of each tumor were frozen in liquid nitrogen for preparation of RNA and the remaining tumor was fixed in formalin and paraffin embedded for histological examination. Lungs from each animal were also embedded for histological examination.

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.

Metabolic Labeling and Immunoprecipitation of TSP-- $2x\ 10^6$ 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 37^0 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 [^{35}S]-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 PMSF 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 TSP with 6 µl of polyclonal anti-TSP antibody at 4^0 C for 1 hr. After 50 µl of Protein A- agarose was added, samples were further incubated at 4^0 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 microelectroporation chambers with a 0.15 cm gap between the two flat-topped

bosses. The chilled mixture of 2x 10^6 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.

Bioassay for inhibition of endothelial cell proliferation-- Proliferation of bovine aortic endothelial cells was determined as previously described (13). Briefly, 15,000 cells/well were plated into 96 well tissue culture plates and exposed to different proteins added to DMEM containing 1% fetal bovine serum. After incubation of the cells at 37^0 C for 72 hours viable cell numbers in the wells were determined using the Cell titer Non-Radioactive Cell Proliferation Assay Kit from Promega.

Production of recombinant TSP -- The breast carcinoma cell line, MDA435, was transfected with mutant or wild type TSP gene. This cell line was chosen for transfection due to the extremely low levels of endogenous TSP production. The transfected cells were grown in RPMI medium containing G418 (Life Technologies). The cells were allowed to attach and grow for 48 to 72 hours and when they became approximately 80% confluent, the medium was replaced with a serum-free medium (CHO-S-SFM II; Life Technologies) containing G418. The cells were maintained in this medium for 48 hours and the conditioned medium was then collected, centrifuged and the supernatant was used for the purification of recombinant TSP.

Purification of recombinant TSP - The conditioned medium was passed through a heparin affinity chromatography column (5 ml HiTrap Heparin column from Pharmacia Biotech) equilibrated in 10 mM Tris, pH 7.5 containing 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mM PMSF. The column was initially washed with the above buffer and subsequently with the same buffer containing 0.35 M NaCl. The column effluent during this step was discarded, and material bound to the column was eluted with the above buffer containing 0.65 M NaCl. Fractions were collected and measured for absorbance at 280 nm. Protein containing fractions were combined and stored at -70° C. This protocol was followed for the purification of different recombinant TSP preparations including W441A mutant TSP, TH50 truncated TSP, and TH26 wild type TSP. In some experiments, conditioned medium from untransfected MDA MB-435, which secreted very low levels of TSP, was also used for the purification of endogenous TSP.

One of the recombinant TSP preparations made by the above method was from MDA cells transfected with TH26, a wild type THBS transfectant. When partially purified TH26-TSP from heparin affinity chromatography was analyzed by gel electrophoresis, a high molecular weight band, which will henceforth be called band-X, was found to co-purify with TSP (see Fig. 2; lane 4). As will be discussed later, this molecule appeared to be a secreted protein, which forms a stable complex with TSP. Several biochemical methods have been employed to separate band-X from TSP. We have also attempted to purify and characterize this molecule.

Electrophoretic analysis – The conditioned medium from transfected MDA cells and purified samples from heparin affinity chromatography were analyzed by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (57). Samples were reduced by β-mercaptoethanol and run on a 4-15% gradient gel. Either Coomassie Blue or Silver staining method was used to visualize the protein bands.

Agarose gel electrophoresis of TSP and band-X – 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 TSP containing band-X purified from heparin affinity chromatography was iodinated using iodogen (Pierce Chemical Co. IL) based on the manufacturer's protocol. The ^{125}I-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.

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

Identification of complex formation between TSP and band-X – Two independent methods were employed to identify the interaction between TSP and band-X. In the first method, TSP/band-X complex purified by heparin affinity column was incubated with anti-TSP 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.

In the second method, TSP purified from human platelets was used to examine its interaction with band-X protein. ^{125}I-radiolabeled TSP 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. ^{125}I-labeled TSP (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).

Proteolytic digestion analysis of band-X protein – The band-X protein purified from

heparin affinity column was digested using trypsin or thrombin. The enzyme digestion was carried out for 1 hour at 30° C. At the end of the reaction, electrophoresis sample buffer containing β-mercaptoethanol was added and the samples were incubated at 95° C for 5 minutes. Samples were then analyzed by SDS-PAGE as described above.

Protein sequencing – A heparin affinity chromatography-purified sample containing TSP/band-X mixture was dialyzed in 0.1 M sodium bicarbonate pH 8. The dialyzed sample was dansylated using 10 mM dansyl chloride at room temperature for 2 h in the dark. The sample was then reduced using β-mercaptoethanol and alkylated using N-isopropyliodoacetamide by the method of Krutzsch et al. (58). The dansylated sample was run on electrophoresis and visualized using a UV light. The fluorescent-labeled band representing band-X protein was cut out from multiple lanes and the gel pieces were electro-eluted to retrieve the protein. The eluted protein solution was lyophilized and digested overnight using EndoLysC in 2 M urea at pH 8. The peptides thus generated were separated by HPLC using a C-8 reverse phase column. Fractions representing a single peptide were selected for peptide sequencing, which was carried out in a Beckman 2090 protein sequencer.

Analysis of TSP/band-X mixture by Sephacryl S-500 gel filtration – TSP/band-X complex was iodinated using iodogen and the radiolabeled material was used for chromatography in a Sephacryl S-500 gel filtration column. The column was equilibrated in 0.01 M Tris pH 7.4 containing 0.15 M NaCl, 100 μg/ml BSA and 1 mM PMSF. Labeled samples were loaded onto the column and the collected fractions were examined for radioactivity using a gamma counter. The elution profile of TSP/band-X complex was compared to the profile of platelet TSP obtained using the same column.

RESULTS

We have completed and published the previously reported studies based on specific aim 1, to define structural elements responsible for activity of the TSP type 1 peptides and to prepare stable analogs with *in vivo* activity (59). We have also completed and published a study of the apoptosis response to the peptides and shown that native TSP has the same activity (60). In the present report we present additional evidence for the specific apoptotic response of endothelial cells, based on loss of membrane phospholipid asymmetry in endothelial cells exposed to the thrombospondin peptides. We examined the effect of aggregation on *in vivo* anti-tumor activity of the peptides and found that monomeric peptide has decreased activity that parallels its lower activity in *in vitro* assays. 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. We present new evidence that transient expression of those mutants that failed to yield stable transfectants, but not wild type thrombospondin, inhibits growth of the breast carcinoma cells. While purifying the recombinant thrombospondins from these cell lines, we discovered that the thrombospondin is tightly complexed with a novel

protein secreted by the breast carcinoma cells. We describe the initial biochemical characterization of this protein and its interactions with thrombospondin. 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.

Mutagenesis of THBS1 cDNA-- In order to study the role of potential anti-angiogenic sequences in the Type I repeats of TSP in regulating angiogenesis of breast and some other tumors, we performed site directed mutagenesis of an expression vector containing a full length THBS cDNA by filling a gapped region in the presence of appropriate mutagenic primers. 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.

Transfections-- MDA435 cell lines stably expressing each mutant were prepared using electroporation and an improved selection protocol (61) to increase the efficiency of stable transfection. Colony formation was optimized by plating pooled transfectants at limiting dilution in 96 well tissue culture plates containing filtered conditioned medium to increase the efficiency of colony formation. This method allowed large numbers of stable transfectants to be rapidly isolated for each mutant.

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 as detecting antibody. Clones with high expression identified by this assay were re-screened by Western blotting to verify the size of the recombinant TSP secreted by the cells using a new antibody raised to reduced and alkylated TSP. Selected clones expressing full length TSP 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.

In our previous report, we described properties of clones over-expressing THBS W441A and THBS F432A for *in vivo* tumorigenesis and *in vitro* functional assays. Clones from THBS W441A transfection had the same or larger tumor masses compared to controls whereas those from THBS F432A clones were smaller than controls. Examination of the lung sections showed that 7 out of 8 (87%) of the W441A clone E.A3 had lung metastases, whereas none of the control animals had detectable lung metastases. We have now 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 (62), and thrombospondin was recently reported to modulate activation of NK lymphocytes (63). 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. 1). 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. 1). 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.

Analysis of the *THBS* W441A transfectant cell lines by RT-PCR confirmed they had the plasmid-derived TSP mRNA. However, a similar analysis of THBS F432A transfectants failed to detect the plasmid-derived mutant TSP mRNA. Since these transfectants produced much smaller tumors than controls and highly expressed thrombospondin, we further examined the origin of the TSP expressed in these clones. 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 (64). The serum induction of expression observed in these experiments indicated that the THBS F432A clones had up-regulated their endogenous *THBS1* gene and were not expressing the stably integrated mutant TSP.

Failure to isolate stable transfectants expressing F432A TSP could indicate instability of the mutant protein or mRNA or inability of the cells to process or secrete this mutant protein. Lack of secretion of erythropoietin receptor following mutation of its sequence homologous to the thrombospondin type I repeats (65, 66) 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 GFP co-transfection experiments. Briefly, 2x 10^6 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 (61). 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. We will further examine this issue by double staining cells transiently co-transfected with the THBS plasmids and β-galactosidase and pulse labeled with bromodeoxyuridine using red-Gal to detect transfected cells and anti-BrdU immunohistochemical staining to detect proliferating cells.

To examine the alternate hypothesis that expression of F432A protein 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. 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}S] methionine and chased with cold methionine for 30, 60 and 180 min. The results of this experiment are shown in Fig. 2. Cells transfected with pCMV control vector showed minimal synthesis and secretion of TSP 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 TSP in the cell lysate after a 60 minute chase (lane 6) and secreted TSP was detected in the medium after 3 h (lane 7). Cells transfected with the F432A mutant THBS plasmid showed similar expression and secretion as the wild type transfectant (lanes 9-12), indicating that synthesis and secretion of F432A mutant TSP is normal.

W385A and W498G stable transfectants: We have transfected MDA435 cells with two other mutant constructs, W385A and W498G involving the first and third type I repeats respectively. Stable clones of W385A, which were selected for high TSP expression by ELISA, were negative for the mutant THBS mRNA by RT-PCR. These clones again might be expressing the endogenous gene like the F432A clones. In contrast, a pool of W498G transfectants in MDA435 cells were positive by RT-PCR. We are in the process of isolating stable clones expressing this mutant TSP.

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 TSP 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 (Figure 3). 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 F432A mutation into the myc-his construct, and use this plasmid to distinguish between the endogenous and exogenous gene expression.

Mapping functional domains for breast carcinoma and endothelial cell interactions with thrombospondin

Because of the multiple biological activities expressed in other domains of thrombospondin-1, use of full length thrombospondin mutants may be limited for defining the biological activities of the type I repeat sequences. Therefore, as proposed in specific aim 2, we have also begun to use truncated recombinant fragments of thrombospondin to define the activities of this domain. We have tested recombinant GST-fusion proteins expressing each of the thrombospondin domains for biological activities toward breast carcinoma and endothelial cells. In adhesion assays, endothelial and breast carcinoma cells recognized different domains of thrombospondin. The endothelial cells bound preferentially to the type 3 repeats (Fig. 4). This adhesion appears to be mediated by the RGD sequence in the last type 3 repeat, since a C-terminal fusion with this repeat also promoted endothelial cell adhesion, but a C-terminal domain fusion without this repeat was inactive. Using a specific integrin inhibitor, this adhesion was shown to be mediated by the integrin $\alpha v\beta 3$ (Fig. 5). In contrast, the MDA435 breast carcinoma cells did not recognize the RGD sequence or any other sequence in the type 3 repeats. MDA435 cells preferentially attached on a fusion protein containing the type 1 repeats (Fig. 6). A fusion protein expressing the second type I repeat that included the flanking sequence KRFK was most active. However, the adhesive activity of the type I repeat fragments was much less than that of intact thrombospondin. To further define the adhesive sequences for the MDA breast carcinoma cells, proteolytic fragments of thrombospondin were also tested (Fig. 7). A 140 kDa fragment lacking the amino terminal heparin-binding domain was as active as intact thrombospondin. The amino terminal heparin-binding domain also showed some activity in this assay, but the recombinant RGD-domain did not.

Adhesion to intact thrombospondin and to the 140 kDa domain was sensitive to inhibition by a $\beta 1$ integrin blocking antibody and stimulated by a $\beta 1$ activating antibody TS2/16 (Fig. 8). However, this same activating antibody did not enhance adhesion of the breast carcinoma cells to the recombinant RGD domain (Fig. 8) or to any of the other recombinant thrombospondin domains (data not shown). Therefore, an RGD-independent $\beta 1$ integrin is responsible for breast carcinoma cell adhesion mediated by the C-terminal 140 kDa fragment of thrombospondin. Because GST fusions spanning this entire sequence failed to induce adhesion of these cells either alone or in the presence of the $\beta 1$ integrin activating antibody, the integrin binding site on thrombospondin may involve a conformational determinant lacking in the bacterial fusions.

The bacterial fusions were also used to map 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 (figure 9). 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.

Synthetic peptides

A peptide derived from the second type I repeat of TSP, KRFKQDGGWSHWSPWSS, inhibits heparin binding to TSP (44), activates latent TGFβ (14), and inhibits proliferation of endothelial cells stimulated by FGF-2 (15). We have prepared a variety of constructs with the D-reverse, or retro-inverso peptidomimetic analogues because of their potential for *in vivo* use due to their enzymatic stability. In the previous report we demonstrated that several of these are potent inhibitors of FGF2 binding and FGF2-dependent endothelial cell proliferation. The retro-inverso analogs also inhibited MDA435 tumor growth when administered to nude mice. These studies have now been published and are included as appendix 2.

Upon further characterization of these peptides, we observed that peptides containing both Trp residues and the basic amino acid motif exist in solution primarily as aggregates. We compared activities of various aggregated forms of the peptide 529 (rv-amKRAKQAGWSHWAAac) separated by gel permeation as reported in the first annual report. In the previous report, we described methods to prepare aggregated and monomeric forms of these peptides and showed that increasing aggregation increased their ability to antagonize bFGF. In the current year, we tested monomeric and aggregated peptides for inhibition of breast carcinoma growth in the nude mouse model (Fig. 10). A monomeric form of the peptide (557L) did not significantly inhibit tumor growth when administered at 6 mg/kg I.V. daily from day 25 to day 40. In contrast, oligomeric forms of this (596) and a retro-inverso form of the native TSP1 sequence (599) significantly inhibited tumor growth when administered using the same dose schedule. Oligomerization therefore seems to be required for anti-tumor activity *in vivo.*

Induction of Apoptosis by Peptides and Thrombospondin: In our previous report, we observed that endothelial cells but not human breast carcinoma cells undergo programmed death in response to the TSP peptides. Intact thrombospondin and the thrombospondin peptides specifically induce apoptosis in endothelial cells and that this activity is independent of their ability to activate latent TGFβ. Programmed cell death was detected by inter-nucleosomal cleavage of genomic DNA in the treated cells (Appendix I), by quantifying increase in DNA fragmentation using BrdU labeling and an ELISA assay (appendix I), and by loss of phospholipid asymmetry in the treated cells (Fig. 11). Loss of phosphatidyl serine asymmetry is an early event observed in cells undergoing apoptotic cell death but not cells undergoing necrosis. Phosphatidyl serine was detected by staining cells using FITC annexin V, and necrotic cells were detected by double staining using propidium iodide. Cells were analyzed by flow cytometry (Fig. 11), and the results are summarized in Table 3. Treatment with the thrombospondin peptide from the type I repeat induced increased apoptotic cells staining with annexin V but did not increase necrosis. Herbimycin was used as a positive control to induce apoptosis in the bovine aortic endothelial cells.

Apoptosis is blocked on a fibronectin matrix. Since loss of matrix adhesion is a known inducer of apoptosis in endothelial cells (67, 68), an anti-adhesive activity was considered as a mechanism for the activity of the peptides. Pre-coating the tissue culture plastic with fibronectin did not alter the morphology of untreated cells (Figure 1, panels a, b of appendix 1) but prevented the morphology changes induced by the TSP1 peptides (Figure 1, panels d, f of appendix 1). The anti-proliferative activities of the native TSP1 sequence (KRFKQDGGWSHWSPWSSC-polysucrose, 407f) and an analog without the TGFβ-activating sequence RFK (KRAKAAGGWSHWSPWSSC-polysucrose, 450f) were also decreased by growth of the endothelial cells on a fibronectin matrix (Fig 3 of appendix 1). In contrast, the antiproliferative activities of intact TSP1 or TGFβ were not significantly decreased by attachment of the endothelial cells on fibronectin (Fig. 3 of appendix 1).

Fibronectin also inhibited appearance of the DNA ladder in endothelial cells treated with a TSP1 peptide analog from the type 1 repeat (Fig. 4 of appendix 1). DNA fragmentation induced by the peptide KRFKQDGGWSHWSPWSSC conjugate was reduced by 84% in cells attached on wells coated with 10 μg/ml fibronectin. A conjugate containing the TSP1 procollagen peptide NGVQYRNC 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 analogs when cells were attached on fibronectin (data not shown)

Purification of recombinant TSP – Recombinant TSP was purified from transfected MDA MB-435 cell line as described under 'Methods' (Fig. 12). All purified TSP preparations were found to contain a novel protein with a higher molecular weight than thrombospondin (band-X) except TH50, a C-terminus-truncated form of TSP (Fig. 12; lane 3). However, unlike other TSP preparations, TH26-TSP was found to contain high levels of band-X secreted into the culture medium (Fig. 12; lane 4). During the purification TH26-TSP, the band-X protein co-eluted with TSP from the heparin affinity chromatography column (Fig. 13; lane 2). Several methods have been 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. (69). 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. 14). The composite results from this experiment and the lectin binding properties of band-X suggest that it is a glycoprotein. Figure 15 shows the results of Western blotting studies using different TSP preparations probed with anti-TSP polyclonal antibody. Although this antibody recognized all of the recombinant TSP 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 TSP interaction. When TSP/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 TSP and band-X, and therefore the two molecules co-precipitated together. Control agarose gels without the antibody did not bind TSP or band-X.

To further examine the interaction between TSP and band-X protein, ^{125}I-labeled TSP 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 TSP binding was noticed up to a dilution of 1:8 (Fig. 16). However, further dilutions of band-X showed a dose dependent decrease in TSP interaction. These results indicate that TSP, independent of its source, binds to band-X. When the radiolabeled proteins were resolved in a gel filtration column, the TH26-TSP/band-X preparation was found to elute at the same point as platelet TSP (Fig. 17), showing the anomalous behavior of the TSP/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-TSP 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. 18; Lane 1). However, due to the difference in their subunit molecular weights, band-X and TH26-TSP appeared as two independent bands after reduction of disulfide bonds (Fig. 18; Lane 2). This result suggests that band-X protein may contain two subunits with similar molecular weights. The electrophoretic mobility of platelet TSP under reducing and non-reducing condition in agarose gel is shown in Fig. 18 lanes 3 and 4 for comparison.

Micro sequencing of band-X protein was carried out as described under 'Methods'. A peptide sequence, KGDQERLD, obtained by this method was compared against the protein and the EST databases from GenBank and against a Yeast genome database. A matching protein could not be found from any of these databases, suggesting the possibility that band-X represents a unique protein.

CONCLUSIONS

Full length thrombospondin expression constructs containing four site-directed mutations of the type I repeat sequences have been prepared. 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 I repeat resulted in reversal of the effect of TSP1 over expression on the tumorigenic potential of MDA435 cells. Suppression of tumorigenesis by expression of wild type TSP but not the W441A mutant was observed both in athymic nude mice and Beige XID mice, demonstrating that the anti-tumor activity of TSP does not require NK, B, or T cell responses by the host. Reversal of the anti-tumor activity of TSP 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 antiangiogenic activity (70). The molecular basis of this change in activity will be correlated with changes in ligand binding and biological activities of the purified recombinant protein *in vitro* and confirmed by further animal experiments *in vivo*. Expression of the F432A mutant inhibits proliferation of MDA435 cells in transient transfections. This may account for failure to isolate any stable transfectants over-expressing this mutant transgene. F432A and W385A mutants are both properly folded and secreted in MDA435 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 (65, 66). Pulse chase experiments will continue to define the stability and fate of these mutant thrombospondins in the breast carcinoma cells.

While purifying recombinant thrombospondins expressed in the MDA435 breast carcinoma cell line, we have identified a novel protein expressed in MDA435 cells that is secreted as a complex with thrombospondin. This is composed of a dimer of 230 kDa subunits which are disulfide linked and noncovalently associate with thrombospondin. The thrombospondin/band X complex is stable to high salt and varying pH. The protein can be separated from thrombospondin in SDS, but not under nondenaturing conditions or denaturing conditions using urea. TSP binding to band X was confirmed by immunoprecipitation and solid phase binding assays. In preliminary experiments, we have not detected this protein in thrombospondin purified from melanoma cells transfected with the same expression vector, suggesting that band X may be specifically secreted by breast carcinoma cells. We will continue to characterize this novel protein and the consequences of its binding to thrombospondin on the biological activities of thrombospondin secreted by the breast carcinoma cells. As proposed in aim 2, to overcome these problems in purification of mutated recombinant thrombospondins and for differentiating expression of the mutants from activation of the endogenous *THBS1* gene, we have constructed an epitope tagged thrombospondin expression plasmid and will use this construct to examine the fate of thrombospondin mutants that can not be stably expressed in breast carcinoma cells.

