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GRANT NUMBER DAMD17-94-J-4499

TITLE: Regulation of Breast Carcinoma Growth and
Neovascularization by Novel Peptide Sequences In Thrombospondin

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REPORT DATE: October 1996

TYPE OF REPORT: Annual

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

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19970327 010

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REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1996	3. REPORT TYPE AND DATES COVERED Annual (30 Sep 95 - 29 Sep 96)	
4. TITLE AND SUBTITLE Regulation of Breast Carcinoma Growth and Neovascularization by Novel Peptide Sequences in Thrombospondin			5. FUNDING NUMBERS DAMD17-94-J-4499	
6. AUTHOR(S) David D. Roberts, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) National Institute 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 96). Other requests for this document shall be referred to Commander, U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RMI-S, Fort Detrick, Frederick, 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 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 <i>in vivo</i> and behavior <i>in vitro</i> .				
14. SUBJECT TERMS Breast Cancer extracellular matrix, thrombospondin, angiogenesis, site-directed mutagenesis, peptidomimetics, growth factor antagonists, apoptosis			15. NUMBER OF PAGES 45	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited	

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INTRODUCTION

Growth of many solid tumors is strongly dependent on recruitment of neovascularization. Increased vascularization of primary breast tumors has been associated with increased rates of metastasis to lymph nodes and poorer prognosis (1, 2). 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, 31).

The major identified simulators of angiogenesis produced by tumors are basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial cell growth factor (VEGF). These are potent simulators 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, and a proteolytic fragment of fibronectin (11). 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, 12).

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 13, 14). Thrombospondin-1 (TSP) is the product of the *THBS1* gene (15) 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 (16). 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 (17) and is a normal component of human milk (18). 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 (19). However, TSP is also expressed in the basement membrane of normal myoepithelial cells, and most invasive ductal carcinoma cells do not express TSP (20). High expression of TSP in breast carcinoma is restricted to invasive lobular carcinoma (20). 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 (21).

TSP may play a role in several steps in the metastatic cascade. We have shown that TSP promotes tumor cell adhesion and motility (22, 23), which are important in several steps of metastasis. Thrombospondin enhances melanoma cell interactions with platelets(24), 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 (25, 26), and so could regulate tumor invasion through matrix. Finally, we and others have recently identified TSP as an inhibitor of angiogenesis (6, 5, 27), 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* (28, 29). 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 (30). Inhibition of adhesion to fibronectin is associated with disruption of focal adhesion contacts (31). 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* (27, 48) 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. (32), 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, 12]. 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 [33] 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 TGF β .

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 (22, 23). The carboxyl-terminal domain mediates attachment and haptotaxis, and the amino-terminal domain mediates cell spreading and chemotaxis (22, 23]. 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 [34]. Integrin and non-integrin receptors for the carboxyl-terminus of TSP have been characterized in several types of tumor and normal cells (35, 36, 37).

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 (38, 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 overexpression 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* (6, 5, 48), 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, 12). 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 signalling 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* (11) and *in vivo* (49).

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 (50, 51, 52). 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 53). 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* (54). 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 (43). 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 C₁₈ 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 (55). 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 μ l 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 $\epsilon = 5540 \text{ M}^{-1}\text{cm}^{-1}$ per Trp residue.

Ligand binding assays -- TSP binding to heparin-BSA was determined using a solid phase assay. Heparin-BSA (0.075 μ g/well) was adsorbed onto 96-well polyvinyl chloride microtiter plate wells by incubation in 50 μ l 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 μ l of various concentrations of inhibitors diluted in tris-BSA buffer or buffer alone and 30 μ l of ^{125}I -TSP (0.1-0.2 μ g/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 (56), 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 (14). 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 labelling the cells with bromodeoxyuridine and exposure to the peptides for 24 h.

Tumorigenesis assay in nude mice-- NIH Nu/Nu 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 μ l 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 1/2" needle, 10 μ l of cell suspension, 1×10^5 cells in HBSS, 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 (57). This method introduces a mutation directly into the double stranded plasmid template. Briefly, a frame of single stranded DNA encompassing only the region of interest is created within a double stranded plasmid (Fig. 1). The mutagenic oligonucleotide is used as a primer for the Klenow fragment of DNA Polymerase I that synthesizes the second strand of the target region and ligation is done using T4 DNA ligase. The schematic representations of the methods used are shown in the figure (Fig. 1). The ligation mixtures were transformed into a *mutS* strain of E. coli, BMH 17- 81, with a defect in mismatch repair.

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

THBS W385A - Primers span bases 1025 to 1264 yielding a fragment size of 239 bp.

Forward primer : 5'- ATGAGCTGAGGCGGCC- 3'

Reverse Touchdown primer : 5'- AGGTCCACTCGGACGC- 3'

THBS W441A -Primers span bases 1250 to 1432 yielding a fragment size of 182 bp.

Forward primer : 5'- GGTCCGAGTGGACCTCCTG- 3'

Reverse Touchdown primer : 5'- ATGACCACGGGGACGC- 3'

THBS W498G - Primers span bases 1349 to 1605 yielding a fragment of 256 bp.

Forward primer : 5'- TCCAGACACGGACCTGC- 3'

Reverse Touchdown primer : 5'- GATGTCCCATGGTGACCC- 3'

THBS F432A - Primers span bases 1250 to 1405 yielding a fragment size of 155 bp.

Forward primer is the forward primer used to make THBS W441A

Reverse Touchdown primer : 5'- CACCATCCTGTTTAGC- 3'

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

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

Transfection--MDA435s cells were transfected by electroporation using 10 μ g 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 μ g/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 μ l of PBS by incubating overnight at 4°. A 50 μ l 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 μ l 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 μ l was added to each well and incubated for 7-10 minutes. Development was stopped by addition of 100 μ l 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 1×10^5 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.

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

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

Further purification of thrombospondin was carried out by gel filtration using Sephacryl S-300. A 35 ml column was packed with Sephacryl S-300 and equilibrated with 10 mM Tris, pH 7.5 containing 150 mM NaCl, 0.1 mM CaCl₂, 0.1 mM PMSF, 5 mM benzamidine and 1 mM N-ethylmaleimide. Approximately 1.5 ml of the partially purified thrombospondin from the HiTrap heparin column was loaded on to the column. The column was run at 0.5 ml/min and 1ml fractions were collected. Protein containing fractions as monitored by absorbance at 280 nm from the void volume peak were pooled and stored at -70° C.

Transwell endothelial cell sprouting assays-- MDA-435 cells and transfectants (TH26, FA pool, WA pool) were grown to confluency in RPMI 1640 media supplemented with 10% fetal calf serum, 2mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Biofluids, Gaithersburg, MD). Transfectants were cultured with the addition of 700 µg/ml G418 (Gibco-BRL, Gaithersburg, MD). Bovine aortic endothelial (BAE) cells were grown to subconfluency in complete DMEM containing 10% fetal calf serum, 1.0 mg/ml glucose, 2mM glutamine, 50 µg/ml ascorbic acid, 100 units/ml penicillin, and 100 µg/ml streptomycin.

On day one, a subconfluent BAE culture was washed once with PBS (GIBCO-BRL) and 0.2% EDTA; trypsinized; diluted in complete media; and plated at a density of 40,000 cells/well in a 24 well plate (Costar, Cambridge, MA). The final volume of media in each well was 500 µl. Cells were incubated overnight at 37° C and 5% CO₂.

On Day two, confluent transfectant cultures were washed once with PBS/EDTA; trypsinized; diluted in complete endothelial cell media; and 100 µl of a 50,000 cell/ml suspension was added to a 0.4 µm Transwell membrane (NUNC, Naperville, IL). The Transwell membranes and 400 µl of complete BAE media were added to each well before the addition of cells. All

conditions were preformed in triplicate.

Cells were fed every two days. 0.5 ml of media was removed from each well, and replaced with 0.5 ml of fresh complete BAE cell media. On day 8, Transwells were removed, and images of BAE cells were captured using ImagePro Plus.

RESULTS

We have continued 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. Based on retro-inverso peptide analogs shown previously to have strong antiproliferative activities *in vitro* and *in vivo*, we have further examined the apoptosis response to the peptides and shown that native TSP has the same activity. In addition, we further examined the effect of aggregation on activity of the peptides and developed a synthetic method to produce peptide without aggregation. For specific aims 2 and 3, we have prepared full length THBS1 cDNA expression vectors containing four point mutations. These constructs have been thoroughly verified, and several stable transfected cell lines expressing high levels of two of these constructs have been prepared. These have been tested *in vivo* for tumorigenesis and *in vitro* for effects on endothelial cell behavior. We have also purified the recombinant thrombospondins secreted in serum-free medium by two of these cell lines and begun the characterization of their biochemical properties and biological activities.

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 at Trp(385)Ala, Trp(441)Ala, Trp(498)Gly, and Phe(432)Ala.

Touchdown PCR of mutant PCR fragments of thrombospondin using the respective specific primers showed distinct differences in the pattern of amplified products compared to native THBS sequence amplified using the same set of primers (Fig. 2). Lanes 2, 4 and 6 show the correct size product being amplified for the mutant PCR fragments. Comparing these to lanes 3, 5 and 7 respectively, either only the mutant product gets amplified (lane 4 versus lane 5) or there is a difference in the patterns of mutant versus wild type (e.g. lanes 2 and 3). Once we established that we could differentiate mutant sequences using Touchdown PCR, we screened the bacterial colonies from the linker scanning method of mutagenesis. Figs. 3 and 4 show agarose gel electrophoreses of Touchdown PCR products from bacterial colonies screened for mutant sequences THBS W441A and THBS F432A. The positive clones were then sequenced between the fill-in sites to make sure other errors were not introduced by the enzyme used in the reaction. The mutant clone THBS W441A has a G > T substitution which changed a. a. 58 from Ala > Ser, and all other plasmids encoded the native TSP sequence except for the desired mutations.

