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

CONTRACTING ORGANIZATION: National Institutes of Health
Bethesda, Maryland 20892

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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 reproduce the growth inhibitory activity of the intact protein. Stable analogues of these peptides inhibit tumor growth *in vivo*, endothelial cell proliferation, and selectively induce apoptosis of endothelial cells. Over-expression of TSP in breast carcinoma cells suppresses tumorigenesis and angiogenesis in murine xenografts. The roles of the TGFβ-activating and FGF2-antagonist sequences in this activity were examined using site-directed mutagenesis of the residues required for activity of the type I repeat peptides. Stable transfected breast carcinoma cell lines expressing these mutant TSPs were characterized for tumorigenic potential *in vivo* and behavior *in vitro*. Endothelial cell responses to the mutant TSPs were examined by transient transfection of the mutated expression plasmids. Adhesion and chemotaxis of the breast carcinoma cells to TSP were shown to be mediated by an activation-dependent β1 integrin.

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Daniel Roberts 8/28/98

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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).

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 (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*. We have characterized polysucrose conjugates as proposed for Task 2 of our Statement of Work. Results describing their effects on breast carcinoma and endothelial cells *in vitro* and lack of antitumor activity *in vivo* for breast tumor xenografts have been published (52, 53).

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 (54). 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* (55). The retro-inverso analog of the thrombospondin type 1 peptide sequence KRFKQDGGWSHWSPWSSC was chosen as the starting point for preparation of retro inverso analogs. Work proposed in Task 2 resulted in two publications demonstrating the activities of these retro-inverso peptides *in vitro* and *in vivo* to inhibit breast carcinoma and endothelial cell growth (52, 53).

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 some of these mutants were used to produce the recombinant proteins for *in vitro* characterization. The same cell lines were simultaneously tested *in vivo* for tumorigenic, angiogenic, and metastatic phenotypes. Correlations between these assays provide insight into the role of specific sequences in TSP in regulating tumor behavior. Preparation of most of the

mutants for Task 3 has been completed, and stable transfectants have been prepared where possible. The present annual report focuses on the characterization of the effects of these mutant thrombospondins on breast and endothelial cells as described in Task 3 and Task 4. Because several of the constructs could not be expressed in stable transfectants, we have modified our experimental plan to utilize transient expression assays to examine their biological activities. In the course of these experiments, we have also discovered that breast carcinoma cells preferentially use the $\alpha 3\beta 1$ integrin to mediate adhesion and chemotactic responses to thrombospondin-1. The activity of this thrombospondin receptor was found to be specifically induced by insulin-like growth factor receptor ligands. These new data are also summarized in this report.

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 (56). 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 E = 5540 $M^{-1}cm^{-1}$ per Trp residue.

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 (57). 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° C 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° C. A 50 µl sample of each conditioned medium was added to the wells in 3-fold serial dilutions and incubated for 2 h at 37° C. The stably transfected clone containing the full length wild type THBS1 sequence (TH26 or 29) was used as the positive control and a pCMVneo transfectant was used as a negative control. The wells were blocked by incubation in tris-BSA. The wells were aspirated and incubated with 50 µl of 1:500 dilution of rabbit anti-TSP in tris-BSA for 2 h at 37° C. The wells were aspirated and washed 3 times with DPBS, 0.02% BSA, 0.02 mM 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 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 4^0C for 1 hr. After 50 µl of Protein A- agarose was added, samples were further incubated at 4^0C 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.

Bioassay for inhibition of endothelial cell proliferation-- Proliferation of bovine aortic endothelial cells was determined as previously described (Vogel, T. et al., J. cell Biochem 53, 74-84, 1993). Briefly, 5000 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.

Transient transfection and uptake of ^{3}H- thymidine: Bovine aortic endothelial cells (BAE Cells) between passages 3 and 13 or MDA435 breast cancer cells between passages 4 and 20 were used for transient transfections by plasmid expression vectors carrying the wild type and type I repeat mutant cDNAs of TSP. Transfections were done by electroporation. The protocol used for transfection is briefly outlined as follows.

MDA435 cells or BAE cells were trypsinized and resuspended in growth medium. $2x10^6$ cells were aliquoted into sterile microfuge tubes containing varying amounts of THBS DNA constructs used for transfection and 5 µg of a plasmid expression vector for β-galactosidase. The total volume was adjusted to 25 µl and incubated on ice for 20 minutes. The cell-DNA mixtures were then transferred into prechilled electroporation chambers taking care to suspend them between the two electrode bosses. The mixtures were exposed to a 1 sec. pulse from a 50 µF capacitor charged to 150 volts. Electroporation chambers were immediately placed on ice for another 20 min. The mixture was then resuspended into 3 ml of medium containing 1% FCS (for BAE cells) or 10% FCS (for MDA435) and plated into two wells of a Nunc 6 well plate. One well was used for ^{3}H-thymidine labeling and the other was used for protein and β-galactosidase activity assays.

24 hr after the transfection, the medium was replaced with 1 ml of medium supplemented with 1 or 10% FCS and one well from each transfection was labeled with 2.5 µCi of ^{3}H-thymidine/ml medium. Labeling was continued for 4 hr at 37^0 C in a CO_2 incubator. At the end of incubation the cells were washed twice with DPBS, fixed with methanol/acetic acid and extracted with 600 µl of trypsin/versene at 37^0 C for 1 hr and at RT for 30 min. 200 µl of 1% SDS were added to the cell extracts which were then counted using a scintillation counter to determine the total uptake of ^{3}H-thymidine.

The well containing the other half of the transfected cells was washed and extracted

with 300 µl of ice cold extraction buffer (0.1 M sodium phosphate, pH 7.4 containing 0.5% triton X-100). The cell extracts were freeze-thawed three times and cell lysates were cleared by centrifugation at 12,000g for 15 min at 4^0 C. Aliquots of cell lysates were assayed for total protein content by the BCA method and for β-galactosidase activity using nitrophenyl β-galactopyranoside as substrate. The β-galactosidase activities of the cell lysates were expressed as mU/µg protein standardized using purified *E coli* β-galactosidase (Sigma).

Quantitation of uPA and uPAR: Approximately 500,000 cells were plated per well of a 6 well plate and allowed to attach and grow to 70-80% confluency in serum-containing medium. The cells were weaned off the serum over a period of 48 hr by incubating in medium supplemented with 0.1% BSA. At the end of the incubation period the cells were washed with PBS and cell extracts were obtained by the addition of 1 ml of cold 1% Triton X-100 in TBS, pH 8.5. The extracts were centrifuged at 12,000g at 4^0 C to remove cell debris. uPA and uPAR concentrations were measured using the respective IMUBIND ELISA kits from American Diagnostica Inc.

RESULTS

We have completed and published the 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 (58). We have also completed and published a study of the apoptosis response to the peptides and shown that native TSP has the same activity (52). For specific aims 2 and 3, we have completed transfections with four full length *THBS1* cDNA expression vectors containing point mutations in the type I repeats. Stable transfected cell lines expressing high levels of two of these constructs have been prepared, but stable lines expressing the other two constructs could not be obtained (W385A and F432A). In the present report, we present further characterization of the *in vitro* interactions of mutant and wild type TSP1 with breast carcinoma and endothelial cells. We have also discovered that breast carcinoma cells preferentially use the α3β1 integrin to spread on TSP1 and that the activity of this integrin in breast carcinoma cells is regulated by insulin-like growth factor I and CD98. This contrasts with endothelial cells, which have been previously demonstrated to use the αvβ3 integrin as a TSP1 receptor (6, 59). Use of different integrins may account for some of the differential responses of breast carcinoma and endothelial cells to TSP1 and the TSP1 mutants. A manuscript based on these results is attached. To overcome the limitations in using full length recombinant thrombospondins to characterize functions of the type I repeats, we have used truncated recombinant thrombospondins to continue mapping the functional domains for its interactions with the endothelial and breast carcinoma cells.

In our previous report, we described properties of clones over-expressing TSP W441A and TSP F432A using *in vivo* tumorigenesis and *in vitro* functional assays. Clones from TSP W441A transfection had the same or larger tumor masses compared to controls, whereas those from TSP 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. These results were also replicated using Beige XID mice, deficient in NK, T and B lymphocytes, to verify that inhibition of tumor growth by over expression of thrombospondin and the lack of inhibition by W441A

thrombospondin was mediated by its anti-angiogenic activity rather than interactions with components of mouse immune system. Our data demonstrated that the *THBS*-transfected MDA435 cell lines retain their inhibition of tumor growth in Beige mice, which lack NK cells, relative to control transfectants.

Analysis of the TSP W441A expressing 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 (60). 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 (61, 62) is consistent with this hypothesis. Alternatively, the mutant protein may be expressed but could be toxic to the cells. In our last annual report, we demonstrated that the F432A mutant was synthesized and secreted normally in transient expression assays. However, the protein did not accumulate in the medium, because transfected cells were rapidly lost from the cultures as assessed by co-transfection with a GFP expression vector. 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*1 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 death or strongly inhibit growth of the cells expressing this transgene.

MDA435 cells were transiently transfected with varying amounts of *THBS1* wild type (WT) DNA or the F432A mutant DNA constructs to study their effects on the proliferation of the cells. Proliferation was measured as the total uptake of ^{3}H- thymidine by transiently transfected MDA435 cells. As shown in Figure 1A, when the assay was done in the presence of 10% FCS, dose-dependent inhibition of proliferation was seen which was maximal at a DNA concentration of 24 μg in the case of cells transfected with THBS-WT DNA. Under similar conditions F432A DNA transfected cells showed very little inhibition of proliferation, even though the transfection efficiency remained above 100% of that for the β-galactosidase indicator vector alone (Figure 1B). Inhibition of thymidine incorporation in this assay is a measure of DNA synthesis in the bulk population, the majority of which are not transfected, as assessed by X-Gal staining using the β-galactosidase reporter. Thus inhibition following transfection using wild type THBS vector results from accumulation of TSP1 in the medium which is secreted by the minority of transfected cells. Failure of the F432A mutant to inhibit thymidine incorporation indicates that this protein does not accumulate. This probably results from the rapid loss of cells expressing this mutant as detected by the GFP reporter studies.

Although we have demonstrated that platelet thrombospondin-1 inhibits proliferation of MDA435 cells (53), endothelial cells are much more sensitive to thrombospondin and are

believed to be the physiological target for its anti-angiogenic activities. Effects of transient expression of wild type TSP, the KRFK mutant F432A and the type I repeat mutant thrombospondins on bovine aortic endothelial (BAE) cells were assayed in a similar manner, and the results are presented in Table 2. With increasing amounts of wild type *THBS* DNA used for transfection, a dose dependent inhibition of BAE cell proliferation is seen, while the transfection efficiency remains above control levels using the β-galactosidase vector alone. We also transfected BAE cells with 15-20 μg of the mutant DNAs. In the case of BAE cells transfected with W385A mutant construct, 15 μg of the DNA used for transfection did not show inhibition of incorporation of ^{3}H- thymidine. On the contrary, comparable amounts of W441A and W498G mutants had inhibitory effects similar to that of 18 μg of WT DNA.

WG1a is a mutant of wild type THBS clone that we obtained while preparing the mutant W498G DNA construct by the linker scanning method. Due to a two base pair deletion (C at #1629 and A at # 1630), a frame shift and a premature stop codon were introduced at amino acid 516. Therefore, this protein lacks the carboxy terminus of TSP beyond the three type I repeats. Transient transfection of BAE cells with 16.5 μg of the WG1a mutant strongly inhibited BAE cell proliferation, with only 20% ^{3}H-thymidine being incorporated into the cells compared to the control. Transfection efficiencies in all the cases were high (Table 2), indicating that expression of this construct was not toxic to the cells.

It has been shown previously that TSP-1 up regulates the urokinase plasminogen activator (uPA) and its receptor (uPAR) and promotes tumor cell invasion in MDA-MB-231 human breast cancer cells (63). We assayed uPA and uPAR in TH26, a stable MDA435 breast carcinoma clone expressing wild type TSP, as well as in MDA435 cells transiently transfected with the wild type DNA (Table 3). There was no change in the level of expression of uPA between the parent MDA435 cells and TH26 although the uPAR level of TH26 was about 10 fold that of the parent MDA435 cells. Transient transfection was used to determine whether the upregulation of uPAR was a direct response to TSP over expression. MDA435 cells transiently transfected with THBS-WT DNA did not show any change in the uPAR level compared to the parent cell line or vector-transfected cells.

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

In our report for 1997, we described use of bacteriological fusion proteins expressing thrombospondin fragments to map functional domains for its interactions with 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, mediated by the RGD sequence in the last type 3 repeat. This adhesion was shown to be mediated by the integrin αvβ3. In contrast, the MDA435 breast carcinoma cells did not recognize the RGD sequence or any other sequence in the type 3 repeats. We presented preliminary evidence that the primary interaction of breast carcinoma cells with thrombospondin-1 was mediated by a β1 integrin. We have now identified the α3β1 integrin as the primary receptor on two breast carcinoma cell lines for thrombospondin-1. A submitted manuscript describing this work is attached and is summarized below.

Integrin Expression on Breast Carcinoma Cells

Flow cytometric analysis (Table 4) and immunoprecipitation using subunit-specific

integrin antibodies (data not shown) demonstrated that MDA-MB-435 cells express several β1 integrins and αvβ3. Integrin expression on MDA-MB-231 and MCF-7 cells have been reported previously (64-66). MDA-MB-231 cells express α2, α3, α4, α5, α6, αv, and β1 subunits. The MDA-MB-231 and MCF-7 cell lines express only low levels of β3 subunits (66).

Binding of Soluble TSP1

Previous studies using MDA-MB-231 breast carcinoma cells (67) concluded that sulfated glycoconjugates including heparan sulfate and chondroitin sulfate proteoglycans play a dominant role in both binding of soluble TSP1 and adhesion on immobilized TSP1. We observed a similar dependence for ^{125}I-TSP1 binding to MDA-MB-435 cells (Fig. 2). Binding was strongly inhibited by heparin or a recombinant 18 kD amino terminal heparin-binding fragment of TSP1, but the peptide GRGDS and β1 or α3 integrin function blocking antibodies had no effect. Conversely, binding of ^{125}I-TSP1 to MDA-MB-435 cells was not enhanced by incubation with the β1 integrin-activating antibody TS2/16, either alone or in the presence of 10 μg/ml heparin to inhibit TSP1 binding to sulfated ligands (data not shown). Therefore, high affinity binding to soluble TSP1 to these cells is mediated by sulfated glycoconjugates and is independent of integrin binding.

β1 Integrin-mediated Adhesion and Chemotaxis to TSP1

Although heparin and recombinant heparin-binding domain from TSP1 partially inhibited attachment of MDA-MB-435 cells on immobilized TSP1, the fraction of spread cells was unaffected (Fig. 3A). In the presence of a β1 integrin function-blocking antibody at 2 μg/ml, however, only spreading was inhibited, and a combination of heparin and the β1 blocking antibody abolished spreading and markedly inhibited attachment. At 50 μg/ml, the β1 antibody completely inhibited adhesion to TSP1 (Fig. 3A). Thus, interaction with a β1 integrin is essential for spreading, but sulfated ligands may also contribute to adhesion of these cells on TSP1. This was confirmed by inhibition of sulfation following growth in chlorate. Adhesion was inhibited by 60% for MDA-MB-435 cells with a 90% reduction in $^{35}SO_4$ incorporation (Fig. 3B). RGD peptides did not inhibit adhesion of MDA-MB-435 cells on TSP1 (results not shown).

Although MDA-MB-435 cells express some αvβ3 integrin (Table 4), a function-blocking antibody or an αvβ3-specific RGD mimetic blocked adhesion of the cells on vitronectin but had no effect on adhesion on TSP1 (Fig. 3C and results not shown). Conversely, in the presence of the β1 activating antibody TS2/16, adhesion of MDA-MB-435 cells was enhanced on TSP1 but not on vitronectin (Fig 3C). Therefore, the αvβ3 integrin is functional in MDA-MB-435 cells, but it is apparently unable to recognize the RGD motif in intact platelet TSP1.

The β1-blocking antibody MAb13 inhibited chemotaxis to TSP1, but heparin did not (Fig. 3D). For these experiments, the filters were coated with polylysine to provide an integrin-independent substrate for adhesion of the cells. Therefore, chemotaxis of MDA-MB-435 cells to TSP1 is also primarily dependent on the β1 integrin receptor.

Several human breast cancer cell lines showed similar involvement of β1 integrins in their adhesion to TSP1 (Fig. 4). MDA-MB-231 cells attached poorly and did not spread on substrates coated with low concentrations of TSP1. In the presence of the β1-activating antibody, however, the cells attached avidly on TSP1 and exhibited spreading (Fig. 4A). A

third breast carcinoma cell line, MCF-7, behaved similarly to the MDA-MB-231 cells and showed spreading on TSP1 only in the presence of the β1 activating antibody (Fig. 4A). The apparent low avidity state of the integrin that recognizes TSP1 on MDA-MB-435 cells was not an artifact from using EDTA to dissociate the cells, because cells suspended by scraping from the dish in the presence of divalent cations showed the same degree of enhancement by TS2/16 for adhesion to TSP1 or type I collagen as cells harvested using EDTA (Fig. 4B).

α3β1 is the Major TSP1-binding Integrin on Breast Carcinoma Cells

Of the α subunit antibodies tested for inhibiting adhesion to TSP1, only an α3 subunit blocking antibody, P1B5, significantly inhibited of adhesion of MDA-MB-231 cells to TSP1 (Fig. 5A, p= 0.0007, 2-tailed t test). An α4 integrin blocking antibody slightly inhibited adhesion, but mixing this antibody with the α3 blocking antibody produced no further inhibition than the latter antibody alone (Fig 5A). MDA-MB-435 cell spreading on TSP1 was also inhibited by the α3 blocking antibody, and somewhat by the α4 antibody (Fig. 5B). Function blocking antibodies for α1β1, α2β1, and α5β1 integrins had no effect on TSP1 adhesion, although the α2β1, and α5β1 antibodies inhibited adhesion of the same cells to known ligands for these integrins (Fig. 5 and results not shown).

Integrin localization and effects on actin cytoskeleton

Activation of α3β1 using TS2/16 altered the morphology of cells attaching on TSP1 (Fig. 6). MDA-MB-435 cells extended a few processes but exhibited no F-actin organization when attached on TSP1 alone (Fig. 6A), but addition of antibody TS2/16 stimulated spreading with redistribution of F-actin to the cell periphery (Fig. 6B). F-Actin was also present in short spikes protruding from the spread cells. Staining with the β1 integrin antibody revealed numerous filopodia extending from these points (Fig. 6C). In some cells, these filopodia were terminated with punctate β1 integrin staining, possibly at sites of contact with the TSP1 substrate. Formation of filopodia was specific to the TSP1 substrate, as TS2/16-induced spreading of these cells on type I collagen (Fig. 6D) or fibronectin (results not shown) only rarely evoked filopodia. These cytoskeletal rearrangement changes were specific for β1-dependent adhesion to intact TSP1 and were not observed in cells attaching on heparin-binding peptides or recombinant fragments of TSP1 (results not shown). Similar induction of filopodia or microspikes by TSP1 have been observed in other cell types (68).

Structural requirements for α3β1 mediated adhesive activity of TSP1

We attempted to localize the region of TSP1 recognized by the α3β1 integrin. A 140 kDa fragment lacking the amino terminal heparin-binding domain also exhibited β1-dependent adhesion, but no smaller proteolytic or recombinant fragments of TSP1 supported β1-dependent adhesion (Fig. 7). Among the recombinant TSP1 fragments tested, an 18 kDa fragment of the amino-terminal heparin-binding domain had the strongest activity, and recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments. A recombinant GST-fusion of the type 3 repeats of TSP1 including the RGD sequence had minimal adhesive activity for MDA-MB-435 cells (Fig. 7A), in contrast to human melanoma cells, which avidly attached on substrates coated with the same concentrations of this fragment (Sipes, and Roberts, manuscript in preparation). Synthetic heparin-binding peptides from the type 1 repeats (peptide 246) and the CD47 binding peptide 4N1K also promoted adhesion, but TS2/16 partially inhibited adhesion of MDA-MB-435 cells to these peptides (Fig. 7B). Two CD36-binding peptides from the procollagen domain

(peptide 500) or the type 1 repeats (Mal-II) had weaker adhesive activity, but were insensitive to TS2/16. The focal adhesion disrupting peptide from the amino terminal domain (Hep1) did not promote MDA-MB-435 cell adhesion.