Truncated GST fusion proteins expressing individual TSP domains were used to further map functional domains for its interactions with endothelial and breast carcinoma cells. These data demonstrate a specific antiproliferative activity for endothelial cells in the type I repeats and demonstrate a differential role of $\beta 1$ and $\beta 3$ 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 TSP fragments by these two cell types.

We have found that in addition to inhibiting proliferation of endothelial cells, thrombospondin and the thrombospondin peptides specifically induce apoptosis of endothelial cells. This was verified by DNA fragmentation and loss of phospholipid asymmetry. Although the MDA cells are completely resistant to induction of apoptosis by the peptides, a breast carcinoma cell line with wild type p53 was sensitive. This suggests that the stable

peptide analogs may also have direct anti-tumor activity towards breast cancers lacking p53 mutations. Induction of apoptosis in endothelial cells was shown to be dependent on the extracellular matrix. Newly plated cells on plastic were the most sensitive to apoptosis, whereas cells plated on a preformed fibronectin matrix were resistant. This suggests that the observed anti-tumor activity of these peptides and their lack of systemic toxicity may result from selective sensitivity of endothelial cells on provisional extracellular matrix in tumor neovasculature top induction of apoptosis. Thus, mature vasculature is unaffected, but neovascularization of the tumor is selectively inhibited.

LITERATURE CITED

1. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma, N Engl J Med. *324:* 1-8, 1991.
2. Horek, Horak, E. R., Leek, R. K. N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., and Harris, A. L. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer, Lancet. *340:* 1120-1124, 1992.
3. Folkman, J. and Shing, Y. Angiogenesis, J. Biol. Chem. *267:* 10931-10934, 1992.
4. Folkman, J. and Klagsbrun, M. Angiogenic factors, Science. *235:*, 1987.
5. Good, D. J., Polverini, P. J., Rastinejad, F., Le, B. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin, Proc Natl Acad Sci U S A. *87:* 6624-6628, 1990.
6. Taraboletti, G., Roberts, D., Liotta, L. A., and Giavazzi, R. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor, J Cell Biol. *111:* 765-772, 1990.
7. Ezekowitz, R. A. B., Mulliken, J. B., and Folkman, J. Interferon alfa-2a therapy for life-threatening hemangiomas of infancy, N. Engl. J. Med. *326:* 1456-1463, 1992.
8. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides, Science. *247:* 77-79, 1990.
9. Funk, S. E. and Sage, H. The Ca2+-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells, Proc. Natl. Acad. USA. *88:* 2648-2652, 1991.
10. Vogel, T., Guo, N. H., Guy, R., Drezlich, N., Krutzsch, H. C., Blake, D. A., Panet, A., and Roberts, D. D. Apolipoprotein E: a potent inhibitor of endothelial and tumor cell proliferation, J Cell Biochem. *54:* 299-308, 1994.
11. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat Med. *1:* 27-31, 1995.
12. Homandberg, G. A., Kramer-Bjerke, J., Grant, D., Christianson, G., and Eisenstein, R. Heparin-binding fragments of fibronectin are potent inhibitors of endothelial cell growth: structure-function correlations, Biochim. Biophys. Acta. *874:* 61-71, 1986.
13. Vogel, T., Guo, N. H., Krutzsch, H. C., Blake, D. A., Hartman, J., Mendelovitz, S., Panet, A., and Roberts, D. D. Modulation of endothelial cell proliferation, adhesion, and motility by recombinant heparin-binding domain and synthetic peptides from the type I repeats of thrombospondin, J Cell Biochem. *53:* 74-84, 1993.
14. Frazier, W. A. Thrombospondins, Curr Opin Cell Biol. *3:* 792-799, 1991.
15. Mosher, D. F. Physiology of thrombospondin, Annu Rev Med. *41:* 85-97, 1990.
16. Wolf, F. W., Eddy, R. L., Shows, T. B., and Dixit, V. M. Structure and chromosomal

localization of the human thrombospondin gene, Genomics. *6:* 685-691, 1990.
17. Aznavoorian, S., Murphy, A. N., Stetler-Stevenson, W. G., and Liotta, L. Molecular aspects of tumor invasion and metastasis, Cancer. *71:* 1368-1383, 1993.
18. Baley, P., Lutzelschwab, I., Scott-Burden, T., Kung, W., and Eppenberger, U. Modulation of extracellular-matrix synthesized by cultured stromal cells from normal human breast tissue by epidermal growth factor, J Cell Biochem. *43:* 111-125, 1990.
19. Dawes, J., Clezardin, P., and Pratt, D. A. Thrombospondin in milk, other breast secretions, and breast tissue, Semin Thromb Hemost. *13:* 378-384, 1987.
20. Wong, S. Y., Purdie, A. T., and Han, P. Thrombospondin and other possible related matrix proteins in malignant and benign breast disease. An immunohistochemical study, Am J Pathol. *140:* 1473-1482, 1992.
21. Clezardin, P., Frappart, L., Clerget, M., Pechoux, C., and Delmas, P. D. Expression of thrombospondin (TSP1) and its receptors (CD36 and CD51) in normal, hyperplastic, and neoplastic human breast, Cancer Res. *53:* 1421-1430, 1993.
22. Zajchowski, D. A., Band, V., Trask, D. K., Kling, D., Connolly, J. L., and Sager, R. Suppression of tumor-forming ability and related traits in MCF-7 human breast cancer cells by fusion with immortal mammary epithelial cells, Proc Natl Acad Sci U S A. *87:* 2314-2318, 1990.
23. Roberts, D. D., Sherwood, J. A., and Ginsburg, V. Platelet thrombospondin mediates attachment and spreading of human melanoma cells, J Cell Biol. *104:* 131-139, 1987.
24. Taraboletti, G., Roberts, D. D., and Liotta, L. A. Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains, J Cell Biol. *105:* 2409-2415, 1987.
25. Katagiri, Y., Hayashi, Y., Baba, I., Suzuki, H., Tanoue, K., and Yamazaki, H. Characterization of platelet aggregation induced by the human melanoma cell line HMV-I: roles of heparin, plasma adhesive proteins, and tumor cell membrane proteins, Cancer Res. *51:* 1286-1293, 1991.
26. Hogg, P. J., Owensby, D. A., and Chesterman, C. N. Thrombospondin 1 is a tight-binding competitive inhibitor of neutrophil cathepsin G. Determination of the kinetic mechanism of inhibition and localization of cathepsin G binding to the thrombospondin 1 type 3 repeats, J Biol Chem. *268:* 21811-21818, 1993.
27. Hogg, P. J., Owensby, D. A., Mosher, D. F., Misenheimer, T. M., and Chesterman, C. N. Thrombospondin is a tight-binding competitive inhibitor of neutrophil elastase, J Biol Chem. *268:* 7139-7146, 1993.
28. Iruela Arispe, M., Bornstein, P., and Sage, H. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro, Proc Natl Acad Sci U S A. *88:* 5026-5030, 1991.
29. Wight, T. N., Raugi, G. J., Mumby, S. M., and Bornstein, P. Light microscopic immunolocation of thrombospondin in human tissues, J Histochem Cytochem. *33:* 295-302, 1985.

30. Munjal, I. D., Crawford, D. R., Blake, D. A., Sabet, M. D., and Gordon, S. R. Thrombospondin: biosynthesis, distribution, and changes associated with wound repair in corneal endothelium [published erratum appears in Eur J Cell Biol 1991 Aug;55(2):IV], Eur J Cell Biol. *52:* 252-263, 1990.
31. Lahav, J. Thrombospondin inhibits adhesion of endothelial cells, Exp Cell Res. *177:* 199-204, 1988.
32. Murphy-Ullrich, J. E. and Höök, M. Thrombospondin modulates focal adhesions in endothelial cells, J Cell Biol. *109:* 1309-1319, 1989.
33. Murphy-Ullrich, J. E., Schultz-Cherry, S., and Höök, M. Transforming growth factor-beta complexes with thrombospondin, Mol Biol Cell. *3:* 181-188, 1992.
34. Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Polverini, P. J., and Bouck, N. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity, J Cell Biol. *122:* 497-511, 1993.
35. Roberts, D. D. Interactions of thrombospondin with sulfated glycolipids and proteoglycans of human melanoma cells, Cancer Res. *48:* 6785-6793, 1988.
36. Yabkowitz, R. and Dixit, V. M. Human carcinoma cells bind thrombospondin through a Mr 80,000/105,000 receptor, Cancer Res. *51:* 3648-3656, 1991.
37. Yabkowitz, R., Dixit, V. M., Guo, N., Roberts, D. D., and Shimizu, Y. Activated T-cell adhesion to thrombospondin is mediated by the α4 ß 1 (VLA-4) and α5 ß 1 (VLA-5) integrins, J Immunol. *151:* 149-158, 1993.
38. Asch, A. S., Tepler, J., Silbiger, S., and Nachman, R. L. Cellular attachment to thrombospondin. Cooperative interactions between receptor systems, J Biol Chem. *266:* 1740-1745, 1991.
39. Kaesberg, P. R., Ershler, W. B., Esko, J. D., and Mosher, D. F. Chinese hamster ovary cell adhesion to human platelet thrombospondin is dependent on cell surface heparan sulfate proteoglycan, J Clin Invest. *83:* 994-1001, 1989.
40. Cardin, A. D. and Weintraub, H. J. R. Molecular modeling of protein-glycosaminoglycan interactions, Arteriosclerosis. *9:* 21-32, 1989.
41. Jackson, R. L., Busch, S. J., and Cardin, A. D. Glycosaminoglycans: molecular properties, protein interactions and role in physiological processes, Physiol. Rev. *71:* 481-539, 1991.
42. Prater, C. A., Plotkin, J., Jaye, D., and Frazier, W. A. The properdin-like type I repeats of human thrombospondin contain a cell attachment site, J Cell Biol. *112:* 1031-1040, 1991.
43. Guo, N. H., Krutzsch, H. C., Nègre, E., Zabrenetzky, V. S., and Roberts, D. D. Heparin-binding peptides from the type I repeats of thrombospondin. Structural requirements for heparin binding and promotion of melanoma cell adhesion and chemotaxis, J Biol Chem. *267:* 19349-19355, 1992.
44. Guo, N. H., Krutzsch, H. C., Nègre, E., Vogel, T., Blake, D. A., and Roberts, D. D. Heparin- and sulfatide-binding peptides from the type I repeats of human

thrombospondin promote melanoma cell adhesion, Proc Natl Acad Sci U S A. *89:* 3040-3044, 1992.

45. Asch, A. S., Silbiger, S., Heimer, E., and Nachman, R. L. Thrombospondin sequence motif (CSVTCG) is responsible for CD36 binding, Biochem Biophys Res Commun. *182:* 1208-1217, 1992.
46. Zabrenetzky, V. S., Harris, C. C., Steeg, P. S., and Roberts, D. D. Reduced thrombospondin expression correlates with tumor cell malignant progression, Proc Am Assoc Cacner Res. *33:* A404, 1992.
47. Weinstat-Saslow, D. L., Steeg, P. S., Roberts, D. D., and Zabrenetzky, V. S. Thrombospondin expression correlates inversely with tumor metastatic potential, Proc Am Assoc Cancer Res. *34:* A394, 1993.
48. Browning, P. J., Roberts, D. D., Zabrenetzky, V., Bryant, J., Kaplan, M., Washington, R. H., Panet, A., Gallo, R. C., and Vogel, T. Apolipoprotein E (ApoE), a novel heparin-binding protein inhibits the development of Kaposi's sarcoma-like lesions in BALB/c nu/nu mice, J Exp Med. *180:* 1949-1954, 1994.
49. Braatz, J. A., Yasuda, Y., Olden, K., Yamada, K. M., and Heifetz, A. H. Functional peptide-polyurethane conjugates with extended circulatory half-lives, Bioconjug Chem. *4:* 262-267, 1993.
50. Komazawa, H., Saiki, I., Nishikawa, N., Yoneda, J., Yoo, Y. C., Kojima, M., Ono, M., Itoh, I., Nishi, N., Tokura, S., and Azuma, I. Inhibition of tumor metastasis by Arg-Gly-Asp-Ser (RGDS) peptide conjugated with sulfated chitin derivative, SCM-chitin-RGDS, Clin Exp Metastasis. *11:* 482-491, 1993.
51. Komazawa, H., Saiki, I., Igarasi, Y., Azuma, I., Tokura, S., Kojima, M., Orikaza, A., and Itoh, I. The conjugation of RGDS peptide with CM-chitin augments the peptide-mediated inhibition of tumor metastasis, Carbohydr Polymers. *21:* 299-307, 1993.
52. Chorev, M. and Goodman, M. A dozen years of retro-inverso peptidomimetics, Acc Chem Res. *26:* 266-273, 1993.
53. Nomizu, M., Utani, A., Shiraishi, N., Kibbey, M. C., Yamada, Y., and Roller, P. P. The all-D-configuration segment containing the IKVAV sequence of laminin A chain has similar activities to the all-L-peptide in vitro and in vivo, J Biol Chem. *267:* 14118-14121, 1992.
54. Brunswick, M., Finkelman, F. D., Highet, P. F., Inman, J. K., Dintzis, H. M., and Mond, J. J. Picogram quantities of anti-Ig antibodies coupled to dextran induce B cell proliferation, J Immunol. *140:* 3364-3372, 1988.
55. Neufeld, G. and Gospodarowicz, D. The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells, J. Biol. Chem. *260:* 13860-13868, 1985.
56. Viville, S. Methods in Molecular Biol. *31:* 57-66, 1994.
57. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4., Nature. *227:* 680-685, 1970.

58. Krutzsch, H. C. and Inman, J. K. N-Isopropyliodoacetamide in the reduction and alkylation of proteins: Use in microsequence analysis., Anal. Biochem. *209:* 109-116, 1993.
59. Guo, N., Krutzsch, H. C., Inman, J. K., and Roberts, D. D. Anti-proliferative and anti-tumor activities of D-reverse peptide mimetics derived from the second type-1 repeat of thrombospondin-1., J. Peptide Res. *49:* in press, 1997.
60. Guo, N., Krutzsch, H. C., Inman, J. K., and Roberts, D. D. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells, Cancer Res. *57:* 1735-1742, 1997.
61. Templeton, N. S., Roberts, D. D., and Safer, B. Efficient gene targeting in mouse embryonic stem cells., Gene Therapy. *4:* 700-709, 1997.
62. Weinstat-Saslow, D. L., Zabrenetzky, V. S., VanHoutte, K., Frazier, W. A., Roberts, D. D., and Steeg, P. S. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis, Cancer Res. *54:* 6504-6511, 1994.
63. Pierson, B. A., Gupta, K., Hu, W.-S., and Miller, J. S. Human natural killer cell expansion is regulated by thrombospondin-mediated activation of transforming growth factor beta-1 and independent accessory cell-derived contact and soluble factors, Blood. *87:* 180-189, 1996.
64. Bornstein, P. Thrombospondins: structure and regulation of expression, FASEB J. *6:* 3290-3299, 1992.
65. Yoshimura, A., Zimmers, T., Neumann, D., Longmore, G., Yoshimura, Y., and Lodish, H. F. Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor., J. Biol. Chem. *267:* 11619- 11625, 1992.
66. Hilton, D. J., Watowich, S. S., Katz, L., and Lodish, H. F. Saturation mutagenesis of the WSXWS motif of the erythropoietin receptor, J Biol Chem. *271:* 4699-4708, 1996.
67. Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. The extracellular matrix as a cell survival factor, Mol Biol Cell. *4:* 953-961, 1993.
68. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells, J Cell Biol. *127:* 537-546, 1994.
69. Alexander, R. J. and Detwiler, T. C. Quantitative adsorption of platelet glycoprotein G (thrombin-sensitive protein, thrombospondin) to barium citrate, Biochem J. *217:* 67-71, 1984.
70. Dawson, D. W., Pearce, S. F., Zhong, R., Silverstein, R. L., Frazier, W. A., and Bouck, N. P. CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells, J Cell Biol. *138:* 707-717, 1997.

Table 1. List of Thrombospondin Mutants

Mutant	Comments
THBS W385A	Type I repeat 1 mutation of central Trp required for heparin binding to synthetic peptides
THBS W441A	Type I repeat 2 mutation of essential central Trp residue
THBS W498G	Type I repeat 3 mutation of essential central Trp residue
THBS F432A	TGF beta activation sequence mutant

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.

Time	Wild Type			F432A Mutant		
	Total	GFP +ve	Percent	Total	GFP +ve	Percent
24 hr	93	12	12.9	43	3	6.9
	138	21	15.2	54	3	5.5
	47	7	14.8	95	6	6.3
		Average	14.3		Average	6.2
	Total	GFP +ve	Percent	Total	GFP +ve	Percent
48 hr	135	12	8.8	40	1	2.5
	126	13	10.3	65	0	0
	39	4	10.2	109	7	6.4
		Average	9.8		Average	2.9

Table 3. Differential detection of apoptotic and necrotic cell death by flow cytometry. Bovine aortic endothelial cells were treated with 2 μM of the thrombospondin peptide KRAKAAGGWSHWSPWSSC-polysucrose or 1 μM herbimycin for 24 h. Appearance of phosphatidylserine in the outer leaflet of the plasma membrane of bovine aortic endothelial cells was detected by flow cytometry using FITC-annexin V. Necrotic cells were detected by simultaneous staining with propidium iodide.

BAE cells	Apoptotic cells	Necrotic cells
control	3.6 %	16.0 %
+TSP peptide	21.3 %	16.5 %
+herbimycin	54.9 %	13.5 %

FIGURE LEGENDS

Figure 1: 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 thrombospondin (▽), clone AE9 expressing W441A thrombospondin (○), or clone EA3 expressing W441A thrombospondin (▲). Results are mean ± SD, n = 10.

Figure 2. Synthesis and secretion of TSP mutants: MDA435 cells grown in 10 % fetal bovine serum were electroporated with 10 μg 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 μCi 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- TSP antibody as described in the 'Methods' section.

Figure 3. Immunoprecipitation of myc-his tagged TSP: 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 [^{35}S] methionine for 3 hr. The spent medium (lane 1) and cell lysate (lane 2) were immunoprecipitated with anti-TSP antibody as described in the 'Methods' section.

Figure 4: Adhesion of bovine aortic endothelial cells to recombinant thrombospondin fusion proteins. GST fusion proteins expressing the Procollagen, (amino acid residues 278 - 355); Type I repeat, 385 -522; Type II repeat, 559 - 669; Type III repeat, 784 - 932; COOH-terminal domain plus the last type III repeat containing the RGD sequence, 877 - 1152; or the COOH-terminal domain alone, 933 -1152, were adsorbed on polystyrene dishes. Unbound proteins were aspirated and the dishes were incubated in DPBS containing 1% BSA for 30 min. BAE cells were harvested by replacing their medium with PBS containing 2.5 mM EDTA and incubating for 10 min at 37°. Cells were suspended in serum-free medium containing 1 mg/ml BSA and added to the plates at a density of 200 cells/mm^2. After incubating for 50 min at 37°, the plates were washed and attached cells were fixed with 1% glutaraldehyde in PBS and stained with Diffquick. Adhesion is presented as the mean ± SD of triplicate determinations.

Figure 5: Inhibition of endothelial cell adhesion by integrin antagonists. BAE cell adhesion to thrombospondin (TSP1), the 140 kDa fragment of thrombospondin (TSP-140), recombinant type III repeats of thrombospondin (TSP-RGD), or vitronectin (VN)

was determined in the presence of the indicated concentrations of integrin αvβ3 or αIIbβ3 antagonists.

Figure 6. Adhesion of MDA435 breast carcinoma cells to recombinant thrombospondin fusion proteins. GST fusion proteins expressing the Procollagen, (amino acid residues 278 - 355); Type I repeat, 385 -522; the second type I repeat without the TGFβ activating sequence RFK (1(2)); the second type I repeat with RFK (1(2')); Type II repeat, 559 - 669; Type III repeat, 784 - 932; COOH-terminal domain plus the last type III repeat containing the RGD sequence, 877 - 1152; or the COOH-terminal domain alone, 933 - 1152, were adsorbed on polystyrene dishes. Unbound proteins were aspirated and the dishes were incubated in DPBS containing 1% BSA for 30 min. MDA435 cells were harvested by replacing their medium with PBS containing 2.5 mM EDTA and incubating for 10 min at 37°. Cells were suspended in serum-free medium containing 1 mg/ml BSA and added to the plates at a density of 200 cells/mm^2. After incubating for 50 min at 37°, the plates were washed and attached cells were fixed with 1% glutaraldehyde in PBS and stained with Diffquick. Adhesion is presented as the mean ± SD of triplicate determinations.