Transfections-- Electroporation and an improved selection protocol (58) were substituted for the previous calcium phosphate method (47) 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 (Fig. 5). Using peroxidase conjugated secondary antibody and o-phenylenediamine for development, conditioned serum free media from 20 clones were screened in one day. Clones with high expression identified by this assay were re-screened by Western blotting to verify the size of the recombinant TSP secreted by the cells using a new antibody raised to reduced and alkylated TSP (Fig. 6). In previous transfections using wild type pCMVTHBS1, a single clone was obtained that had undergone rearrangement resulting in expression of a truncated TSP (47). In the present screenings we identified several additional clones with similar rearrangements (e.g. Fig 6, clone A.A11). Several of these were saved for examining the activity of type I repeat mutants in the context of C-terminal deletions, which we have previously shown to abrogate the anti-tumor activity of TSP over expression. 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.

Three transfectant clones of MDA MB435 with high levels of TSP expression, AA11, AE9 and EA3 from THBS W441A transfection and AE10, DE8 and GD3 from THBS F432A transfection, were selected for *in vivo* animal experiments and *in vitro* functional assays. In the animal experiment, each group had 8 animals that received one clone of transfected cells. After 84 days, the animals were sacrificed and the tumors were removed. Fig. 7A and 7B show the growth curves for the different clones in athymic nude mice. Clones from THBS W441A transfection had the same or larger tumor masses compared to controls (Fig. 7C), whereas those from THBS F432A clones were smaller than controls.

Histopathological analyses of these tumors showed that the tumors produced by the W441A clone producing the largest tumors, E.A3, had relatively little necrotic area in the tumors compared to the control transfectants, although the former tumors were much larger. The lack of necrosis in these large tumors implies that angiogenesis in the W441A tumors is more efficient than in the control transfectants. Examination of the lung sections showed that 7 out of 8 (87%) of the W441A clone E.A3 had lung metastases, whereas none of the control animals had detectable lung metastases.

Clones selected as mentioned above were analyzed by Northern blotting to verify that the increased TSP expression is due to the transgene and not the activation of the endogenous *THBS1* gene. The transfectant cell lines were also analyzed by RT-PCR to confirm they had the plasmid-derived TSP mRNA. In the first step of RT-PCR, an antisense primer was annealed to the mRNA at the 5' end of the thrombospondin sequence which would help transcription of the sequence upstream of the primer. The single stranded cDNA was then subjected to PCR using a nested antisense primer and a sense primer from the rabbit beta globin gene which is immediately upstream of the *THBS* gene in our expression vector. The rationale for doing this RT-PCR was to amplify only the transcript that was derived from the expression vector and not the endogenous *THBS* gene. Tumor samples were also analyzed by RT-PCR for the plasmid-derived mRNA to verify that expression of the mutated TSP was retained throughout tumor growth in the animals.

Figure 8 shows the products of RT-PCR of tumor RNA from animals which received *THBS* W441A clones. Every one of the tumors showed the expected product, although the amount of expression varied from tumor to tumor. The mean expression of the plasmid-derived TSP RNA in the tumors shown in lanes 3, 4 and 5 are lower than the mean from tumors shown in lanes 6, 7 and 8. Tumors in lanes 3, 4 and 5 and 6, 7 and 8 came from mice injected with clones AA11 and EA3 of *THBS* W441A respectively. This is interesting because while wild-type TSP expression in tumors has shown to produce smaller primary tumors and a reduction in capillary densities (47, 58) the tumors from the clone AA11 with a mutation in the second Type I repeat are bigger compared to the ones from other clones. Immunohistological examination of the tumors and the adjacent tissues might shed more light on the effect of the mutation on metastasis.

Purification of mutant thrombospondin from MDA-conditioned medium and analysis of purified fractions by SDS-PAGE

MDA-conditioned medium was passed through the heparin affinity column and the column was washed with a buffer containing 0.35 M NaCl as described under methods. During this step several proteins were removed from the column (Fig. 9), none of which was found to bind thrombospondin polyclonal antibody on a Western blot (Results not shown). However, when the column was further eluted with 0.6 M NaCl containing buffer, several proteins including thrombospondin was eluted from the column. Fractions from this step were combined and used for further purification by gel filtration. An aliquot of this sample subjected to electrophoresis on an SDS-polyacrylamide gel and stained with Coomassie Blue is shown in Fig. 11; lane 1.

The partially purified thrombospondin from heparin affinity column was further purified using Sephacryl S-300 gel filtration. Several fractions showing strong absorbance at 280 nm was eluted from the column in the void volume (Fig. 10). These fractions were combined and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 11). This fraction contained the thrombospondin band (Fig 11; lane 2), migrating to the same position as purified human thrombospondin (Fig 11; lane 4). Fractions eluted later to the void volume fractions contained mostly small molecule weight proteins (Fig. 11; lane 3). The purified thrombospondin, when analyzed by electrophoresis, contained a band migrating slightly above the thrombospondin band (see Fig. 11; lane 2). This band was not present in the sample that was loaded on to the column. In addition, this band did not show any positive reaction to thrombospondin antibodies in Western blots (Result not shown), suggesting that it may be a polysaccharide from the column material or an artifact from the staining procedure. Currently we are looking at ways to eliminate this artifact to obtain thrombospondin purified to homogeneity.

The functional differences of the proteins secreted by these two clones were studied for activity to promote MDA cell adhesion and to inhibit BAE cell proliferation. Figure 12 shows that, compared to the adhesion to wild-type TSP secreted by clone TH26, adhesion of MDA cells to the two mutant TSPs is only 25%. The mutant proteins partially purified by heparin affinity chromatography inhibited BAE cell proliferation to a similar extent (Fig. 13). Following further purification by gel filtration, the F432A mutant protein showed the maximum inhibition of BAE cell proliferation of about 80% compared to the control.

Transwell endothelial cell sprouting assay

In our previous work, over-expression of TSP in MDA cells had no detectable effect on any *in vitro* behavior of the breast cancer cell line, including growth rate, motility, or ability to

form colonies in soft agar (47, 58). Because our hypothesis is that TSP secreted by the tumor cells inhibits neovascularization of the tumors *in vivo*, we examined co-cultures of these two cell types to reproduce this interaction *in vitro*. Preliminary experiments demonstrated that wild type MDA cells grown in Transwell chambers over confluent monolayers of BAE cells induced sprouting of the BAE cells, a behavior that has been correlated with early events during angiogenesis *in vivo* (27, 48?). Similar cultures using the TSP-over expressing clone TH26 failed to induce sprouting, whereas the control transfectant C5 induced similar sprouting as the untransfected MDA cells. This assay therefore, may replicate the tumor endothelial cell interactions during the tumorigenesis assay that result in the suppressive activity of TSP over expression in the tumor.

This recently developed assay was used to examine the effects of over expression of TSP mutants on endothelial cell sprouting (Fig. 14). Although the magnitude of the response was less than for TH26, both mutant transfectants inhibited sprouting relative to the control. Thus both mutant TSPs retain some anti-angiogenic activity in this assay.

Synthetic peptides

A peptide derived from the second type I repeat of TSP, KRFKQDGGWSHWSPWSS, inhibits heparin binding to TSP (43), activates latent TGF β (13), and inhibits proliferation of endothelial cells stimulated by FGF-2 (14). 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.

During further characterization of these peptides, we observed that peptides containing Trp and basic amino acid residues 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 (Fig 15A). Ability to antagonize TSP binding to heparin increased with increasing molecular weight of the aggregates (Fig. 16), although the monomer also retained activity. Using purified monomeric peptide, we observed that treatment with trifluoroacetic acid (TFA) resulted in formation of some aggregates. This suggested that exposure to TFA during synthesis or cleavage from the resin was responsible for the aggregation. Because acidic conditions may induce dimerization of Trp-containing peptides, we synthesized the same peptide using N- α -BOC-N-formyl-D-tryptophan to prevent electrophilic attack on the indole ring. The resulting peptide (557) had similar heparin binding activity as the original peptide (529) but was less aggregated when analyzed by gel filtration (Fig. 15B). The major peak of the peptide comigrated with the purified monomer fraction from peptide 529 (Fig. 15C). Use of fmoc synthesis was used as a second approach to prepare the peptide in monomeric form. Peptide prepared by this chemistry is not exposed to strongly acidic conditions during synthesis and produced mostly monomeric peptide (Fig. 15D).

Induction of Apoptosis by Peptides and Thrombospondin: We 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 [Guo, #953]. Although the peptides also inhibited 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 towards endothelial cells or that they trigger programmed death of these cells. We have further examined the effects of the peptides on endothelial and breast carcinoma cells and report here that the thrombospondin peptides specifically induce apoptosis in endothelial cells and that this activity is independent of their ability to activate latent TGF β .

Analysis of low molecular weight DNA extracted from endothelial cells treated with the active TSP1 peptides demonstrated a characteristic ladder pattern resulting from inter-nucleosomal cleavage of the genomic DNA (Fig. 17). Polysucrose conjugates containing 0.4 μ M 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 1) 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, as 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 μ g/ml TSP1 did not result in detectable DNA fragmentation by this method (lane h).

An ELISA assay for detecting DNA fragmentation was used to quantify the activity of the peptides. Based on this sensitive and quantitative assay for DNA fragmentation, both TSP1 and the type I repeat peptides induced significant DNA fragmentation in endothelial cells (Fig. 18A). 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, as no DNA fragmentation was induced in MDA435 breast carcinoma cells by the peptide conjugates (Fig. 18B). DNA fragmentation was induced in MDA435 cells by the topoisomerase I inhibitor camptothecin, indicating that these cells can initiate programmed cell death. Since the same peptides inhibited growth of the MDA cells [Guo, #953], induction of apoptosis can be independent of the anti-proliferative 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. 18B). The magnitude of the peptide response was similar to the DNA fragmentation induced in the same cells by camptothecin.

CONCLUSIONS

Expression constructs containing four directed mutations of the type I repeat sequences in TSP have been prepared. Stably transfected human breast carcinoma cell lines have been prepared expressing the mutants FA and WA. Whereas disrupting the essential Phe residue for TGF β activation did not alter the anti-tumor activity of TSP over expression, 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. 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*.