Differences in the conformation or folding of TSP1 could account for discrepancies in its reported adhesive activity. The conformation of TSP1 and formation of specific intra-chain disulfide bonds are sensitive to the levels of divalent cations present during its purification. Disulfide bonding also influences interactions of TSP1 with several proteases and regulates the accessibility of the RGD sequence to the αvβ3 integrin (69, 70). We therefore examined the influence of conformation on α3β1-dependent adhesion by absorbing TSP1 with or without divalent cations, at low pH (39), or by reducing disulfide bonds using dithiothreitol (Fig. 8). Coating TSP1 at pH 4 in acetate buffer enhanced MDA-MB-435 cell adhesion relative to TSP1 adsorbed in PBS with Ca^{2+} and Mg^{2+}, but use of PBS with 2.5 mM EDTA did not significantly affect β1-mediated adhesion. Although heparin only partially inhibited MDA-MB-435 cell adhesion to TSP1 (20-50%) when the TSP1 was adsorbed in Dulbecco's PBS (e.g. Fig. 3A), adhesion to TSP1 adsorbed in pH 4 acetate buffer was inhibited 98% by 10 μg/ml heparin. Conversely, TS2/16 did not reproducibly increase adhesion of MDA-MB-435 cells to TSP1 adsorbed in acetate buffer (data not shown). Therefore, the enhanced adhesion to TSP coated at pH 4 was due primarily to enhancement of heparin-dependent adhesion, whereas β1-integrins contributed less to adhesion on TSP1 coated at the lower pH. Adhesion of MDA-MB-435 cells and MDA-MB-231 cells (results not shown) was strongly inhibited following reduction of TSP1 with dithiothreitol. This contrasts with αvβ3-dependent adhesion to TSP1, which was reported to be enhanced following disulfide reduction using the same conditions as used in Fig. 8 (70). Thus, α3β1-dependent adhesion of breast carcinoma cells does not require Ca-replete TSP, but some intact disulfide bonds are essential.

Regulation of β1 integrin activation in breast carcinoma cells

Adhesion of T lymphocytes to TSP1, mediated by α4β1 and α5β1 integrins, is stimulated by phorbol esters (37). PMA activation of protein kinase C in MDA-MB-435 cells increased αvβ3-mediated adhesion to vitronectin but had no effect on β1 integrin-mediated adhesion to TSP1 (Fig. 9). Integrin associated protein (CD47) also regulates integrin function in several cell types (71, 72). The carboxyl-terminal domain of TSP1 contains two peptide motifs that activate integrin function through binding to CD47 (71). The CD47-binding TSP1 peptide 7N3 activated adhesion of MDA-MB-435 cells on vitronectin and a recombinant TSP1 fragment containing the RGD sequence (results not shown) but had no effect on adhesion to native TSP1 (Fig. 9). Thus MDA-MB-435 cells express functional αvβ3 that can be activated by PMA or the TSP1 7N3 peptide. This αvβ3 integrin can recognize the TSP1 RGD sequence in the context of a bacterial fusion protein, but it does not play a significant role in adhesion of resting or stimulated breast carcinoma cells to native platelet TSP1.

Several pharmacological agents stimulated β1-dependent adhesion to TSP1 (Table 5). The broad spectrum Ser/Thr protein kinase inhibitor staurosporine increased spreading of all three cell lines. However, this activation in MDA-MB-435 cells was only partially replicated by specific inhibitors of protein kinase C (bisindoyl maleimide) or protein kinase A (KT5720), or protein kinase G (KT5823 and guanosine-3',5'-cyclic monophosphorothioate, 8-(4-chloro-phenylthio)-, Rp-isomer). Inhibition of PI 3-kinase using wortmannin had no significant

effect on MDA-MB-435 cell spreading and weakly enhanced MDA-MB-231 cell spreading on TSP1. Two calcium ionophores, ionomycin and A23187, strongly enhanced spreading of MDA-MB-435 cells but had no effect on MDA-MB-231 cell spreading on TSP1.

Modulation of TSP1 adhesion by G-protein signaling

Although TSP1 peptides promote PT-sensitive integrin activation through binding to CD47 (71, 73), we showed above that this pathway does not function in MDA-MB-435 cells to activate $\alpha 3\beta 1$. However, PT did influence MDA-MB-231 and MDA-MB-435 cell adhesion and spreading on TSP1 or collagen (Fig. 10). PT increased adhesion of MDA-MB-231 cells to TSP1 (Fig. 10A) but inhibited both basal and TS2/16-stimulated adhesion of MDA-MB-435 cells on the same substrate (Fig. 10B). The effects of PT in both cell lines were specific, since PT B-oligomer at the same concentration had no effect (Fig. 10). The enhancement of MDA-MB-231 cell adhesion by PT is mediated by the $\beta 1$ integrin, because the $\beta 1$ blocking antibody MAb13 inhibited the PT-induced adhesion of MDA-MB-231 cells but heparin did not (results not shown). However, not all $\beta 1$ integrins in these breast carcinoma cells were activated by PT. Adhesion of MDA-MB-231 cells to collagen mediated by $\alpha 2\beta 1$ (verified by the blocking antibody 6D7, results not shown) was not altered by PT, although the same adhesive pathway could be further activated by TS2/16 (Fig. 10A). In MDA-MB-435 cells, PT partially inhibited $\alpha 2\beta 1$-mediated spreading on collagen stimulated by TS2/16 (Fig. 10B).

Physiological activators of TSP1 adhesion and chemotaxis

We noted that freshly passaged breast carcinoma cells exhibited stronger $\beta 1$ integrin mediated adhesion on TSP1. This suggested that proliferation regulates $\alpha 3\beta 1$-mediated TSP1 adhesion. Serum induced a dose-dependent increase in $\beta 1$ integrin-mediated attachment (Fig. 11A) and spreading of MDA-MB-435 cells to TSP1 or type I collagen (results not shown) . A similar serum response was observed in MDA-MB-231 cells for adhesion on TSP1, although adhesion of the latter cell line to type I collagen was maintained in the absence of serum (data not shown).

Several growth factors were examined to define the basis of the serum response for TSP1 adhesion (Fig. 11B). Addition of EGF to serum-depleted medium increases adhesion of breast carcinoma cells to some substrates (74) but in several experiments showed only a slight stimulatory activity for spreading of MDA-MB-435 cells on TSP1 (Fig. 11B). FGF2 and TGF$\beta 1$ were also ineffective, but addition of insulin stimulated MDA-MB-435 cell adhesion as well as 10% serum after 24 h (Fig. 11B). Insulin was also the only growth factor tested that stimulated adhesion of MDA-MB-231 cells to TSP1 (results not shown).

Acute addition of insulin, but not EGF, during the adhesion assay produced a similar enhancement in adhesion of both cell lines to TSP1 as the 24 h pretreatment of the cells in culture (Fig. 11C and results not shown). The dose-dependence for the insulin response was consistent with that for signaling through the IGF1 receptor (Fig. 11C), which is expressed in these breast carcinoma cells (75). Both insulin and IGF1 strongly stimulated MDA-MB-435 cell spreading on TSP1, moderately stimulated adhesion on type I collagen, but did not stimulate adhesion on laminin-1 (Fig. 11C). EGF (2 nM) was also inactive in this assay (results not shown). IGF1 (EC_{50} = 1 nM) was 100-fold more potent than insulin, as expected for a response mediated by the IGF1 receptor (75). A similar difference in the potencies of IGF1 and insulin was also observed in stimulation of TSP1 attachment of MDA-MB-231 cells

(results not shown). Thus, occupancy of the IGF1 receptor specifically stimulates the activity of the TSP1-binding integrin in both cell lines.

IGF1 also enhanced the chemotactic response to TSP1. Addition of IGF1 to MDA-MB-435 cells in the upper well of a modified Boyden chamber did not alter motility of the cells, but it stimulated (2- to 5-fold) the chemotactic response to TSP1 added to the lower chamber (Fig. 11D). This IGF1-stimulated motility to TSP1 was mediated by the $\alpha 3\beta 1$ integrin, because MAb13 ($\beta 1$) and P1B5 antibodies ($\alpha 3$) strongly inhibited direct TSP1 chemotaxis and that stimulated by IGF1. IGF1-stimulated chemotaxis to TSP1 was also sensitive to PT inhibition (Fig. 11D).

Modulation of TSP1 adhesion by CD98

Expression of the transmembrane protein CD98 is induced by serum, and this protein was recently shown to activate function of some $\beta 1$ integrins (76). Clustering of CD98 using the antibody 4F2 stimulates small cell lung carcinoma adhesion on fibronectin and laminin (76) and similarly activated $\alpha 3\beta 1$-mediated spreading of breast carcinoma cells on TSP1 and $\alpha 2\beta 1$-mediated adhesion on type I collagen (Fig. 12). In MDA-MB-435 cells spreading on TSP1, both CD98 and $\beta 1$ integrins are present on filopodia extending on the TSP1 substrate (Fig. 13A-C). This co-localization suggested that the induction of $\alpha 3\beta 1$-mediated TSP1 adhesion by growth with serum or insulin could be mediated by induction of CD98 expression. This may be the case for the serum response, because a 24 h exposure to 10% serum increased surface expression of CD98 in MDA-MB-435 cells (Fig. 13D). IGF1 treatment for the same time, however, decreased rather than increased CD98 expression (Fig. 13D).

CONCLUSIONS

Full length thrombospondin expression constructs containing four site-directed mutations of the type I repeat sequences have been prepared. These disrupt the WSXW motifs in each type 1 repeat and the latent TGFβ-activating sequence at the border of the first and second type 1 repeats. Stably transfected human breast carcinoma cell lines have been prepared expressing two of these mutants. Mutation of the central Trp residue in the second type 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 anti-angiogenic activity (77). Based on transient expression, disruption of the WSXW motifs in the first but not the second or third type 1 repeats abolishes the ability of TSP1 to inhibit endothelial cell proliferation. The mutant TSP1 with the latent TGFβ-activating sequence disrupted, however, retains its antiproliferative activity for endothelial cells. Therefore, this sequence is not required for the antiproliferative effect of TSP1 on endothelial cells. Analysis of a truncated mutant TSP1

(WG1a) suggests that TSP1 fragments lacking the carboxyl-terminal domains may have increased anti-angiogenic activity.

Expression of the F432A mutant inhibits proliferation of endothelial cells in transient transfections, but the same mutant can not accumulate to sufficient levels to significantly inhibit thymidine incorporation in transiently transfected breast carcinoma cells. This may explain our failure to isolate any stable MDA435 cell 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 (61, 62). We will continue to examine the stability and fate of these mutant thrombospondins in the breast carcinoma cells.

Thrombospondin type I repeat mutants have been shown to inhibit proliferation of bovine aortic endothelial cells to different degrees. While the W385A mutant showed little to no inhibitory effect in our thymidine incorporation assay, both W441A and W498G showed 50-60% inhibition of cell proliferation. We were also unable to isolate stable clones that express the mutant W385A protein. In this regard we think that the W385A protein might be functioning in a way similar to the F432A mutant protein. We have made a truncation mutant of wild type TSP, WG1a, which lacks the entire sequence 3' to the type I repeats. We have also shown that this mutant is a potent inhibitor of proliferation. Based on a recent report that gene therapy using a nonviral construct expressing a similar fragment of TSP1 in conjunction with p53 decreased breast tumor xenograft growth in nude mice (78), we will further examine the activities of this truncated TSP1.

We have reported in our earlier annual report that the F432A mutant protein is synthesized and secreted normally by the MDA435 cells. We had also reported that at 24 and 48 hours post transfection 50% and 30% cells compared to control were respectively positive for GFP protein, an indicator of cells that were transfected. So we had concluded that the mutant protein F432A is toxic to the MDA435 cells. Next, we wanted to see the effects of this mutant on incorporation of ^{3}H-thymidine, an indicator of cell proliferation. The TSP mutant F432A does not show a dose-dependent effect on inhibition of thymidine incorporation in MDA435 cells (Figure 1). When MDA435 cells are transfected with 24 μg of F432A DNA, the thymidine uptake stays at about 90% of control. This could mean that at higher concentrations the mutant F432A protein is toxic to the cells expressing it and the measured thymidine incorporation is that of untransfected cells. We have seen some inhibition of endothelial cell proliferation by the F432A mutant. In preliminary experiments, lower doses of this plasmid have produced stronger inhibition. This inverted dose-response suggests that transient transfection using lower doses of the F432A plasmid may allow us to examine the anti-proliferative activity of this mutant in both cell types.

Since it had been shown earlier that in MDA231 cells uPA and uPAR were up-regulated by thrombospondin (63), we wanted to see if TSP1expression had similar effects in MDA435 breast cancer cells. Compared to the parent cell line, stable over-expression of TSP1 was associated with a ten-fold increase in the uPAR level in the stable clone TH26, there was no significant change in the uPA level (Table 3). The interesting observation was that when MDA435 cells were transiently transfected with THBS-WT DNA or the empty vector, the uPAR level remained unchanged. Thus it seems that the increased uPAR level seen

in the stable clone TH26 may not be directly due to the over-expression of thrombospondin.

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

The α3β1 integrin in MDA-MB-435 cells does not recognize the RGD sequence in the TSP1 type 3 repeats. Because we have tested 85% of the TSP1 primary sequence using recombinant fragments or synthetic peptides, the β1 recognition motif may not be a linear epitope in TSP1. One caveat in interpreting the negative results using the recombinant TSP1 fragments, however, is that misfolding of fragments expressed in bacteria could selectively mask a linear recognition sequence for the α3β1 integrin in the GST- or T7-fusion proteins .

Several β1 integrins have been implicated as TSP1 receptors in other cell types, including α2β1 on activated platelets (79), α3β1 on neurons (80), and α4β1 and α5β1 on activated T lymphocytes (37). α3β1 is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas α2β1 mediates adhesion of these cells to type I collagen but not to TSP1. The integrin α4β1 may play some role in breast carcinoma adhesion to TSP1, as we previously reported for T lymphocytes (37). The mechanism for the apparent differential recognition of TSP1 by β1 integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by "inside-out" signaling (81), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to αvβ3 integrin, the α3β1 integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 "VVM" peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β1-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via αvβ3 but not α3β1 integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of α3β1-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically

associated with the αvβ3 integrin but not with β1 integrins in fibroblasts (82). The αvβ3 integrin also co-immunoprecipitated with insulin receptor substrate-1 (83). Engagement of αvβ3 integrin by vitronectin but not α2β1 integrin by collagen increased mitogenic signaling through the insulin receptor (82, 83). Thus, the specific activation of α3β1 mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the α3β1 integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific coupling of α3β1 activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

CD98 was recently identified as an activator of β1 integrins by its ability to overcome Tac-β1 suppression of β1 integrin function (76, 84). Our data demonstrate that clustering of CD98 can also increase α3β1-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated α3β1 integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis can not be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the α3β1 integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could be result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including H-Ras (85), integrin-linked kinase, and protein kinase C (81). Additional proteins are known to associate with the α3β1 integrin, including some members of the TM4SF family and EMMPRIN (86, 87), but their roles in regulating function are unknown. In MDA-MB-231 cells, suppression of α3β1 appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the α3β1 integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using α2β1 integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better α3β1-dependent adhesion to TSP1 than α2β1-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores demonstrates that regulation of α3β1 activity for TSP1 may differ even between two cell lines derived from the same type of human cancer.

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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

Table 2: **Effects of wild type and mutant TSPs on proliferation of BAE cells.**
Effects of the different transiently expressed proteins on proliferation of BAE cells was measured as ^{3}H-thymidine incorporation. Varying amounts of THBS wild type expression vector (WT) and 15-20 µg of the KRFK mutant (F432A), the first type I repeat mutant (W385A), the second type I repeat mutant (W441A), the third type I repeat mutant (W498G) or the truncation mutant (WG1a) were used for transfection and thymidine incorporation was measured as mentioned in the methods section. The transfection efficiencies are represented as β-galactosidase/µg total protein. The numbers in parentheses indicate number of samples assayed per group.

Sample	**^{3}H-thymidine uptake (% Control ± SEM)**	**Transfection Efficiency as mU β-gal/µg protein (% Control ± SEM)**
WT - 6 µg	90.41 ± 14.16	118.20 ± 13.61 (4)
WT - 12 µg	72.82 ± 9.01	124.19 ± 18.71 (4)
WT - 18 µg	48.34 ± 13.41	122.16 ± 41.99 (4)
F432A -18 µg	64.30 ± 19.35	143.25 ± 15.60 (2)
W385A - 15µg	128.01 ± 40.75	84.43 ± 17.59 (3)
W441A -19.5 µg	53.88 ± 8.33	127.36 ± 18.99 (5)
W498G - 16.5 µg	66.31 ± 13.49	129.15 ± 57.47 (3)
WG1a - 16.5 µg	19.06 ± 7.45	259.60 ± 104.35(2)

Table-3: **uPA/uPAR levels of stable clones and transient transfectants of MDA435 cells.** For transient transfection 12.5 µg of vector DNA or wild type THBS expression vector were used. uPAR levels were assayed 48 hours post transfection. The numbers in parentheses indicate number of samples assayed per group.•

	uPAR ng/mg protein ± SD (# of expts)	**uPA ng/mg protein ± SD**
Cell lines		
MDA435	9.0 ± 1.41 (2)	0.93 ± 0.64 (2)
TH26	97.71 ± 19.46 (4)	0.88 ± 0.16 (2)
Transient Transfectants		
Control vector	8.88 ± 1.6 (4)	
THBS-WT	10.03 ± 2.92 (4)	

Table 4. **Integrin expression in MDA-MB-435 breast carcinoma cells.**

Integrin	Antibody	Mean fluorescence intensity
α2β1	6D7	200
α3β1	M-KID2	127
α4β1	HP2/1	98
α5β1	SAM1	122
αv	LM142	256
αvβ3	LM609	157
β1	MAB13	158
-	mouse IgG	31

Table 5. **Modulation of breast carcinoma spreading on TSP1.**

	Cell line		
Inhibitor	MDA-MB-435	MDA-MB-231	MCF-7
	spreading (% of TS2/16)		
staurosporine	112 ± 16	13 ± 3	39 ± 8
KT5720	35 ± 25	1 ± 2	
bisindoylmaleimide	15 ± 10	0 ± 1	
KT5823	17.2 ± 7.9	0 ± 1	
RP8-pCPT-cGMPS	-2 ± 6	3 ± 1	
wortmannin-2	-18 ± 18	16 ± 4	
ionomycin	52 ± 3	0 ± 0	
A23187	83 ± 29	0 ± 0	
herbimycin	-16 ± 1	0 ± 1	
vanadate	-9 ± 2	8 ± 2	

MDA-MB-435 or MDA-MB-231 cell spreading on TSP1 was measured in untreated cells in the presence or absence of the β1-activating antibody TS2/16 to measure basal and total β1-dependent adhesion and in cells pretreated with and maintained in the following inhibitors: 10 nM staurosporine (Ser/Thr kinase inhibitor), 100 nM KT5720 (protein kinase A), 200 nM bis-indoylmaleimide (protein kinase C), 1 μM KT5823 or 2 μM Guanosine-3',5'-cyclic monophosphorothioate, 8-(4-chloro-phenylthio)-, Rp-isomer (protein kinase G), 2 nM wortmannin (PI 3-kinase), 1 μg/ml ionomycin or A23187 (calcium ionophores), 1 μM herbimycin (tyrosine kinase), or 20 μM vanadate. The net increase in cell spreading in the presence of the indicated drugs is expressed as a percent of that induced by the β1-activating antibody TS2/16, mean ± SD, n = 3.

FIGURE LEGENDS

Figure 1: Dose response for inhibiting proliferation of MDA435 breast carcinoma cells by expression of wild type TSP1 or TSP1 with mutation in the TGFβ activation sequence (KRFK). (Panel A) ^{3}H-thymidine incorporation by MDA435 cells transiently transfected with varying amounts of THBS wild type expression vector (WT) and the KRFK mutant (F432A) were measured as described in the methods section. (Panel B) The transfection efficiencies were determined by co-transfection with a constant amount of β-galactosidase vector. Efficiency was calculated as β-galactosidase/ μg total protein and is presented as a percent of control transfections without addition of the THBS vectors.

Figure 2. Specificity of ^{125}I-TSP binding to MDA-MB-435 cells. Cells were harvested using 2.5 mM EDTA in PBS, resuspended in RPMI 1640 medium containing 0.1% BSA, and incubated with ^{125}I-TSP for 1 h at 25^{0} C with the indicated inhibitors. Cells were centrifuged through oil to remove unbound labeled protein. The mean ± SD for triplicate determinations is presented for binding determined in the absence (control) or presence of 4 μM 18 kDa recombinant TSP1 heparin-binding domain (HBD), 204 μM GRGDS peptide, 100 μg/ml heparin, 10 μg/ml MAb13 (anti-β1) or P1B5 (anti-α3β1).