Figure 7: Adhesion of breast carcinoma cells to 140 kDa fragment of thrombospondin. Platelet TSP1 or the 140 kDa fragment of TSP1 cells were adsorbed on polystyrene dishes. Unbound proteins were aspirated and the dishes were incubated in DPBS containing 1% BSA for 30 min. MDA435 cells were harvested by replacing their medium with PBS containing 2.5 mM EDTA and incubating for 5 min at 37°. Cells were suspended in serum-free medium containing 1 mg/ml BSA (black and white bars) or with activating antibody TS2/16 (striped and gray bars) and added to the plates at a density of 200 cells/mm^2. After incubating for 55 min at 37°, the plates were washed and attached cells were fixed and stained with Diffquick. Adhesion is presented as the mean ± SD of triplicate determinations.

Figure 8. Effect of β1 integrin activating antibody on adhesion of breast carcinoma cells on recombinant thrombospondin fragments. Fibronectin (FN), type I collagen (Coll I), recombinant T7-thrombospondin RGD domain fusion protein (TSP-RGD), platelet thrombospondin (TSP1) or recombinant thrombospondin heparin-binding domain (TSP-N) were cells were adsorbed on polystyrene dishes. Unbound proteins were aspirated and the dishes were incubated in DPBS containing 1% BSA for 30 min. MDA435 cells were suspended in serum-free medium containing 1 mg/ml BSA (solid bars) or with 20 μg/ml of the β1-activating antibody TS2/16 (gray bars) and added to the plates at a density of 200 cells/mm^2. After incubating for 50 min at 37°, the plates were washed and attached cells were fixed and stained. Adhesion is presented as the mean ± SD of triplicate determinations.

Figure 9: Proliferation Assay using bovine aortic endothelial cells with recombinant fragments of different domains of TSP. The amounts indicated are µg/ml. Values are averages of triplicate wells. The amino acid residues of TSP 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 10. Inhibition of breast carcinoma growth in mouse xenografts. MDA435 breast carcinoma cells were implanted in the mammary fat pads of female nu/nu athymic mice on day 0. Beginning on day 25 and continuing to day 40, the animals were administered daily I.V. injections of Hanks buffered salt solution (HBSS, ●) or HBSS with monomeric retro-inverso TSP peptide mimetic (557L, ○), a mixture of oligomers (▲), or oligomeric retro-inverso analog of the native thrombospondin sequence (❑).

Figure 11. Thrombospondin Type 1 repeat peptide induces loss of phospholipid asymmetry in the endothelial cell plasma membrane. Loss of phosphatidylserine asymmetry is an early marker of apoptosis. Appearance of phosphatidylserine in the outer leaflet of the plasma membrane of bovine aortic endothelial cells was detected by flow cytometry using FITC-annexin V. Cells were incubated in medium containing 3% FCS for 24 h with effectors, washed with PBS, and harvested by brief trypsinization (3 min). Cells were collected in medium with 10% serum, centrifuged, washed with PBS, and resuspended at 10^6 cells/ml in binding buffer (R&D Systems). Cells were incubated with FITC annexin V and propidium iodide and analyzed by flow cytometry. 5000 events were analyzed for each sample. Left panel: negative control, center panel: 2 µM KRAKAAGGWSHWSPWSSC-polysucrose, right panel: 1 µM herbimycin.

Figure 12: Electrophoretic analysis of TSP 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 TSP (lane 2), TH50 mutant TSP (lane 3) and TH26 wild type TSP (lane 4). The gel was stained using Coomassie blue. The electrophoretic pattern of platelet thrombospondin is shown in lane 5.

Figure 13: Purification of TH26-TSP 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 TSP band and the high molecular weight protein band-X (Lane 2). A pre-elution step with 0.35 M NaCl did not elute TSP or band-X (Lane 3). Platelet TSP run in the same gel is also shown (Lane 4).

Figure 14: 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).

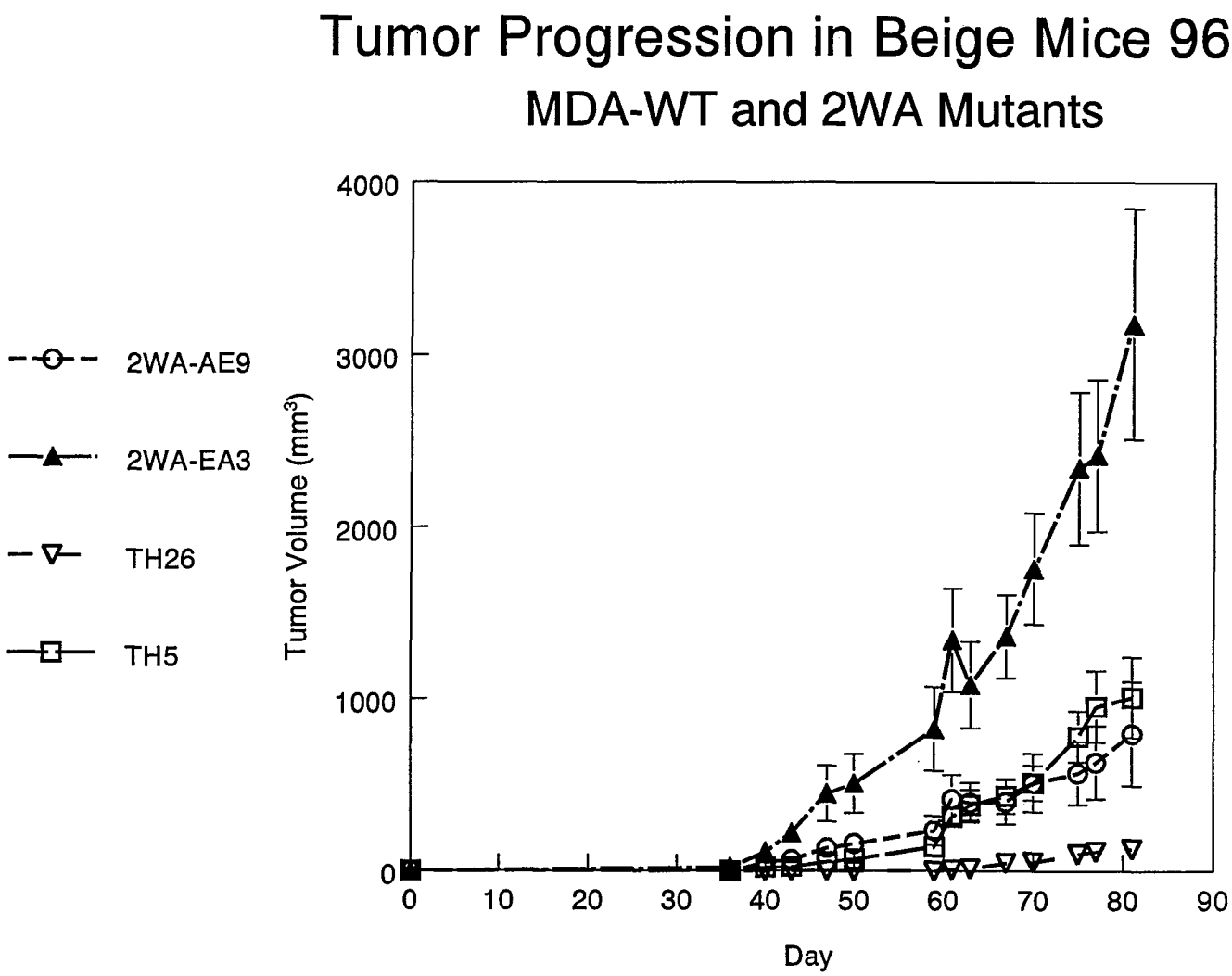
Figure 15: Western blotting analysis of thrombospondin. Heparin affinity chromatography purified thrombospondin from untransfected MDA (lane 1), TH26 wild type thrombospondin-transfected MDA (lane 2), 2WA- mutant-transfected MDA (lane 3), and thrombospondin purified from human platelets (lane 4) were 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 16: 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 17: Gel filtration profile of TSP/band-X complex compared to platelet TSP. ^{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 18: Agarose gel electrophoresis. Agarose gel (3%) was used to resolve TSP and TSP/band-X complex. TSP 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 TSP under reducing and non-reducing conditions respectively.

Figure 1


Tumor Progression in Beige Mice 96-02
MDA-WT and 2WA Mutants
2WA-AE9
2WA-EA3
TH26
TH5
Tumor Volume (mm3)
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
Day

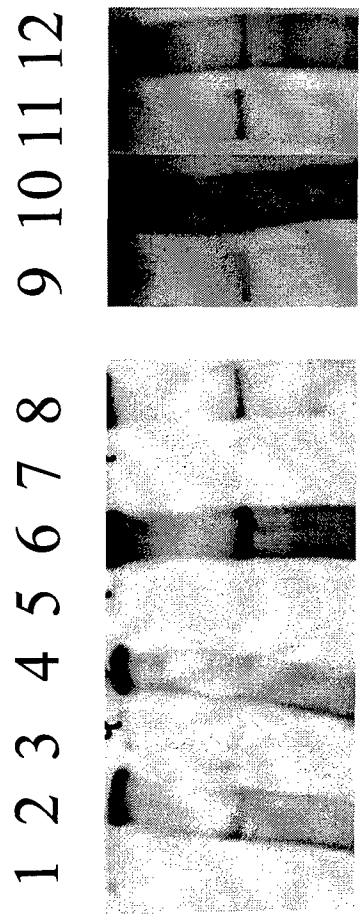


Figure 2

Figure 3

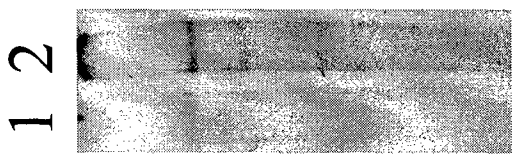


Figure 4

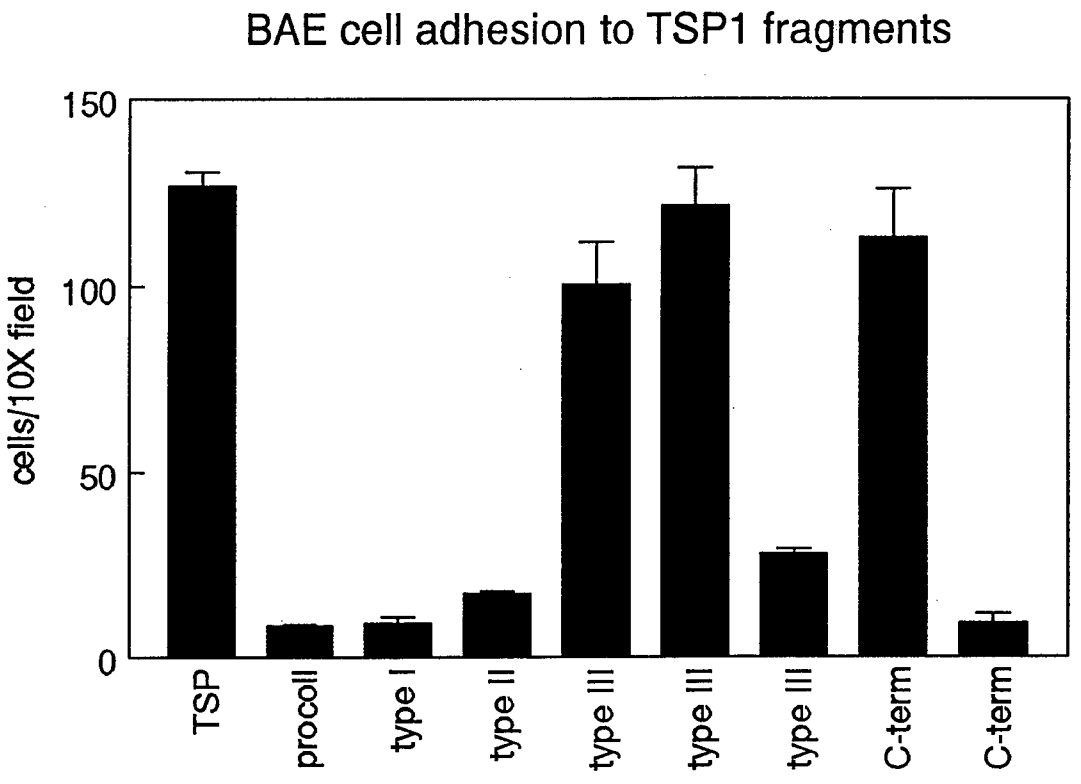

BAE cell adhesion to TSP1 fragments
cells/10X field
150
100
50
0
TSP
procoll
type I
type II
type III
type III
type III
C-term
C-term

Figure 5

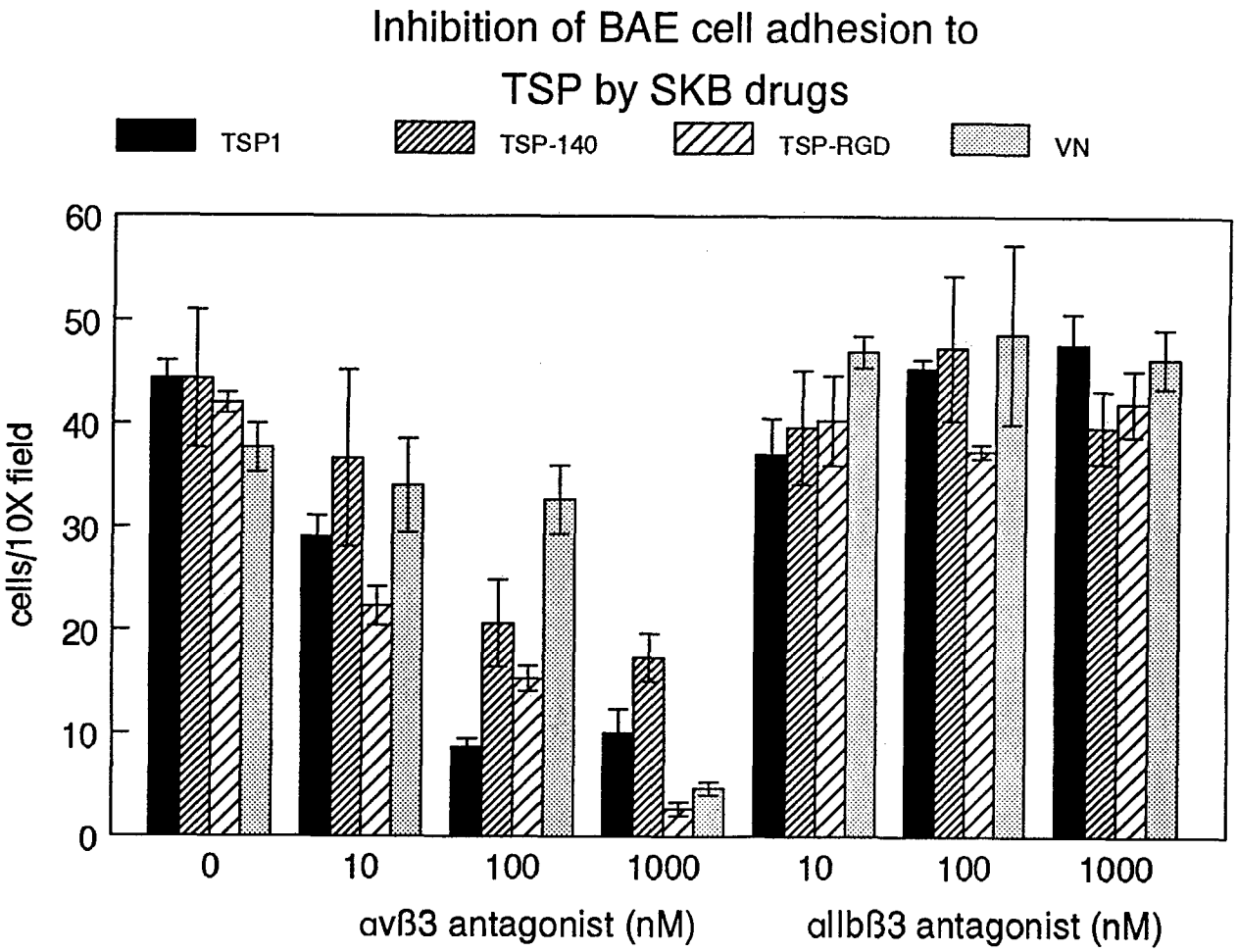

Inhibition of BAE cell adhesion to
TSP by SKB drugs
TSP1
TSP-140
TSP-RGD
VN
cells/10X field
60
50
40
30
20
10
0
0
10
100
1000
10
100
1000
αvβ3 antagonist (nM)
αIIbβ3 antagonist (nM)

Figure 6

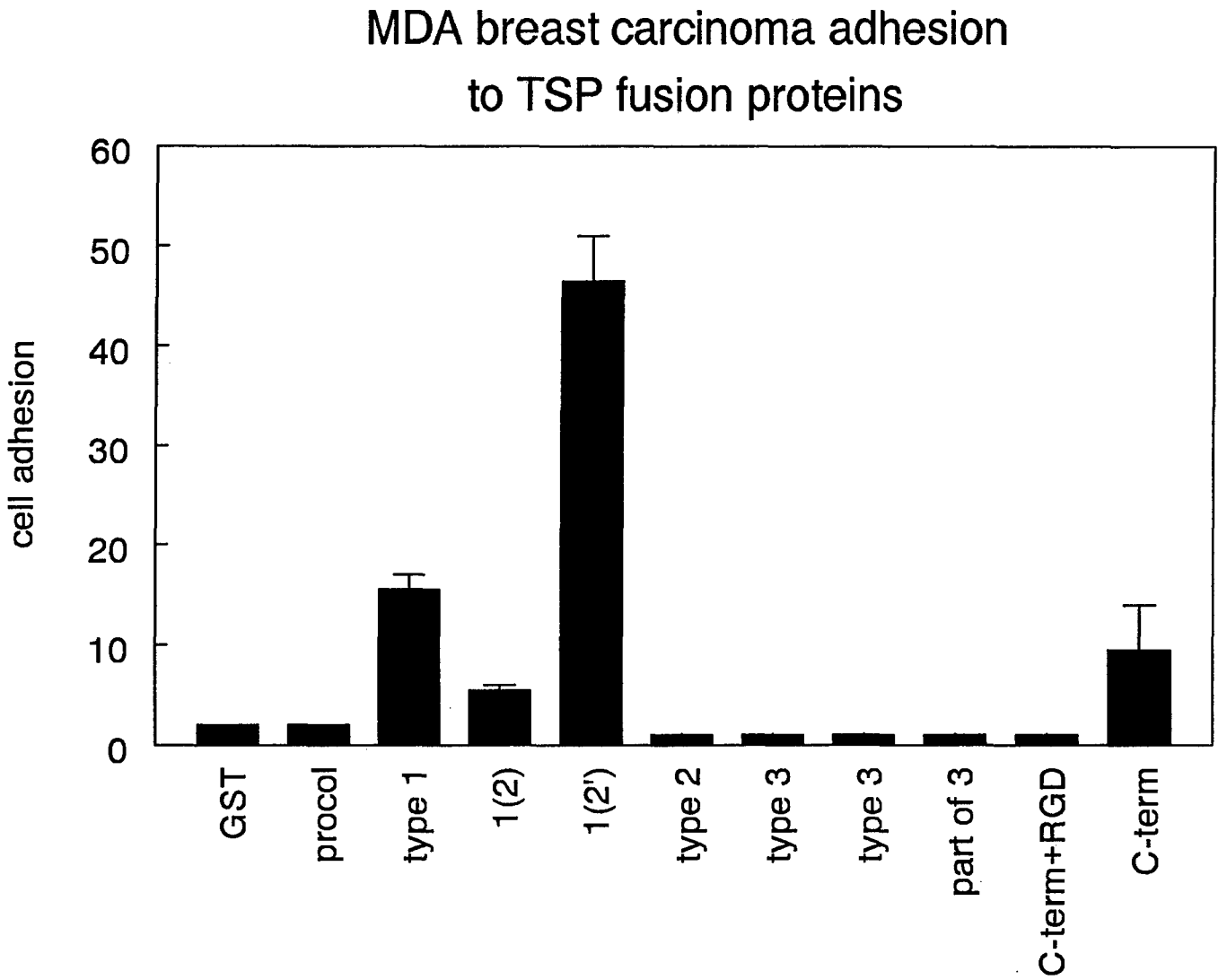

MDA breast carcinoma adhesion
to TSP fusion proteins
cell adhesion
60
50
40
30
20
10
0
GST
procol
type 1
1(2)
1(2')
type 2
type 3
type 3
part of 3
C-term+RGD
C-term