Our new endothelial morphogenesis assay will be useful for characterizing the nature and magnitude of endothelial cell responses to MDA435 cells expressing mutant thrombospondins.

These will be compared to responses of the same cells to the purified mutant proteins to determine which responses are directly mediated by the mutant proteins and which may be mediated by activation or modulation of activities of growth factors secreted by the tumor cells and modulating sprouting of the endothelial cells. Stable transfectants expressing corresponding mutants from the first and third type I repeats are being prepared and compared to the activities of the second type I repeat mutation to define the relative role of each repeat in the activities of intact TSP.

Several retro-inverso analogues of the TSP type I sequence have been prepared with increased potency and specificity of inhibiting FGF-2 dependent proliferation of endothelial cells and binding of FGF-2 to heparin/heparan sulfate. These peptides were also shown to specifically induce apoptosis of endothelial cells. Both the anti-proliferative and apoptotic activities of these sequences are independent of the TGF β activating activity of the type I repeats. Inhibitory activity in *in vitro* assays and anti-tumor activity in a breast cancer xenograft model were found to be dependent on the aggregation state of the peptide analogs. Aggregated forms of the peptides showed potent anti-proliferative activity *in vitro* and significantly inhibited growth of established breast carcinomas in nude mice. Future studies will optimize dose, schedule, and route of administration of these molecules and mechanism of action *in vivo*.

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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

FIGURE LEGENDS

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

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

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

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

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

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

Figure 7. Panel A: growth curves for MDA435(F432A) transfectants in athymic nude mice. Panel B: growth curves for MDA435(W441A) transfectants in athymic nude mice; Panel C: Tumor mass at sacrifice on day 84.

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

Figure 9: HiTrap heparin affinity chromatography. MDA-conditioned medium was passed through a 1 ml heparin affinity column and the column was washed with Tris buffer as described under methods. The column was run at 1 ml/min and 0.5 ml fractions were collected. Buffers containing 0.35 M NaCl and 0.65 M NaCl in the starting buffer were used for the elution of bound materials. The points at which these buffers were used are indicated by the down arrow.

Figure 10. Sephacryl S-300 gel filtration. Fractions eluted with 0.6 M NaCl containing buffer from the heparin affinity column were pooled, concentrated and used for purification by gel filtration. About 1.5 ml of the concentrated sample was loaded and the column was run at 0.5 ml/min. Fractions of 1ml each were collected.

Figure 11. SDS polyacrylamide gel electrophoresis. Samples from the HiTrap heparin affinity column and Sephacryl S-300 gel filtration column are analyzed by electrophoresis on a 4-15% polyacrylamide gradient gel. Samples were boiled in the presence of β -mercaptoethanol before loading on the gel. The gel was stained with Coomassie blue. Lane 1, unconcentrated 0.6 M eluate from HiTrap heparin affinity column; lane 2, an aliquot from the void volume peak (concentrated 10 fold); lane 3, an aliquot of the peak that followed the void volume fraction (concentrated 10 fold); lane 4, human thrombospondin purified from platelets; and lane 5, molecular weight markers.

Figure 12: Cell adhesion assay showing MDA MB435 binding to different expressed protein at a concentration of 5 ug/ml. TH26, wild-type TSP expressed in MDA MB435 cells; TH50, a spontaneous truncated mutant TSP; F432A, thrombospondin fraction purified from the conditioned medium of MDA cells transfected with THBS F432A; W441A, thrombospondin fraction purified from the conditioned medium of MDA cells transfected with THBS W441A; MDA CM, proteins purified from the conditioned medium of wild-type MDA cells.

Figure 13: Proliferation assay using bovine aortic endothelial cells with mutant thrombospondins. Serial dilutions of the partially purified W441A ($\blacktriangle$); F432A (Δ) and gel filtration column purified F432A (O) proteins were used in this assay.

Figure 14: Transwell assay of tumor stimulation of endothelial cell sprouting. The indicated MDA435 transfectants were co-cultured with BAE cells separated by Transwells. The micrographs show induction of sprouting by wild type MDA cells which is suppressed in the TH26 transfectant over expressing TSP.

Figure 15. Aggregation state of peptide 529 (rv-amKRAKQAGWSHWAAac) prepared by alternative synthetic methods. Peptide 529 was prepared by standard tBOC chemistry (Panel A), by tBOC chemistry using N- α -BOC-N-formyl-D-tryptophan (Panel B), or by FMOC chemistry (Panel D). 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. Panel C shows rechromatography of purified monomer prepared from the crude peptide in panel B.

Figure 16. Effect of peptide aggregation state on activity of peptide 529. Isolated fractions of peptide 529 obtained as shown in panel A of figure 12 were tested for ability to inhibit thrombospondin binding to heparin.

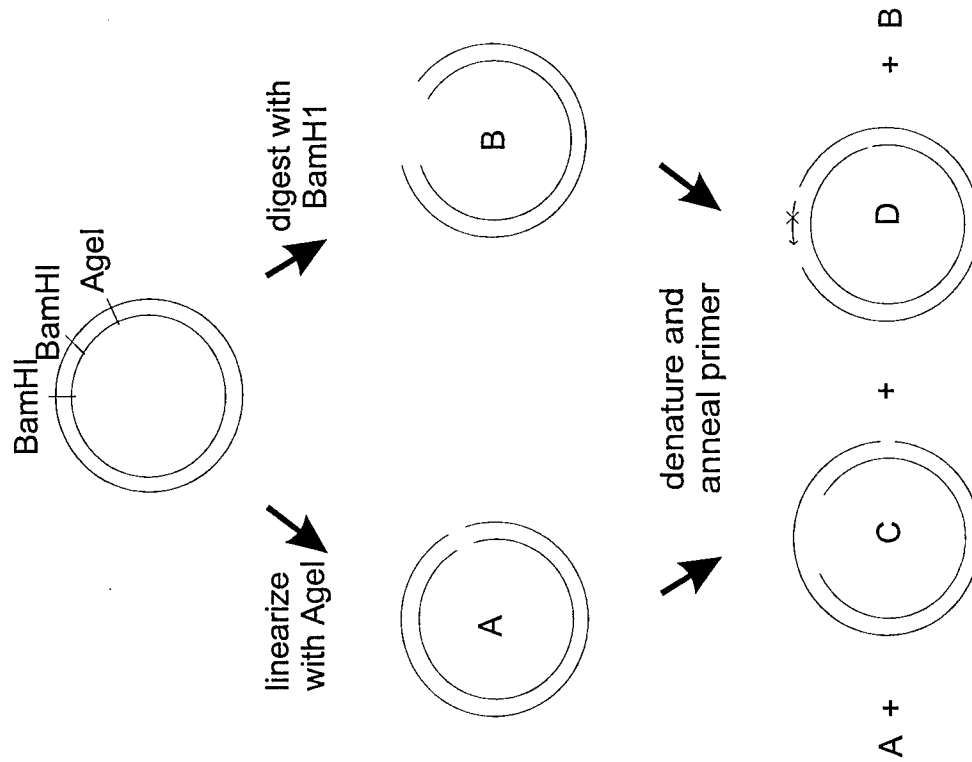
Figure 17. Type I repeat peptide analogs from TSP1 induce DNA fragmentation in aortic endothelial cells. BAE cells (5×10^5) were treated with 0.4 μ M of the indicated TSP1 peptide

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: KRFKQDGGASHASPSSC, lane a; KRFKQDGGWSHWSPWSSC, lane b; retro-inverso amKRFKQDGGWSHWSPWSSCac, lane c; GGWSHWSPWAAC, lane d; KRAKAAGGWSHWSPWSSC, lane e; NGVQYRNC, lane f; retro-inverso amKRFKQDGGWSHWSPWSS-thiopropionyl, lane g; TSP1, lane h; and control, lane I. Migration of DNA size markers is indicated in the left margins.

Fig. 18. Detection of TSP1 and peptide-induced apoptosis in bovine aortic endothelial and human breast carcinoma cells by a DNA fragment ELISA. DNA fragmentation in BAE cells (panel A), MCF7 breast carcinoma cells (panel B, solid bars), or MDA435 breast carcinoma cells (panel B, striped bars) was quantified by an ELISA assay using bromodeoxyuridine labeled cells. Target cells (10 ml; 2×10^5 cells/ml) were labeled overnight using 10 µM bromodeoxyuridine. 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 TGFβ) or medium only (Control). After incubation for 24 h at 37°, the cells in the wells were lysed and centrifuged. Released DNA fragments in 100 µl of the supernatant was quantified using a sandwich ELISA using anti-DNA capture antibody and anti-BrdU peroxidase conjugate for detection. After washing, immune-complexed anti-bromodeoxyuridine peroxidase was detected using TMB substrate. Absorbance was measured at 450 nm and is presented as mean $\pm$ SD, n=3.