Figure 3. Role of integrins and sulfated glycoconjugates in breast carcinoma cell adhesion and chemotaxis to TSP1. Panel A: MDA-MB-435 cell attachment (solid bars) and spreading (striped bars) was measured on polystyrene coated with TSP (50 μg/ml) and blocked with 1% BSA to reduce nonspecific adhesion. Heparin-dependent adhesion was assessed by inhibition using 4 μM 18 kDa recombinant TSP1 heparin-binding domain (HBD) or 50 μg/ml heparin. β1 Integrin-dependent adhesion was inhibited using 2 μg/ml or 50 μg/ml MAb13 (anti-β1). Results are presented as mean ± SD, n = 3. Panel B: Effect of inhibiting sulfation on attachment of MDA-MB-435 cells. MDA-MB-435 cells were grown in Ham's F-12 medium (low sulfate) containing 10% dialyzed fetal calf serum for 48 h. The medium was replaced with the same medium containing 1% dialyzed serum with or without sodium chlorate at the indicated concentrations. The cells were cultured for 24 h, harvested, and resuspended in F-12 medium containing 1 mg/ml BSA with or without chlorate at the indicated concentrations. Cell adhesion was quantified to polystyrene coated with 50 μg/ml of TSP (striped bars) or 10 μg/ml FN (gray bars). ^{35}S-Incorporation in MDA-435 cell macromolecules (-o-). was assessed in duplicate cultures supplemented with 25 μCi/ml [^{35}S] sulfate. The cells were fixed and washed in acetic acid/methanol, and incorporation of radioactivity in macromolecules was determined by scintillation counting after solubilization in 1% sodium dodecyl sulfate. Panel C: Integrin αvβ3 mediates breast carcinoma cell adhesion to vitronectin but not to TSP1. Adhesion of MDA-MB-435 cells to 30 μg/ml TSP1 (solid bars) or 10 μg/ml vitronectin (striped bars) was measured in the presence of the αvβ3 function blocking antibody LM609 or the β1 activating antibody TS2/16. Panel D: Chemotaxis to TSP1 is β1 integrin-dependent. MDA-MB-435 chemotaxis to 50 μg/ml TSP1 was determined in modified Boyden chambers. Cells were added in the upper chamber with the incubated concentrations of β1 integrin blocking antibody MAb13 (●) or heparin (o). Spontaneous motility (▲) was determined in the absence of TSP1. Migrated cells were counted microscopically, and results from triplicate wells are presented as a percent of migration to TSP1 without inhibitors, mean ± SD.

Figure 4. β1 Integrins recognizing TSP1 and type I collagen are partially inactive in human breast carcinoma cell lines. Panel A: Spreading of three breast carcinoma cell lines on 50 μg/ml TSP1 (solid

bars) or on TSP1 in the presence of 5 μg/ml TS2/16 (striped bars). Panel B: Comparison of β1 integrin activity in MDA-MB-435 cells harvested by scraping in RPMI medium or by a 5 min treatment with 2.5 mM EDTA in PBS. Cells were resuspended in RPMI medium with 0.1% BSA (solid bars) or with 20 μg/ml TS2/16 (striped bars), and cell spreading was assessed on substrates coated with 20 μg/ml of TSP1 or 5 μg/ml of type I collagen.

Figure 5. Integrin α-subunit specificity of TSP1 adhesion. Panel A: MDA-MB-231 cell attachment was quantified using substrates coated with 40 μg/ml TSP1 (solid bars) or 5 μg/ml type I collagen (gray bars) in the presence of 5 μg/ml of TS2/16 to activate β1 integrins and 5 μg/ml of the indicated function blocking antibodies. Panel B: Inhibition of MDA-MB-435 cell spreading on TSP1 (solid bars) or type I collagen (gray bars) in the presence of TS2/16 and the indicated α subunit blocking antibodies.

Figure 6. Actin organization and filopodia formation on TSP1 is stimulated by β1 integrin activation. Actin was visualized using BODIPY-phallacidin in MDA-MB-435 cells attached on TSP1 (panel A) or TSP1 in the presence of 5 μg/ml TS2/16 (panel B). β1 Integrin localization of the TS2/16 treated cells was visualized using BODIPY FL-anti-mouse IgG on TSP1 (panel C) or type I collagen substrates (panel D). Bar in panel A = 20 μm.

Figure 7. Adhesion to recombinant TSP1 fragments and synthetic TSP1 peptides. Panel A: Cell attachment to TSP1 or a 140 kDa proteolytic fragment of TSP1 at the indicated concentrations (solid bars) was enhanced by addition of 20 μg/ml of the β1-activating antibody TS2/16 (striped bars). Panel B: Adhesion to synthetic TSP1 peptides adsorbed at 10 μM (246, KRFKQDGGWSHWSPWSS; 500, NGVQYRNC; Mal II, SPWSSCSVTCGDGVITRIR; 4N1K, KRFYVVMWKK; HepI, ELTGAARKGSGRRLVKGPD), TSP1 (0.11 μM), recombinant 18 kDa heparin-binding domain (HBD, 2.7 μM), or GST-fusion proteins expressing the TSP1 procollagen domain, type 1, type 2, or type 3 repeats (2 μM) was measured in the absence (solid bars) or presence of 20 μg/ml of TS2/16 (striped bars).

Figure 8. Tertiary structure dependence for MDA-MB-435 cell adhesion on TSP1. MDA-MB-435 cell adhesion to 20 μg/ml TSP1 coated on polystyrene in 10 mM sodium acetate, 150 mM NaCl, pH 4 (39), Dulbecco's PBS with calcium and magnesium (PBS-Ca), PBS with 2.5 mM EDTA, or PBS with 2.5 mM EDTA and 2 mM dithiothreitol (DTT). Attachment (solid bars) and spreading (striped bars) were assessed in the absence or presence of 5 μg/ml TS2/16.

Figure 9. Differential regulation of β1 and β3 integrin activity in MDA-MB-435 cells. Attachment of MDA-MB-435 cells on 5 μg/ml vitronectin (striped bars) or 40 μg/ml TSP1 (solid bars) was measured using cells treated with 20 μg/ml TS2/16, 10 ng/ml PMA, or 3 μM of the CD47-binding TSP1 peptide 7N3 (FIRVVMYEGKK). Results are presented as a % of cell attachment without additions, mean ± SD, n = 3.

Figure 10. Pertussis toxin differentially regulates MDA-MB-435 and MDA-MB-231 cell adhesion on TSP1. Panel A: MDA-MB-231 cell attachment on 40 μg/ml TSP1 (solid bars) or 5 μg/ml type I collagen (gray bars) was measured alone or in the presence of 5 μg/ml TS2/16, 1 μg/ml PT, or 1 μg/ml PT B-oligomer. Results are mean ± SD for triplicate determinations. Panel B: MDA-MB-435 cell spreading was determined on TSP1 (solid bars) or type I collagen substrates (gray bars) in the presence of PT or PT

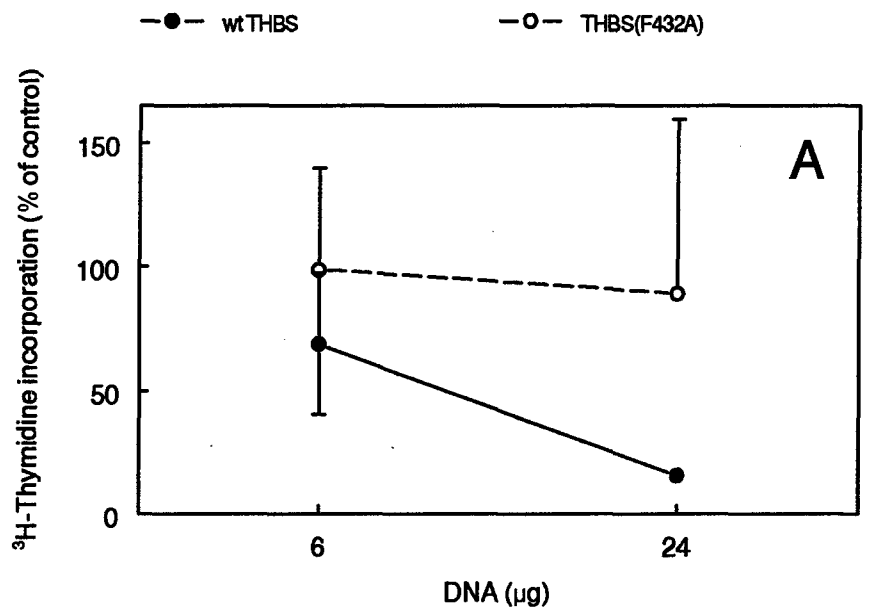
B-oligomer added alone or combined with 5 μg/ml TS2/16.

Figure 11. Regulation of β1 integrin-mediated TSP1 interactions by serum and growth factors. Panel A: Serum induces attachment of MDA-MB-435 cells to TSP1(solid bars) and type I collagen (striped bars). Cells were grown for 24 h in RPMI medium containing the indicated concentration of FCS. Panel B: Insulin specifically induces adhesion of breast carcinoma cells to TSP1. MDA-MB-435 cell spreading on surfaces coated with 40 μg/ml of TSP1 was determined using cells grown for 24 h in RPMI medium containing 2% serum and supplemented with the indicated growth factors (10 ng/ml EGF, 100 ng/ml FGF2, 5 ng/ml TGFβ, or 10 μg/ml insulin) or RPMI medium containing 10% serum. Spreading of cells grown in 2% serum was also tested in the presence of 5 μg/ml of antibody TS2/16 (2%+TS2/16) to assess maximal β1 integrin-mediated spreading activity. Panel C: Dose dependence for induction of TSP1 adhesion by insulin and IGF1. Cell spreading (expressed as a % of maximal spreading elicited on each substrate in the presence of 5 μg/ml TS2/16 antibody) was determined in RPMI medium containing 0.1% BSA and supplemented with the indicated concentrations of insulin (closed symbols) or IGF1 (open symbols) using substrates coated with 40 μg/ml TSP1 (o,●), 20 μg/ml laminin (▲,△), or 5 μg/ml type I collagen (■, □). Panel D: IGF1 synergizes with TSP1 to promote chemotaxis of MDA-MB-435 cells. Chemotaxis to 50 μg/ml TSP1 was determined in the presence of the indicated inhibitors or stimulators at the following concentrations: 10 nM IGF1 5 μg/ml MAb13 (anti-β1), 5 μg/ml P1B5 (anti-α3), and 1 μg/ml PT. Results are mean ± SD, n = 3-6.

Figure 12. CD98 ligation stimulates breast carcinoma cell adhesion to TSP1. Basal (solid bars) or stimulated MDA-MB-231 or MDA-MB-435 cell spreading on 25 μg/ml TSP1 or 5 μg/ml type I collagen was determined in the presence of 5 μg/ml TS2/16 (striped bars) or 20 μg/ml 4F2 (gray bars).

Figure 13. CD98 localization and expression in MDA-MB-435 cells. Panel A: CD98 is present with β1 integrin in filopodia of cells spreading on TSP1. MDA-MB-435 cells were allowed to attach on TSP1, fixed, and incubated with β1 integrin (Panel A) or CD98 antibodies (Panel B) or control without primary antibody (Panel C) followed by BODIPY FL-anti mouse IgG. Bar in panel A represents 10 μm. Panel D: Serum induces but IGF1 inhibits CD98 expression. MDA-MB-435 cells grown 24 h in RPMI medium containing 1% FCS, 10% FCS or 1% FCS and 10 nM IGF1 as described in Fig. 10A were biotinylated and immunoprecipitated with antibody 4F2. The immunoprecipitates were analyzed by SDS gel electrophoresis and Western blotting using streptavidin-peroxidase and chemiluminescent detection. Markers indicate the migration of the 80 and 45 kDa subunits of CD98.

Fig.1


wt THBS
THBS(F432A)
^{3}H-Thymidine incorporation (% of control)
150
100
50
0
6
24
DNA (μg)
A

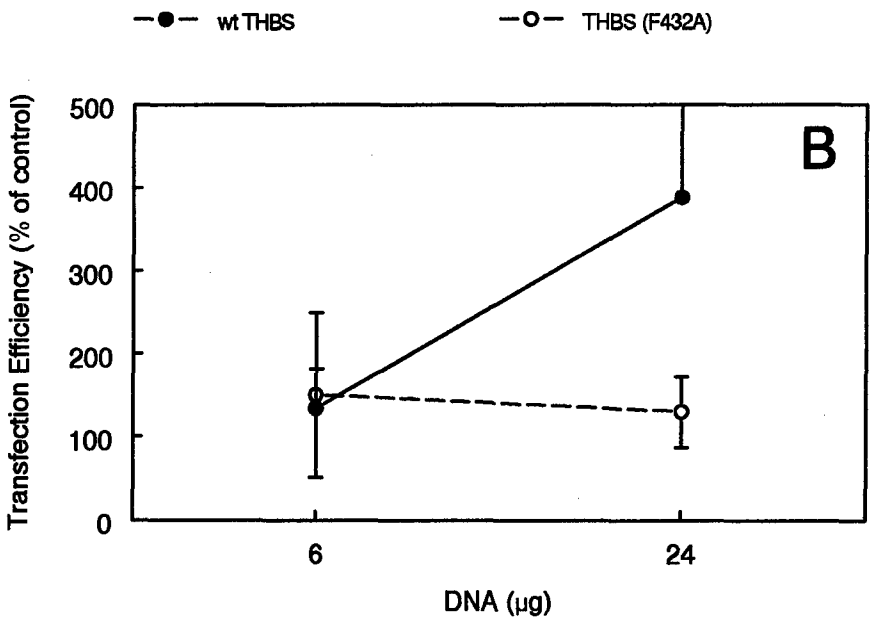

wt THBS
THBS (F432A)
Transfection Efficiency (% of control)
500
400
300
200
100
0
6
24
DNA (μg)
B

Fig.2

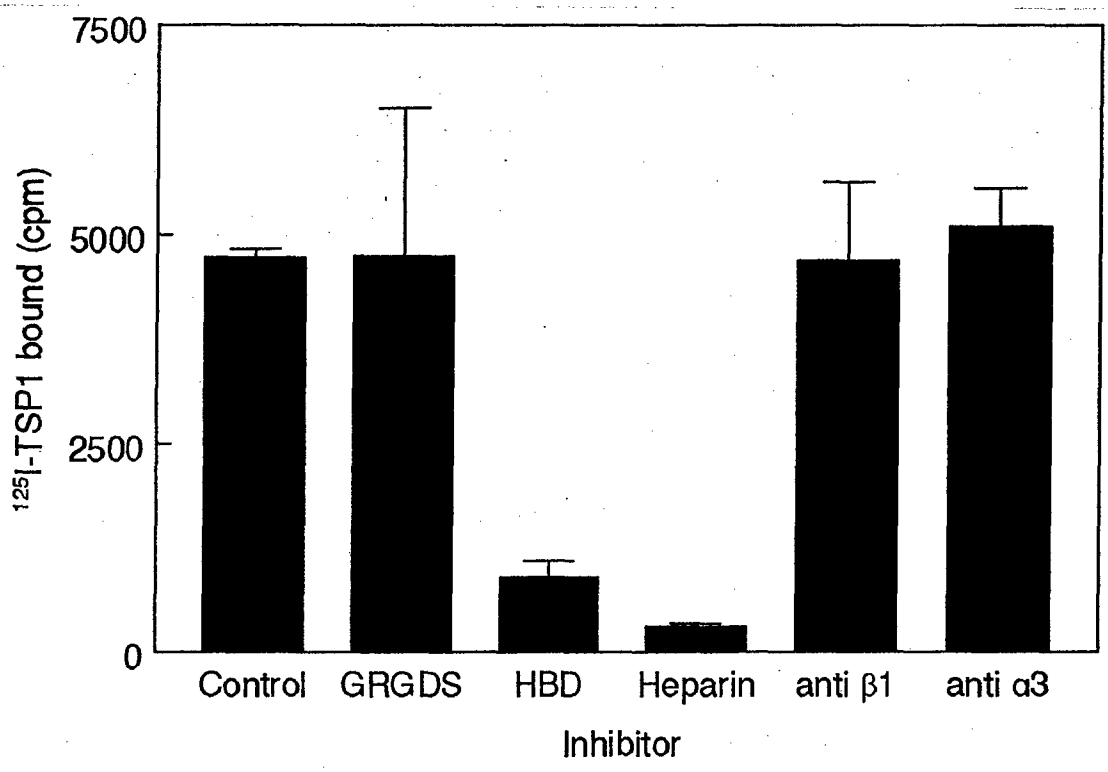

7500
5000
2500
0
^{125}I-TSP1 bound (cpm)
Control
GRGDS
HBD
Heparin
anti β1
anti α3
Inhibitor

Fig.3

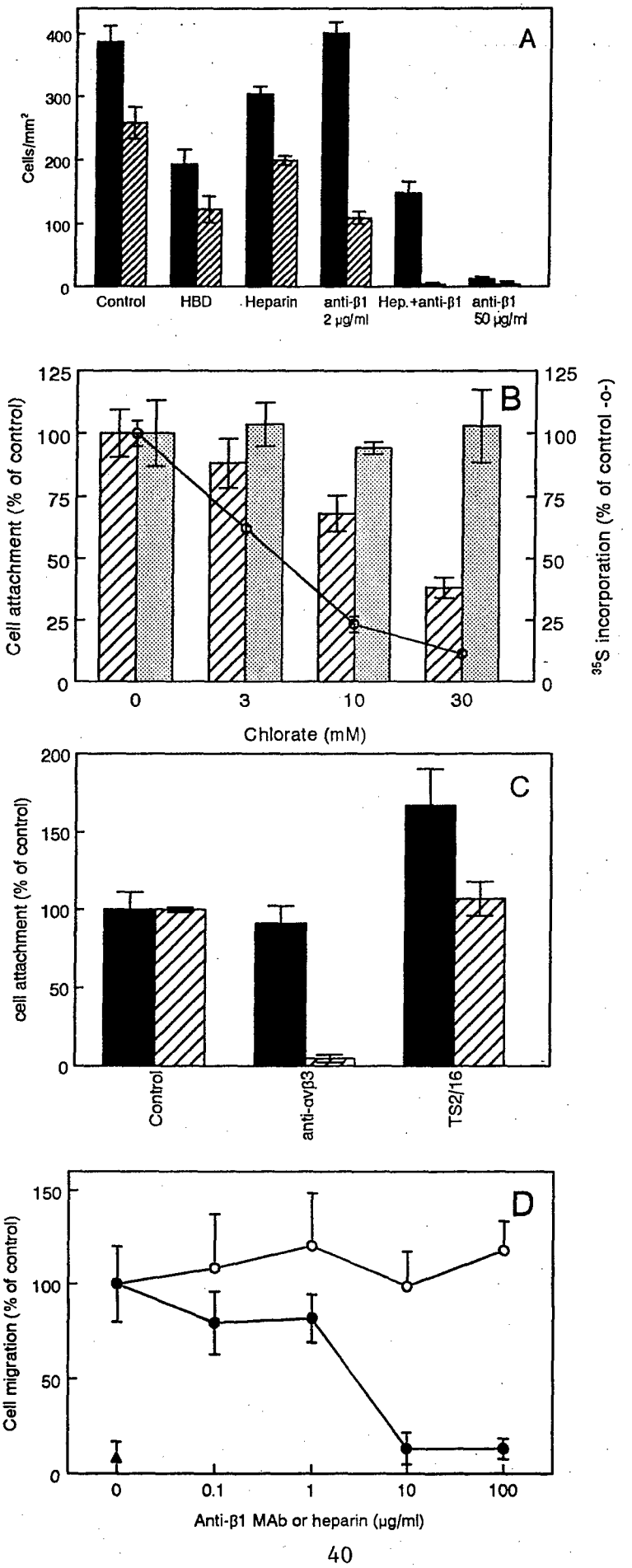

A
Cells/mm²
400
300
200
100
0
Control
HBD
Heparin
anti-β1 2 μg/ml
Hep.+anti-β1
anti-β1 50 μg/ml
B
Cell attachment (% of control)
125
100
75
50
25
0
^{35}S incorporation (% of control -o-)
0
3
10
30
Chlorate (mM)
C
cell attachment (% of control)
200
150
100
50
0
Control
anti-αvβ3
TS2/16
D
Cell migration (% of control)
150
100
50
0
0
0.1
1
10
100
Anti-β1 MAb or heparin (μg/ml)