Figure 7

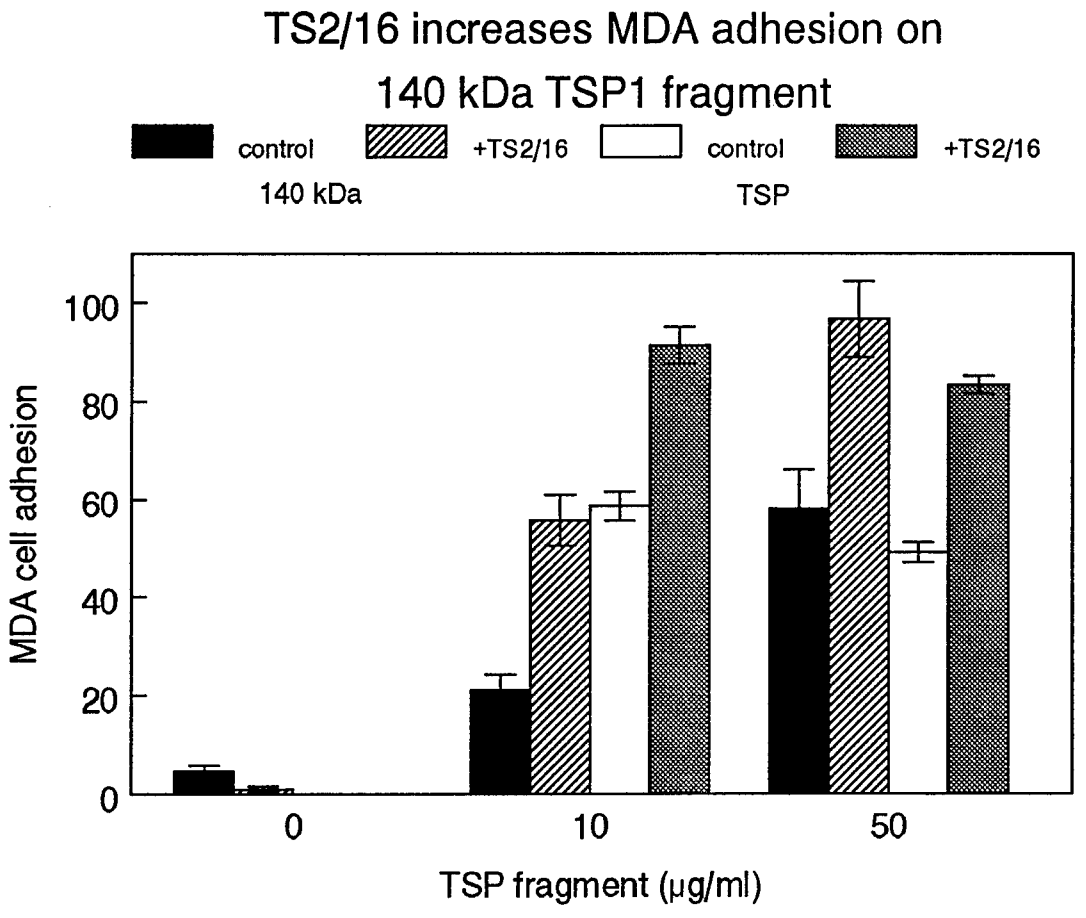

TS2/16 increases MDA adhesion on
140 kDa TSP1 fragment
control
+TS2/16
control
+TS2/16
140 kDa
TSP
MDA cell adhesion
100
80
60
40
20
0
0
10
50
TSP fragment (µg/ml)

Figure 8

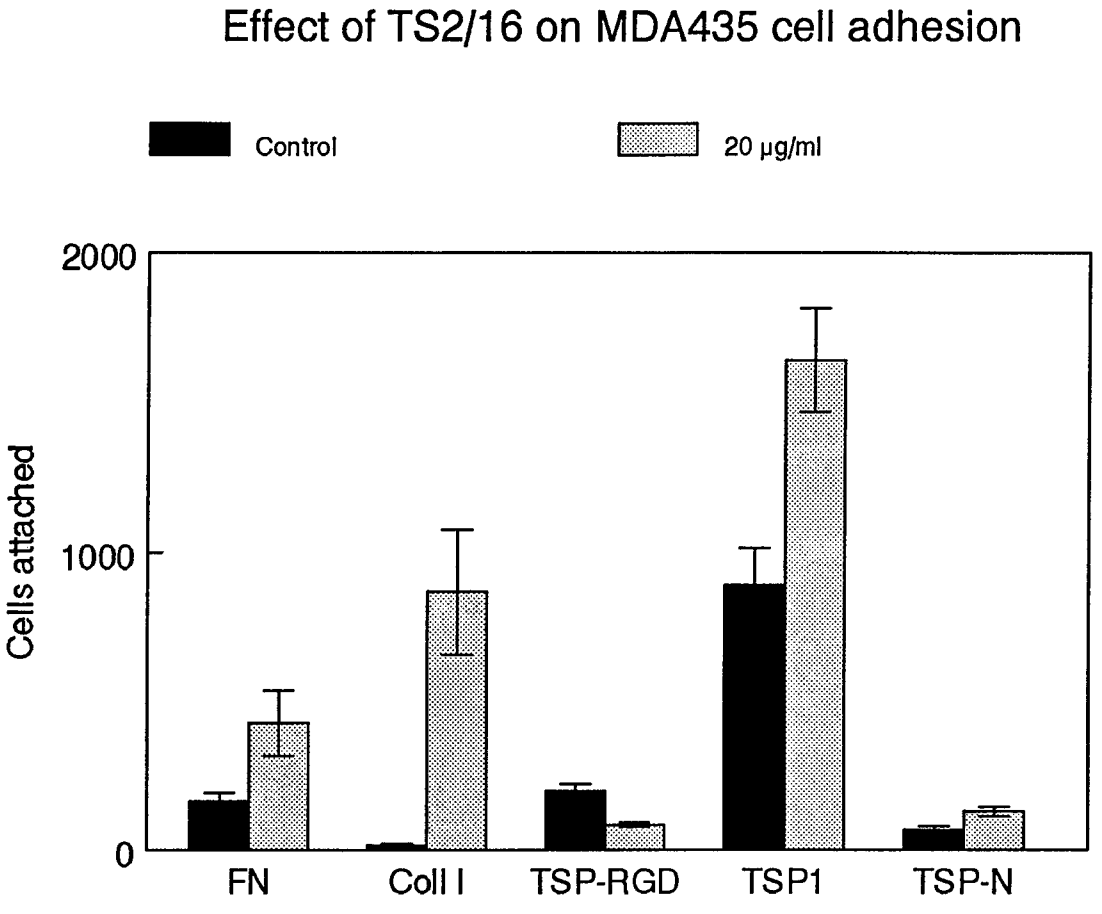

Effect of TS2/16 on MDA435 cell adhesion
Control
20 µg/ml
Cells attached
2000
1000
0
FN
Coll I
TSP-RGD
TSP1
TSP-N

Figure 9

Proliferation of BAE Cells in the presence of recombinant TSP proteins

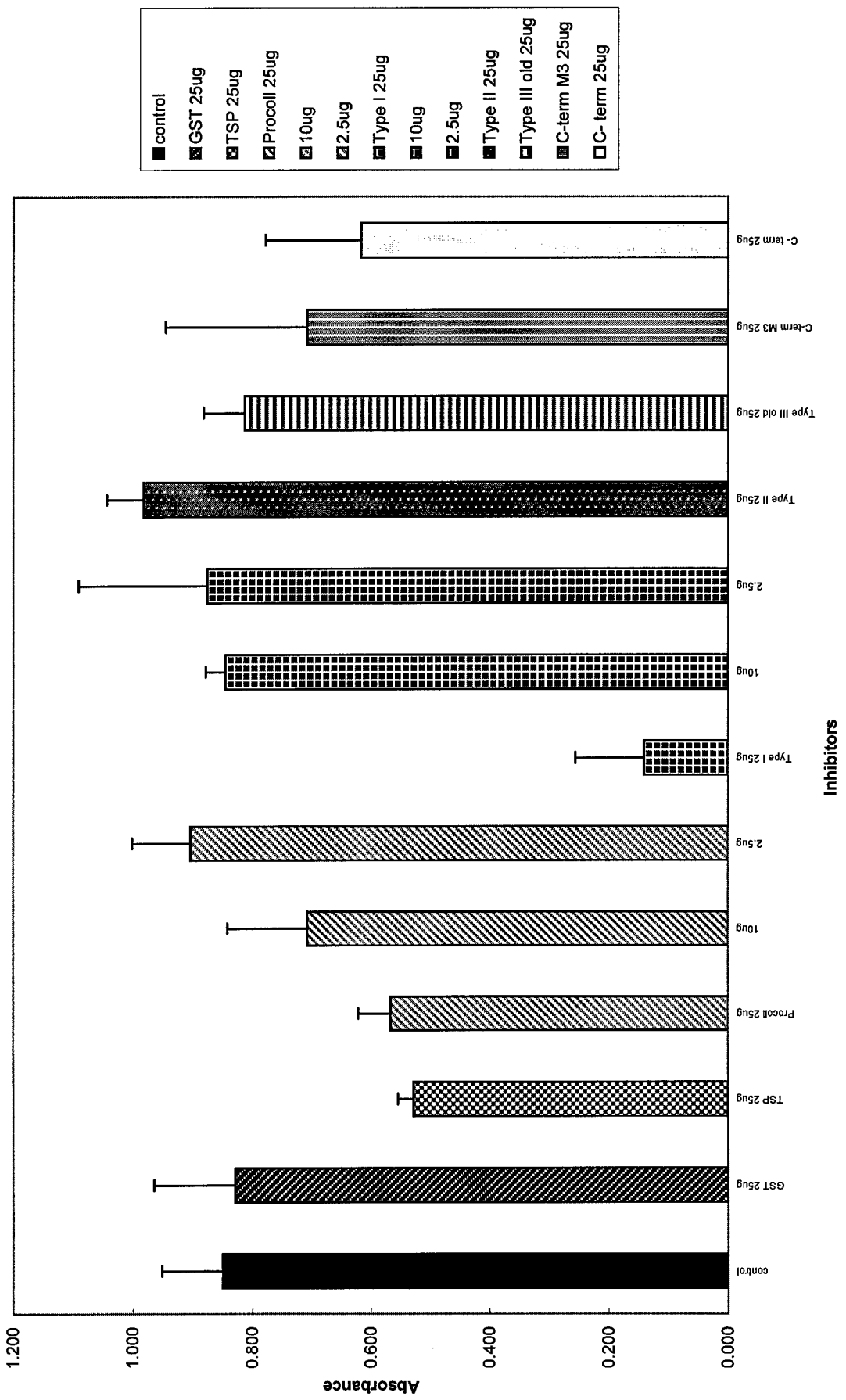
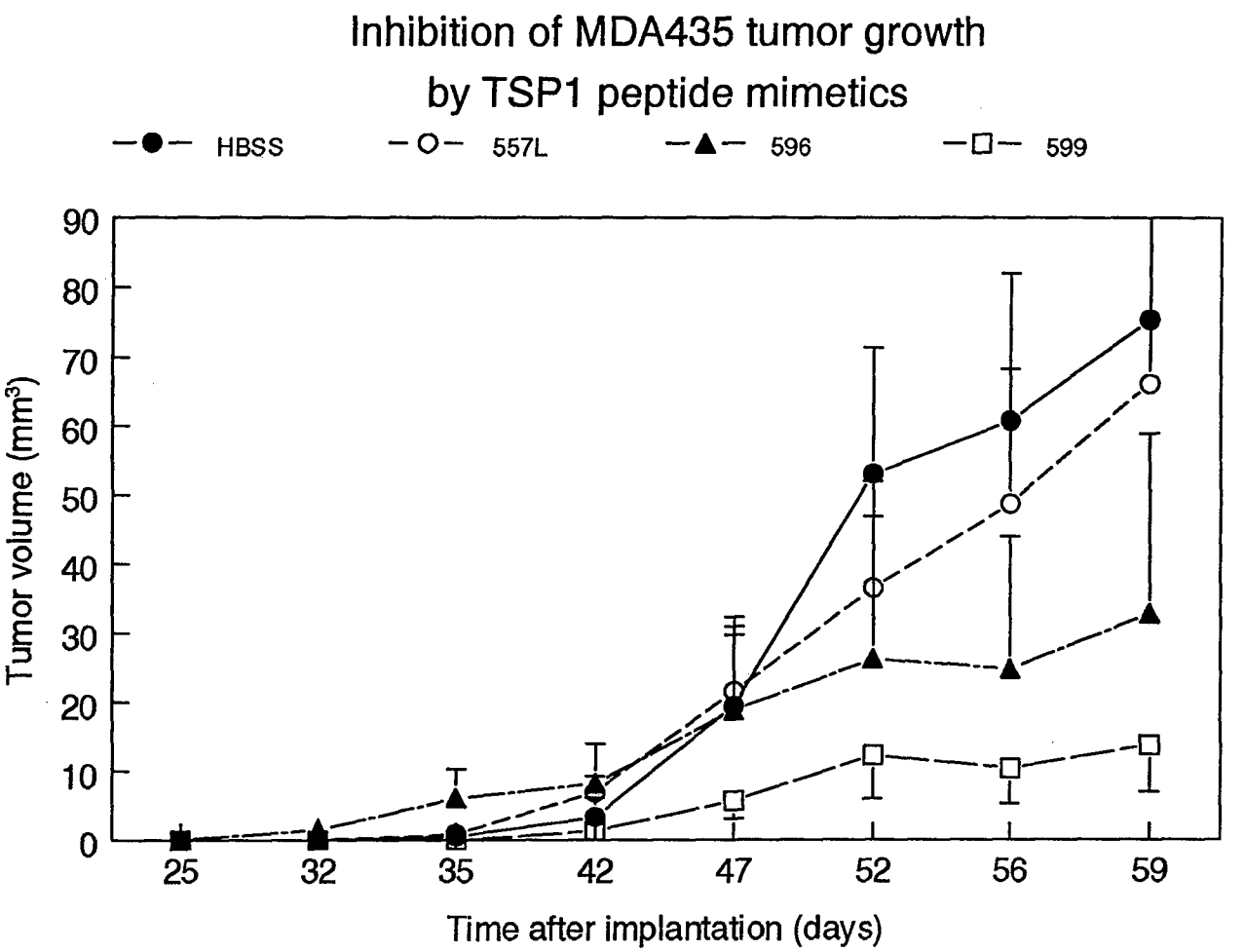


Figure 10


Inhibition of MDA435 tumor growth
by TSP1 peptide mimetics
HBSS
557L
596
599
Tumor volume (mm³)
0
10
20
30
40
50
60
70
80
90
25
32
35
42
47
52
56
59
Time after implantation (days)

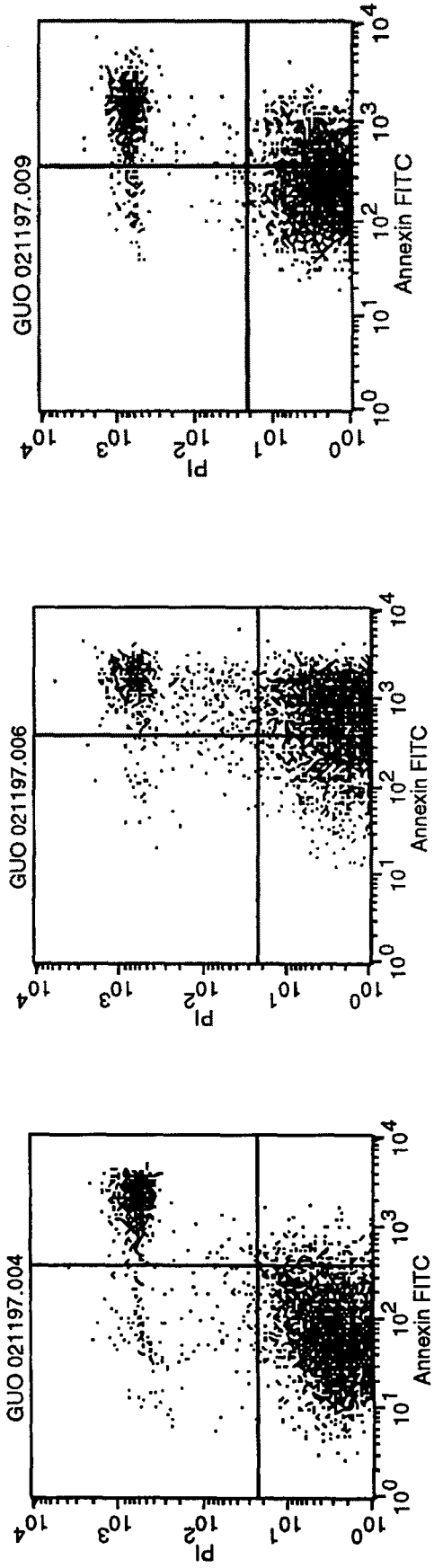

GUO 021197.009
GUO 021197.006
GUO 021197.004
Annexin FITC
PI
10^0
10^1
10^2
10^3
10^4

Figure 11

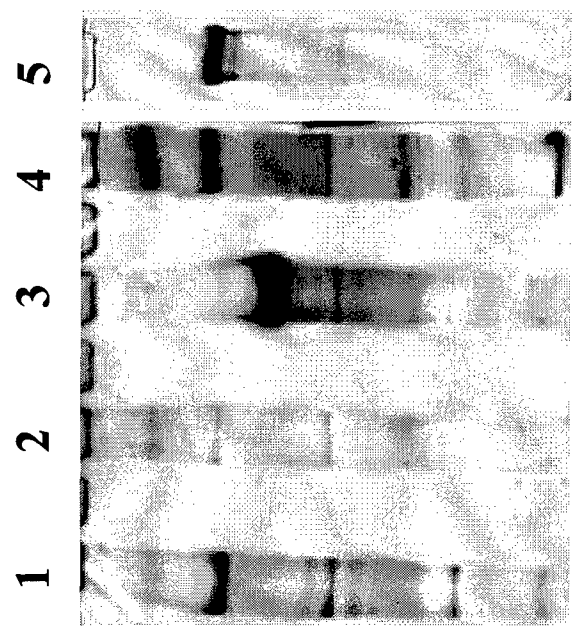
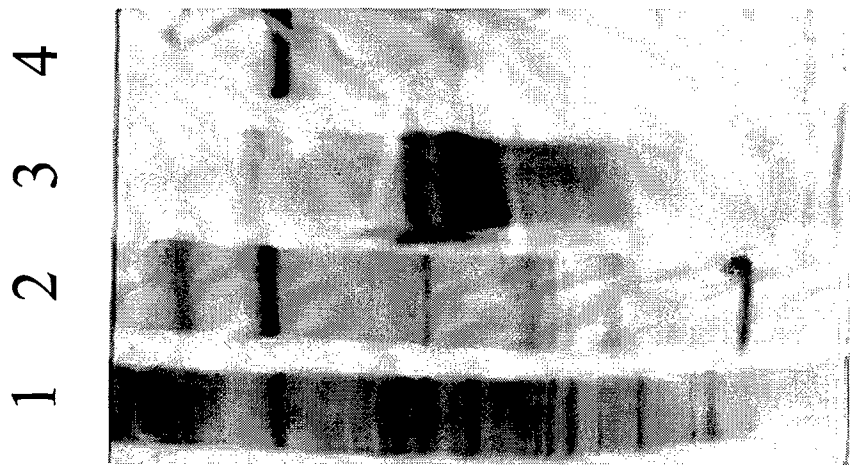


Figure 12

Figure 13



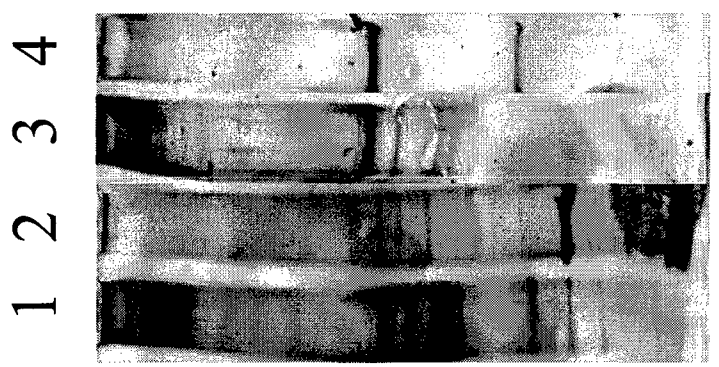
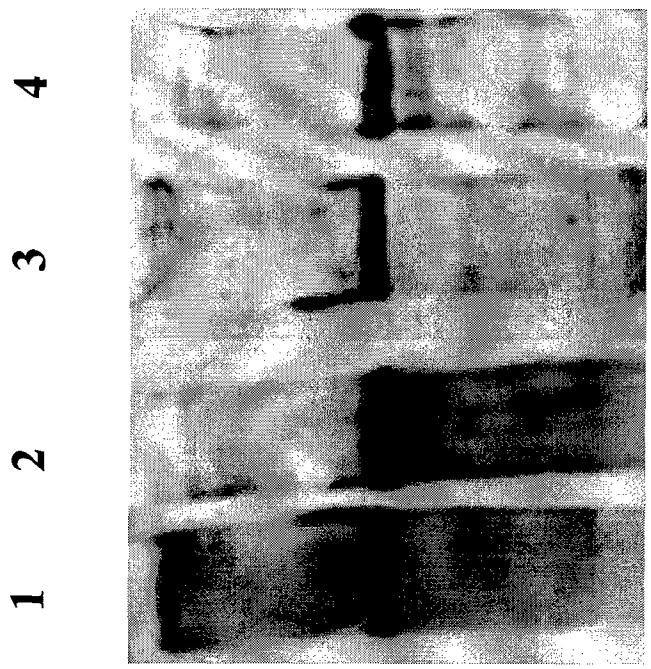