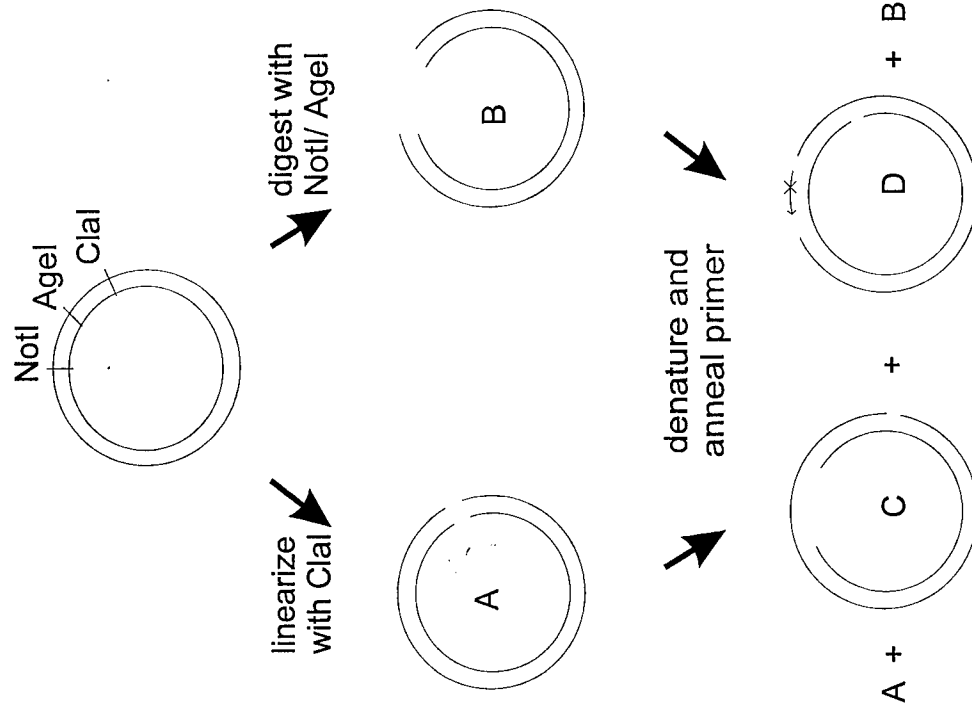
Fig. 1

Type I repeat # 1, 2 and TGF- β activation
sequence mutations



Fill in with Klenow, ligate and transform.

Type I repeat # 3 mutation



Fill in with Klenow, ligate and transform.

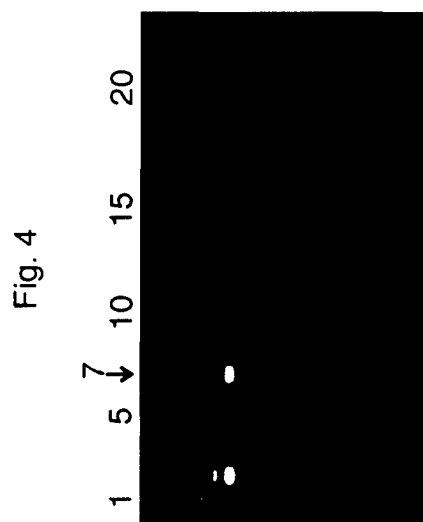
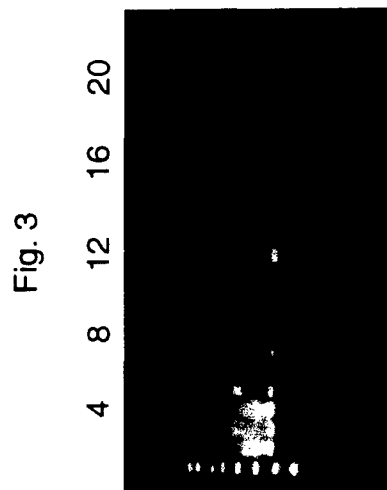
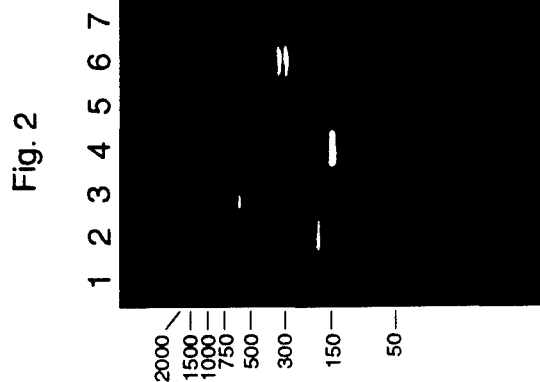


Fig. 5

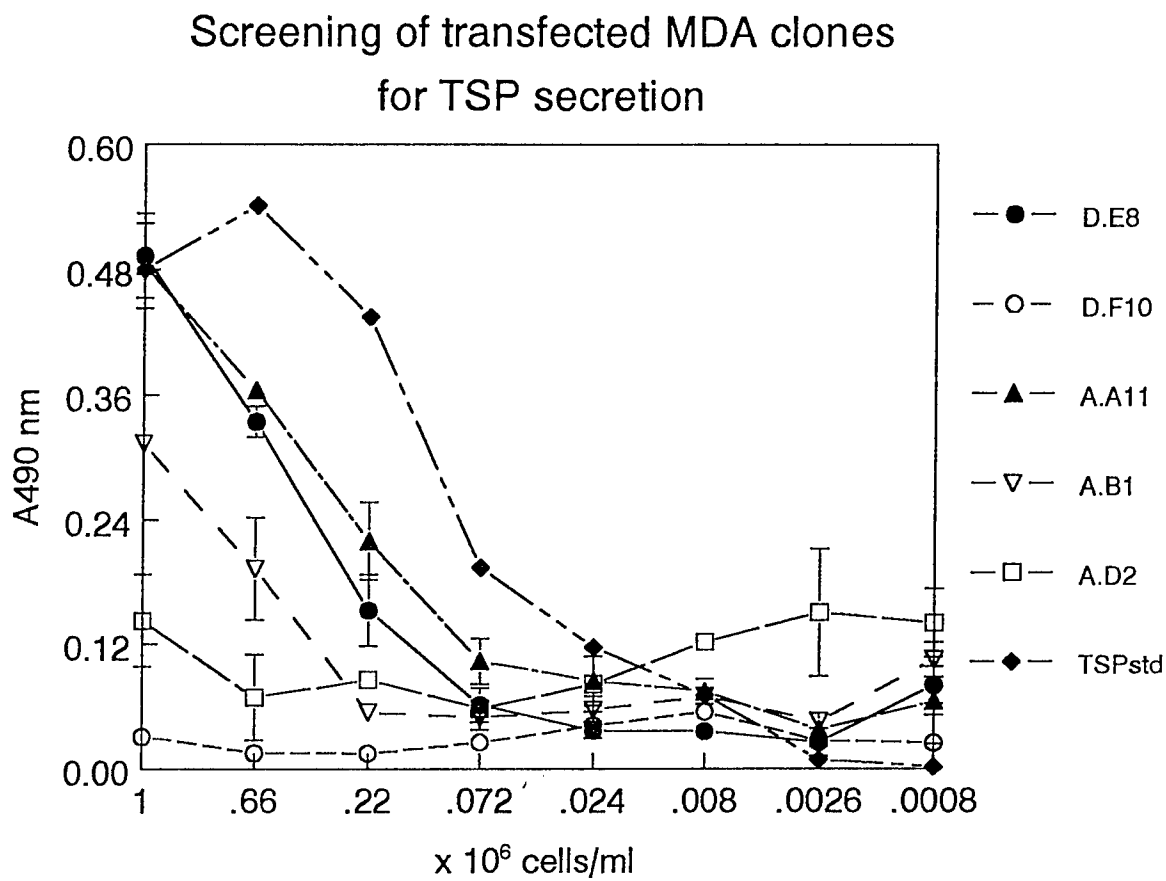
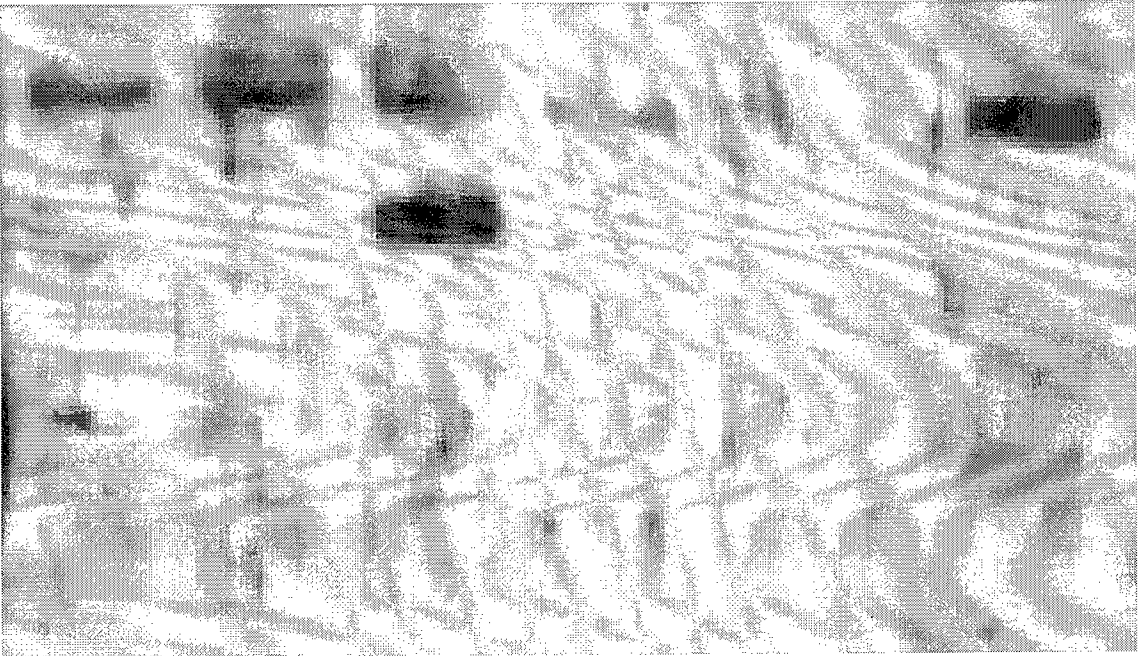