Fig.4

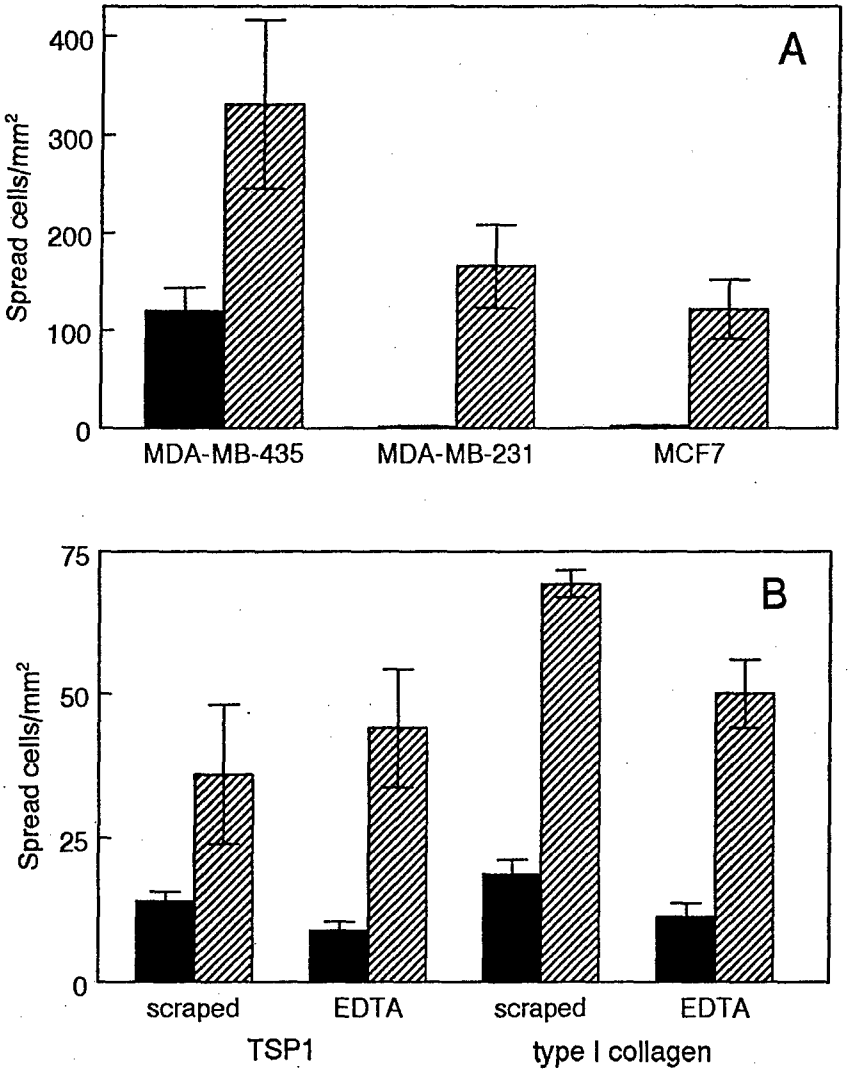
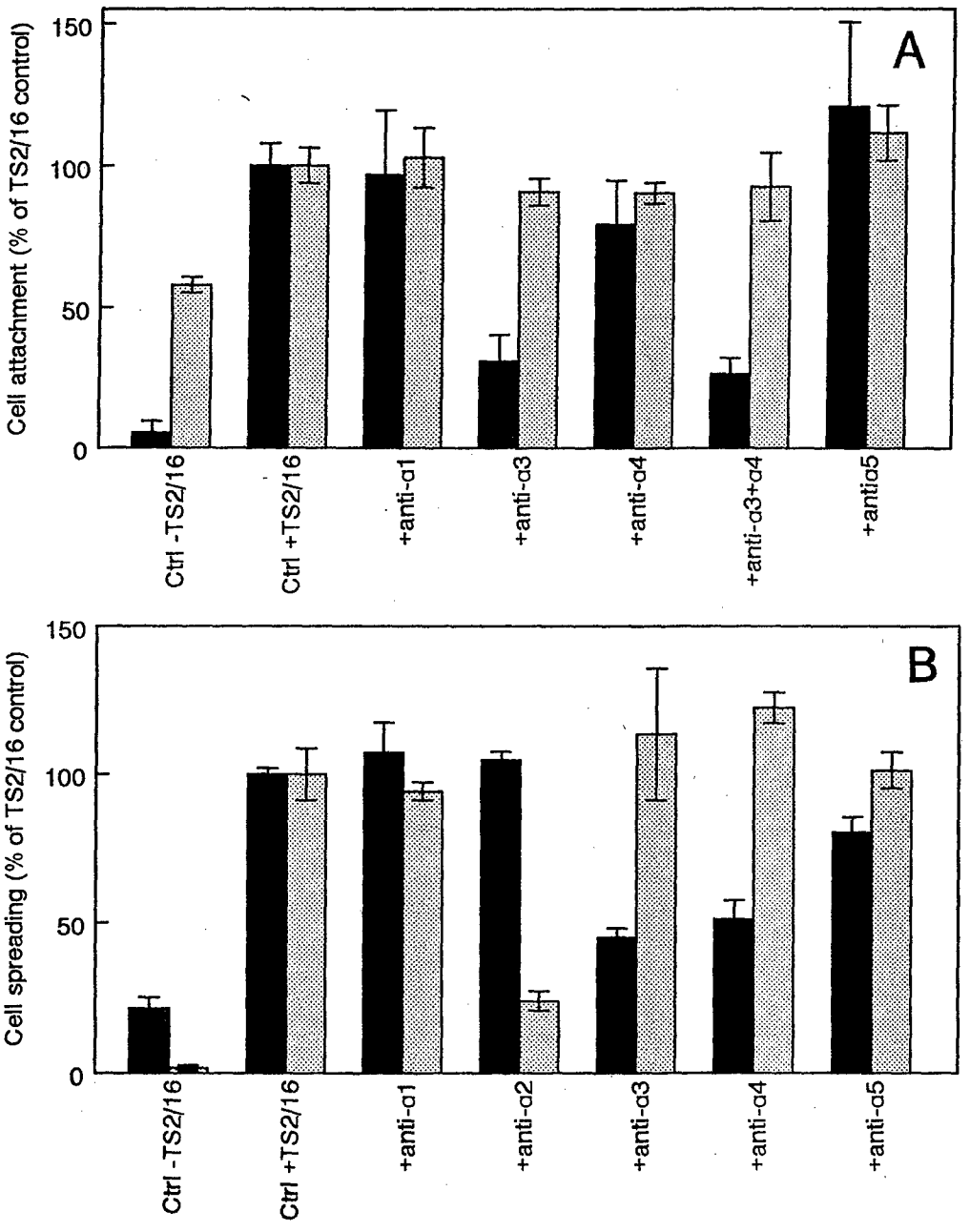

A
Spread cells/mm²
400
300
200
100
0
MDA-MB-435
MDA-MB-231
MCF7
B
Spread cells/mm²
75
50
25
0
scraped
EDTA
scraped
EDTA
TSP1
type I collagen

Fig.5


A
Cell attachment (% of TS2/16 control)
150
100
50
0
Ctrl -TS2/16
Ctrl +TS2/16
+anti-α1
+anti-α3
+anti-α4
+anti-α3+α4
+antiα5
B
Cell spreading (% of TS2/16 control)
150
100
50
0
Ctrl -TS2/16
Ctrl +TS2/16
+anti-α1
+anti-α2
+anti-α3
+anti-α4
+anti-α5

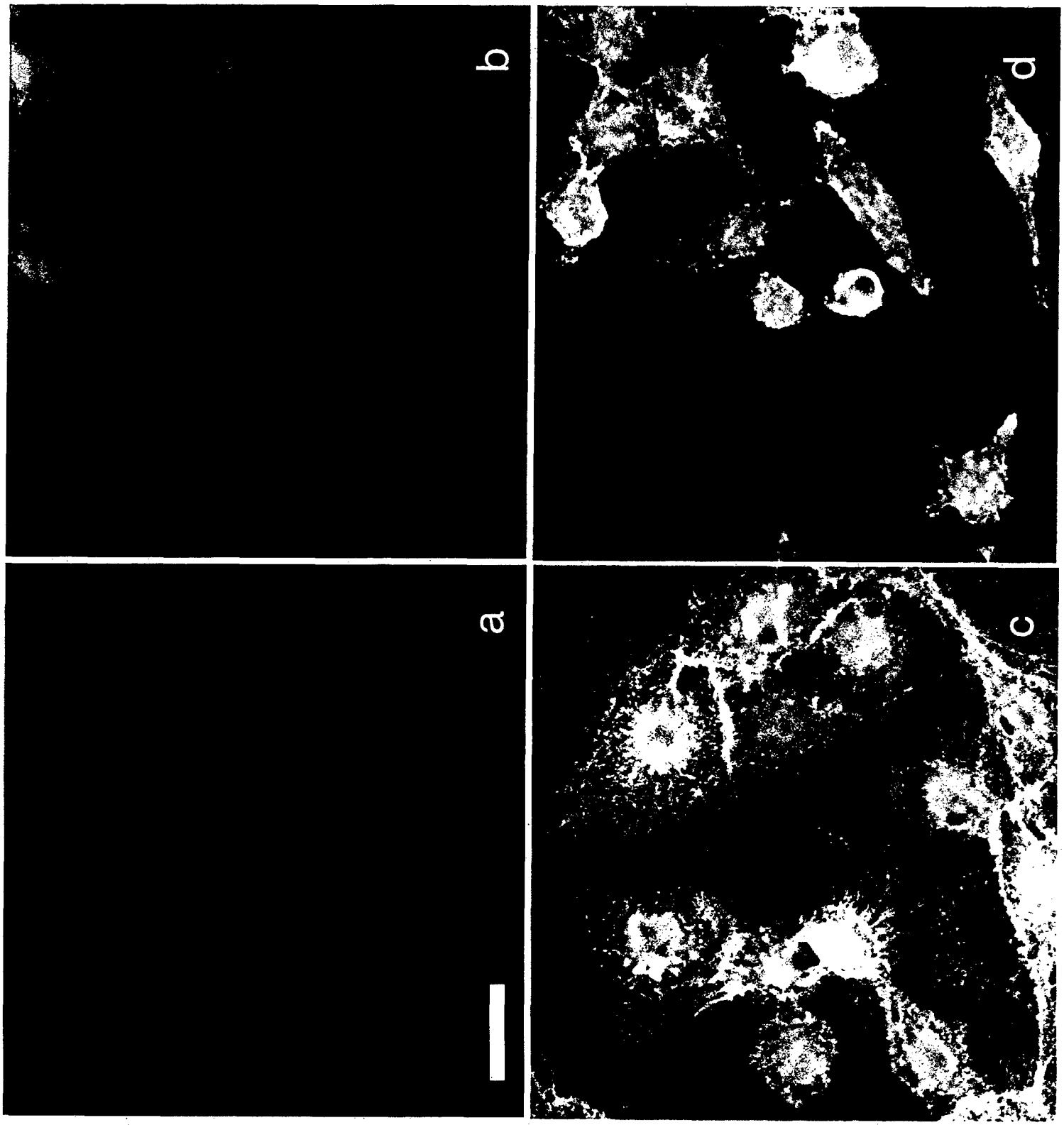


Fig.6

Fig.7

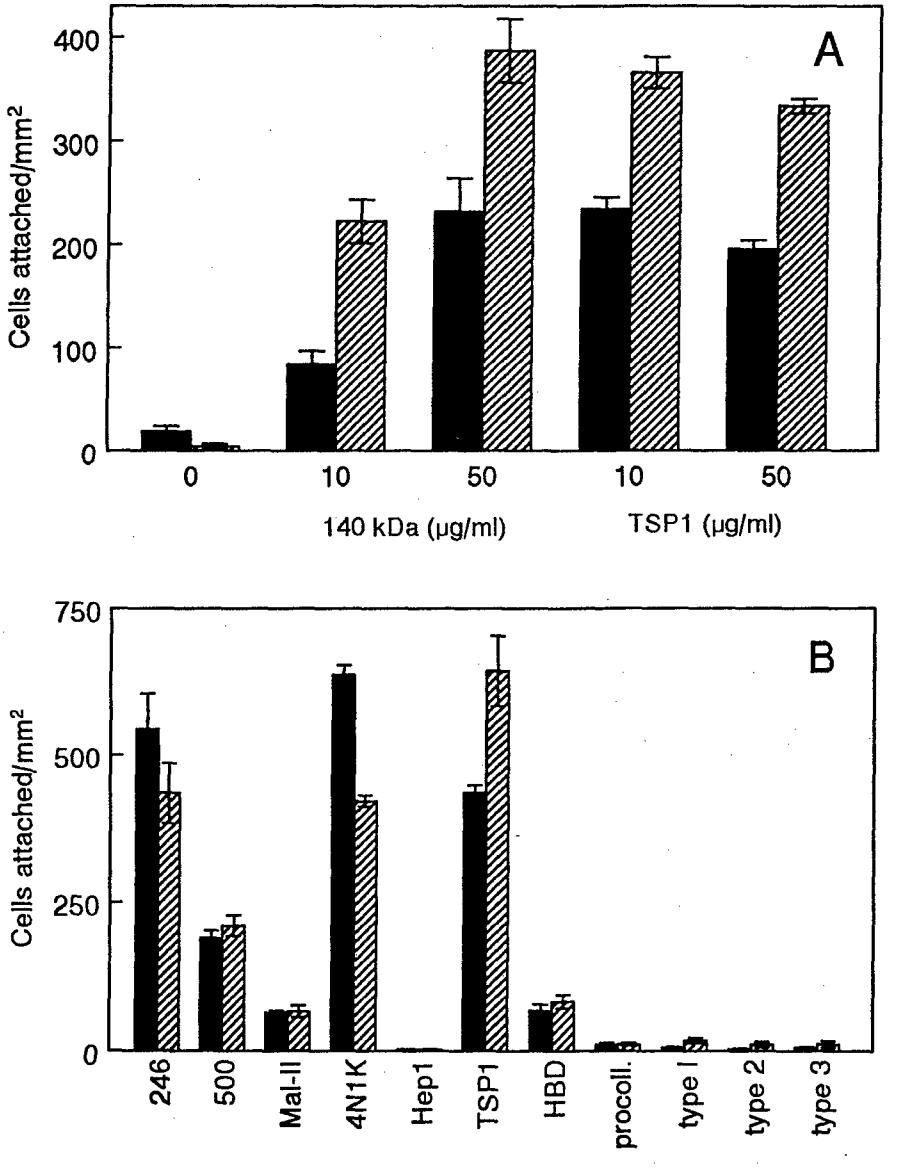

A
Cells attached/mm²
400
300
200
100
0
0
10
50
10
50
140 kDa (µg/ml)
TSP1 (µg/ml)
B
Cells attached/mm²
750
500
250
0
246
500
Mal-II
4N1K
Hep1
TSP1
HBD
procoll.
type I
type 2
type 3

Fig.8

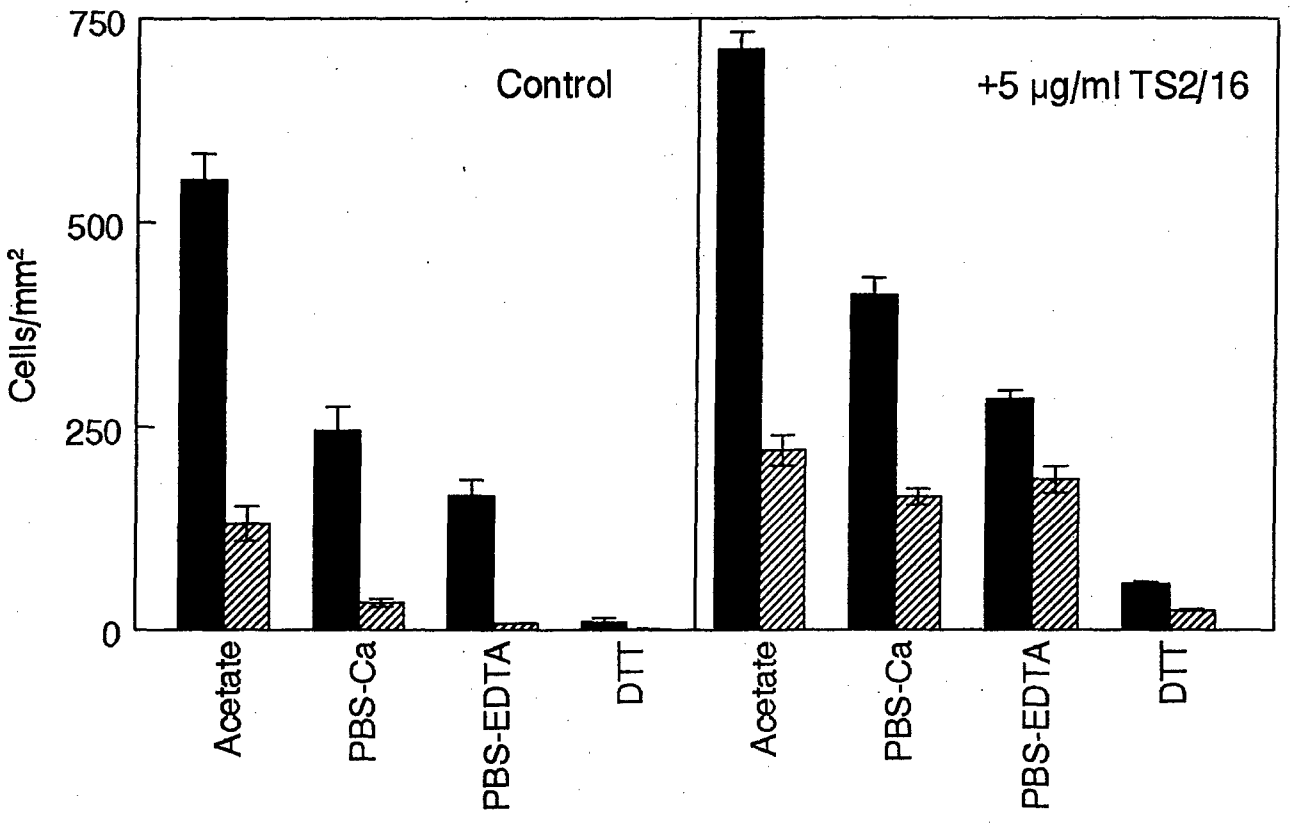

750
500
250
0
Cells/mm²
Control
+5 µg/ml TS2/16
Acetate
PBS-Ca
PBS-EDTA
DTT
Acetate
PBS-Ca
PBS-EDTA
DTT

Fig.9

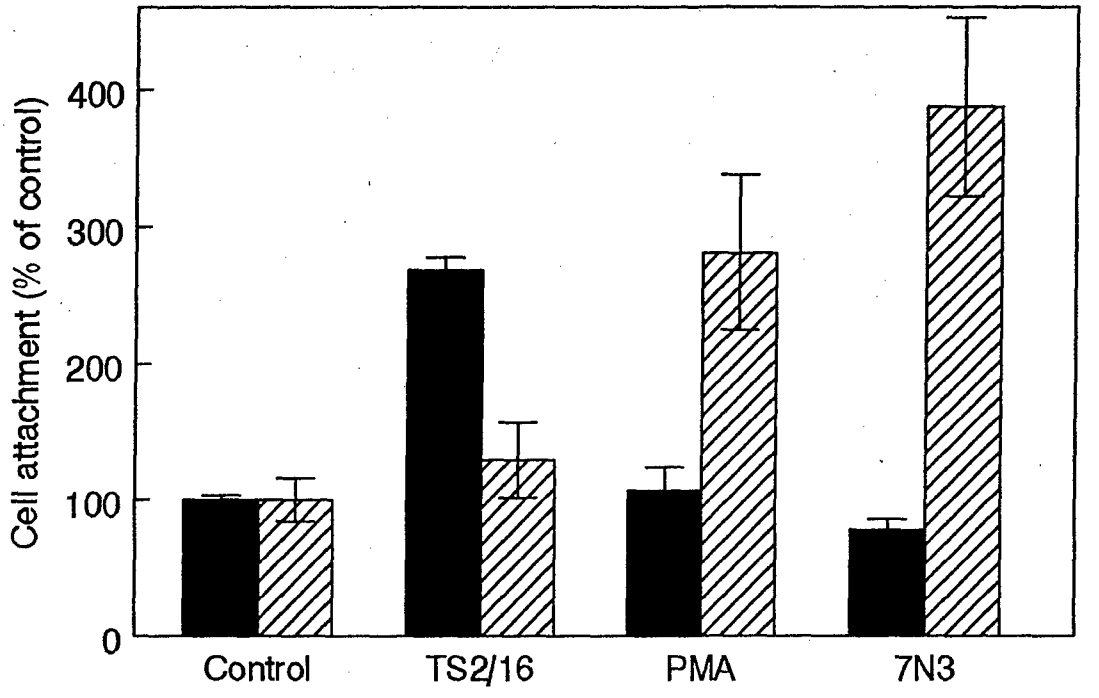
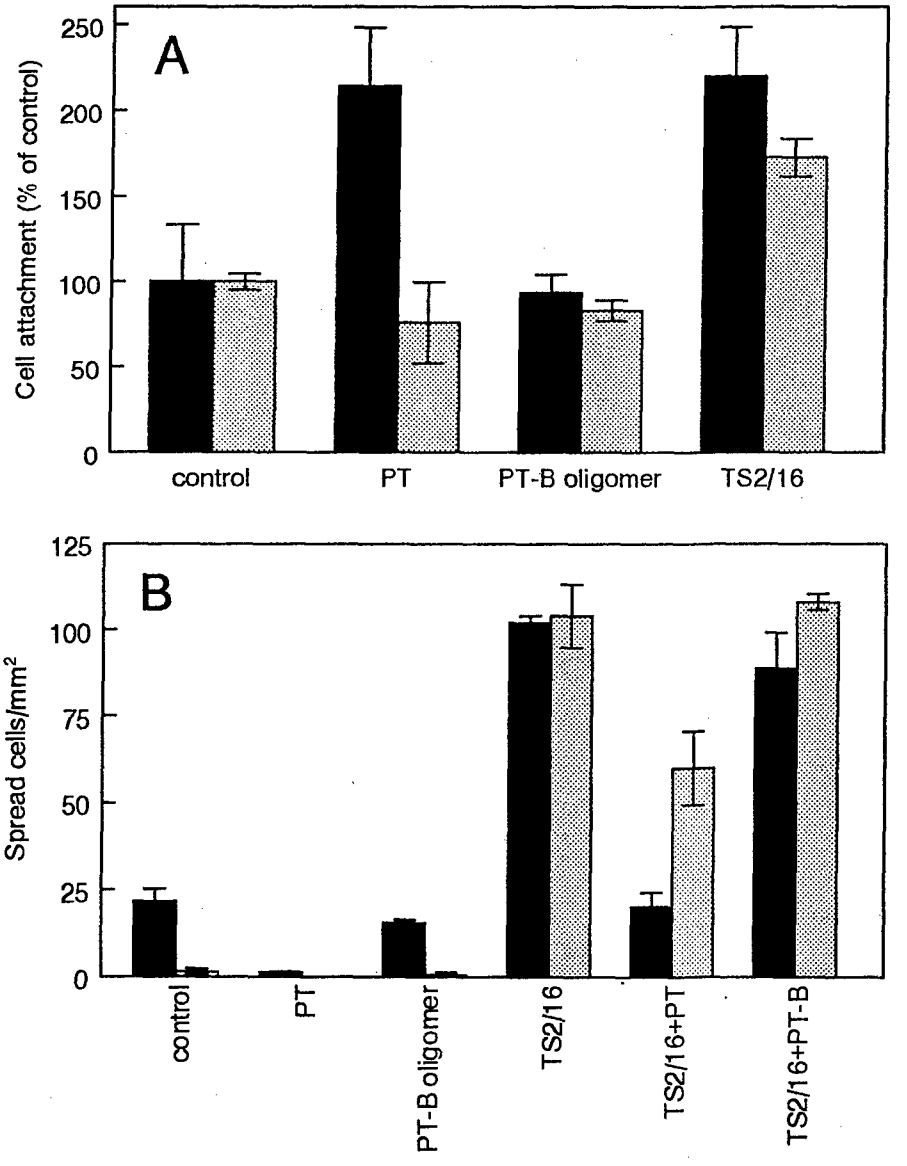