Figure 14

Figure 15



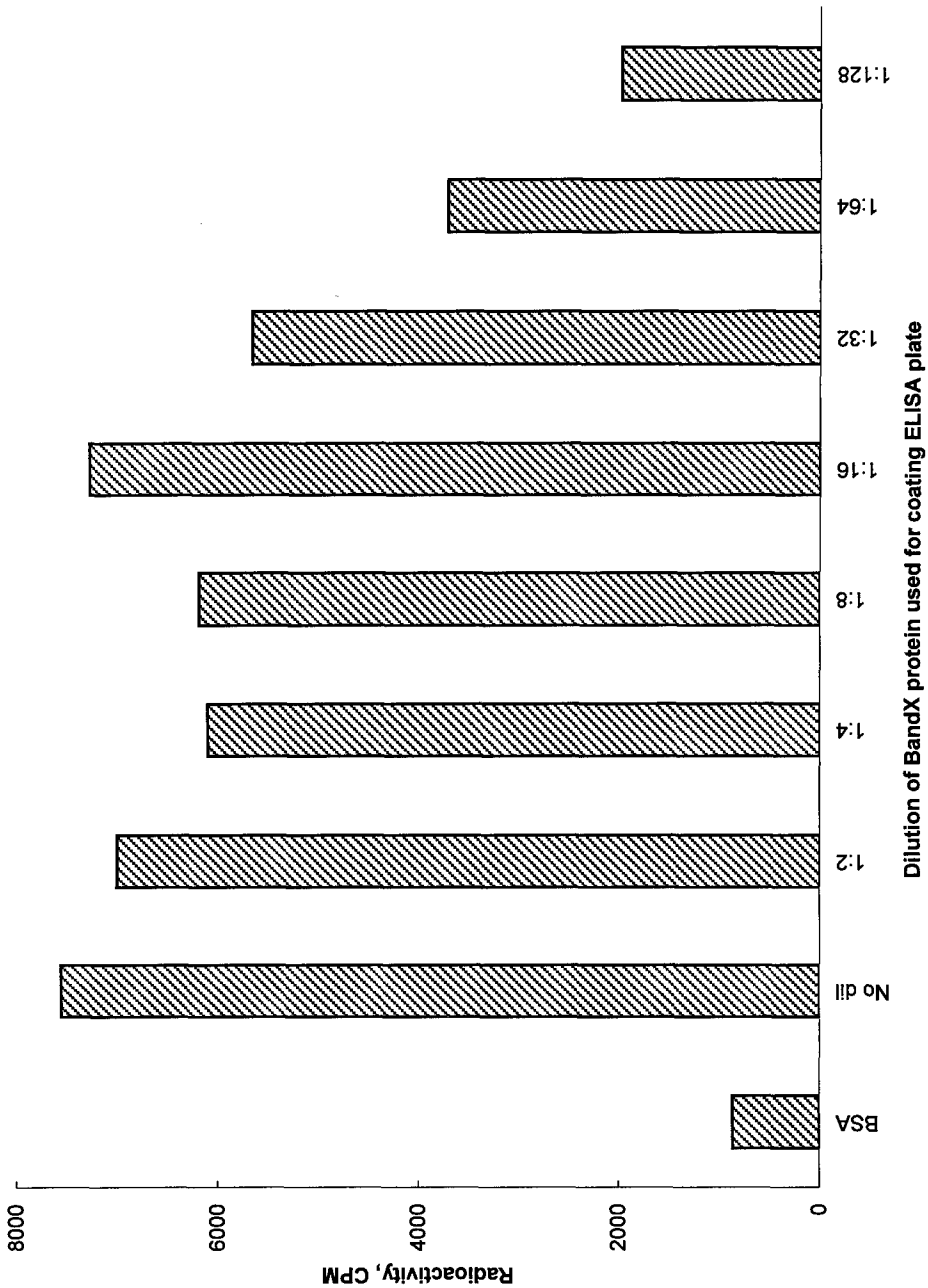

Radioactivity, CPM
0
2000
4000
6000
8000
BSA
No dil
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Dilution of BandX protein used for coating ELISA plate

Figure 16

Figure 17

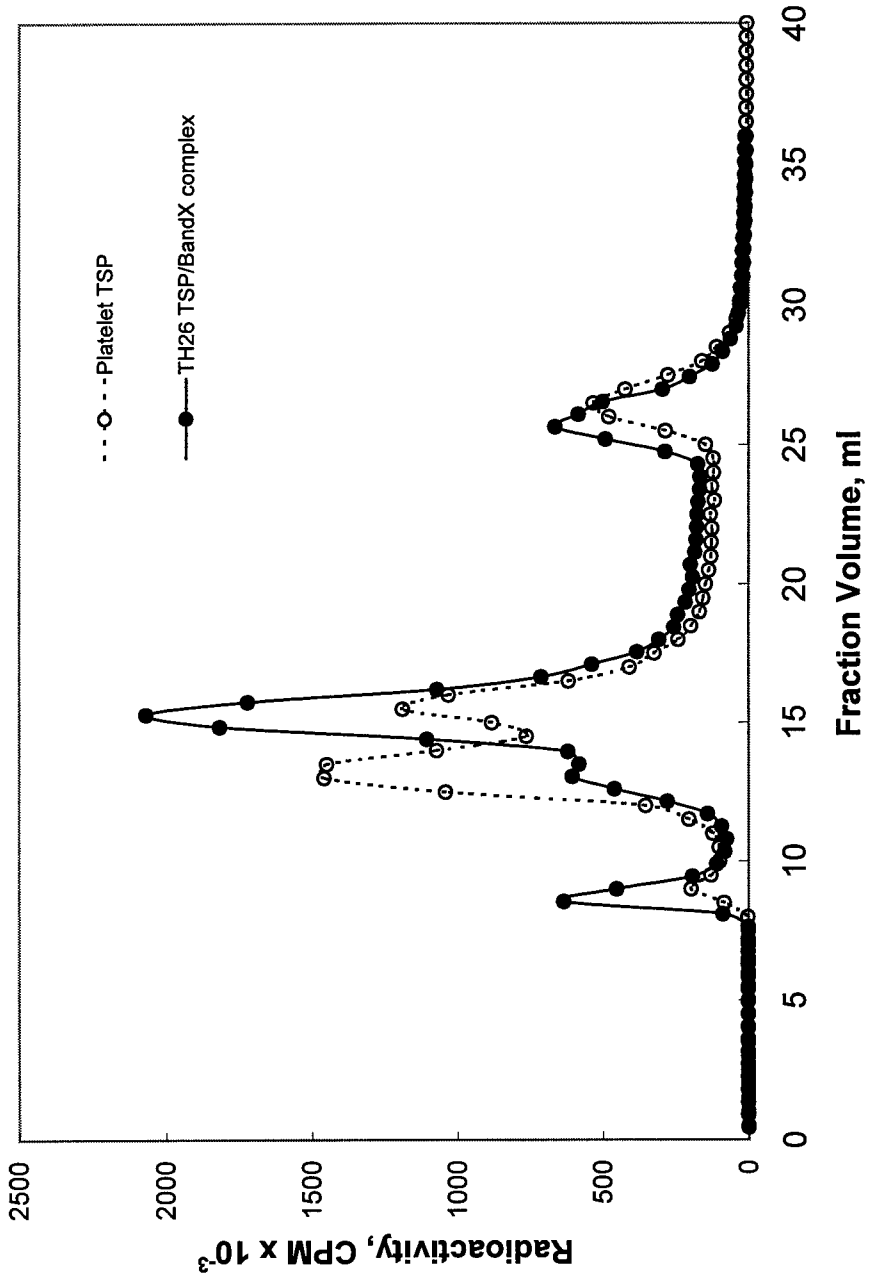
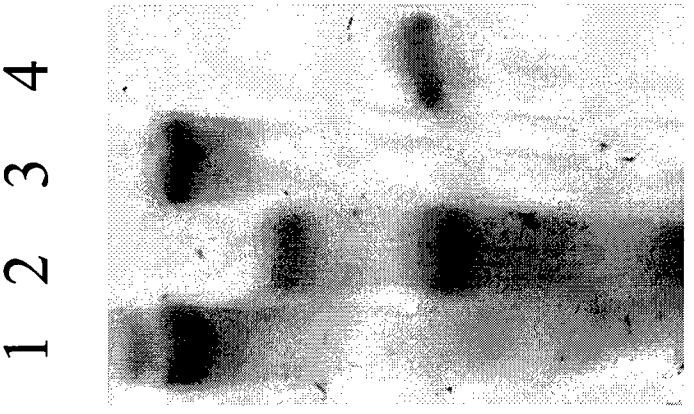

Platelet TSP
TH26 TSP/BandX complex
Radioactivity, CPM x 10-3
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Fraction Volume, ml

Figure 18



[CANCER RESEARCH 57, 1735–1742, May 1, 1997]

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

Neng-hua Guo, Henry C. Krutzsch, John K. Inman, and David D. Roberts[2]

Laboratory of Pathology, National Cancer Institute [N-h. G., H. C. K., D. D. R.], and National Institute of Allergy and Infectious Diseases [J. K. I.], NIH, Bethesda, Maryland 20892-1500

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 specific because no DNA fragmentation was induced in MDA-MB-435S 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[3] 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 receptor $\alpha_5\beta_1$ 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 fibroblasts (7). The absence of appropriate matrix signals can induce programmed cell death or apoptosis of endothelial cells (8, 9). Inhibitors of a second integrin, $\alpha v\beta 3$, also induce apoptosis of angiogenic blood vessels and regression of tumors dependent on this neovascularization (10, 11).

In addition to these positive signals, the extracellular matrix may also provide negative signals to regulate endothelial cell growth. TSP1 (reviewed in Refs. 12 and 13) is one of several matricellular components that, under defined conditions, can inhibit endothelial cell adhesion (14, 15), motility (16), and growth (16–19). TSP1 specifically inhibits endothelial cell adhesion on a fibronectin matrix (14). However, TSP1 can also act as a positive stimulator of endothelial cell adhesion and motility (16, 20), and both positive and negative effects of TSP1 have been reported on angiogenesis *in vivo* (reviewed in Refs. 21–23). Conflicting signals may therefore arise from the interaction of endothelial cells with TSP1, and further work is needed to define the integration and regulation of these responses. In our experience, however, expression of TSP1 in human breast carcinoma cells suppresses their tumorigenic and angiogenic activity in mouse xenografts (24, 25).

Three domains of TSP1 are implicated to date in the inhibitory activities of TSP1 on endothelial cell growth and motility. The amino-terminal domain of TSP1 mimics the inhibitory activity of intact TSP1 on focal adhesion contacts, and this activity is suppressed by an antibody to this domain (26). The recombinant amino-terminal domain of TSP1 also inhibits proliferation and motility of endothelial cells in response to FGF-2 (27). The TSP1 procollagen domain peptide NGVQYRN inhibits motility of endothelial cells to FGF-2 *in vitro* and angiogenesis *in vivo* (28), but its mechanism of action is not known. The type I repeats of TSP1 contain additional inhibitory peptide sequences (27, 28). Peptides from the type I repeats compete with FGF-2 for binding to endothelial cells and inhibit both proliferative and motility responses to this growth factor (27). Because the type I repeat peptides also compete for binding of FGF-2 to heparin or to intact endothelial cells, we proposed that these peptides inhibit endothelial cell responses to FGF-2 by competing with the growth factor for binding to the heparan sulfate proteoglycans that are required for presenting FGF-2 to its high affinity tyrosine kinase receptor (27). On the basis of our recent identification of a TGF-β-activating sequence in this same peptide (29), a second possible mechanism for the observed growth inhibition by TSP1 is by activation of latent TGF-β produced by the endothelial cells (30).

We also observed that endothelial cells lose their normal morphology when treated with peptides from the type I repeats of TSP1 and that cell numbers decreased after incubation with the peptides.[4] Although the peptides also inhibited the growth of a human breast carcinoma cell line, we did not observe a decrease in cell number. This suggested that the peptides may either have a specific cytotoxic activity toward endothelial cells or trigger programmed death of these cells. We have further examined the effects of TSP1 and the peptides on endothelial and breast carcinoma cells, and we report here that TSP1 and the thrombospondin peptides specifically induce apoptosis

Received 10/7/96; accepted 3/8/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by Department of Defense Grant 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.

[2] To whom requests for reprints should be addressed, at Building 10, Room 2A33, 10 Center Drive MSC 1500, Bethesda, MD 20892-1500. Phone: (301) 496-6264; Fax: (301) 402-0043.

[3] The abbreviations used are: FGF-2, fibroblast growth factor 2; BAE, bovine aortic endothelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TGF-β, transforming growth factor β1; TSP1, thrombospondin 1; BrdUrd, bromodeoxyuridine.

[4] N. Guo, H. C. Krutzsch, J. K. Inman, and D. D. Roberts. Antiproliferative and antitumor activities of D-reverse peptide mimetics derived from the second type-1 repeat of thrombospondin 1, submitted for publication.

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 (KRAKAAGGWSHWSPWSSC, KRFKQDGGASHASPASSC) were prepared with appropriate Ala substitutions to eliminate the essential Phe residue for TGF-β activation or the Trp residues required for heparin binding and contain a carboxyl-terminal 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% CO_2 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 sulfate. 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 B1, 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.). BAE cells secrete latent TGF-β1 and TGF-β2 (36), and MDA-MB-435S cells produce TGF-β1, TGF-β2, and TGF-β3 (37). Using the TGF-β1-specific ELISA, 24-h conditioned media from BAE, MCF7, and MDA-MB-435S cells contained 2.05, 2.53, and 1.68 ng/ml acid-activatable TGF-β1. More than 90% of total TGF-β activity was latent in media from each cell line as assessed by the NRK bioassay.

Endothelial Cell Proliferation. Cell proliferation was measured using the Cell-Titer colorimetric assay (Promega) as described previously (27). In brief, cells were trypsinized and suspended in complete medium (BAE cells in DMEM; MDA-MB-435S cells in RPMI 1640) containing 10% FCS at 1–1.5 $\times$ 10^5 cells/ml. Inhibitors were added to a 96-well plate (Costar Corp.) in 50 μl of medium without FCS followed by 50 μl of the endothelial cell suspension. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 h. For determining the effect of okadaic acid, phorbol, herbimycin, fumonisin B1, or TPA on proliferation, the reagents were prepared in medium and added into wells together with the protein or peptide inhibitors.

DNA Fragmentation ELISA. DNA fragmentation was quantified by an ELISA assay (Boehringer Mannheim, Indianapolis, IN) using BrdUrd-labeled cells. A 10-ml suspension of target cells (BAE cells or MDA-MB-435S or MCF7 cells, 10 ml at 2 $\times$ 10^5 cells/ml) was plated and incubated overnight at 37°C in a humidified atmosphere with 10 μM BrdUrd added to 10 ml of low-glucose DMEM with 10% FCS. After labeling, the cells were trypsinized and centrifuged at 250 $\times$ g for 3 min and resuspended in culture medium. The cell concentration was adjusted to 1 $\times$ 10^5 cells/ml, and 100 μl/well were transferred to replicate wells of a microtiter plate containing either medium with peptides or culture medium only (100 μl/well) to yield a final volume of 200 μl/well. The cells were incubated for 24–72 h as indicated at 37°C in a humidified atmosphere with 5% CO_2. For determining the effect of inhibitors on confluent endothelial cells, cells were cultured for 24–48 h at 37°C until the cells reached confluence, and inhibitors were added into the wells and incubated as described above.

After incubation, the cells in the plate were lysed by adding 20 μl of washing buffer (10×) for 30 min at room temperature. The microtiter plate was centrifuged at 250 $\times$ g for 10 min, and 100 μl of supernatant were transferred into the wells of a microtiter plate precoated with anti-DNA antibody. 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-BrdUrd peroxidase conjugate solution was added and incubated for an additional 90 min at room temperature. After washing, immune-complexed anti-BrdUrd 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 $\times$ 10^5/well in 1.5 ml) were cultured on 6-well Nunc tissue 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 in complete medium. Lysis buffer [0.5 ml; containing 5 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol, and 0.5 mg/ml proteinase K] was added to the cell pellet and incubated 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 $\times$ 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 Tris-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 Tris-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).[4] Some of these peptides also promote cell adhesion (34).[4] Because these two activities may elicit opposing signals in cells,[4] 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. 1*c*), including membrane blebbing, nuclear condensation, and loss of adhe-

Table 1 *Structures of TSP1 peptides and mimetics*

Peptide	Origin	Sequence[a]
407	TSP1 type 1 (residues 429–447)	KRFKQDGGWSHWSPWSSC
389	peptide 407 ($Trp^3 \rightarrow Ala^3$)	KRFKQDGGASHASPASSC
450	peptide 407 (Phe, GlnAsp $\rightarrow$ Ala^3)	acKRAKAAGGWSHWSPWSSCam
416	peptide 407 (retro-inverso)	all D-acCSSWPSWHSWGGDQKFRKam
500	TSP1 procollagen (residues 321–327)	NGVQYRNC
493	TSP1 residues 436–444 (retro-inverso)	all D-tpAAWPSWHSWGGam
521	TSP1 residues 429–446 (retro-inverso)	all D-tpSSWPSWHSWGGDQKFRKam

[a] Sequences are depicted using single-letter codes and are L-amino acids except where indicated; tp, thiopropionyl; am, amide; ac, acetyl.

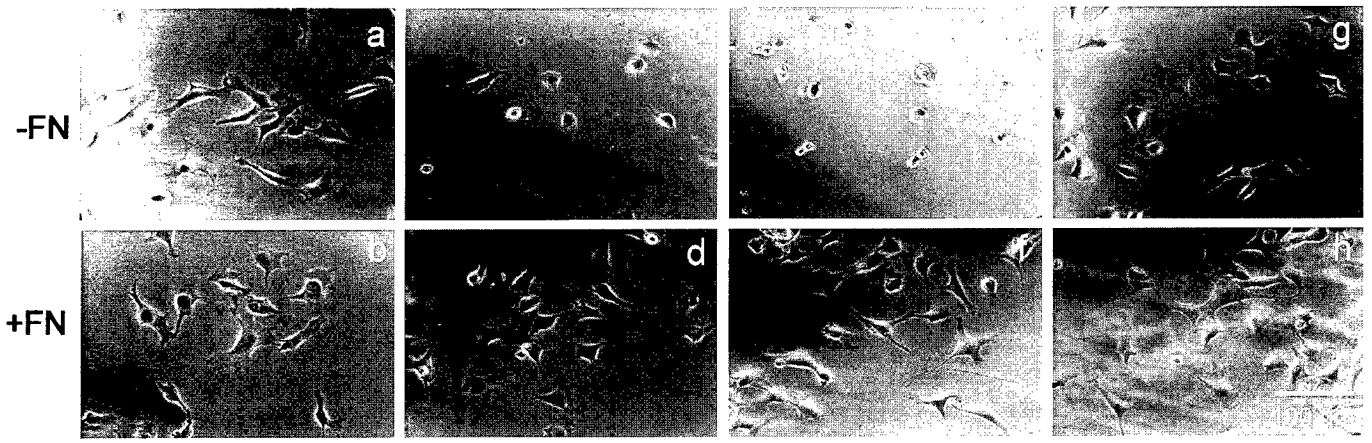


Fig. 1. Effect of TSP1 type I repeat peptides on morphology of BAE cells. Cells were photographed 22 h after plating on tissue culture plastic (*a, c, e,* and *g*) or on plastic coated with 10 μg/ml human plasma fibronectin (*b, d, f,* and *h*). Cells in *c–h* were treated with polysucrose conjugates containing 4 μM of the TSP1 type 1 repeat peptide KRFKQDGGWSHWSPWSSC (*c* and *d*), an analogue without the TGF-β-activating sequence acKRAKAAGGWSHWSPWSSCam (*e* and *f*), or the TSP1 procollagen domain peptide NGVQYRNC (*g* and *h*).

sion. 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. 1*g*).