Fig. 6



TSP(FA) A.E10

TSP

TSP(WA) D.C5

TSP(WA) A.A11

TSP(WA) D.F11

TSP(WA) E.B4

686.V TSP-FA transfected MDA
cell lines in nude mice

PROPRIETARY DATA

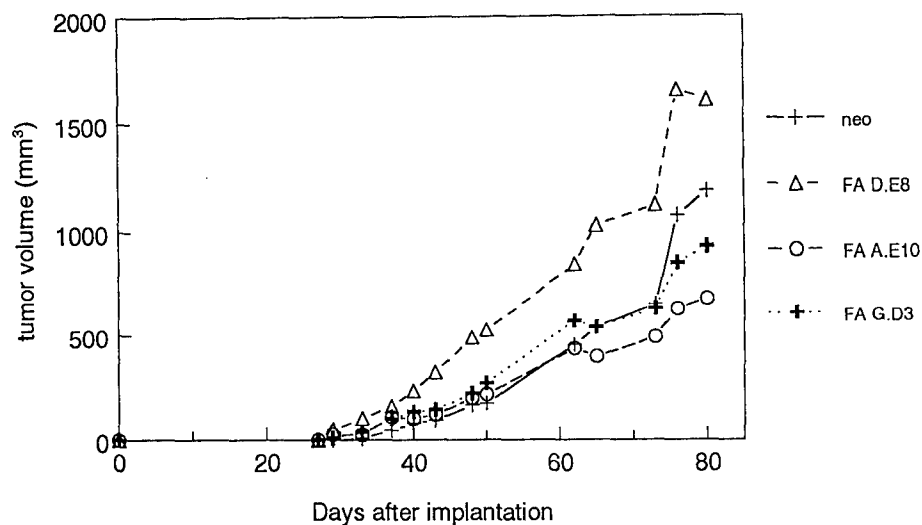


Fig. 7A

686.V TSP-WA transfected MDA
cell lines in nude mice

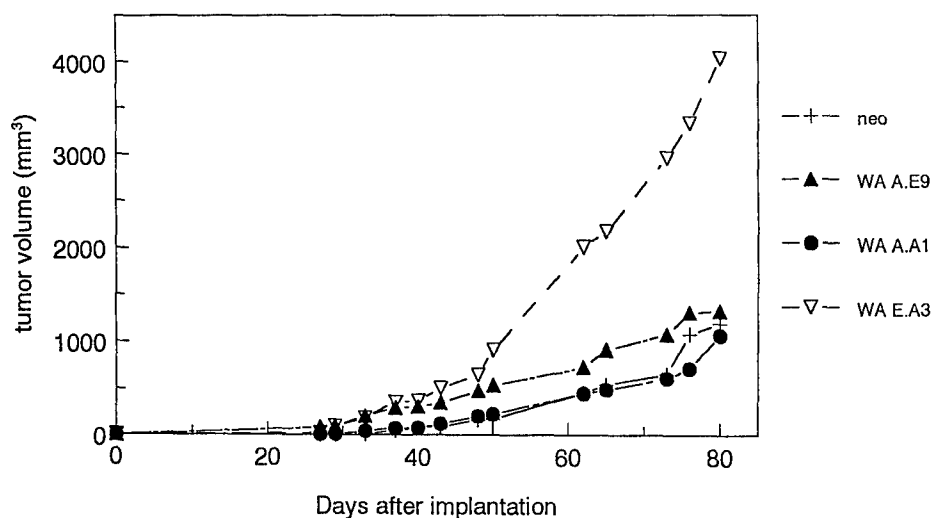


Fig. 7B

686.V MDA transfectants
Tumor mass at sacrifice

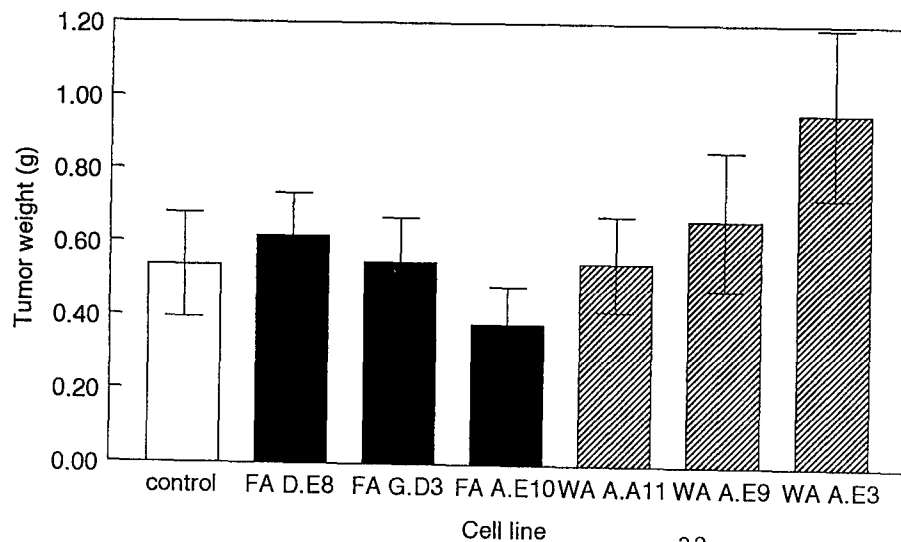


Fig. 7C

PROPRIETARY DATA

Fig. 8

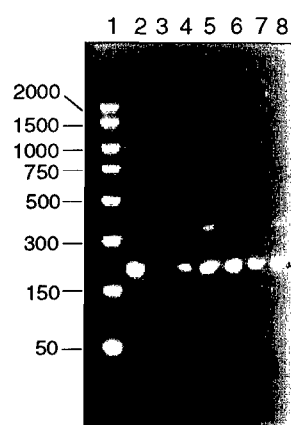


Fig. 9
Purification of thrombospondin from 2WA mutant MDA-conditioned medium
HITRAP HEPARIN AFFINITY CHROMATOGRAPHY

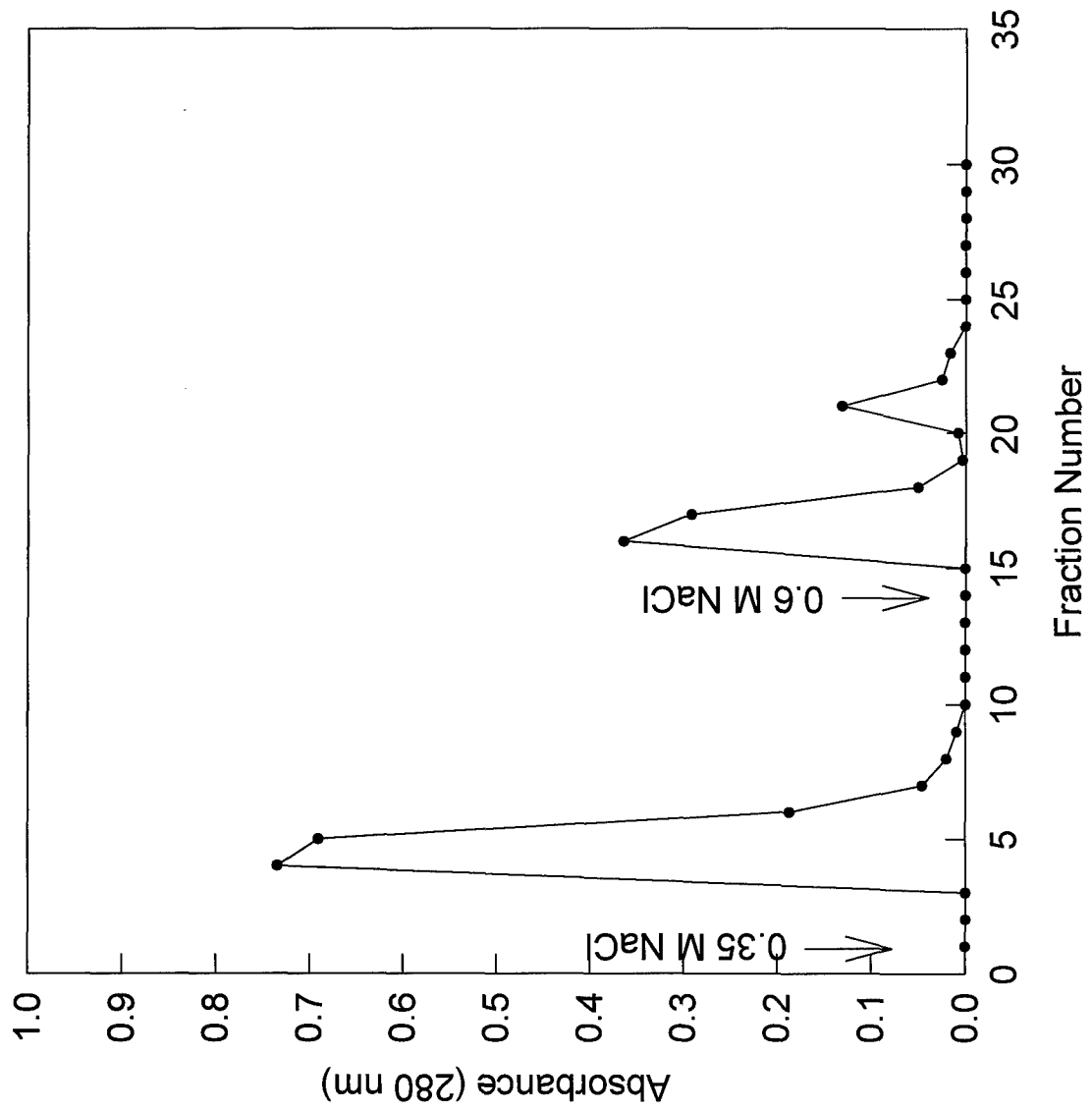


Fig. 10

Purification of thrombospondin from 2WA mutant MDA-conditioned medium
SEPHACRYL S-300 GEL FILTRATION