Cell attachment (% of control)
400
300
200
100
0
Control
TS2/16
PMA
7N3

Fig.10


A
Cell attachment (% of control)
250
200
150
100
50
0
control
PT
PT-B oligomer
TS2/16
B
Spread cells/mm²
125
100
75
50
25
0
control
PT
PT-B oligomer
TS2/16
TS2/16+PT
TS2/16+PT-B

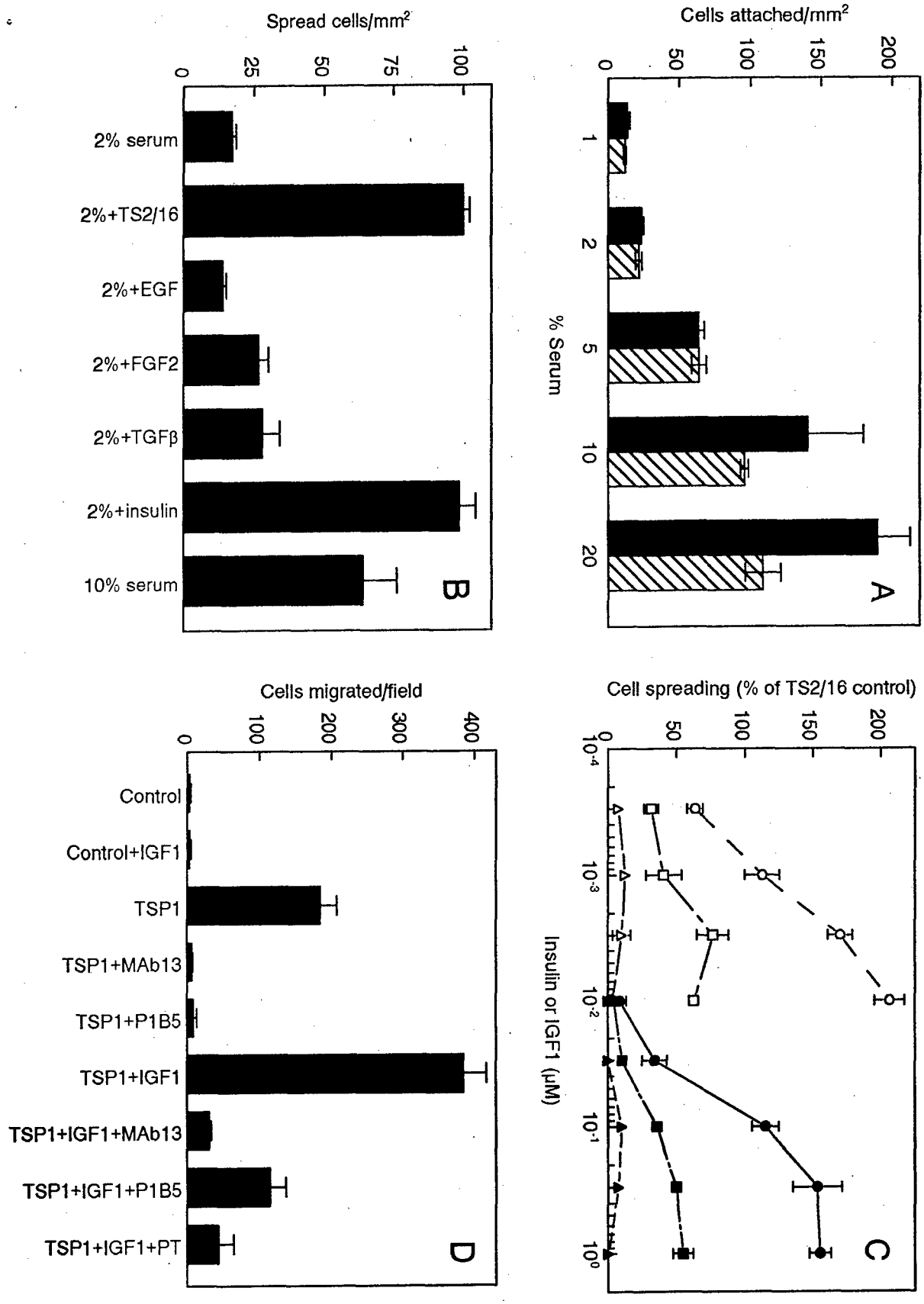

Cells attached/mm²
0
50
100
150
200
1
2
5
10
20
% Serum
A
Spread cells/mm²
0
25
50
75
100
2% serum
2%+TS2/16
2%+EGF
2%+FGF2
2%+TGFβ
2%+insulin
10% serum
B
Cell spreading (% of TS2/16 control)
0
50
100
150
200
10⁻⁴
10⁻³
10⁻²
10⁻¹
10⁰
Insulin or IGF1 (μM)
C
Cells migrated/field
0
100
200
300
400
Control
Control+IGF1
TSP1
TSP1+MAb13
TSP1+P1B5
TSP1+IGF1
TSP1+IGF1+MAb13
TSP1+IGF1+P1B5
TSP1+IGF1+PT
D

Fig.11

Fig.12

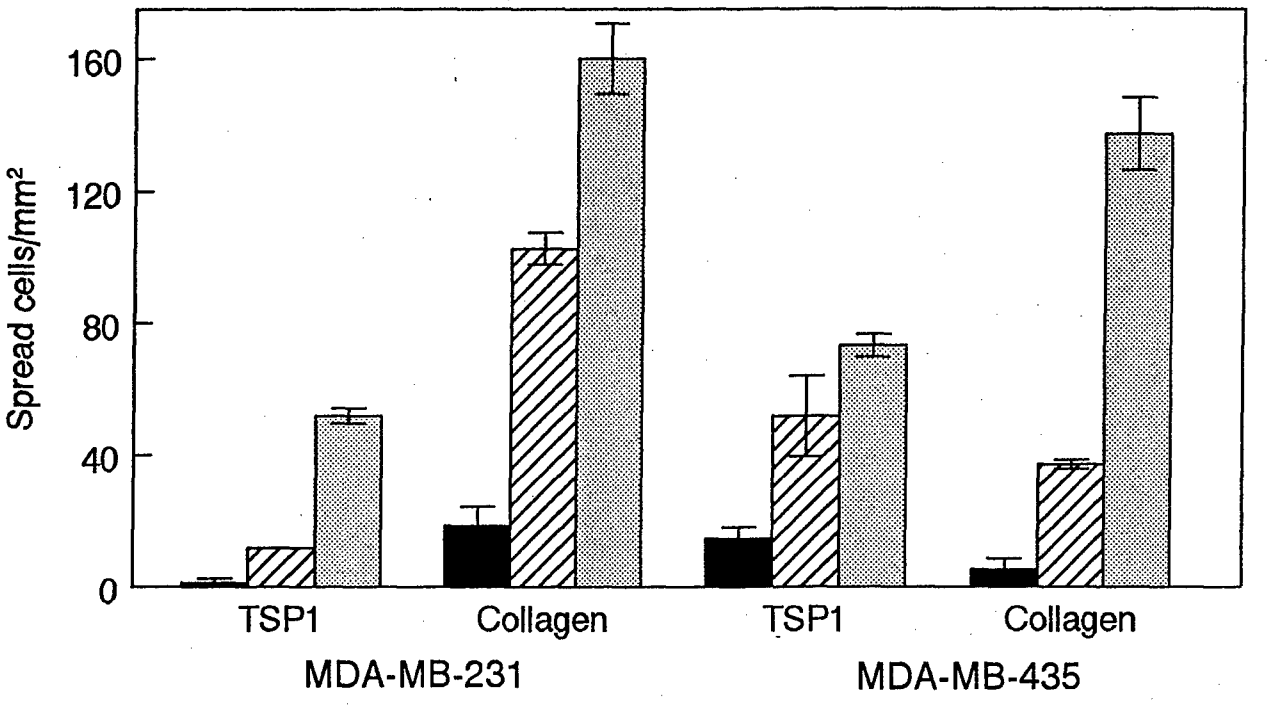

Spread cells/mm²
160
120
80
40
0
TSP1
Collagen
TSP1
Collagen
MDA-MB-231
MDA-MB-435

Fig.13

Pro-adhesive and Chemotactic Activities of Thrombospondin-1 for Breast Carcinoma Cells are Mediated by α3β1 Integrin and Regulated by Insulin-like Growth Factor-1 and CD98

S. Chandrasekaran, Neng-hua Guo, Rui G. Rodrigues, James Kaiser, and David D. Roberts

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Address correspondence to David D. Roberts, Building 10, Room 2A33, 10 CENTER DR. MSC 1500, NIH, Bethesda, MD 20892-1500. Tel: 301-496-6264; Fax: 301-402-0043; E-mail: droberts@helix.nih.gov

Dr. Kaiser's present address is CBER/OTRR/DCTDA, FDA, Woodmont Office Complex 1, 1401 Rockville Pike, Rockville MD 20852

Condensed title: Regulation of thrombospondin-1 adhesion by α3β1 integrin

Keywords: adhesion, motility, integrin, breast cancer,

Abstract.

Thrombospondin-1 (TSP1) is a matricellular protein that displays both pro- and anti-adhesive activities. Binding to sulfated glycoconjugates mediates most high affinity binding of soluble TSP1 to MDA-MB-435 cells, but attachment and spreading of these cells on immobilized TSP1 is primarily β1 integrin-dependent. The integrin α3β1 is the major mediator of breast carcinoma cell adhesion and chemotaxis to TSP1. This integrin is partially active in MDA-MB-435 cells but is mostly inactive in MDA-MB-231 and MCF-7 cells, which require β1 integrin activation to induce spreading on TSP1. Adhesion of breast carcinoma cells on TSP1 is Arg-Gly-Asp-independent and localized to the 140 kDa fragment of TSP1. Integrin-mediated cell spreading on TSP1 is accompanied by extension of filopodia containing β1 integrins and CD98. TSP1-binding activity of the α3β1 integrin is not stimulated by CD47 binding peptides from TSP1 or by protein kinase C activation, which activate αvβ3 function in the same cells. In MDA-MB-231 but not MDA-MB-435 cells, this integrin is activated by pertussis toxin, whereas serum, insulin, IGF1, and ligation of CD98 increase activity of this integrin in both cell lines. Serum stimulation is accompanied by increased surface expression of CD98, whereas IGF1 does not increase CD98 expression. Thus, the pro-adhesive activity of TSP1 for breast carcinoma cells is controlled by several signals that regulate activity of the integrin α3β1.

Thrombospondin-1 (TSP1[1]) is an extracellular matrix glycoprotein that has diverse effects on cell behavior (reviewed in Bornstein, 1995, Frazier, 1991). The five known thrombospondin genes display distinct patterns of expression during development and in several disease states. Disruption of the *thbs1* gene in mice results in lordosis of the spine and abnormal proliferation and inflammatory responses in the lung (Lawler, et al., 1998). Suppression of *THBS1* expression by loss of wild type p53, by activated Ras, Myc, nickel, and in metastatic clones of several tumor cell lines suggested that loss of TSP1 expression may contribute to tumor progression (reviewed in Roberts, 1996). Consistent with this hypothesis, over-expression of *THBS1* in breast carcinoma cells (Weinstat-Saslow, et al., 1994), a transformed endothelial cell line (Sheibani and Frazier, 1995), fibroblasts from Li Fraumini patients (Dameron, et al., 1994), and glioblastoma cells (Hsu, et al., 1996) decreases tumor growth in animal models. This suppressive activity is due at least in part to the anti-angiogenic activity of TSP1 (reviewed in Iruela-Arispe and Dvorak, 1997, Roberts, 1996, Volpert, et al., 1995). TSP1 antagonizes growth factor-stimulated proliferation and migration of endothelial cells. Its anti-angiogenic activity is thought to be the major mechanism for suppression of tumor growth in *THBS1*-transfected MDA-MB-435 breast carcinoma cells, because TSP over-expression strongly inhibited tumor growth *in vivo* but did not significantly alter *in vitro* proliferation, motility, or the ability of the tumor cells to form colonies in soft agar (Weinstat-Saslow, et al., 1994). However, higher doses of exogenous TSP1 and some TSP1 peptides can directly inhibit proliferation of these cells *in vitro* (Guo, et al.,).

Defining the receptors the recognize TSP1 on endothelial and tumor cells may provide insights into the differential effects of this protein on each cell type. Receptors that mediate cell interactions with TSP1 include integrins, proteoglycans, CD36, CD47, the LDL receptor-related

protein, and sulfated glycolipids. Binding of TSP1 to each of these receptors may elicit different cellular responses. Thus both the relative levels of expression of each receptor and, potentially, the activation state of each receptor may determine the nature of the adhesive, motility, and proliferative responses of cells to TSP1.

We have examined the role of integrins in the pro-adhesive activity of TSP1 for human breast carcinoma cells. Although the integrin $\alpha v\beta 3$ is important for adhesion of several cell types to TSP1 (Lawler, et al., 1988), we found that adhesion of breast carcinoma cells on TSP1 substrates is not mediated by this integrin. We report here that the $\alpha 3\beta 1$ integrin rather than $\beta 3$ integrins play a dominant role in adhesion of several breast carcinoma cell lines on TSP1. The activation state of these integrins varies among the human breast carcinoma cell lines examined and can be modulated by inside-out signaling, suggesting that the ability to receive pro-adhesive and motility signals from TSP1 is tightly regulated in these breast carcinoma cell lines.

Materials and Methods

Proteins and peptides- Calcium replete TSP1 was purified from human platelets as described (Roberts, et al., 1994). Proteolytic fragments of TSP1 were prepared as previously described (Roberts, et al., 1987). Recombinant fragments and GST fusion proteins expressing the procollagen, type 1, type 2, or type 3 repeats of TSP1 were provided by Dr. Jack Lawler or prepared as described (Guo, et al., in press, Legrand, et al., 1992, Vogel, et al., 1993). Synthetic peptides containing TSP1 sequences were prepared as previously described (Gao, et al., 1996, Guo, et al., 1997, Guo, et al., 1992, Guo, et al., 1992, Murphy-Ullrich, et al., 1993, Prater, et al., 1991). Bovine type I collagen was obtained from Collaborative Research, and vitronectin was

from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (Akiyama and Yamada, 1985). Murine laminin-1 purified from the EHS tumor was provided by Dr. Sadie Aznavoorian. Recombinant human EGF and TGFβ1 were obtained from R&D Systems. Insulin was from Biofluids, and recombinant human insulin-like growth factor-1 (IGF1) was from Bachem.

Monoclonal antibodies- Hybridomas producing the β1 integrin activating antibody TS2/16 (Hemler, et al., 1984) and the CD98 antibody 4F2 were obtained from the American Type Culture Collection. Antibodies secreted in PFHM-II medium (GibcoBRL) were purified by protein G affinity chromatography (Pierce). Integrin function blocking antibodies used include: LM609 (αvβ3, provided by Dr. David Cheresh), 05-246 (α1β1, Upstate Biotechnology), 6D7 (α2β1, Dr. Harvey Gralnick, NIH), P1B5 (α3β1, GibcoBRL), 407279 (α4β1, Calbiochem), P1D6 (α5β1, GibcoBRL), and MAb13 (β1, Dr. Kenneth Yamada, NIH). Non-blocking antibodies recognizing α3β1 (M-KID2), α4β1 (HP2/1), and α5β1 (SAM1) were obtained from AMAC, and αv (LM142) was provided by Dr. David Cheresh.

Cell lines and reagents- MDA-MB-435, MDA-MB-231, and MCF-7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% FCS. Okadaic acid, 12-O-tetradecanoyl phorbol-13-acetate (PMA), pertussis toxin (PT), herbimycin A, heparin, and sodium vanadate were purchased from Sigma. Pertussis toxin B oligomer, staurosporine, wortmannin, KT5823, guanosine-3',5'-cyclic monophosphothioate, 8-(4-chloro-phenylthio)-, Rp-isomer, and bis-indoyl maleimide were from Calbiochem. KT5720 was from Kamiya Biomedical (Thousand Oaks, CA).

Adhesion assays- Cells were detached by replacing the growth medium with PBS

containing 2.5 mM EDTA and incubating 5-10 min at 37° C. The cells were collected by centrifugation, suspended in RPMI containing 0.1% BSA, and assayed for adhesion to bacteriological polystyrene substrates coated with proteins as previously described (Roberts, et al., 1987)

Chemotaxis- Chemotaxis was measured in 48-well chambers using Nucleopore 8 μm, polyvinylpyrrolidone-free filters (Neuroprobe Inc, Gaithersburg, MD). To provide an integrin-independent substrate for motility, the filters were coated with 10 μg/ml polylysine for 16 h at 4° C prior to use. Motility was measured after 6.5 h and scored microscopically by counting nuclei of migrated cells on the lower surface of the membrane.

Fluorescence microscopy – To examine integrin localization and cytoskeletal rearrangement, 8-well glass chamber slides (Nalge Nunc International, Naperville, IL) were coated with type I collagen, TSP1, or fibronectin overnight at 4^0 C. The chambers were then blocked with 1% BSA in PBS, and cells were added in RPMI containing 0.1% BSA. In some cases, antibodies were included in the medium. Cells were allowed to attach and spread for 90 min. The unbound cells were then removed along with the medium, and the chambers were rinsed with PBS and fixed with 3.7% formaldehyde. Cells were stained with BODIPY TR-X phallacidin (Molecular Probes, Inc. Eugene, OR) to visualize F-actin or using primary antibodies followed by BODIPY FL anti-mouse IgG to localize integrins or CD98. All staining procedures were carried out according to the manufacturer's directions. Stained cells were observed and photographed under a Zeiss fluorescent microscope using appropriate filters.

Unstimulated MDA-MB-435 cells were evaluated for expression of integrins or their subunits one day after plating in RPMI medium containing 10% FCS (Biofluids) by indirect

immunofluorescence and flow cytometry. Cells were washed with PBS/0.2% BSA and incubated at 37 degrees C for 6 min. with Puck's saline A with 0.2% EDTA and 10% FCS. All subsequent procedures were performed on ice, and all washes were with PBS containing 0.2% BSA. Cells were dislodged with a scraper, and the resultant cell suspension was washed. Cell pellets were exposed to mouse IgG or primary antibodies to integrins or integrin subunits in PBS/0.2% BSA , washed, and incubated with FITC-conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA) Following a wash, the cells were fixed in 1% paraformaldehyde and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Initial gating was done using forward and side scatter to identify a population of intact cells without debris.

Ligand binding-TSP1 was iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (Vogel, et al., 1993). For some experiments, cells were grown in sulfate-deficient medium containing chlorate to inhibit proteoglycan and glycolipid sulfation as previously described (Guo, et al., 1992).

Results

Integrin Expression on Breast Carcinoma Cells

Flow cytometric analysis (Table 1) and immunoprecipitation using subunit-specific integrin antibodies (data not shown) demonstrated that MDA-MB-435 cells express several β1 integrins and αvβ3. Integrin expression on MDA-MB-231 and MCF-7 cells have been reported previously (Coopman, et al., 1996, Doerr and Jones, 1996, van der, et al., 1997). MDA-MB-231 cells express α2, α3, α4, α5, α6, αv, and β1 subunits. The MDA-MB-231 and MCF-7 cell lines express only low levels of β3 subunits (Doerr and Jones, 1996).

Binding of Soluble TSP1

Previous studies using MDA-MB-231 breast carcinoma cells (Incardona, et al., 1996) concluded that sulfated glycoconjugates including heparan sulfate and chondroitin sulfate proteoglycans play a dominant role in both binding of soluble TSP1 and adhesion on immobilized TSP1. We observed a similar dependence for ^{125}I-TSP1 binding to MDA-MB-435 cells (Fig. 1). Binding was strongly inhibited by heparin or a recombinant 18 kD amino terminal heparin-binding fragment of TSP1, but the peptide GRGDS and β1 or α3 integrin function blocking antibodies had no effect. Conversely, binding of ^{125}I-TSP1 to MDA-MB-435 cells was not enhanced by incubation with the β1 integrin-activating antibody TS2/16, either alone or in the presence of 10 μg/ml heparin to inhibit TSP1 binding to sulfated ligands (data not shown). Therefore, high affinity binding to soluble TSP1 to these cells is mediated by sulfated glycoconjugates and is independent of integrin binding.