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. 1*e*), 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 KRFKQDGGWSHWSPWSSC (*Lane b*) or the modified sequence KRAKAAGGWSHWSPWSSC (*Lane e*), 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 KRFKQDGGASHASPASSC (*Lane a*) or GGWSHWSPWSSC (*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 NGVQYRNC 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 (KRFKQDGGWSHWSPWSSC-polysucrose, 407f) and an analogue without the TGF-β-activating sequence RFK (KRAKAAGGWSHWSPWSSC-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 1 repeat (Fig. 4). DNA fragmentation induced by the peptide KRFKQDGGWSHWSPWSSC conjugate was reduced by 84% in cells attached on wells coated with 10 μg/ml fibronectin. A conjugate containing the TSP1 procollagen peptide NGVQYRNC 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

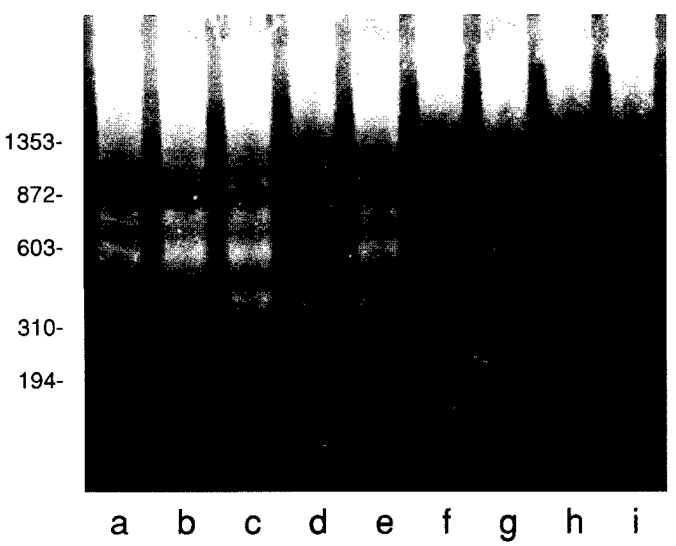


Fig. 2. Type I repeat peptide analogues from TSP1 induce DNA fragmentation in aortic endothelial cells. BAE cells (5×10^5) were treated with 0.4 μM of the indicated TSP1 peptide conjugates or 25 μg/ml of intact TSP1 for 24 h. Low molecular weight DNA was extracted from the cells and analyzed by electrophoresis on a 2% agarose gel. DNA fragments were visualized by staining with SYBR green. Cells were treated with polysucrose conjugates of the following peptides: KRFKQDGGASHASPASSC, *Lane a*; KRFKQDGGWSHWSPWSSC, *Lane b*; retro-inverso amKRFKQDGGWSHWSPWSS-Cac, *Lane c*; GGWSHWSPWAAC, *Lane d*; KRAKAAGGWSHWSPWSSC, *Lane e*; NGVQYRNC, *Lane f*; retro-inverso amKRFKQDGGWSHWSPWSS-thioproprionyl, *Lane g*; TSP1, *Lane h*; and control, *Lane i*. *Left margin*, migration of DNA size markers.

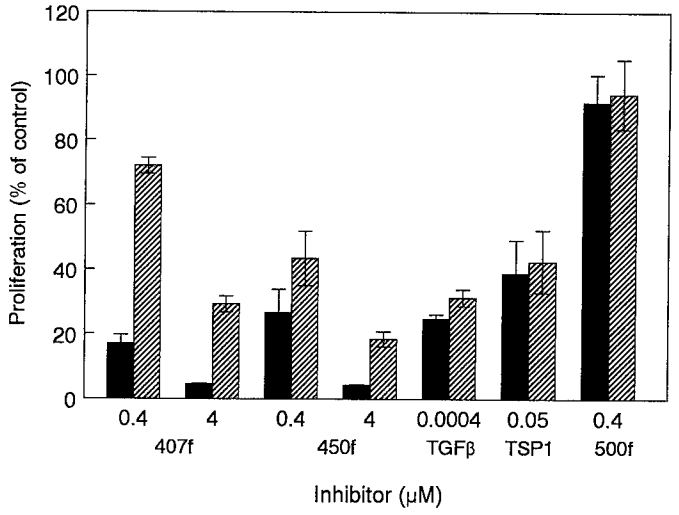


Fig. 3. Attachment of endothelial cells on fibronectin partially reverses the inhibition of proliferation by type 1 repeat peptides but not by TSP1 or TGF-β. Proliferation of BAE cells was determined on untreated tissue culture plastic (■) or on plastic coated with 10 μg/ml fibronectin (▨) in DMEM medium containing 1% FBS and the indicated concentrations of the TSP1 peptide KRFKQDGGWSHWSPWSSC (*407f*), an analogue without the TGF-β-activating sequence acKRAKAAGGWSHWSPWSSCam (*450f*), TGF-β1 (*TGF-β*), TSP1, or the TSP1 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, $n = 3$.

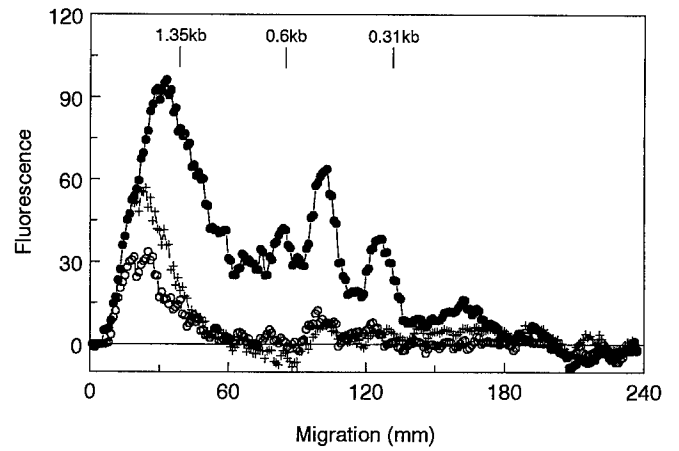


Fig. 4. Fibronectin inhibits TSP1 peptide-induced DNA fragmentation. DNA fragmentation was determined as described in the legend to Fig. 2. Band intensity was determined by image analysis and is plotted with background subtraction using control cells as a reference for endothelial cells treated with a conjugate of peptide KRFKQDGGWSHWSPWSSC (●), cells treated with the same peptide in a well coated with 10 μg/ml fibronectin (+), or cells treated with a conjugate of the TSP1 procollagen peptide NGVQYRNC (○). The migration of DNA size standards is as indicated.

the peptides. On the basis of this sensitive and quantitative assay for DNA fragmentation, both TSP1 and the type I repeat peptides induced significant DNA fragmentation in endothelial cells (Fig. 5*A*). The activity of TSP1 was weaker than that of the synthetic peptide conjugates to elicit DNA fragmentation but was consistently observed in several independent experiments. The TSP1 procollagen domain peptide, however, was inactive. Treatment of BAE cells with TGF-β induced DNA fragmentation to a similar extent as TSP1. The stimulation of DNA fragmentation by TSP1 and the peptide conjugates was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells by the peptide conjugates (Fig. 5*B*). DNA fragmentation was induced in MDA-MB-435S cells by the topoisomerase I inhibitor camptothecin, indicating that these cells can initiate programmed cell death. Because the same peptides inhibited the growth of MDA-MB-435S cells,[4] the induction of apoptosis can be independent of the antiproliferative effects of the TSP1 peptides. A second breast carcinoma cell line, MCF7, showed DNA fragmentation in response to the peptide 407 conjugate but not to intact TSP1 (Fig. 5*B*). 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 on a fibronectin matrix inhibited DNA fragmentation induced by TSP1 (Fig. 6*A*) or by a TSP1 peptide analogue from the type I repeats (Fig. 6*B*). 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 TSP1 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

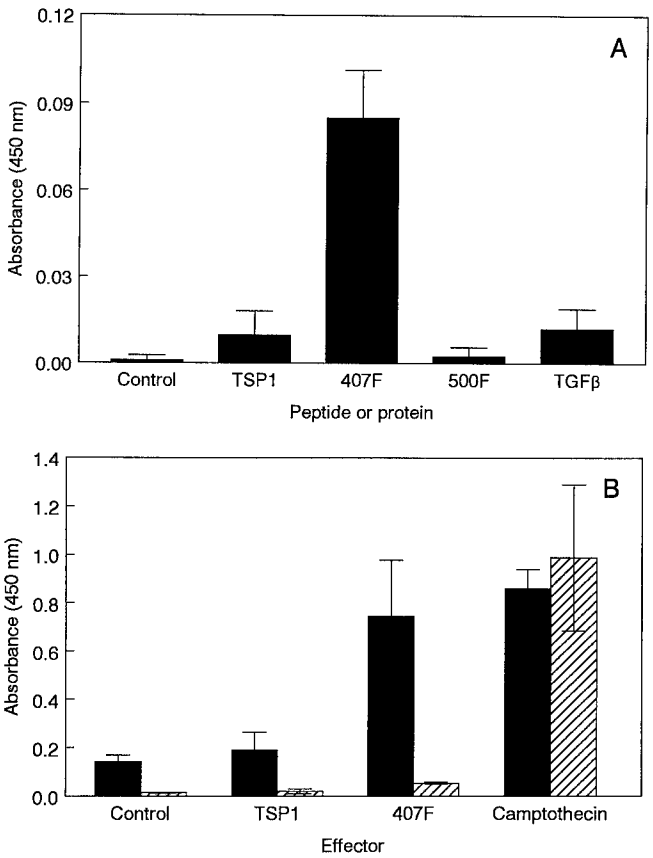


Fig. 5. Detection of TSP1- and peptide-induced apoptosis in BAE and human breast carcinoma cells by a DNA fragment ELISA. DNA fragmentation in BAE cells (*A*), MCF7 breast carcinoma cells (*B*, ■), or MDA-MB-435S breast carcinoma cells (*B*, ▨) was quantified by an ELISA assay using BrdUrd-labeled cells. Target cells (10 ml; 2×10^5 cells/ml) were labeled overnight using 10 μM BrdUrd. After labeling, a cell suspension containing 1×10^5 cells/ml was transferred to replicate wells of a microtiter plate (100 μl/well) containing 100 μl of culture medium containing inhibitors (25 μg/ml TSP1, 4 μM KRFKQDGGWSHWSPWSSC (*407F*), NGVQYRNC (*500F*), 400 ng/ml camptothecin, or 10 ng/ml TGF-β) or medium only (*Control*). After incubation for 24 h at 37°C, the cells in the wells were lysed and centrifuged. Released DNA fragments in 100 μl of the supernatant were quantified using a sandwich ELISA using anti-DNA capture antibody and anti-BrdUrd peroxidase conjugate for detection. After washing, immune-complexed anti-BrdUrd peroxidase was detected using 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was measured at 450 nm and is presented as mean ± SD, $n = 3$.

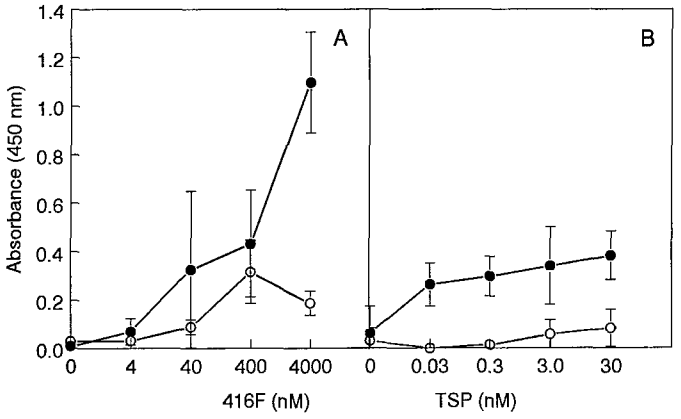


Fig. 6. Fibronectin protects endothelial cells from DNA fragmentation induced by TSP1 and a peptide from the type I repeat. DNA fragmentation in BrdUrd-labeled endothelial cells was detected using an ELISA assay. BAE cells were grown in DMEM with 5% FCS in tissue culture wells (●) or wells coated with 10 μg/ml fibronectin (○) with the indicated concentrations of a TSP1 peptide analogue (retro-inverso amKRFKQDGGWSHWSPWSSCac, *A*) or human platelet TSP1 (*B*). TSP1 concentration is expressed on a subunit molar basis. Colorimetric detection of DNA fragment release was determined in triplicate and is presented as mean ± SD.

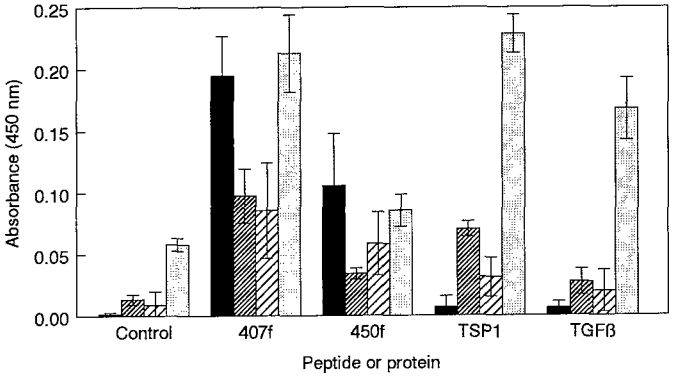


Fig. 7. Modulation of TSP1- and peptide-induced DNA fragmentation in BAE cells. Cells were plated in DMEM containing 5% FCS (■) or the same medium containing 50 μM vanadate (*narrow stripes*), 5 nM okadaic acid (*wide stripes*), or 25 nM fumonisin B1 (▨). The indicated peptides or proteins (4 μM 407f or 450f, 50 nM TSP1, and 0.4 nM TGF-β) were added and incubated for 36 h at 37°C in 5% CO_2. DNA fragmentation was detected by ELISA, and the results are presented as mean ± SD, $n = 3$.

synthase inhibitor fumonisin B1 did not. In contrast, the tyrosine and serine/threonine phosphatase inhibitors unexpectedly increased DNA fragmentation induced by intact TSP1, as did fumonisin B1. Alterations in the DNA fragmentation response to TGF-β in the presence of these inhibitors paralleled those of TSP1, suggesting a common mechanism of action or that TGF-β bound to the TSP1 is responsible for the observed response. The TGF-β concentration in the TSP1 was quantified using an ELISA assay specific for TGF-β1 (Genzyme Corp.). No TGF-β1 immunoreactivity was detected without acid treatment of the TSP1 used in these experiments. After acid treatment, 0.07 ng of TGF-β1 was detected per microgram of TSP1. Assuming that all of this TGF-β1 is active, it would account for only 17% of the observed TSP1 response.

A role for adhesion in regulating the sensitivity of endothelial cells to the TSP1 peptides was also suggested by its dependence on cell density (Fig. 8). Maximal sensitivity to induction of DNA fragmentation by the TSP1 peptide KRFKQDGGWSHWSPWSSC was observed using subconfluent cells, whereas the induction of DNA fragmentation was suppressed when confluent endothelial cells were used. The addition of the phosphatase inhibitor vanadate to the subconfluent cells produced a similar suppression of the DNA fragmentation response to the peptide (Fig. 8).

Phosphorylation Differentially Modulates Antiproliferative Responses to Peptides 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 effect 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).

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

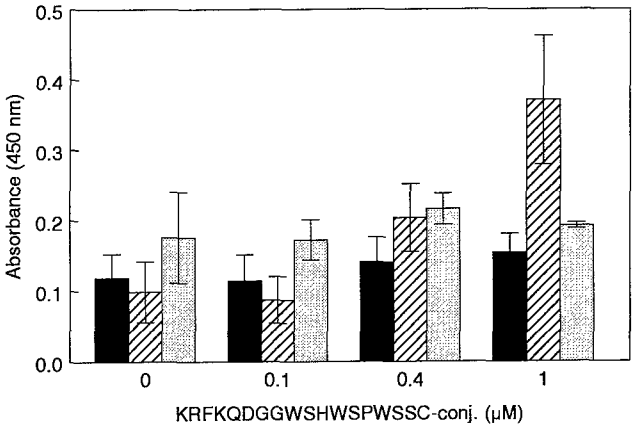


Fig. 8. Endothelial cell density and vanadate modulate DNA fragmentation induced by a peptide from the type I repeat of TSP1. Subconfluent BAE cells (1×10^4 cells/well) with (▨) or without (▨) 100 μM vanadate or confluent BAE cells (1×10^4 cells/well and precultured for 48 h, ■) were labeled with BrdUrd and incubated for 24 h in DMEM containing 5% FCS and the indicated concentrations of the TSP1 type 1 repeat peptide conjugate. DNA fragment release was quantified by ELISA and is presented as mean ± SD, $n = 3$.

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

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.

	% Inhibition of proliferation				
	450F	500F	407F	TSP1	TGFβ
	(0.4 μM)	(0.4 μM)	(0.4 μM)	(150 nM)	(0.4 nM)
Control	49 ± 4	−4 ± 4	97 ± 1	70 ± 4	71 ± 2
Herbimycin (1 μM)	0 ± 5	0 ± 9	50 ± 7	−14 ± 12	−5 ± 12
Fumonisin (15 μM)	24 ± 3	6 ± 4	97 ± 1	74 ± 2	84 ± 3
Control	59 ± 4	−5 ± 19	74 ± 5	61 ± 3	72 ± 1
Vanadate (20 μM)	61 ± 3	−3 ± 8	83 ± 5	−8 ± 12	26 ± 7
Okadaic acid (5 nM)	77 ± 5	17 ± 4	96 ± 1	65 ± 4	80 ± 1
Control		1 ± 5	43 ± 2	52 ± 1	
TPA (50 nM)		1 ± 2	64 ± 6	48 ± 1	
Phorbol (50 nM)		2 ± 6	77 ± 2	57 ± 2	

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 (K_i = 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 matricellular 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 discreet 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-β1 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 procollagen domain peptide (28). Mutagenesis of the type I repeat sequences will be required to confirm the role of the type 1 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 ionizing radiation 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 $\alpha5\beta1$ 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 $\beta1$ or $\beta3$ 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 KRFKQDGGWSHWSPWSSC 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 $\alpha v\beta 3$ 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 $\alpha v\beta 3$ (20), the active TSP1 peptides do not contain the Arg-Gly-Asp sequence recognized by the $\alpha v\beta 3$ integrin.