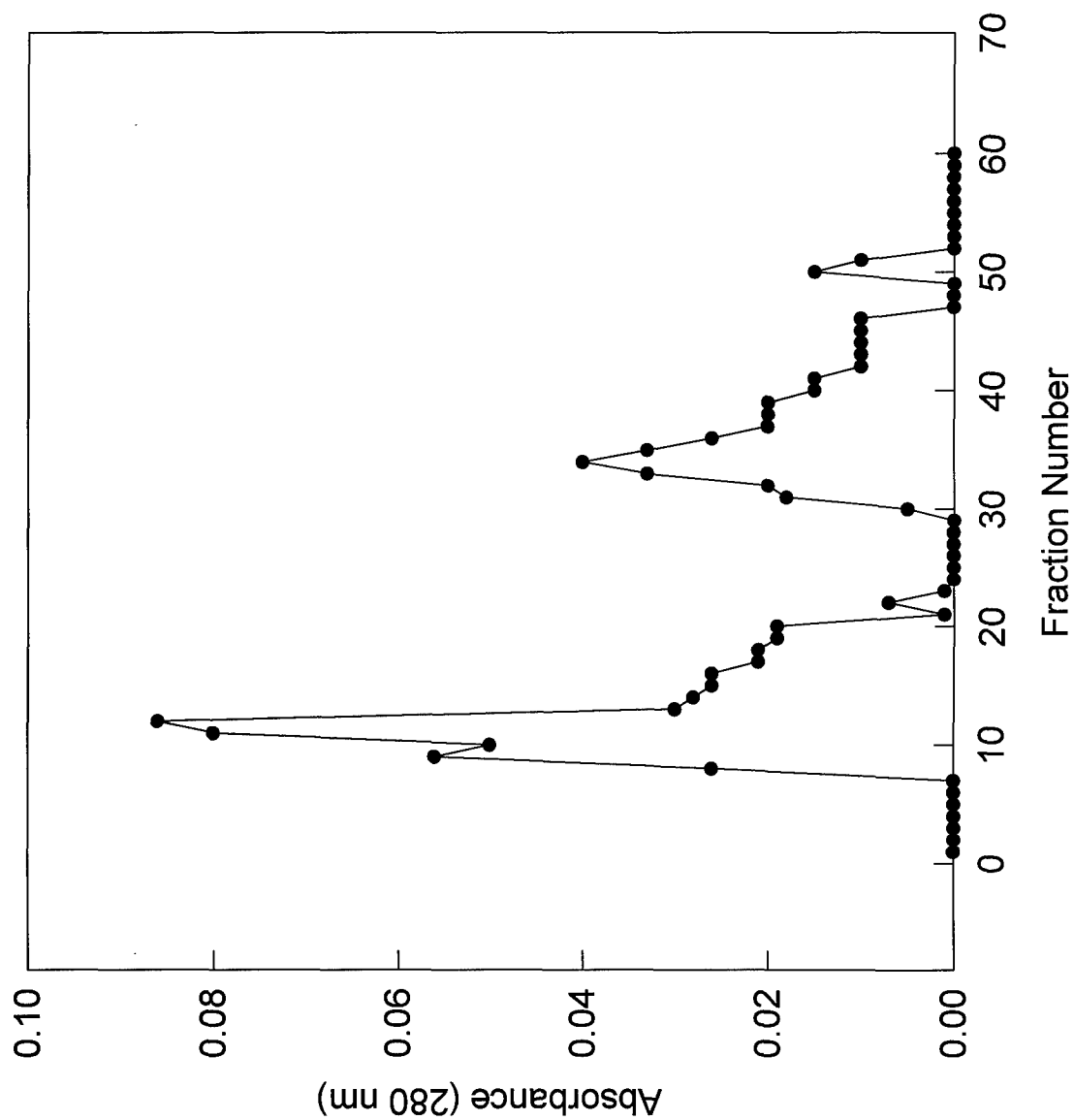


Fig. 11

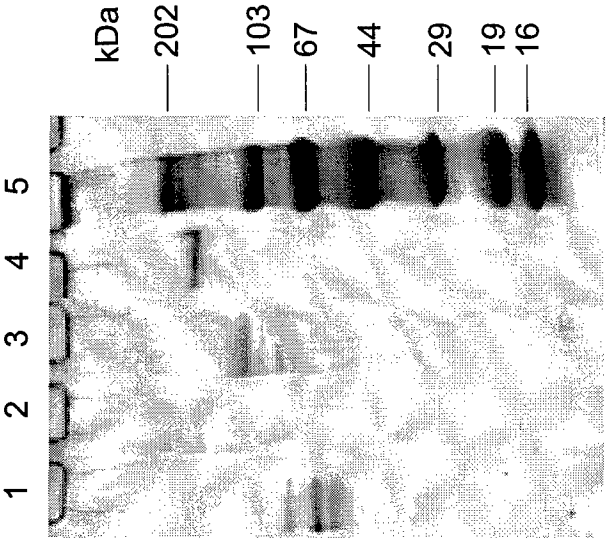


Fig. 12

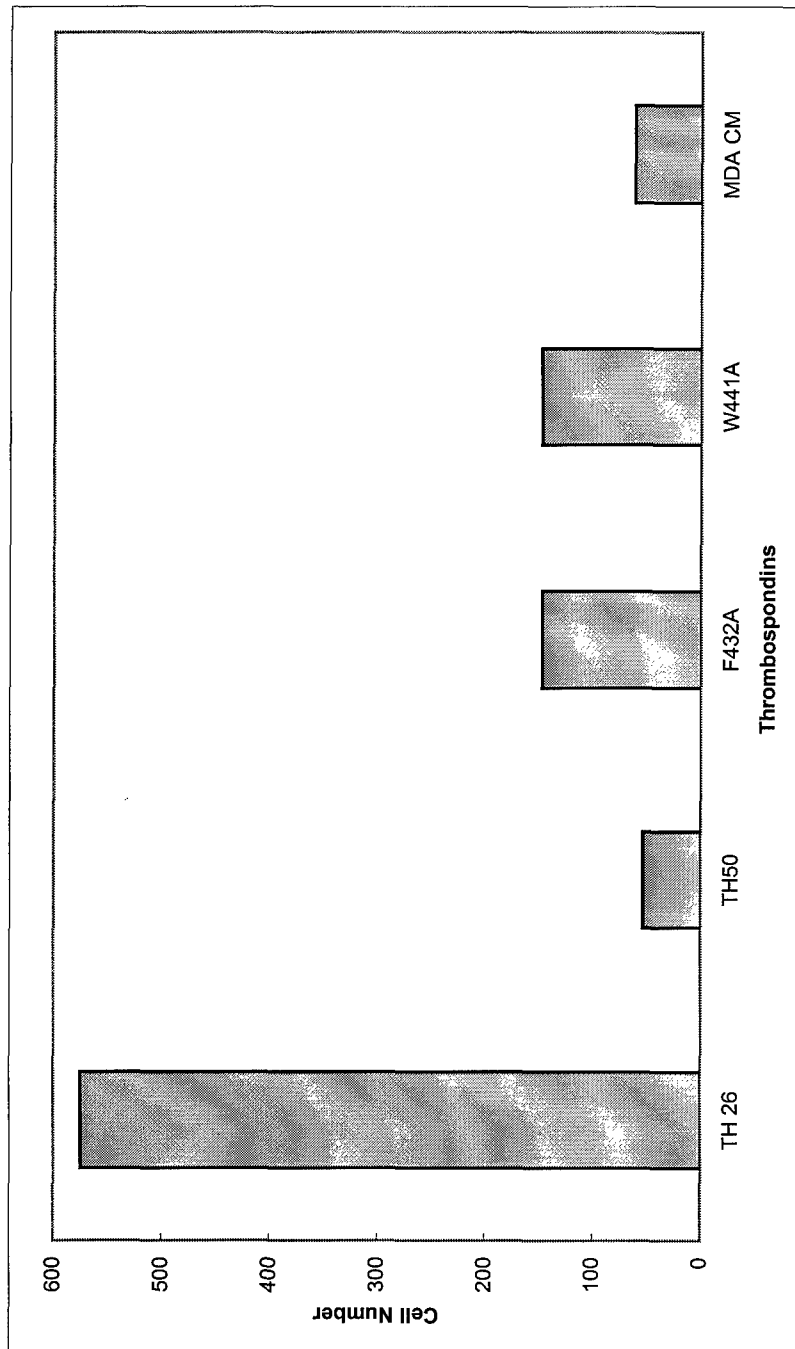


Fig.13

Proliferation of BAE cells with mutant thrombospondins

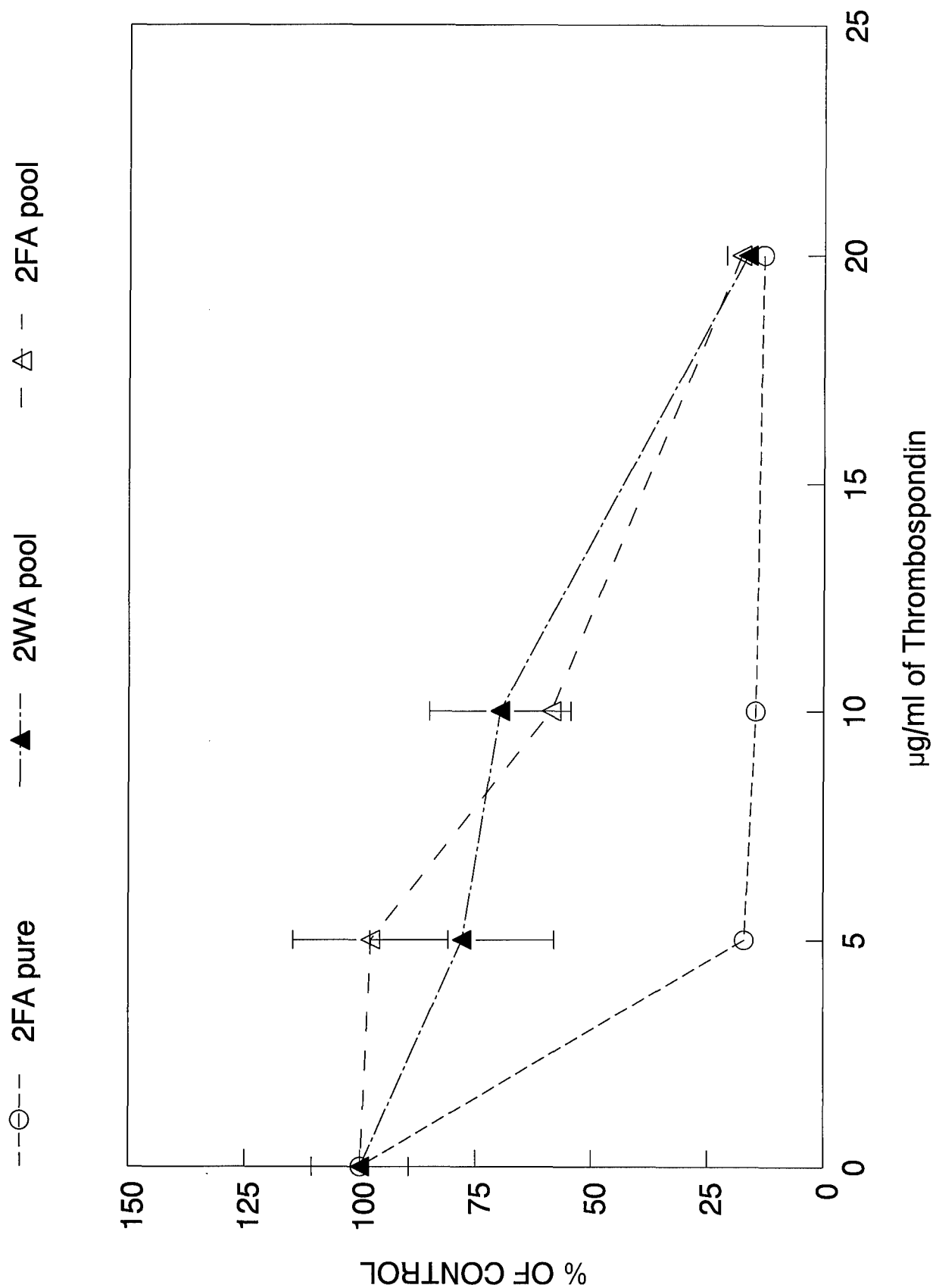
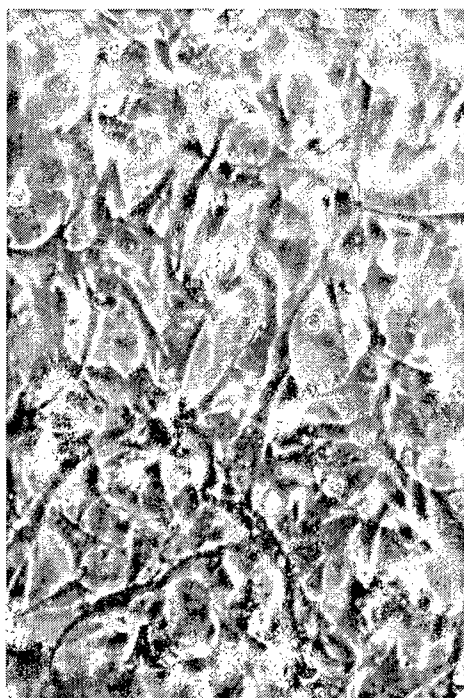
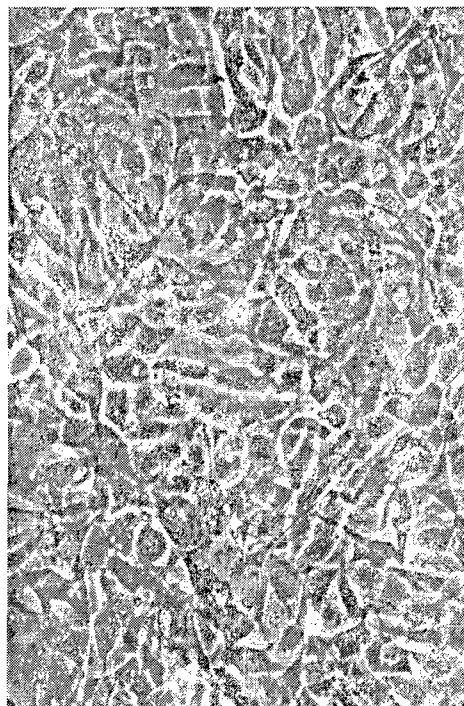