β1 Integrin-mediated Adhesion and Chemotaxis to TSP1

Although heparin and recombinant heparin-binding domain from TSP1 partially inhibited attachment of MDA-MB-435 cells on immobilized TSP1, the fraction of spread cells was unaffected (Fig. 2A). In the presence of a β1 integrin function-blocking antibody at 2 μg/ml, however, only spreading was inhibited, and a combination of heparin and the β1 blocking antibody abolished spreading and markedly inhibited attachment. At 50 μg/ml, the β1 antibody completely inhibited adhesion to TSP1 (Fig. 2A). Thus, interaction with a β1 integrin is essential for spreading, but sulfated ligands may also contribute to adhesion of these cells on TSP1. This was confirmed by inhibition of sulfation following growth in chlorate. Adhesion was inhibited by 60% for MDA-MB-435 cells with a 90% reduction in $^{35}SO_4$ incorporation (Fig. 2B). RGD

peptides did not inhibit adhesion of MDA-MB-435 cells on TSP1 (results not shown).

Although MDA-MB-435 cells express some αvβ3 integrin (Table 1), a function-blocking antibody or an αvβ3-specific RGD mimetic blocked adhesion of the cells on vitronectin but had no effect on adhesion on TSP1 (Fig. 2C and results not shown). Conversely, in the presence of the β1 activating antibody TS2/16, adhesion of MDA-MB-435 cells was enhanced on TSP1 but not on vitronectin (Fig 2C). Therefore, the αvβ3 integrin is functional in MDA-MB-435 cells, but it is apparently unable to recognize the RGD motif in intact platelet TSP1.

The β1-blocking antibody MAb13 inhibited chemotaxis to TSP1, but heparin did not (Fig. 2D). For these experiments, the filters were coated with polylysine to provide an integrin-independent substrate for adhesion of the cells. Therefore, chemotaxis of MDA-MB-435 cells to TSP1 is also primarily dependent on the β1 integrin receptor.

Several human breast cancer cell lines showed similar involvement of β1 integrins in their adhesion to TSP1 (Fig. 3). MDA-MB-231 cells attached poorly and did not spread on substrates coated with low concentrations of TSP1. In the presence of the β1-activating antibody, however, the cells attached avidly on TSP1 and exhibited spreading (Fig. 3A). A third breast carcinoma cell line, MCF-7, behaved similarly to the MDA-MB-231 cells and showed spreading on TSP1 only in the presence of the β1 activating antibody (Fig. 3A). The apparent low avidity state of the integrin that recognizes TSP1 on MDA-MB-435 cells was not an artifact from using EDTA to dissociate the cells, because cells suspended by scraping from the dish in the presence of divalent cations showed the same degree of enhancement by TS2/16 for adhesion to TSP1 or type I collagen as cells harvested using EDTA (Fig. 3B).

α3β1 is the Major TSP1-binding Integrin on Breast Carcinoma Cells

Of the α subunit antibodies tested for inhibiting adhesion to TSP1, only an α3 subunit blocking antibody, P1B5, significantly inhibited of adhesion of MDA-MB-231 cells to TSP1 (Fig. 4A, p= 0.0007, 2-tailed t test). An α4 integrin blocking antibody slightly inhibited adhesion, but mixing this antibody with the α3 blocking antibody produced no further inhibition than the latter antibody alone (Fig 4A). MDA-MB-435 cell spreading on TSP1 was also inhibited by the α3 blocking antibody, and somewhat by the α4 antibody (Fig. 4B). Function blocking antibodies for α1β1, α2β1, and α5β1 integrins had no effect on TSP1 adhesion, although the α2β1, and α5β1 antibodies inhibited adhesion of the same cells to known ligands for these integrins (Fig. 4 and results not shown).

Integrin localization and effects on actin cytoskeleton

Activation of α3β1 using TS2/16 altered the morphology of cells attaching on TSP1 (Fig. 5). MDA-MB-435 cells extended a few processes but exhibited no F-actin organization when attached on TSP1 alone (Fig. 5A), but addition of antibody TS2/16 stimulated spreading with redistribution of F-actin to the cell periphery (Fig. 5B). F-Actin was also present in short spikes protruding from the spread cells. Staining with the β1 integrin antibody revealed numerous filopodia extending from these points (Fig. 5C). In some cells, these filopodia were terminated with punctate β1 integrin staining, possibly at sites of contact with the TSP1 substrate. Formation of filopodia was specific to the TSP1 substrate, as TS2/16-induced spreading of these cells on type I collagen (Fig. 5D) or fibronectin (results not shown) only rarely evoked filopodia. These cytoskeletal rearrangements changes were specific for β1-dependent adhesion to intact TSP1 and were not observed in cells attaching on heparin-binding peptides or recombinant fragments of TSP1 (results not shown). Similar induction of filopodia or microspikes by TSP1 have been

observed in other cell types (Adams, 1995).

Structural requirements for α3β1 mediated adhesive activity of TSP1

We attempted to localize the region of TSP1 recognized by the α3β1 integrin. A 140 kDa fragment lacking the amino terminal heparin-binding domain also exhibited β1-dependent adhesion, but no smaller proteolytic or recombinant fragments of TSP1 supported β1-dependent adhesion (Fig. 6). Among the recombinant TSP1 fragments tested, an 18 kDa fragment of the amino-terminal heparin-binding domain had the strongest activity, and recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments. A recombinant GST-fusion of the type 3 repeats of TSP1 including the RGD sequence had minimal adhesive activity for MDA-MB-435 cells (Fig. 6), in contrast to human melanoma cells, which avidly attached on substrates coated with the same concentrations of this fragment (Sipes, and Roberts, manuscript in preparation). Synthetic heparin-binding peptides from the type 1 repeats (peptide 246) and the CD47 binding peptide 4N1K also promoted adhesion, but TS2/16 partially inhibited adhesion of MDA-MB-435 cells to these peptides. Two CD36-binding peptides from the procollagen domain (peptide 500) or the type 1 repeats (Mal-II) had weaker adhesive activity, but were insensitive to TS2/16. The focal adhesion disrupting peptide from the amino terminal domain (Hep1) did not promote MDA-MB-435 cell adhesion.

Differences in the conformation or folding of TSP1 could account for discrepancies in its reported adhesive activity. The conformation of TSP1 and formation of specific intra-chain disulfide bonds are sensitive to the levels of divalent cations present during its purification. Disulfide bonding also influences interactions of TSP1 with several proteases and regulates the accessibility of the RGD sequence to the αvβ3 integrin (Hotchkiss, et al., 1996, Sun, et al., 1992).

We therefore examined the influence of conformation on α3β1-dependent adhesion by absorbing TSP1 with or without divalent cations, at low pH (Kaesberg, et al., 1989), or by reducing disulfide bonds using dithiothreitol (Fig. 7). Coating TSP1 at pH 4 in acetate buffer enhanced MDA-MB-435 cell adhesion relative to TSP1 adsorbed in PBS with Ca^{2+} and Mg^{2+}, but use of PBS with 2.5 mM EDTA did not significantly affect β1-mediated adhesion. Although heparin only partially inhibited MDA-MB-435 cell adhesion to TSP1 (20-50%) when the TSP1 was adsorbed in Dulbecco's PBS (e.g. Fig. 2A), adhesion to TSP1 adsorbed in pH 4 acetate buffer was inhibited 98% by 10 μg/ml heparin. Conversely, TS2/16 did not reproducibly increase adhesion of MDA-MB-435 cells to TSP1 adsorbed in acetate buffer (data not shown). Therefore, the enhanced adhesion to TSP coated at pH 4 was due primarily to enhancement of heparin-dependent adhesion, whereas β1-integrins contributed less to adhesion on TSP1 coated at the lower pH. Adhesion of MDA-MB-435 cells (Fig 7) and MDA-MB-231 cells (results not shown) was strongly inhibited following reduction of TSP1 with dithiothreitol. This contrasts with αvβ3-dependent adhesion to TSP1, which was reported to be enhanced following disulfide reduction using the same conditions as used in Fig. 7 (Sun, et al., 1992). Thus, α3β1-dependent adhesion of breast carcinoma cells does not require Ca-replete TSP, but some intact disulfide bonds are essential.

Regulation of βl integrin activation in breast carcinoma cells

Adhesion of T lymphocytes to TSP1, mediated by α4β1 and α5β1 integrins, is stimulated by phorbol esters (Yabkowitz, et al., 1993). PMA activation of protein kinase C in MDA-MB-435 cells increased αvβ3-mediated adhesion to vitronectin but had no effect on β1 integrin-mediated adhesion to TSP1 (Fig. 8). Integrin associated protein (CD47) also regulates integrin

function in several cell types (Gao, et al., 1996, Wang and Frazier, 1998). The carboxyl-terminal domain of TSP1 contains two peptide motifs that activate integrin function through binding to CD47 (Gao, et al., 1996). The CD47-binding TSP1 peptide 7N3 activated adhesion of MDA-MB-435 cells on vitronectin (Fig. 8) and a recombinant TSP1 fragment containing the RGD sequence (results not shown) but had no effect on adhesion to native TSP1 (Fig. 8). Thus MDA-MB-435 cells express functional $\alpha v\beta 3$ that can be activated by PMA or the TSP1 7N3 peptide. This $\alpha v\beta 3$ integrin can recognize the TSP1 RGD sequence in the context of a bacterial fusion protein, but it does not play a significant role in adhesion of resting or stimulated breast carcinoma cells to native platelet TSP1.

Several pharmacological agents stimulated $\beta 1$-dependent adhesion to TSP1 (Table 2). The broad spectrum Ser/Thr protein kinase inhibitor staurosporine increased spreading of all three cell lines. However, this activation in MDA-MB-435 cells was only partially replicated by specific inhibitors of protein kinase C (bisindoyl maleimide) or protein kinase A (KT5720), or protein kinase G (KT5823 and guanosine-3',5'-cyclic monophosphorothioate, 8-(4-chloro-phenylthio)-, Rp-isomer). Inhibition of PI 3-kinase using wortmannin had no significant effect on MDA-MB-435 cell spreading and weakly enhanced MDA-MB-231 cell spreading on TSP1. Two calcium ionophores, ionomycin and A23187, strongly enhanced spreading of MDA-MB-435 cells but had no effect on MDA-MB-231 cell spreading on TSP1.

Modulation of TSP1 adhesion by G-protein signaling

Although TSP1 peptides promote PT-sensitive integrin activation through binding to CD47 (Chung, et al., 1997, Gao, et al., 1996), we showed above that this pathway does not function in MDA-MB-435 cells to activate $\alpha 3\beta 1$. However, PT did influence MDA-MB-231 and

MDA-MB-435 cell adhesion and spreading on TSP1 or collagen (Fig. 9). PT increased adhesion of MDA-MB-231 cells to TSP1 (Fig. 9A) but inhibited both basal and TS2/16-stimulated adhesion of MDA-MB-435 cells on the same substrate (Fig. 9B). The effects of PT in both cell lines were specific, since PT B-oligomer at the same concentration had no effect (Fig. 9). The enhancement of MDA-MB-231 cell adhesion by PT is mediated by the β1 integrin, because the β1 blocking antibody MAb13 inhibited the PT-induced adhesion of MDA-MB-231 cells but heparin did not (results not shown). However, not all β1 integrins in these breast carcinoma cells were activated by PT. Adhesion of MDA-MB-231 cells to collagen mediated by α2β1 (verified by the blocking antibody 6D7, results not shown) was not altered by PT, although the same adhesive pathway could be further activated by TS2/16 (Fig. 9A). In MDA-MB-435 cells, PT partially inhibited α2β1-mediated spreading on collagen stimulated by TS2/16 (Fig. 9B).

Physiological activators of TSP1 adhesion and chemotaxis

We noted that freshly passaged breast carcinoma cells exhibited stronger β1 integrin mediated adhesion on TSP1. This suggested that proliferation regulates α3β1-mediated TSP1 adhesion. Serum induced a dose-dependent increase in β1 integrin-mediated attachment (Fig. 10A) and spreading of MDA-MB-435 cells to TSP1 or type I collagen (results not shown) . A similar serum response was observed in MDA-MB-231 cells for adhesion on TSP1, although adhesion of the latter cell line to type I collagen was maintained in the absence of serum (data not shown).

Several growth factors were examined to define the basis of the serum response for TSP1 adhesion (Fig. 10B). Addition of EGF to serum-depleted medium increases adhesion of breast carcinoma cells to some substrates (Genersch, et al., 1996) but in several experiments showed

only a slight stimulatory activity for spreading of MDA-MB-435 cells on TSP1 (Fig. 10B). FGF2 and TGFβ1 were also ineffective, but addition of insulin stimulated MDA-MB-435 cell adhesion as well as 10% serum after 24 h (Fig. 10B). Insulin was also the only growth factor tested that stimulated adhesion of MDA-MB-231 cells to TSP1 (results not shown).

Acute addition of insulin, but not EGF, during the adhesion assay produced a similar enhancement in adhesion of both cell lines to TSP1 as the 24 h pretreatment of the cells in culture (Fig. 10C and results not shown). The dose-dependence for the insulin response was consistent with that for signaling through the IGF1 receptor (Fig. 10C), which is expressed in these breast carcinoma cells (Peyrat, et al., 1989). Both insulin and IGF1 strongly stimulated MDA-MB-435 cell spreading on TSP1, moderately stimulated adhesion on type I collagen, but did not stimulate adhesion on laminin-1 (Fig. 10C). EGF (2 nM) was also inactive in this assay (results not shown). IGF1 (EC_{50} = 1 nM) was 100-fold more potent than insulin, as expected for a response mediated by the IGF1 receptor (Peyrat, et al., 1989). A similar difference in the potencies of IGF1 and insulin was also observed in stimulation of TSP1 attachment of MDA-MB-231 cells (results not shown). Thus, occupancy of the IGF1 receptor specifically stimulates the activity of the TSP1-binding integrin in both cell lines.

IGF1 also enhanced the chemotactic response to TSP1. Addition of IGF1 to MDA-MB-435 cells in the upper well of a modified Boyden chamber did not alter motility of the cells, but it stimulated (2- to 5-fold) the chemotactic response to TSP1 added to the lower chamber (Fig. 10D). This IGF1-stimulated motility to TSP1 was mediated by the α3β1 integrin, because MAb13 (β1) and P1B5 antibodies (α3) strongly inhibited direct TSP1 chemotaxis and that stimulated by IGF1. IGF1-stimulated chemotaxis to TSP1 was also sensitive to PT inhibition

(Fig. 10D).

Modulation of TSP1 adhesion by CD98

Expression of the transmembrane protein CD98 is induced by serum, and this protein was recently shown to activate function of some β1 integrins (Fenczik, et al., 1997). Clustering of CD98 using the antibody 4F2 stimulates small cell lung carcinoma adhesion on fibronectin and laminin (Fenczik, et al., 1997) and similarly activated α3β1-mediated spreading of breast carcinoma cells on TSP1 and α2β1-mediated adhesion on type I collagen (Fig. 11). In MDA-MB-435 cells spreading on TSP1, both CD98 and β1 integrins are present on filopodia extending on the TSP1 substrate (Fig. 12A-C). This co-localization suggested that the induction of α3β1-mediated TSP1 adhesion by growth with serum or insulin could be mediated by induction of CD98 expression. This may be the case for the serum response, because a 24 h exposure to 10% serum increased surface expression of CD98 in MDA-MB-435 cells (Fig. 12D). IGF1 treatment for the same time, however, decreased rather than increased CD98 expression (Fig. 12D).

Discussion

The α3β1 integrin, with some cooperation of sulfated glycoconjugates and α4β1 integrin, mediates adhesion of MDA-MB-435 and MDA-MB-231 breast carcinoma cells to TSP1. This β1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1 and ligation of CD98. In MDA-MB-231 cells, the inactive state of the α3β1 integrin is maintained by a G-protein mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β1-dependent adhesion to TSP1 do not stimulate β3-dependent adhesion to TSP1, even though the cells express

the known TSP1 receptor αvβ3 and this integrin is functional and inducible for vitronectin adhesion. We do not know why the αvβ3 integrin on MDA-MB-435 cells can not recognize the RGD sequence in the type III repeat of platelet TSP1. Other cell types, however, can utilize the same TSP1 preparations used for these experiments to support αvβ3-dependent adhesion (unpublished results).

The α3β1 integrin in MDA-MB-435 cells does not recognize the RGD sequence in the TSP1 type 3 repeats. Because we have tested 85% of the TSP1 primary sequence using recombinant fragments or synthetic peptides, the β1 recognition motif may not be a linear epitope in TSP1. One caveat in interpreting the negative results using the recombinant TSP1 fragments, however, is that misfolding of fragments expressed in bacteria could selectively mask a linear recognition sequence for the α3β1 integrin in the GST- or T7-fusion proteins .

Several β1 integrins have been implicated as TSP1 receptors in other cell types, including α2β1 on activated platelets (Tuszynski and Kowalska, 1991), α3β1 on neurons (DeFreitas, et al., 1995), and α4β1 and α5β1 on activated T lymphocytes (Yabkowitz, et al., 1993). α3β1 is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas α2β1 mediates adhesion of these cells to type I collagen but not to TSP1. The integrin α4β1 may play some role in breast carcinoma adhesion to TSP1, as we previously reported for T lymphocytes (Yabkowitz, et al., 1993). The mechanism for the apparent differential recognition of TSP1 by β1 integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-

adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by "inside-out" signaling (Kolanus and Seed, 1997), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to αvβ3 integrin, the α3β1 integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 "VVM" peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β1-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via αvβ3 but not α3β1 integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of α3β1-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically associated with the αvβ3 integrin but not with β1 integrins in fibroblasts (Schneller, et al., 1997). The αvβ3 integrin also co-immunoprecipitated with insulin receptor substrate-1 (Vouri and Ruoslahti, 1994). Engagement of αvβ3 integrin by vitronectin but not α2β1 integrin by collagen increased mitogenic signaling through the insulin receptor (Schneller, et al., 1997, Vouri and Ruoslahti, 1994). Thus, the specific activation of α3β1 mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the α3β1 integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific

coupling of α3β1 activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

CD98 was recently identified as an activator of β1 integrins by its ability to overcome Tac-β1 suppression of β1 integrin function (Fenczik, et al., 1997, Lasky, 1998). Our data demonstrate that clustering of CD98 can also increase α3β1-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated α3β1 integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis can not be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the α3β1 integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could be result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including H-Ras (Hughes, et al., 1997), integrin-linked kinase, and protein kinase C (Kolanus and Seed, 1997). Additional proteins are known to associate with the α3β1 integrin, including some members of the TM4SF family and EMMPRIN (Berditchevski, et al., 1997, Tachibana, et al., 1997), but their roles in regulating function are unknown. In MDA-MB-

231 cells, suppression of α3β1 appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the α3β1 integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using α2β1 integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better α3β1-dependent adhesion to TSP1 than α2β1-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores demonstrates that regulation of α3β1 activity for TSP1 may differ even between two cell lines derived from the same type of human cancer.

TSP1 has diverse effects on breast carcinoma cell behavior, altering their adhesion, motility, proliferation, protease expression, and invasion. These cellular responses result in alterations of their *in vivo* tumorigenic, angiogenic, and metastatic potentials (reviewed in (Roberts, 1996). We have defined specific roles for the α3β1 integrin in spreading, induction of filopodia, and chemotactic responses to TSP1. In other cell types, LRP has been assigned a role in internalization of TSP1 (Mikhailenko, et al., 1995), and CD36 has been shown to play an essential role in angiogenesis inhibition (Dawson, et al., 1997). The receptors that mediate many responses to TSP1 remain to be defined. These responses may require coordinated signaling through two or more TSP1 receptors. Defining the role of IGF1 and CD98 in regulating β1 integrin interactions with TSP1 provides our first insight into a breast carcinoma TSP1 receptor that can be turned on or off in response to known environmental stimuli. The ability to regulate the activity of this TSP1 receptor will facilitate analysis of the signals resulting from this interaction.

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Footnotes

[1]The abbreviations used are: BSA, bovine serum albumin; IGF1, insulin-like growth factor-1; PMA, phorbol 12-myristate 13-acetate; PT, pertussis toxin; RGD, Arg-Gly-Asp; TSP1, human thrombospondin-1

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Table I. Integrin expression in MDA-MB-435 breast carcinoma cells.

Integrin	Antibody	Mean fluorescence intensity
α2β1	6D7	200
α3β1	M-KID2	127
α4β1	HP2/1	98
α5β1	SAM1	122
αv	LM142	256
αvβ3	LM609	157
β1	MAB13	158
-	mouse IgG	31

Table II. Modulation of breast carcinoma spreading on TSP1.