The TSP1 peptides are potent inducers of DNA fragmentation in BAE cells *in vitro*. This activity may account for the differential effects of these peptides on endothelial and breast carcinoma cell proliferation *in vitro*. We recently found that stable analogues of the TSP1 peptides inhibit tumor growth *in vivo* in MDA-MB-435S xenografts in nude mice.[4] 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

1. Juliano, R. L., and Haskill, S. Signal transduction from the extracellular matrix. J. Cell Biol., *120:* 577–585, 1993.
2. Schaller, M. D., and Parsons, J. T. Focal adhesion kinase and associated proteins. Curr. Opin. Cell Biol., *6:* 705–710, 1994.
3. Hannigan, G. E., Leung-Hagesteijn, C., Fitz-Gibbon, L., Coppolino, M. G., Radeva, G., Filmus, J., Bell, J. C., and Dedhar, S. Regulation of cell adhesion and anchorage-dependent growth by a new β1-integrin-linked protein kinase. Nature (Lond.), *379:* 91–96, 1996.
4. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol., *131:* 791–805, 1995.
5. Vuori, K., and Ruoslahti, E. Activation of protein kinase C precedes α5β1 integrin-mediated cell spreading on fibronectin. J. Biol. Chem., *268:* 21459–21462, 1993.
6. Zhang, Z., Vuori, K., Reed, J. C., and Ruoslahti, E. The α5β1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. Proc. Natl. Acad. Sci. USA, *92:* 6161–6165, 1995.
7. Chen, Q., Kinch, M. S., Lin, T. H., Burridge, K., and Juliano, R. L. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. J. Biol. Chem., *269:* 26602–26605, 1994.
8. Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. The extracellular matrix as a cell survival factor. Mol. Biol. Cell, *4:* 953–961, 1993.
9. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. J. Cell Biol., *127:* 537–546, 1994.
10. Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., and Cheresh, D. A. Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell, *79:* 1157–1164, 1994.
11. Brooks, P. C., Stromblad, S., Klemke, R., Visscher, D., Sarkar, F. H., and Cheresh, D. A. Anti-integrin αvβ3 blocks human breast cancer growth and angiogenesis in human skin. J. Clin. Invest., *96:* 1815–1822, 1995.
12. Bornstein, P. Diversity of function is inherent in matricellular proteins: an appraisal of thrombospondin 1. J. Cell Biol., *130:* 503–506, 1995.
13. Lahav, J. The functions of thrombospondin and its involvement in physiology and pathophysiology. Biochim. Biophys. Acta, *1182:* 1–14, 1993.
14. Lahav, J. Thrombospondin inhibits adhesion of endothelial cells. Exp. Cell Res., *177:* 199–204, 1988.
15. Murphy-Ullrich, J. E., and Hook, M. Thrombospondin modulates focal adhesions in endothelial cells. J. Cell Biol., *109:* 1309–1319, 1989.
16. Taraboletti, G., Roberts, D., Liotta, L. A., and Giavazzi, R. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. J. Cell Biol., *111:* 765–772, 1990.
17. Bagavandoss, P., and Wilks, J. W. Specific inhibition of endothelial cell proliferation by thrombospondin. Biochem. Biophys. Res. Commun., *170:* 867–872, 1990.
18. Good, D. J., Polverini, P. J., Rastinejad, F., Le, B. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc. Natl. Acad. Sci. USA, *87:* 6624–6628, 1990.
19. Iruela Arispe, M., Bornstein, P., and Sage, H. Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells *in vitro*. Proc. Natl. Acad. Sci. USA, *88:* 5026–5030, 1991.
20. Lawler, J., Weinstein, R., and Hynes, R. O. Cell attachment to thrombospondin: the role of Arg-Gly-Asp, calcium, and integrin receptors. J. Cell Biol., *107:* 2351–2361, 1988.
21. Roberts, D. D. Regulation of tumor growth and metastasis by thrombospondin-1. FASEB J., *10:* 1183–1191, 1996.
22. Tuszynski, G. P., and Nicosia, R. F. The role of thrombospondin-1 in tumor progression and angiogenesis. BioEssays, *18:* 71–76, 1996.
23. Volpert, O. V., Stellmach, V., and Bouck, N. The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. Breast Cancer Res. Treat., *36:* 119–126, 1995.
24. Weinstat-Saslow, D. L., Zabrenetzky, V. S., VanHoutte, K., Frazier, W. A., Roberts, D. D., and Steeg, P. S. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. Cancer Res., *54:* 6504–6511, 1994.
25. Zabrenetzky, V., Harris, C. C., Steeg, P. S., and Roberts, D. D. Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung, and breast carcinoma cell lines. Int. J. Cancer, *59:* 191–195, 1994.
26. Murphy-Ullrich, J. E., Gurusiddappa, S., Frazier, W. A., and Hook, M. Heparin-binding peptides from thrombospondins 1 and 2 contain focal adhesion-labilizing activity. J. Biol. Chem., *268:* 26784–26789, 1993.
27. Vogel, T., Guo, N. H., Krutzsch, H. C., Blake, D. A., Hartman, J., Mendelovitz, S., Panet, A., and Roberts, D. D. Modulation of endothelial cell proliferation, adhesion, and motility by recombinant heparin-binding domain and synthetic peptides from the type I repeats of thrombospondin. J. Cell. Biochem., *53:* 74–84, 1993.
28. Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Polverini, P. J., and Bouck, N. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have antiangiogenic activity. J. Cell Biol., *122:* 497–511, 1993.
29. Schultz-Cherry, S., Chen, H., Mosher, D. F., Misenheimer, T. M., Krutzsch, H. C., Roberts, D. D., and Murphy-Ullrich, J. E. Regulation of transforming growth factor β activation by discrete sequences of thrombospondin 1. J. Biol. Chem., *270:* 7304–7310, 1995.
30. Schultz-Cherry, S., and Murphy-Ullrich, J. E. Thrombospondin causes activation of latent transforming growth factor β secreted by endothelial cells by a novel mechanism. J. Cell Biol., *122:* 923–932, 1993.
31. Roberts, D. D., Cashel, J., and Guo, N. Purification of thrombospondin from human platelets. J. Tissue Cult. Methods, *16:* 217–222, 1994.
32. Akiyama, S. K., and Yamada, K. M. The interaction of plasma fibronectin with fibroblastic cells in suspension. J. Biol. Chem., *260:* 4492–4500, 1985.
33. Guo, N. H., Krutzsch, H. C., Negre, E., Vogel, T., Blake, D. A., and Roberts, D. D. Heparin- and sulfatide-binding peptides from the type I repeats of human thrombospondin promote melanoma cell adhesion. Proc. Natl. Acad. Sci. USA, *89:* 3040–3044, 1992.
34. Guo, N. H., Krutzsch, H. C., Negre, E., Zabrenetzky, V. S., and Roberts, D. D. Heparin-binding peptides from the type I repeats of thrombospondin. Structural requirements for heparin binding and promotion of melanoma cell adhesion and chemotaxis. J. Biol. Chem., *267:* 19349–19355, 1992.
35. Danielpour, D., Dart, L. L., Flanders, K. C., Roberts, A. B., and Sporn, M. B. Immunodetection and quantitation of the two forms of transforming growth factor β (TGF-β1 and TGF-β2) secreted by cells in culture. J. Cell. Physiol., *138:* 79–86, 1989.
36. Kojima, S., and Rifkin, D. B. Mechanism of retinoid-induced activation of latent transforming growth factor β in bovine endothelial cells. J. Cell. Physiol., *155:* 323–332, 1993.
37. Charpentier, A., Simmons-Menchaca, M., Yu, W., Zhao, B., Qian, M., Heim, K., Sanders, B. G., and Kline, K. RRR-α tocopherol succinate enhances TGF-β1, -β2, and -β3 and TGF-βR-II expression by human MDA-MB-435 breast cancer cells. Nutr. Cancer, *26:* 237–250, 1996.
38. Havrilesky, L. J., Hurteau, J. A., Whitaker, R. S., Elbendary, A., Wu, S., Rodriguez, G. C., Bast, R. C., Jr., and Berchuck, A. Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor β. Cancer Res., *55:* 944–948, 1995.
39. Cohen, P., Holmes, C. F., and Tsukitani, Y. Okadaic acid: a new probe for the study

of cellular regulation. Trends Biochem. Sci., *15:* 98–102, 1990.
40. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., and Kolesnick, R. Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. Cell, *82:* 405–414, 1995.
41. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuks, Z., and Kolesnick, R. N. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J. Exp. Med., *180:* 525–535, 1994.
42. Haimovitz-Friedman, A., Balaban, N., McLoughlin, M., Ehleiter, D., Michaeli, J., Vlodavsky, I., and Fuks, Z. Protein kinase C mediates basic fibroblast growth factor protection of endothelial cells against radiation-induced apoptosis. Cancer Res., *54:* 2591–2597, 1994.
43. Sage, E. H., and Bornstein, P. Extracellular proteins that modulate cell-matrix interactions: SPARC, tenascin, and thrombospondin. J. Biol. Chem., *266:* 14831–14834, 1991.
44. Hannun, Y. A., and Obeid, L. M. Ceramide: an intracellular signal for apoptosis. Trends Biochem. Sci., *20:* 73–77, 1995.
45. Defilippi, P., Bozzo, C., Volpe, G., Romano, G., Venturino, M., Silengo, L., and Tarone, G. Integrin-mediated signal transduction in human endothelial cells: analysis of tyrosine phosphorylation events. Cell Adhes. Commun., *2:* 75–86, 1994.
46. Oikawa, T., Ogasawara, H., Sano, H., Shibata, K., and Omura, S. Possible functional groups responsible for inhibition of *in vivo* angiogenesis by herbimycin A. Biol. Pharm. Bull., *17:* 1430–1432, 1994.
47. Nakashima, I., Pu, M. Y., Hamaguchi, M., Iwamoto, T., Rahman, S. M., Zhang, Y. H., Kato, M., Ohkusu, K., Katano, Y., Yoshida, T., Koga, Y., Isobe, K. I., and Nagase, F. Pathway of signal delivery to murine thymocytes triggered by co-cross-linking CD3 and Thy-1 for cellular DNA fragmentation and growth inhibition. J. Immunol., *151:* 3511–3520, 1993.
48. Lesoon-Wood, L. A., Kim, W. H., Kleinman, H. K., Weintraub, B. D., and Mixson, A. J. Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. Hum. Gene Ther., *6:* 395–405, 1995.

JPEP 70204

J. Peptide Res. 49, 1997, 000–000
Printed in the United States of America - all rights reserved

JOURNAL OF PEPTIDE RESEARCH
ISSN 1397-002X

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

NENG-HUA GUO,[1] HENRY C. KRUTZSCH,[1] JOHN K. INMAN,[2] CAITLIN S. SHANNON,[1] and DAVID D. ROBERTS[1]

[1]*Laboratory of Pathology, National Cancer Institute and* [2]*Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA*

Received 10 January, revised 19 March, accepted for publication 10 May 1997

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 KRFKQDGGWSHWSPWSSC. 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, product of the *THBS1* gene; TGFβ, transforming growth factor β1; 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, TGFβ (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 TSP1 mRNA and protein expression decreased in several tumor cell lines selected for high metastatic potential in mice (18). Expression of activating forms of the *ras* oncogene or loss of the wild-type tumor suppressor *p53* were also associated with loss of TSP1 expression (18, 19). Furthermore, overexpression of TSP1 in breast carcinoma cells suppressed tumor growth in nude mice (20), identifying *THBS1* as a potential tumor and metastasis suppressor gene. These data, combined with evidence that TSP1 inhibits endothelial cell growth and motility *in vitro* and angiogenesis *in vivo* (4–7, 21, 22), suggest that TSP1 may inhibit neovascularization of tumors.

Several sites on the TSP1 molecule may modulate angiogenesis. Two peptide sequences in the type I repeats, a peptide from the procollagen domain and a recombinant amino-terminal fragment of TSP1 inhibit endothelial growth or motility (10, 23). Synthetic peptides derived from the type I repeats of TSP1 inhibited proliferation and motility of endothelial cells stimulated by FGF-2, mimicking the activity of intact TSP1 (10). These peptides act at least in part by competing with FGF-2 for binding to heparan sulfate proteoglycan receptors on the endothelial cells, which are essential for presentation of FGF-2 to its signaling receptor (10). Although these synthetic peptides lack the contaminating TGFβ that may account for some antiangiogenic activities of platelet TSP1 (24), peptides from the second type I repeat contain the sequence RFK which activates purified latent TGFβ (16). The TSP1 peptide KRFKQDGGWSHWSPWSS, therefore, has three defined activities: promotion of adhesion, antagonism of FGF-2 and activation of latent TGFβ.

The strong antiproliferative activity of the TSP1 peptides suggested that they may be useful for inhibition of pathological angiogenesis *in vivo*. To achieve this goal, the multiple activities of the peptides must be resolved, and the role of each in the antiangiogenic activities of the TSP1 peptide must be defined. Furthermore, free peptides often have short half-lives in circulation because of 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 (25, 26). Preparation of enzymatically stable retro-inverso analogs is a second method to increase the duration of activity of peptides *in vivo*. These analogs have been successfully applied to increase the stability and biological activity of peptide sequences for therapeutic applications (27, 28). Of particular relevance to the TSP1 peptides, an all D-amino acid peptide analog of the IKVAV peptide from the A chain of laminin replicated the activity of the natural sequence to influence tumor cell adhesion and growth *in vitro* and *in vivo* (29). Pseudo peptides based on the B1 chain of laminin also inhibited tumorigenesis and metastasis *in vivo* (30).

In this study, we have further examined the basis for the antiproliferative activity of these TSP1 peptides. Using analogs of the TSP1 sequence that separate the heparin-binding activity from the latent TGFβ-activating activity, we demonstrate here that only heparin-binding activity is required for antiproliferative activity. Using polymer conjugates of the peptides, we can also separate the adhesive activity of the peptides from their antiproliferative activities. A stereochemical analysis of the TSP1 peptides demonstrates that retro-inverso peptide analogs retain the heparin-binding activity. This observation allowed us to prepare proteolytically stable forms of the TSP1 peptides and to demonstrate their activity *in vivo* for inhibiting growth of a human breast carcinoma in an mouse orthotopic xenograft model.

EXPERIMENTAL PROCEDURES

Materials. TSP1 was purified from the supernatant of thrombin-stimulated human platelets (31). EGF and TGFβ1 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 *t*-butoxycarbonyl chemistry (32). Peptides were analyzed by reverse-phase HPLC chromatography. Peptides for biological assays were further purified by dialysis using Spectrapor 500 M_r-cutoff membranes or by reverse-phase purification using C_{18} Sep-Pak cartridges. Identities of some peptides were verified ~~or by reverse-phase purification using C_{18} Sep-Pak cartridges. Identities of some peptides were verified~~ by MALDI 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-

ously (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 Na_2CO_3. 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 led 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_r-cutoff tubing. The peptide concentration of the resulting conjugate was determined by measuring its absorbance at 280 nm using $\varepsilon = 5540\ M^{-1}\ cm^{-1}$ per Trp residue. The conjugates had between 8 and 29 moles of peptide covalently bound per mole of M_r 400,000 polysucrose.

Ligand binding assays. TSP1 binding to heparin-BSA was determined using a solid-phase assay (35). Heparin-BSA (0.075 μg/well) was adsorbed onto 96-well polyvinyl chloride 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 $CaCl_2$, 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 ^{125}I-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 ^{125}I-FGF-2 to heparin was determined using an immobilized heparin-BSA conjugate as described previously (10). ^{125}I-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 1×10^4 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 10^5 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 L-reverse and D-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 L-reverse and D-reverse peptides **600** and **599** were equal in activity to the native L-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 L-forward peptide **246** activated latent TGFβ, as assessed by stimulation of normal rat kidney fibroblast colony for-

TABLE 1

Stereospecificity for heparin-binding and TGFβ-activation by the TSP1 type I repeat peptide

Peptide	Sequence	TPS1 binding	FGF-2 binding	Latent TGFβ activation
246	L-KRFKQDGGWSHWSPWSS	0.17	1.3	0.002
600	L-SSWPSWHSWGGDQKFRK	0.14	0.9	0.03
599	D-SSWPSWHSWGGDQKFRK	0.12	0.9	0.01
596	D-SSWPSWHSWGGAQKARK	0.032	0.16	4
597	D-SSAPSAHSAGGDQKFRK	>10	>50	0.01
598	D-SSAPSAHSAGGAQKARK	>10	>50	>10

^{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. 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.

mation in soft agar (Fig. 1). The L-reverse and D-reverse analogs also activated latent TGFβ (Fig. 1) but required higher concentrations than the L-forward peptide for half-maximal activation (Table 1). The dose for half-maximal stimulation by the L-forward peptide KRFKQDGGWSHWSPWSS (10 nM), measured

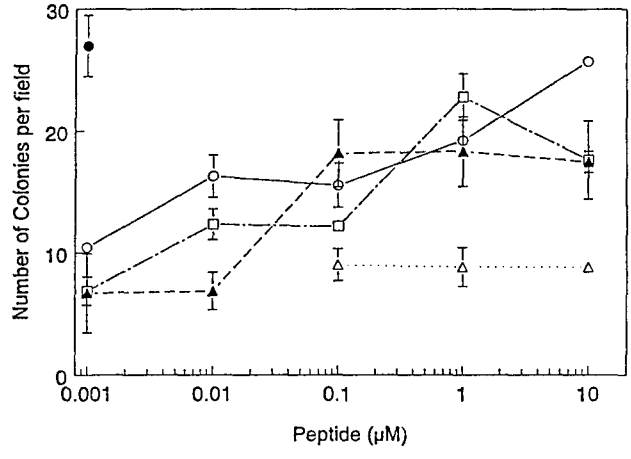


FIGURE 1

Stereochemical specificity for activation of latent TGFβ by peptide analogs of the second thrombospondin-1 type I repeat. The indicated concentrations of the L-forward peptide **246** (KRFKQDGGWSHWSPWSS, ○), the L-reverse peptide **600** (SSWPSWHSWGGDQKFRK, ▲), the D-reverse peptide **599** (ri-amKRFKQDGGWSHWSPWSSac, □) were mixed with serum-free conditioned medium from bovine aortic endothelial cell and added with NRK cells suspended in soft agar. The D-reverse peptide **598** (ri-amKRAKQAGGASHASPASSac, △) was used as a negative control, and conditioned medium heated to 85°C for 5 min to quantitatively activate the endogenous latent TGFβ was used as a positive control (●). Colony formation, determined as described under "Experimental Procedures," is presented as mean ± SD for triplicate determinations. Enhancement of colony formation by peptides 246, 599 and 600 was significant ($p < 0.05$ by a two-sided *t*-test) at 1 and 10 μM.

using endogenous latent TGFβ in endothelial cell conditioned medium, was higher than previously reported for the same peptide using purified latent TGFβ (16).

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 **597,** 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 TSP1 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-amKRAKQAGGWSHWSPWSSCac), indicating that this effect is independent of the RFK motif. The stimulation of proliferation by the peptides was not specific to the Mv1Lu cells. Although some free TSP1 type

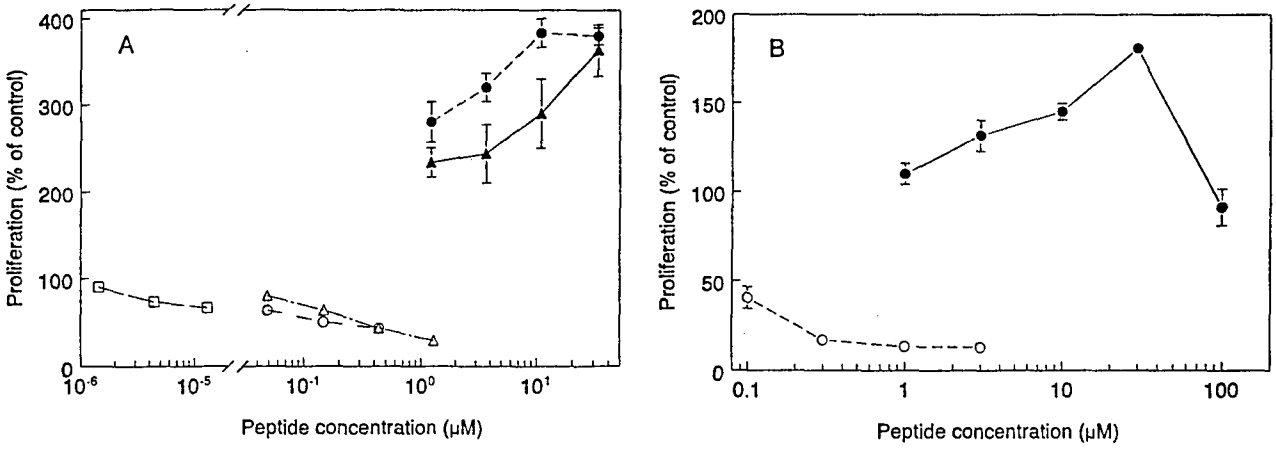


FIGURE 2

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 Mv1Lu cells (1×10^4/well) was determined in the presence of the indicated concentrations of the TGFβ-activating peptide **416** (ri-amKRFKQDGGWSHWSPWSSCac, ●) 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-amKRFKQDGGWSHWSPWSSCac, ●) 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 type 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 mink lung and bovine endothelial cells resulted from this adhesive activity. The peptides that stimulated proliferation also promoted adhesion of Mv1Lu and BAE 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

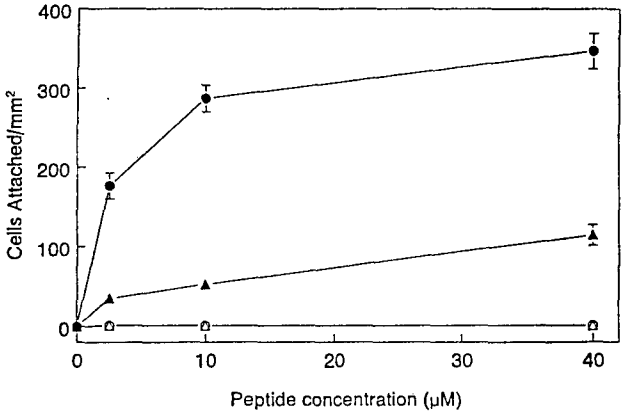


FIGURE 3.

Conjugation of TSP1 peptides to polysucrose ablates their ability to promote cell adhesion. Adhesion of Mv1Lu cells (●, ○) or bovine aortic endothelial cells (▲, △) to plastic coated with the indicated concentrations of TSP1 peptide **246** (KRFKQDGGWSHWSPWSS, ●, ▲) or a polysucrose conjugate of the peptide KRFKQDGGWSHWSPWSS (○, △) was determined. Results are the mean ± SD of triplicate determinations.

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

Peptide sequence	Heparin-binding protein	
	^{125}I-Thrombospondin IC_{50} (μM)[a]	^{125}(I-FGF-2 IC_{50} (μM)[a]
KRFKQDGGWSWHSPWSSC (TSP1)	0.5	3.8
KRFKQDGGWSHW	0.65	2.5
KRFKQDGG	>10	>40
SHWSPWSS	5	>40
TRIRQDGGWSHW (TSP2)	6	13
NGVQYRNCam (TSP1 procoll.)	>10	>40
FIRVVMYEGKK (TSP1 C-term.)	>10	>40
FRYVVMWK (TSP1 C-term.)		>40

^{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.

activity derived from the procollagen domain of TSP1 (NGVQYRNC) (23) and two peptides with chemotactic activities for endothelial cells or from the carboxyl-terminal cell binding domain of TSP1 (FIRVVMYEGKK or RFYVVMWK) (39, 40). Although these peptides contain basic amino acids and have a net positive charge at physiological pH, they did not inhibit FGF-2 binding to heparin (Table 2). A peptide from the second type I repeat of thrombospondin-2 (TRIRQDGGWSHW) homologous to the active TSP1 peptide (KRFKQDGGWSHW) inhibited FGF-2 binding, but was about 5-fold less active than the TSP1 peptide.