Fig. 14

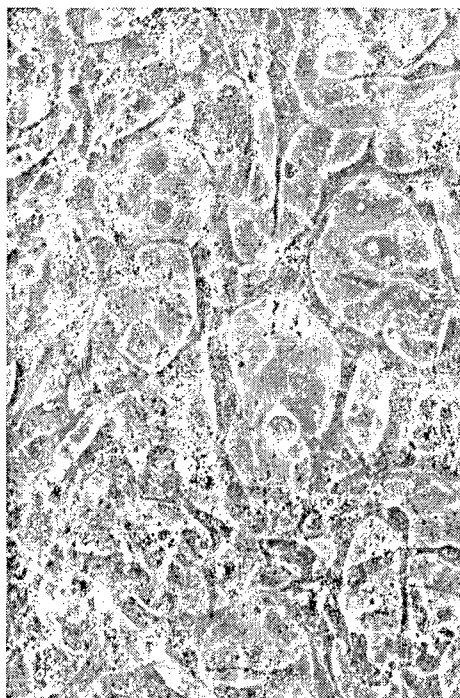
MDA



TH26



F432A



W441A

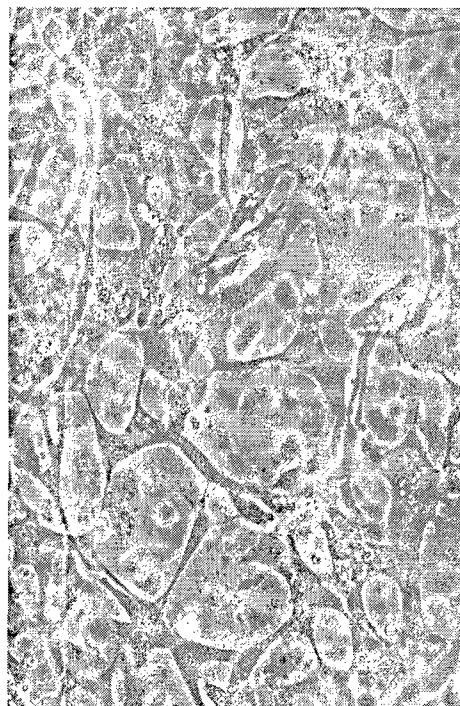


Fig. 15

PROPRIETARY DATA

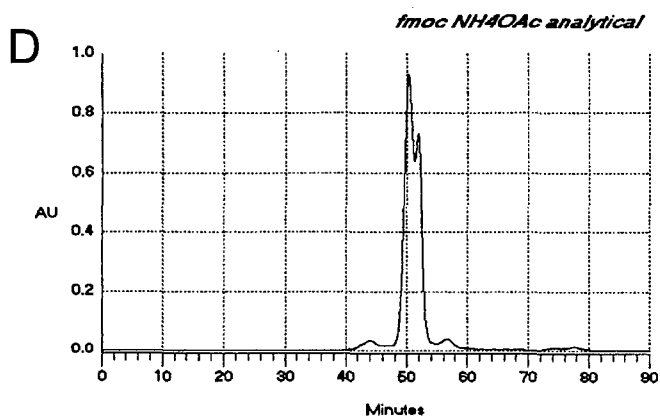
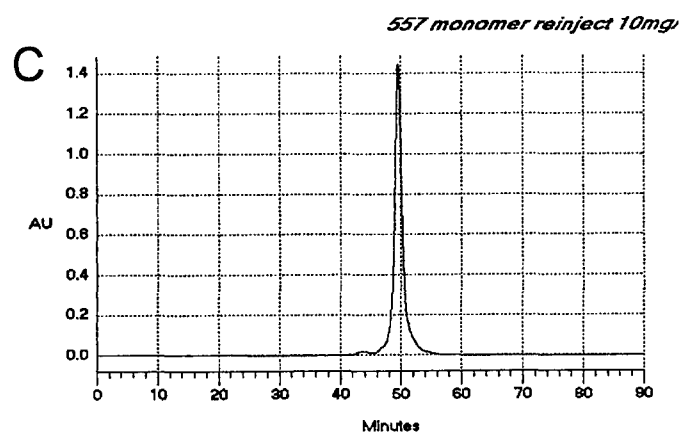
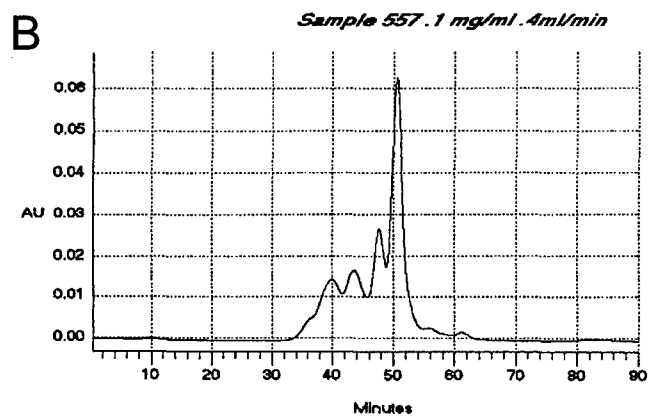
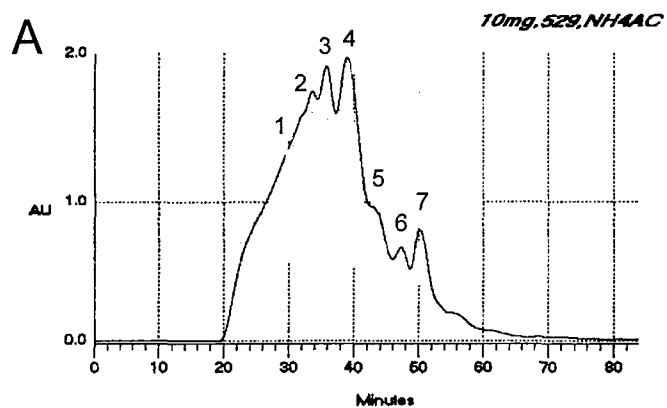