	Cell line		
Inhibitor	MDA-MB-435	MDA-MB-231	MCF-7
		spreading (% of TS2/16)	
staurosporine	112 ± 16	13 ± 3	39 ± 8
KT5720	35 ± 25	1 ± 2	
bisindoylmaleimide	15 ± 10	0 ± 1	
KT5823	17.2 ± 7.9	0 ± 1	
RP8-pCPT-cGMPS	-2 ± 6	3 ± 1	
wortmannin-2	-18 ± 18	16 ± 4	
ionomycin	52 ± 3	0 ± 0	
A23187	83 ± 29	0 ± 0	
herbimycin	-16 ± 1	0 ± 1	
vanadate	-9 ± 2	8 ± 2	

MDA-MB-435 or MDA-MB-231 cell spreading on TSP1 was measured in untreated cells in the presence or absence of the β1-activating antibody TS2/16 to measure basal and total β1-dependent adhesion and in cells pretreated with and maintained in the following inhibitors: 10 nM staurosporine (Ser/Thr kinase inhibitor), 100 nM KT5720 (protein kinase A), 200 nM bis-indoylmaleimide (protein kinase C), 1 μM KT5823 or 2 μM Guanosine-3',5'-cyclic monophosphorothioate, 8-(4-chloro-phenylthio)-, Rp-isomer (protein kinase G), 2 nM wortmannin (PI 3-kinase), 1 μg/ml ionomycin or A23187 (calcium ionophores), 1 μM herbimycin (tyrosine kinase), or 20 μM vanadate. The net increase in cell spreading in the presence of the indicated drugs is expressed as a percent of that induced by the β1-activating antibody TS2/16, mean ± SD, n = 3.

FIGURE LEGENDS

Figure 1. Specificity of ^{125}I-TSP binding to MDA-MB-435 cells. Cells were harvested using 2.5 mM EDTA in PBS, resuspended in RPMI 1640 medium containing 0.1% BSA, and incubated with ^{125}I-TSP for 1 h at 25^0 C with the indicated inhibitors. Cells were centrifuged through oil to remove unbound labeled protein. The mean ± SD for triplicate determinations is presented for binding determined in the absence (control) or presence of 4 μM 18 kDa recombinant TSP1 heparin-binding domain (HBD), 204 μM GRGDS peptide, 100 μg/ml heparin, 10 μg/ml MAb13 (anti-β1) or P1B5 (anti-α3β1).

Figure 2. Role of integrins and sulfated glycoconjugates in breast carcinoma cell adhesion and chemotaxis to TSP1. Panel A: MDA-MB-435 cell attachment (solid bars) and spreading (striped bars) was measured on polystyrene coated with TSP (50 μg/ml) and blocked with 1% BSA to reduce nonspecific adhesion. Heparin-dependent adhesion was assessed by inhibition using 4 μM 18 kDa recombinant TSP1 heparin-binding domain (HBD) or 50 μg/ml heparin. β1 Integrin-dependent adhesion was inhibited using 2 μg/ml or 50 μg/ml MAb13 (anti-β1). Results are presented as mean ± SD, n = 3. Panel B: Effect of inhibiting sulfation on attachment of MDA-MB-435 cells. MDA-MB-435 cells were grown in Ham's F-12 medium (low sulfate) containing 10% dialyzed fetal calf serum for 48 h. The medium was replaced with the same medium containing 1% dialyzed serum with or without sodium chlorate at the indicated concentrations. The cells were cultured for 24 h, harvested, and resuspended in F-12 medium containing 1 mg/ml BSA with or without chlorate at the indicated concentrations. Cell adhesion was quantified to polystyrene coated with 50 μg/ml of TSP (striped bars) or 10 μg/ml FN (gray bars). ^{35}S-Incorporation in MDA-435 cell macromolecules (-o-). was assessed in duplicate cultures

supplemented with 25 μCi/ml [^{35}S] sulfate. The cells were fixed and washed in acetic acid/methanol, and incorporation of radioactivity in macromolecules was determined by scintillation counting after solubilization in 1% sodium dodecyl sulfate. Panel C: Integrin αvβ3 mediates breast carcinoma cell adhesion to vitronectin but not to TSP1. Adhesion of MDA-MB-435 cells to 30 μg/ml TSP1 (solid bars) or 10 μg/ml vitronectin (striped bars) was measured in the presence of the αvβ3 function blocking antibody LM609 or the β1 activating antibody TS2/16. Panel D: Chemotaxis to TSP1 is β1 integrin-dependent. MDA-MB-435 chemotaxis to 50 μg/ml TSP1 was determined in modified Boyden chambers. Cells were added in the upper chamber with the incubated concentrations of β1 integrin blocking antibody MAb13 (●) or heparin (o). Spontaneous motility (▲) was determined in the absence of TSP1. Migrated cells were counted microscopically, and results from triplicate wells are presented as a percent of migration to TSP1 without inhibitors, mean ± SD.

Figure 3. β1 Integrins recognizing TSP1 and type I collagen are partially inactive in human breast carcinoma cell lines. Panel A: Spreading of three breast carcinoma cell lines on 50 μg/ml TSP1 (solid bars) or on TSP1 in the presence of 5 μg/ml TS2/16 (striped bars). Panel B: Comparison of β1 integrin activity in MDA-MB-435 cells harvested by scraping in RPMI medium or by a 5 min treatment with 2.5 mM EDTA in PBS. Cells were resuspended in RPMI medium with 0.1% BSA (solid bars) or with 20 μg/ml TS2/16 (striped bars), and cell spreading was assessed on substrates coated with 20 μg/ml of TSP1 or 5 μg/ml of type I collagen.

Figure 4. Integrin α-subunit specificity of TSP1 adhesion. Panel A: MDA-MB-231 cell attachment was quantified using substrates coated with 40 μg/ml TSP1 (solid bars) or 5 μg/ml type I collagen (gray bars) in the presence of 5 μg/ml of TS2/16 to activate β1 integrins and 5

μg/ml of the indicated function blocking antibodies. Panel B: Inhibition of MDA-MB-435 cell spreading on TSP1 (solid bars) or type I collagen (gray bars) in the presence of TS2/16 and the indicated α subunit blocking antibodies.

Figure 5. Actin organization and filopodia formation on TSP1 is stimulated by β1 integrin activation. Actin was visualized using BODIPY-phallacidin in MDA-MB-435 cells attached on TSP1 (panel A) or TSP1 in the presence of 5 μg/ml TS2/16 (panel B). β1 Integrin localization of the TS2/16 treated cells was visualized using BODIPY FL-anti-mouse IgG on TSP1 (panel C) or type I collagen substrates (panel D). Bar in panel A = 20 μm.

Figure 6. Adhesion to recombinant TSP1 fragments and synthetic TSP1 peptides. Panel A: Cell attachment to TSP1 or a 140 kDa proteolytic fragment of TSP1 at the indicated concentrations (solid bars) was enhanced by addition of 20 μg/ml of the β1-activating antibody TS2/16 (striped bars). Panel B: Adhesion to synthetic TSP1 peptides adsorbed at 10 μM (246, KRFKQDGGWSHWSPWSS; 500, NGVQYRNC; Mal II, SPWSSCSVTCGDGVITRIR; 4N1K, KRFYVVMWKK; HepI, ELTGAARKGSGRRLVKGPD), TSP1 (0.11 μM), recombinant 18 kDa heparin-binding domain (HBD, 2.7 μM), or GST-fusion proteins expressing the TSP1 procollagen domain, type 1, type 2, or type 3 repeats (2 μM) was measured in the absence (solid bars) or presence of 20 μg/ml of TS2/16 (striped bars).

Figure 7. Tertiary structure dependence for MDA-MB-435 cell adhesion on TSP1. MDA-MB-435 cell adhesion to 20 μg/ml TSP1 coated on polystyrene in 10 mM sodium acetate, 150 mM NaCl, pH 4 (Kaesberg, et al., 1989), Dulbecco's PBS with calcium and magnesium (PBS-Ca), PBS with 2.5 mM EDTA, or PBS with 2.5 mM EDTA and 2 mM dithiothreitol (DTT). Attachment (solid bars) and spreading (striped bars) were assessed in the absence or presence of 5

μg/ml TS2/16.

Figure 8. Differential regulation of β1 and β3 integrin activity in MDA-MB-435 cells. Attachment of MDA-MB-435 cells on 5 μg/ml vitronectin (striped bars) or 40 μg/ml TSP1 (solid bars) was measured using cells treated with 20 μg/ml TS2/16, 10 ng/ml PMA, or 3 μM of the CD47-binding TSP1 peptide 7N3 (FIRVVMYEGKK). Results are presented as a % of cell attachment without additions, mean ± SD, n = 3.

Figure 9. Pertussis toxin differentially regulates MDA-MB-435 and MDA-MB-231 cell adhesion on TSP1. Panel A: MDA-MB-231 cell attachment on 40 μg/ml TSP1 (solid bars) or 5 μg/ml type I collagen (gray bars) was measured alone or in the presence of 5 μg/ml TS2/16, 1 μg/ml PT, or 1 μg/ml PT B-oligomer. Results are mean ± SD for triplicate determinations. Panel B: MDA-MB-435 cell spreading was determined on TSP1 (solid bars) or type I collagen substrates (gray bars) in the presence of PT or PT B-oligomer added alone or combined with 5 μg/ml TS2/16.

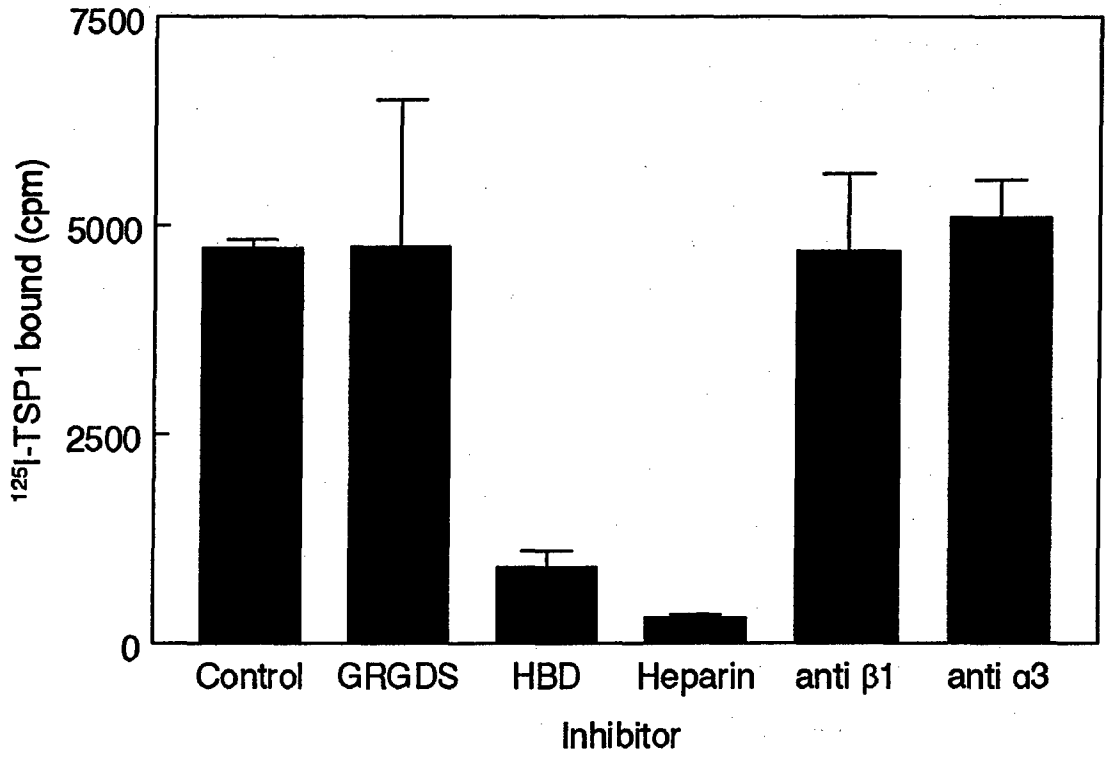
Figure 10. Regulation of β1 integrin-mediated TSP1 interactions by serum and growth factors. Panel A: Serum induces attachment of MDA-MB-435 cells to TSP1(solid bars) and type I collagen (striped bars). Cells were grown for 24 h in RPMI medium containing the indicated concentration of FCS. Panel B: Insulin specifically induces adhesion of breast carcinoma cells to TSP1. MDA-MB-435 cell spreading on surfaces coated with 40 μg/ml of TSP1 was determined using cells grown for 24 h in RPMI medium containing 2% serum and supplemented with the indicated growth factors (10 ng/ml EGF, 100 ng/ml FGF2, 5 ng/ml TGFβ, or 10 μg/ml insulin) or RPMI medium containing 10% serum. Spreading of cells grown in 2% serum was also tested in the presence of 5 μg/ml of antibody TS2/16 (2%+TS2/16) to assess maximal β1 integrin-mediated spreading activity. Panel C: Dose dependence for induction of TSP1 adhesion by insulin

and IGF1. Cell spreading (expressed as a % of maximal spreading elicited on each substrate in the presence of 5 μg/ml TS2/16 antibody) was determined in RPMI medium containing 0.1% BSA and supplemented with the indicated concentrations of insulin (closed symbols) or IGF1 (open symbols) using substrates coated with 40 μg/ml TSP1 (o,●), 20 μg/ml laminin (▲,△), or 5 μg/ml type I collagen (■, □). Panel D: IGF1 synergizes with TSP1 to promote chemotaxis of MDA-MB-435 cells. Chemotaxis to 50 μg/ml TSP1 was determined in the presence of the indicated inhibitors or stimulators at the following concentrations: 10 nM IGF1 5 μg/ml MAb13 (anti-β1), 5 μg/ml P1B5 (anti-α3), and 1 μg/ml PT. Results are mean ± SD, n = 3-6.

Figure 11. CD98 ligation stimulates breast carcinoma cell adhesion to TSP1. Basal (solid bars) or stimulated MDA-MB-231 or MDA-MB-435 cell spreading on 25 μg/ml TSP1 or 5 μg/ml type I collagen was determined in the presence of 5 μg/ml TS2/16 (striped bars) or 20 μg/ml 4F2 (gray bars).

Figure 12. CD98 localization and expression in MDA-MB-435 cells. Panel A: CD98 is present with β1 integrin in filopodia of cells spreading on TSP1. MDA-MB-435 cells were allowed to attach on TSP1, fixed, and incubated with β1 integrin (Panel A) or CD98 antibodies (Panel B) or control without primary antibody (Panel C) followed by BODIPY FL-anti mouse IgG. Bar in panel A represents 10 μm. Panel D: Serum induces but IGF1 inhibits CD98 expression. MDA-MB-435 cells grown 24 h in RPMI medium containing 1% FCS, 10% FCS or 1% FCS and 10 nM IGF1 as described in Fig. 10A were biotinylated and immunoprecipitated with antibody 4F2. The immunoprecipitates were analyzed by SDS gel electrophoresis and Western blotting using streptavidin-peroxidase and chemiluminescent detection. Markers indicate the migration of the 80 and 45 kDa subunits of CD98.

Figure 1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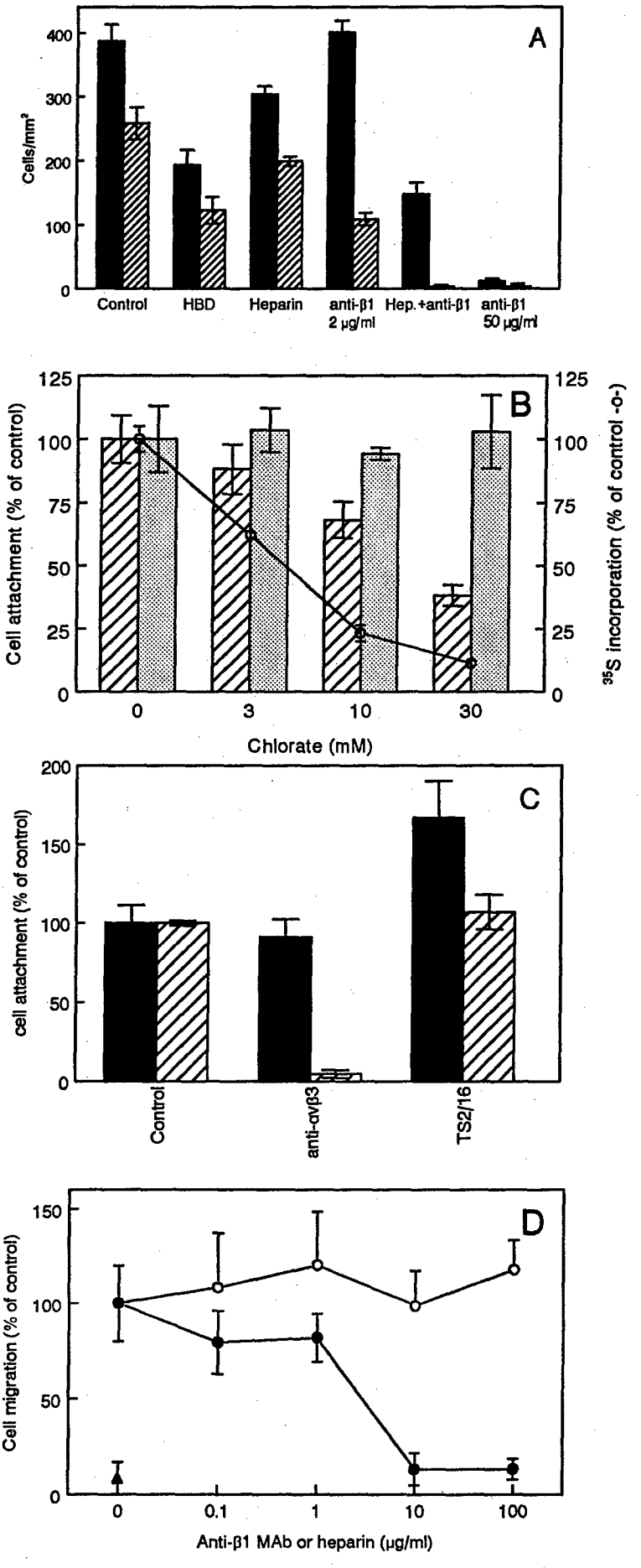
Control
GRGDS
HBD
Heparin
anti β1
anti α3
Inhibitor

Figure 2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300
200
100
0
Control
HBD
Heparin
anti-β1 2 μg/ml
Hep.+anti-β1
anti-β1 50 μg/ml
B
Cell attachment (% of control)
125
100
75
50
25
0
35S incorporation (% of control -o-)
0
3
10
30
Chlorate (mM)
C
cell attachment (% of control)
200
150
100
50
0
Control
anti-αvβ3
TS2/16
D
Cell migration (% of control)
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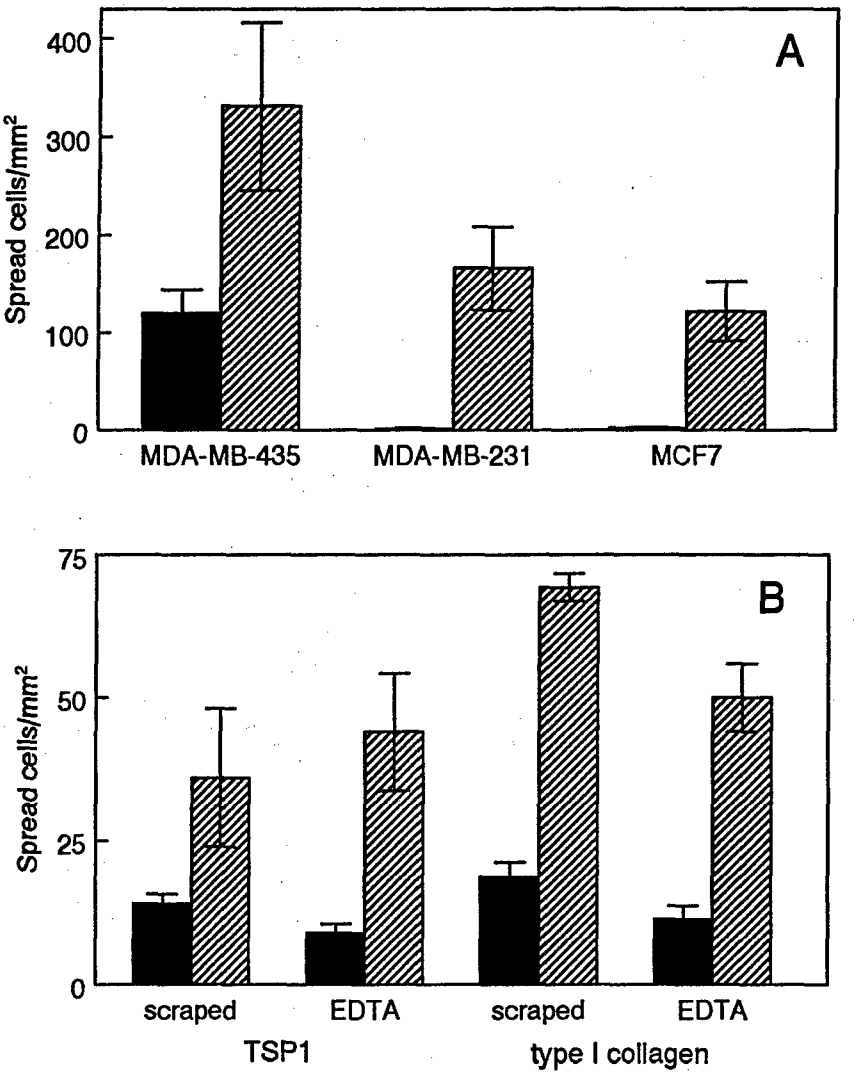
100
50
0
0
0.1
1
10
100
Anti-β1 MAb or heparin (μg/ml)