Activities for inhibiting FGF-2 binding to heparin differed in some cases from those determined using TSP1 as the labeled ligand. The basic amino acid motif and one of the Trp motifs were required for inhibiting FGF-2 binding (Table 2). Peptides containing only Trp motifs were uniformly inactive for inhibiting FGF-2 binding (SHWSPWSSC, Table 2; and SPWSEWTSCSTS and GPWSPWDICSVT from the first and third type I repeats of TSP1, results not shown), as were peptides containing the basic motif without the Trp motif (e.g., KRFKQDGG (Table 2) and KRFKQDGGASHASPASSC [Table 3]).

Substitution of the Asp residue at position 6 (peptide **392**) or the Gln and Asp residues at positions 5 and 6 by Ala residues (peptide **419**) improved the solubility of the peptides and also increased their heparin-binding activities (Table 3). Using the latter sequence, substitution of the Phe at position 3 by Ala to eliminate the TGFβ-activating activity of the TSP1 peptide (16) (peptide **450**), resulted in a peptide that retained the enhanced heparin-binding activity. The polysucrose conjugates of the peptides retained the heparin-binding activity of the free peptides (Table 3). Retro-inverso peptides with Ala substitutions showed a fairly direct correlation with the activities of L-forward peptides for inhibiting TSP1 binding. In general, the Ala substitutions increased the inhibitory activity and solubility. Retro-inverso peptides also showed similar activity as forward peptides for inhibiting FGF-2 binding to heparin (Table 3).

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 μM). 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). 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

Peptide	Sequence	IC_{50} (μM)[a]			
		^{125}I-Thrombospondin binding to heparin		^{125}I-FGF-2 binding	BAE cell proliferation
		Peptide	Conjugate	Peptide	Conjugate
407	KRFKQDGGWSWHSPWSSC (TSP1)	0.5	3.5	3.8	0.5
392	KRFKQAGGWSHWSPWSSC	0.3	0.14	0.45	0.12
419	KRFKAAGGWSHWSPWSSCam	0.028	0.04	0.18	0.12
450	KRAKAAGGWSHWSPWSSC	0.03	0.025	0.22	0.22
389	KRFKQDGGASHASPASSC	N.D.	>5	>40	>10
500	NGVQYRNCam	>10	>10	>40	>10
416	ri-amKRFKQDGGWSHWSPWSSCac	0.4	1.0	2.8	0.13
474	ri-amKRAKQDGGWSHWSPWSSCac	0.1	0.3	0.95	N.D.
476	ri-amKRAKQAGGWSHWSPWSSCac	0.03	0.05	0.22	0.1
475	ri-amKRFKQDGGASHASPASSCac	10	>5	>20	4
513	ri-amKRAKQDGGASHASPASSCac	6.2	>5	18	>17

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. IC_{50} 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 L-forward peptides; ac represents an N-terminal acetyl, and am represents a C-terminal amide.

[a]IC_{50} values represent the concentrations of peptides required for 50% inhibition of net binding to immobilized heparin or proliferation of untreated BAE cells.

polysucrose conjugate containing the antiangiogenic procollagen domain peptide (NGVQYRNC) (23) was inactive (Table 3).

Inhibition of breast carcinoma cell proliferation. The peptide conjugates also inhibited proliferation of the human breast carcinoma cell line MDA MB435 (Fig. 4B). In contrast to the endothelial cells, growth of these cells was inhibited by TSP1 but not by TGFβ or by the free peptide KRFK, which activates latent TGFβ. In general, the peptide conjugates inhibited breast carcinoma cell proliferation to a lesser extent than endothe-

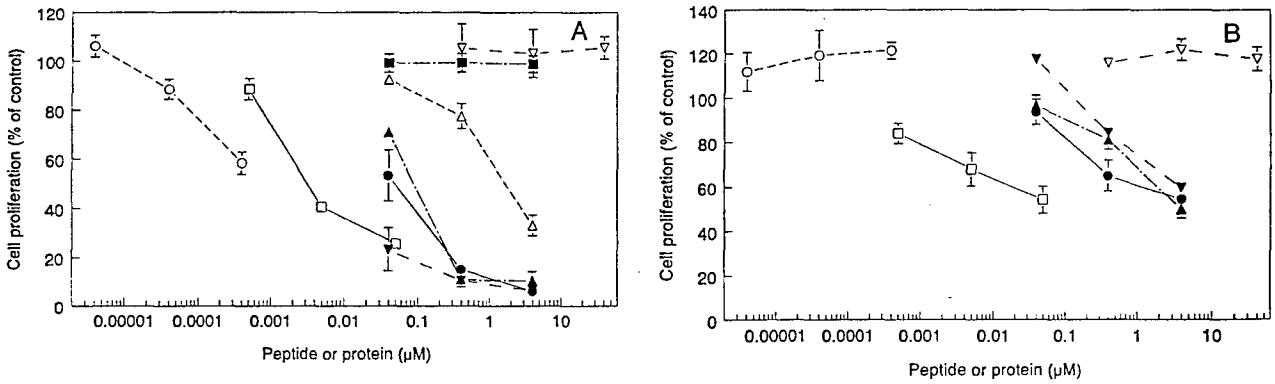


FIGURE 4

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 KRFKQDGGWSHWSPWSSC (●), SHWSPWSSC (△), acKRAKAAGGWSHWSPWSSCam (▼) and ri-amKRFKQDGGWSHWSPWSSCac (▲).

TABLE 4
In vitro screening of antitumor activities of TSP1 peptide analogs

Panel	Peptide **476**		Peptide **476**-polysucrose	
	Susceptible/tested	Mean IC_{50} (range)	Susceptible/tested	Mean IC_{50} (range)
Leukemia	6/6	20 (10–39)	3/6	2.5 (1.0–5.3)
NSCLC	4/9	19 (15–28)	0/9	
Colon	5/6	25 (11–41)	2/6	4.0 (2.3–5.8)
CNS cancer	4/6	15 (14–17)	0/6	
Melanoma	8/8	19 (10–46)	3/8	3.4 (1.0–6.0)
Ovarian	1/6	43	1/6	2.4
Renal	5/8	18 (10–43)	0/8	
Prostate	1/2	17	0/2	
Breast	7/8	15 (8–29)	3/8	2.1 (1.1–2.7)

Free and polysucrose-conjugated forms of peptide **476** (ri-amKRAKQAGGWSHWSPWSSCac) 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 IC_{50} values are presented.

lial cell proliferation, but the doses required for half-maximal inhibition were similar for the two cell types.

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-amKRAKQAGGWSHWSPWSSCac) significantly inhibited growth of many tumor cell lines in the NCI Developmental Therapeutics Program *in vitro* panel (Table 4). As expected, the polysucrose conjugate was more active than the free peptide. Susceptibility varied among the panels, with breast cancer, leukemia and melanoma cell lines showing the broadest inhibition and ovarian cancer lines being generally resistant to growth inhibition.

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 M_r 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-amKRFKQDGGWSHWSPWSSCac, 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-

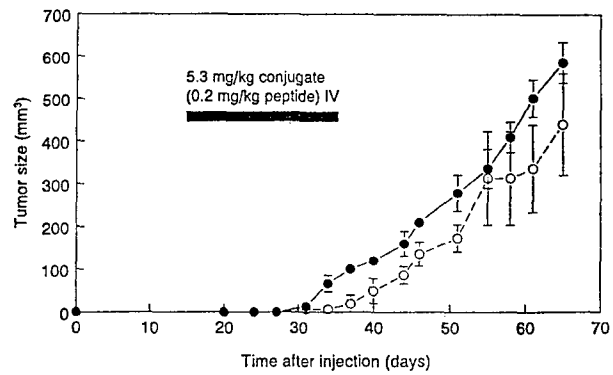


FIGURE 5
Effect of a polysucrose conjugate of the thrombospondin-1 mimetic ri-amKRFKQDGGWSHWSPWSSCac on growth of MDA MB435 human breast carcinoma xenografts in athymic nude mice. Tumor dimensions, determined by external caliper measurements, are presented as a function of time after implantation in 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).

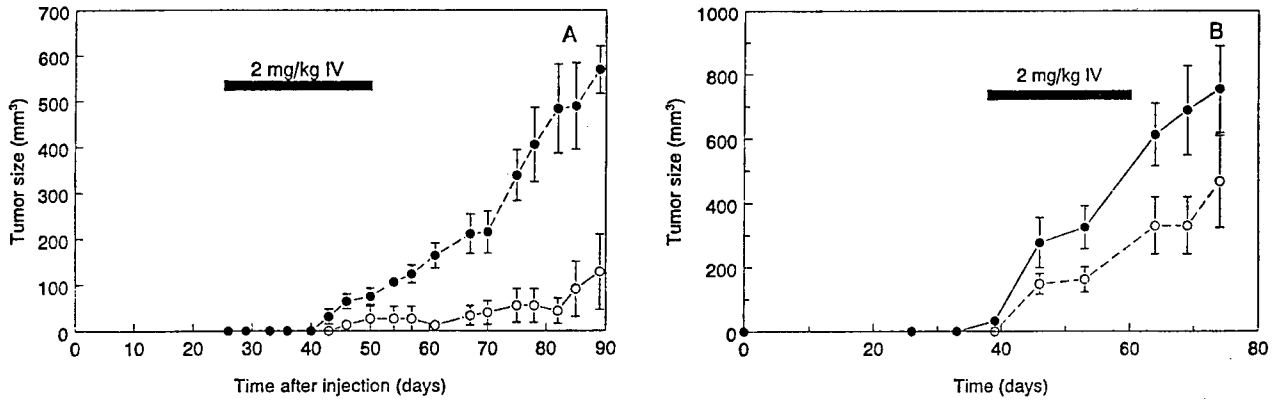


FIGURE 6

Inhibition of MDA MB435 tumor xenograft growth in nude mice by the thrombospondin-1 mimetic ri-amKRFKQDGGWSHWSPWSSCac. Tumor dimensions, determined by external caliper measurements, are presented as a function of time after implantation. Animals were treated by intravenous injection of 2 mg/kg of the peptide in HBSS or a corresponding volume of 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).

tide showed maximal inhibition of tumor growth after treatment with 6 mg/kg of peptide (Fig. 7). Animals were treated with up to 10 mg/kg without overt toxicity, based on relative weight gain in the control and treated groups and gross necropsy performed 10 days after termination of treatment.

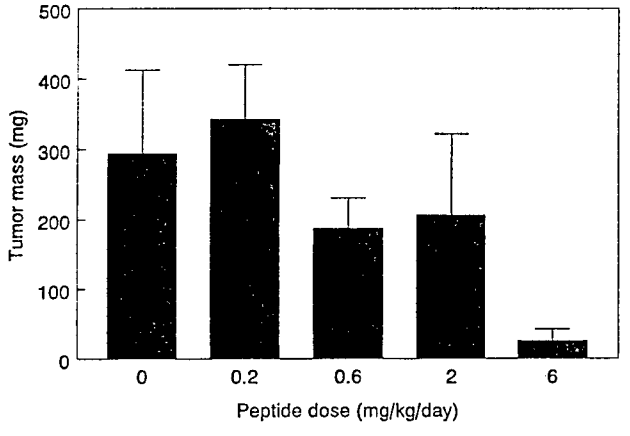


FIGURE 7

Dose dependence for inhibition of MDA MB435 xenograft growth in nude mice by intravenous treatment with the thrombospondin mimetic ri-amKRFKQDGGWSHWSPWSSCac. Animals were implanted with 10^5 MDA 435 cells in one mammary fat pad on day 0. Treated animals were injected daily by tail-vein injection with the indicated doses of peptide dissolved in 0.1 ml of HBSS from day 25 to day 50. Control animals were injected with HBSS alone. Animals were sacrificed on day 67. Tumors were excised and weighed. Results are presented as mean ± SD, $n = 6$. At the 0.6, 2 and 6 mg/kg doses, the p values for the observed inhibition were 0.07, 0.2 and 0.002, respectively, using a two-sided t-test.

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-1 repeat of TSP1 result from antagonism of heparin-dependent growth factors such as FGF-2. This mechanism is supported by the similar activities of the L-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 TSP1 sequence reproduced the antiproliferative activity of TSP1 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 TSP1 peptide analogs *in vitro* does not require the TGFβ-activating sequence KRFK (16), because pepides 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 TSP1 (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,* (25), 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 Roberts, 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. Jiun Woei 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 Chaney 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.

REFERENCES

1. Folkman, J. (1995) *Nat. Med.* **1,** 27–31
2. Weidner, N., Semple, J.P., Welch, W.R. & Folkman, J. (1991) *N. Engl. J. Med.* **324,** 1–8
3. Polverini, P.J. (1995) *Crit. Rev. Oral. Biol. Med.* **6,** 230–247
4. Good, D.J., Polverini, P.J., Rastinejad, F., Le, B.M., Lemons, R.S., Frazier, W.A. & Bouck, N.P. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6624–6628
5. Taraboletti, G., Roberts, D., Liotta, L.A. & Giavazzi, R. (1990) *J. Cell Biol.* **111,** 765–772
6. Iruela Arispe, M., Bornstein, P. & Sage, H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5026–5030
7. Bagavandoss, P. & Wilks, J.W. (1990) *Biochem. Biophys. Res. Commun.* **170,** 867–872
8. Lane, T.F., Iruela Arispe, M. & Sage, E.H. (1992) *J. Biol. Chem.* **267,** 16736–16745
9. Vogel, T., Guo, N.H., Guy, R., Drezlich, N., Krutzsch, H.C., Blake, D.A., Panet, A. & Roberts, D.D. (1994) *J. Cell. Biochem.* **54,** 299–308
10. Vogel, T., Guo, N.H., Krutzsch, H.C., Blake, D.A., Hartman, J., Mendelovitz, S., Panet, A. & Roberts, D.D. (1993) *J. Cell Biochem.* **53,** 74–84
11. Bornstein, P. & Sage, E.H. (1994) *Methods Enzymol.* **245,** 62–85
12. Bornstein, P. (1992) *FASEB J.* **6,** 3290–3299
13. Lahav, J. (1993) *Thrombospondin.* CRC Press, Inc., Boca Raton
14. Bornstein, P. (1995) *J. Cell Biol.* **130,** 503–506
15. Schultz-Cherry, S. & Murphy-Ullrich, J.E. (1993) *J. Cell Biol.* **122,** 923–932
16. Schultz-Cherry, S., Chen, H., Mosher, D.F., Misenheimer, T.M., Krutzsch, H.C., Roberts, D.D. & Murphy-Ullrich, J.E. (1995) *J. Biol. Chem.* **270,** 7304–7310
17. Roberts, D.D. (1996) *FASEB J.* **10,** 1183–1191
18. Zabrenetzky, V., Harris, C.C., Steeg, P.S. & Roberts, D.D. (1994) *Int. J. Cancer* **59,** 191–195
19. Dameron, K.M., Volpert, O.V., Tainsky, M.A. & Bouck, N. (1994) *Science* **265,** 1582–1584
20. Weinstat-Saslow, D.L., Zabrenetzky, V.S., VanHoutte, K., Frazier, W.A., Roberts, D.D. & Steeg, P.S. (1994) *Cancer Res.* **54,** 6504–6511
21. Volpert, O.V., Stellmach, V. & Bouck, N. (1995) *Breast Cancer Res. Treat.* **36,** 119–126
22. Sheibani, N. & Frazier, W.A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6788–6792
23. Tolsma, S.S., Volpert, O.V., Good, D.J., Frazier, W.A., Polverini, P.J. & Bouck, N. (1993) *J. Cell Biol.* **122,** 497–511
24. Murphy-Ullrich, J.E., Schultz-Cherry, S. & Hook, M. (1992) *Mol. Biol. Cell* **3,** 181–188
25. Braatz, J.A., Yasuda, Y., Olden, K., Yamada, K.M. & Heifetz, A.H. (1993) *Bioconjug. Chem.* **4,** 262–267
26. Komazawa, H., Saiki, I., Nishikawa, N., Yoneda, J., Yoo, Y.C., Kojima, M., Ono, M., Itoh, I., Nishi, N., Tokura, S. & Azuma, I. (1993) *Clin. Exp. Metastasis* **11,** 482–491
27. Chorev, M. & Goodman, M. (1993) *Acc. Chem. Res.* **26,** 266–273
28. Chorev, M. & Goodman, M. (1995) *Trends Biotechnol.* **13,** 438–445
29. Nomizu, M., Utani, A., Shiraishi, N., Kibbey, M.C., Yamada, Y. & Roller, P.P. (1992) *J. Biol. Chem.* **267,** 14118–14121
30. Zhao, M., Kleinman, H.K. & Mokotoff, M. (1994) *J. Med. Chem.* **37,** 3383–3388
31. Roberts, D.D., Cashel, J. & Guo, N. (1994) *J. Tissue Cult. Meth.* **16,** 217–222
32. Guo, N.H., Krutzsch, H.C., Négre, E., Vogel, T., Blake, D.A., & Roberts, D.D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3040–3044
33. Inman, J.K. (1975) *J. Immunol.* **114,** 704–709
34. Brunswick, M., Finkelman, F.D., Highet, P.F., Inman, J.K., Dintzis, H.M. & Mond, J.J. (1988) *J. Immunol.* **140,** 3364–3372
35. Guo, N.H., Krutzsch, H.C., Négre, E., Zabrenetzky, V.S. & Roberts, D.D. (1992) *J. Biol. Chem.* **267,** 19349–19355
36. Kosfeld, M.D. & Frazier, W.A. (1992) *J. Biol. Chem.* **267,** 16230–16236
37. Danielpour, D., Dart, L.L., Flanders, K.C., Roberts, A.B. & Sporn, M.B. (1989) *J. Cell Physiol.* **138,** 79–86
38. Grever, M.R., Schepartz, S.A. & Chabner, B.A. (1992) *Semin. Oncol.* **19,** 622–638
39. Kosfeld, M.D. & Frazier, W.A. (1993) *J. Biol. Chem.* **268,** 8808–8814
40. Gao, A.-G., Lindberg, F.P., Finn, M.B., Blystone, S.D., Brown, E.J. & Frazier, W.A. (1996) *J. Biol. Chem.* **271,** 21–24

41. Volpert, O.V., Tolsma, S.S., Pellerin, S., Feige, J.J., Chen, H., Mosher, D.F. & Bouck, N. (1995) *Biochem. Biophys. Res. Commun.* **217,** 326–332
42. Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G. & Merrifield, R.B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4761–4765
43. Dooley, C.T., Chung, N.N., Wilkes, B.C., Schiller, P.W., Bidlack, J.M., Pasternak, G.W. & Houghten, R.A. (1994) *Science* **266,** 2019–2022
44. Guo, N.H., Krutzsch, H.C., Inman, J.K. & Roberts, D.D. (1997) *Cancer Res.*, in press
45. Pierson, B.A., Gupta, K., Hu, W.-S. & Miller, J.S. (1996) *Blood* **87,** 180–189
46. Riser, B.L., Mitra, R., Perry, D., Dixit, V. & Varani, J. (1989) *Cancer Res.* **49,** 6123–6129

Address:

Dr. *David D. Roberts*
Building 10, Room 2A33
National Institutes of Health
10 Center Dr., MSC 1500
Bethesda, MD 20892-1500
USA
Phone: 301-496-6264
Fax: 301-402-0043
E-mail: droberts@helix.nih.gov

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

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

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be changed to "Approved for public release; distribution unlimited"

Grant Number	Accession Document Number
DAMD17-94-J-4147	ADB221256
DAMD17-93-C-3098	ADB231640
DAMD17-94-J-4203	ADB221482
DAMD17-94-J-4245	ADB219584
DAMD17-94-J-4245	ADB233368
DAMD17-94-J-4191	ADB259074
DAMD17-94-J-4191	ADB248915
DAMD17-94-J-4191	ADB235877
DAMD17-94-J-4191	ADB222463
DAMD17-94-J-4271	ADB219183
DAMD17-94-J-4271	ADB233330
DAMD17-94-J-4271	ADB246547
DAMD17-94-J-4271	ADB258564
DAMD17-94-J-4251	ADB225344
DAMD17-94-J-4251	ADB234439
DAMD17-94-J-4251	ADB248851
DAMD17-94-J-4251	ADB259028
DAMD17-94-J-4499	ADB221883
DAMD17-94-J-4499	ADB233109
DAMD17-94-J-4499	ADB247447
DAMD17-94-J-4499	ADB258779
DAMD17-94-J-4437	ADB258772
DAMD17-94-J-4437	ADB249591
DAMD17-94-J-4437	ADB233377
DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900

Encl