Fig. 16

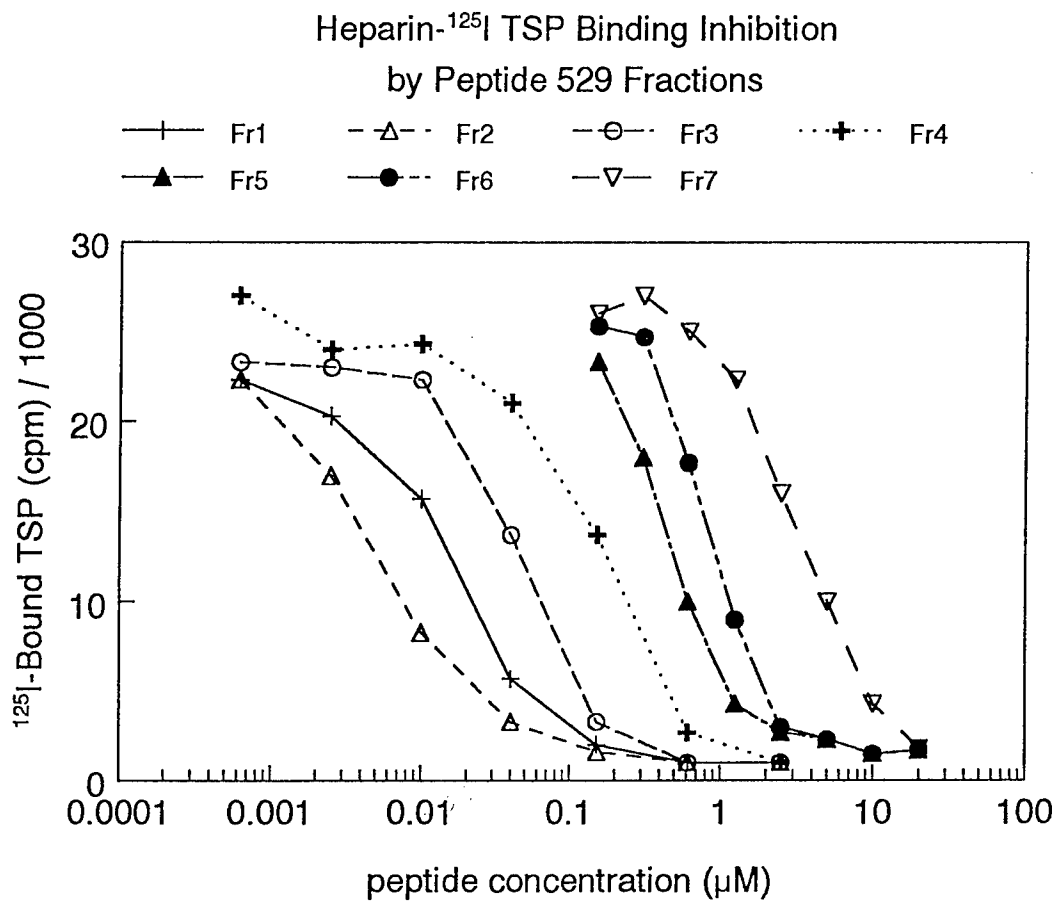


Fig. 17

DNA Fragmentation in Aortic Endothelial Cells Treated with
Thrombospondin-1 Peptides

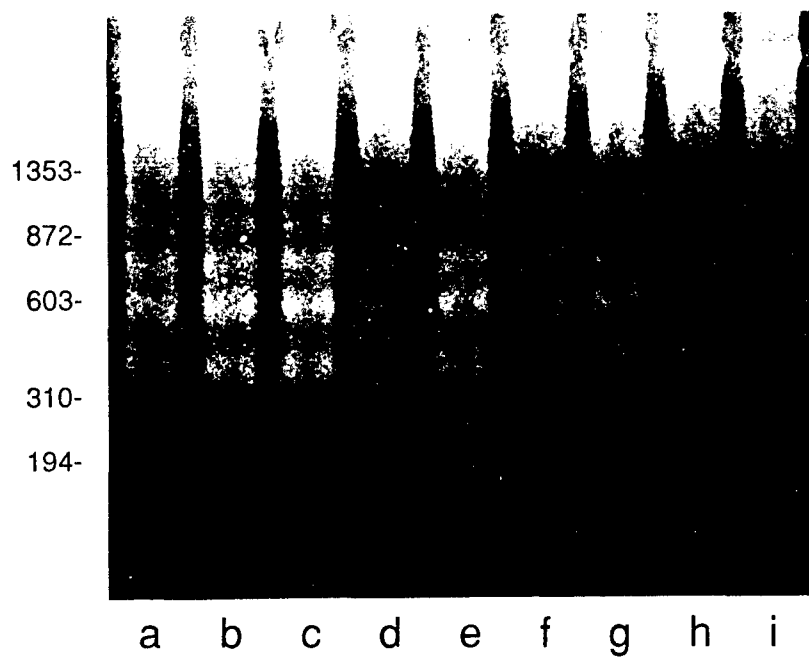
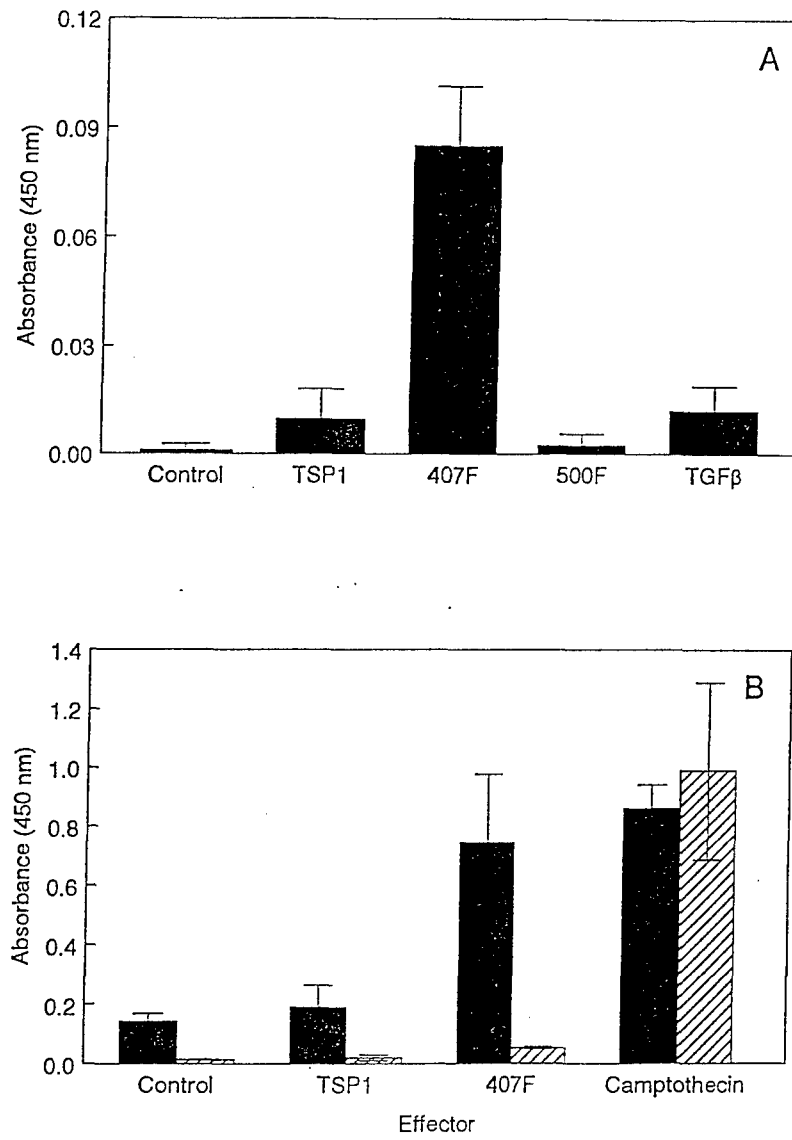


Fig. 18



Personnel Supported by Breast Cancer Research Grant DAMD17-94-J-4499

Personnel with Salary Support:

Dr. Lakshmi Chandrasekaran, Biotechnology Fellow, supported 10-95 to 9-96

Dr. S. Chandrasekaran, Biotechnology Fellow, supported 3-96 to 9-96

Mr. Juin Woei Tseng, Predoctoral IRTA Fellow, supported 10-95 to 11-95

Personnel without salary support:

Dr. David D. Roberts

Dr. Henry Krutzsch

Dr. John Inman

Dr. Neng-hua Guo

Breast Cancer Research Grant DAMD17-94-J-4499

Publications:

1. Roberts, D. D.: Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10:1183-1191, 1996.
2. Guo, N., Kruttsch, 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-I repeat of thrombospondin-1. (submitted)
3. Guo, N., Kruttsch, 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 (submitted)

ABSTRACTS

Roberts, D.D.: Use of retro-inverso analogs of heparin-binding peptides as growth factor antagonists in cancer, Cambridge Healthtech Institute Conference, Development of Small Molecule Mimetic Drugs, May 1996

University of Washington Conference. The thrombospondin gene family and its functional relatives: tenascins, osteopontin, and SPARC, Seattle, WA, June 1996

Roberts, D.D.: Modulation of tumor growth and angiogenesis by thrombospondin-1 and related peptides. 4th International Cancer Symposium, Seoul, Korea, September, 1996



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

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ATTENTION OF:

MCMR-RMI-S (70-1y)

18 June 2001

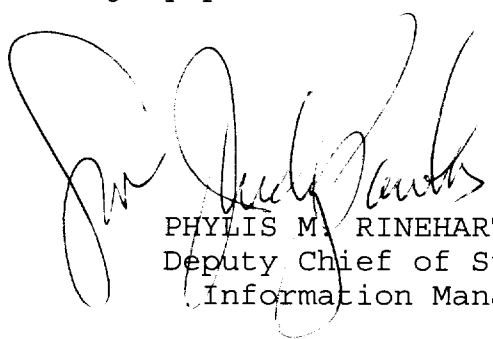
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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical report written for Grant DAMD17-95-J-4499. Request the limited distribution statement for Accession Document Number ADB221833, be changed to "Approved for public release; distribution unlimited." This report 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:



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Deputy Chief of Staff for
Information Management