Figure 3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100
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MDA-MB-231
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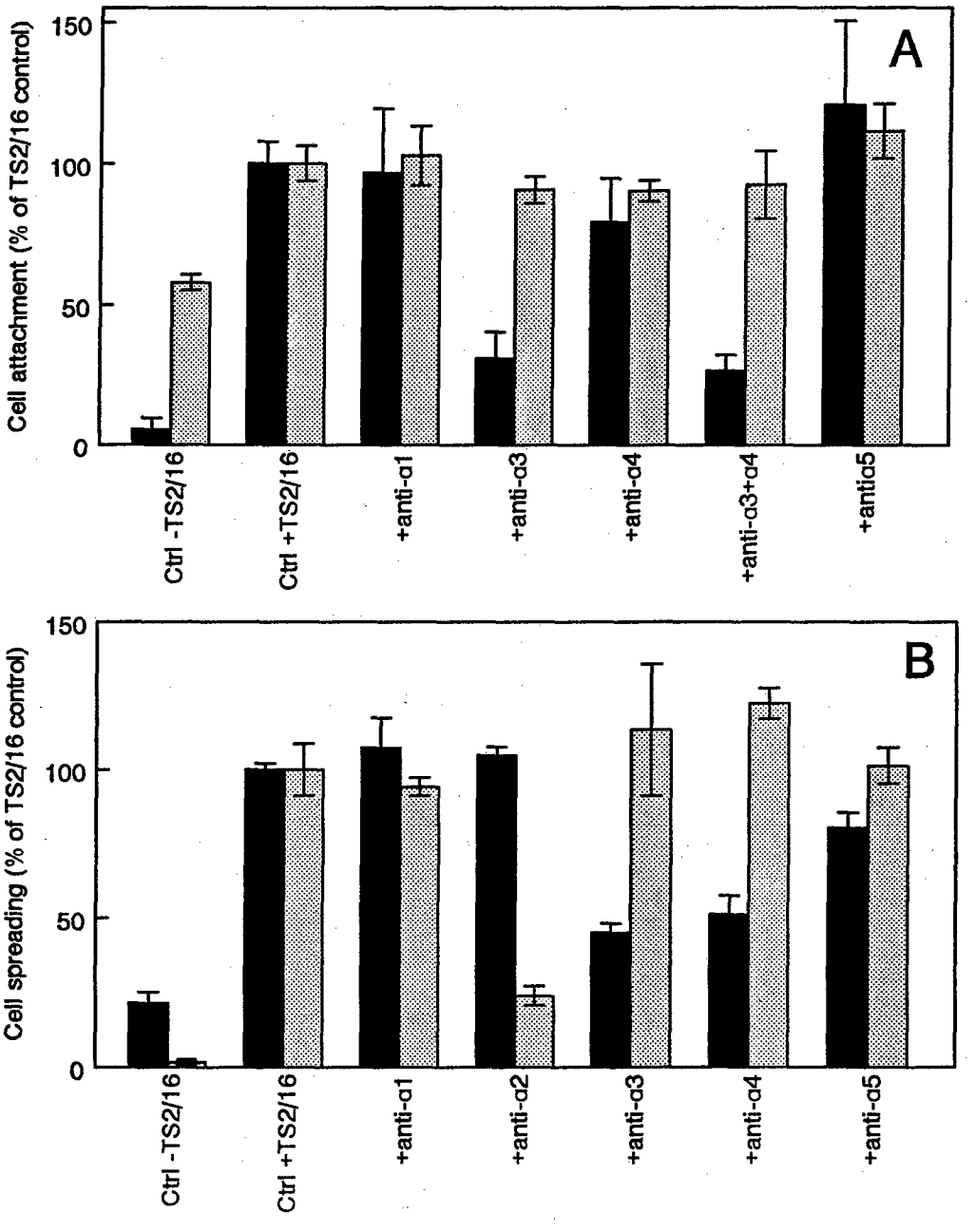
50
25
0
scraped
EDTA
scraped
EDTA
TSP1
type I collagen

Figure 4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+anti-α1
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+anti-α4
+anti-α3+α4
+antiα5
B
Cell spreading (% of TS2/16 control)
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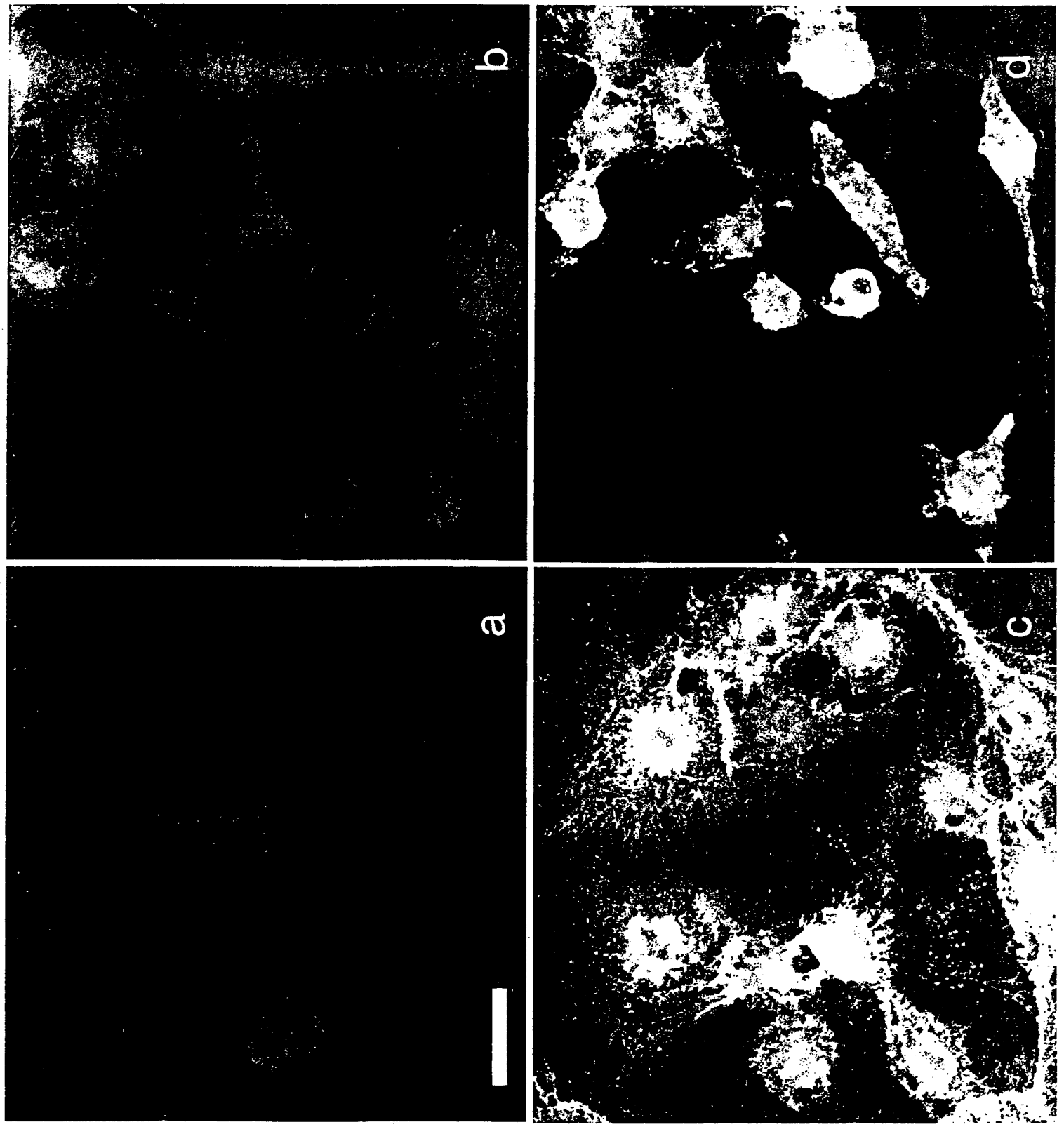
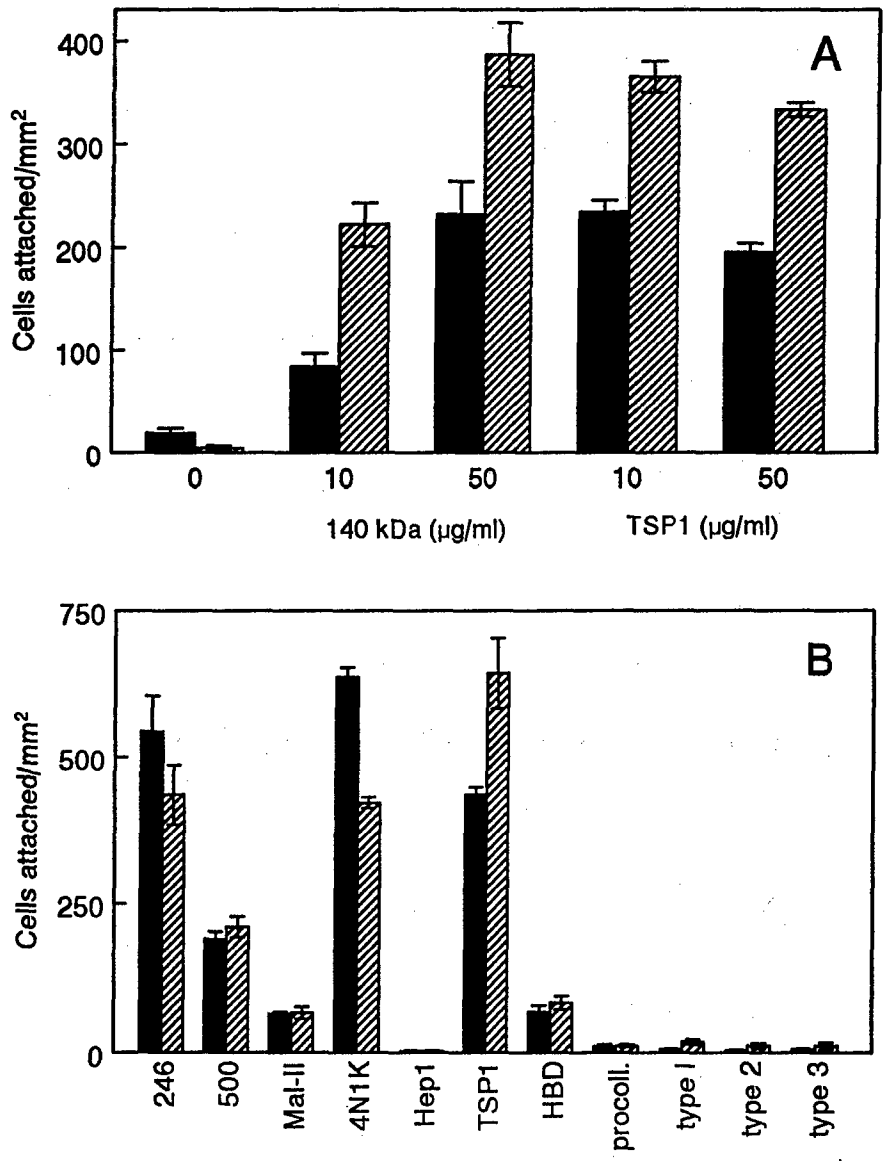


Figure 5

Figure 6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200
100
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0
10
50
10
50
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TSP1 (µg/ml)
B
Cells attached/mm²
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500
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Mal-II
4N1K
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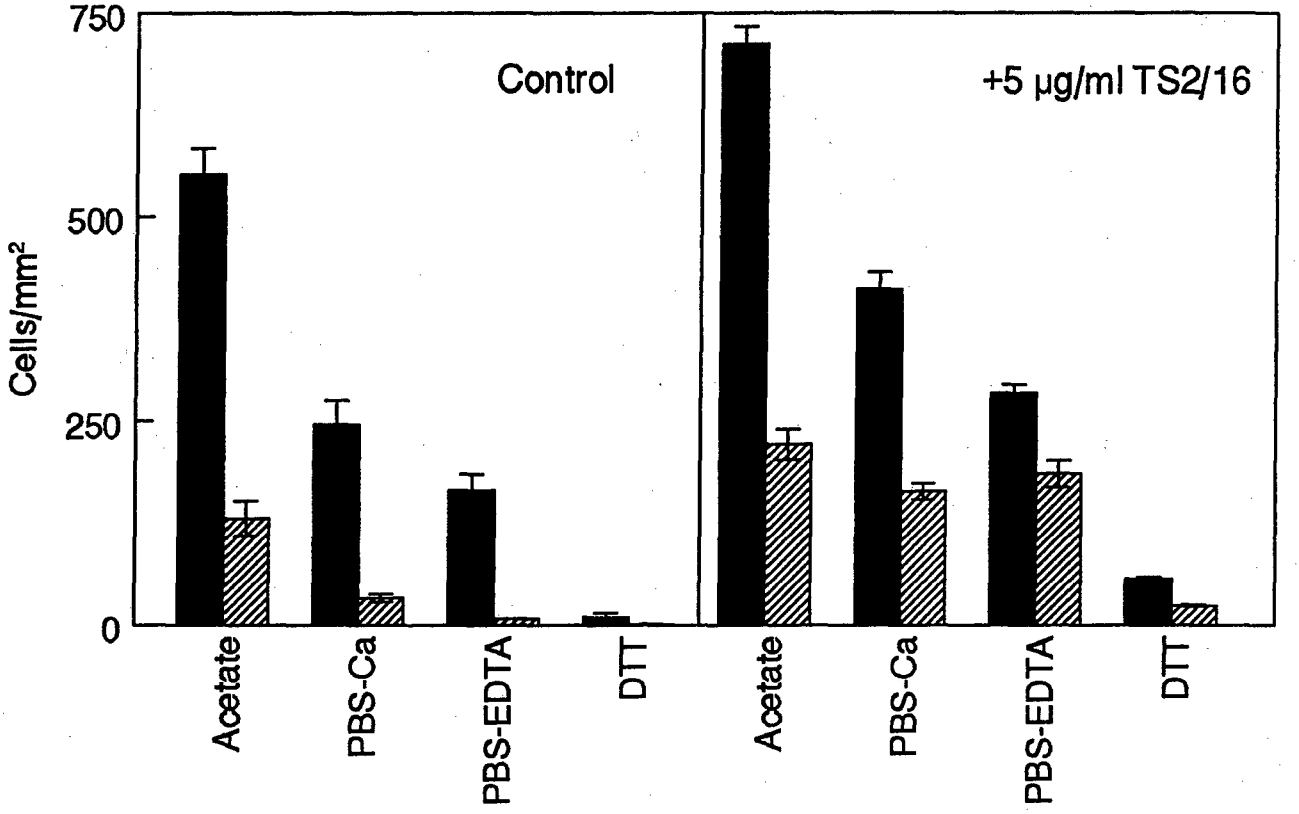
TSP1
HBD
procoll.
type I
type 2
type 3

Figure 7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500
250
0
Cells/mm²
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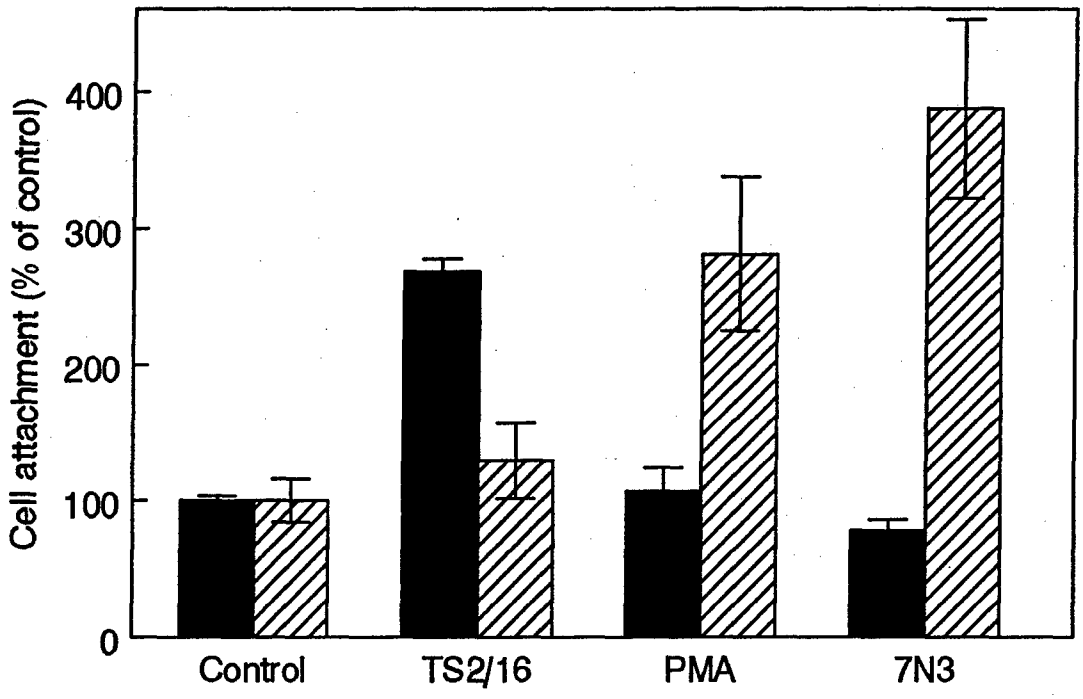
Acetate
PBS-Ca
PBS-EDTA
DTT

Figure 8


Cell attachment (% of control)
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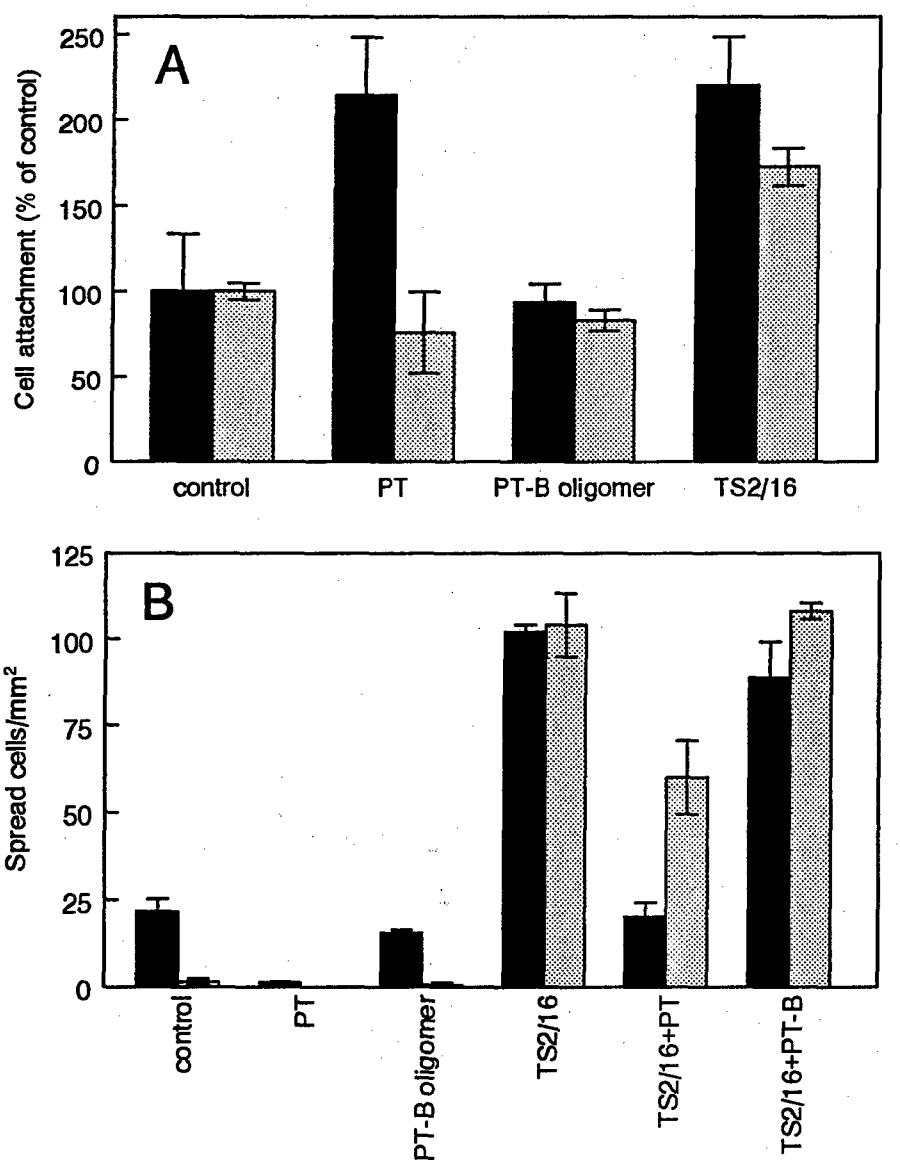
300
200
100
0
Control
TS2/16
PMA
7N3

Figure 9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Cell attachment (% of control)
250
200
150
100
50
0
control
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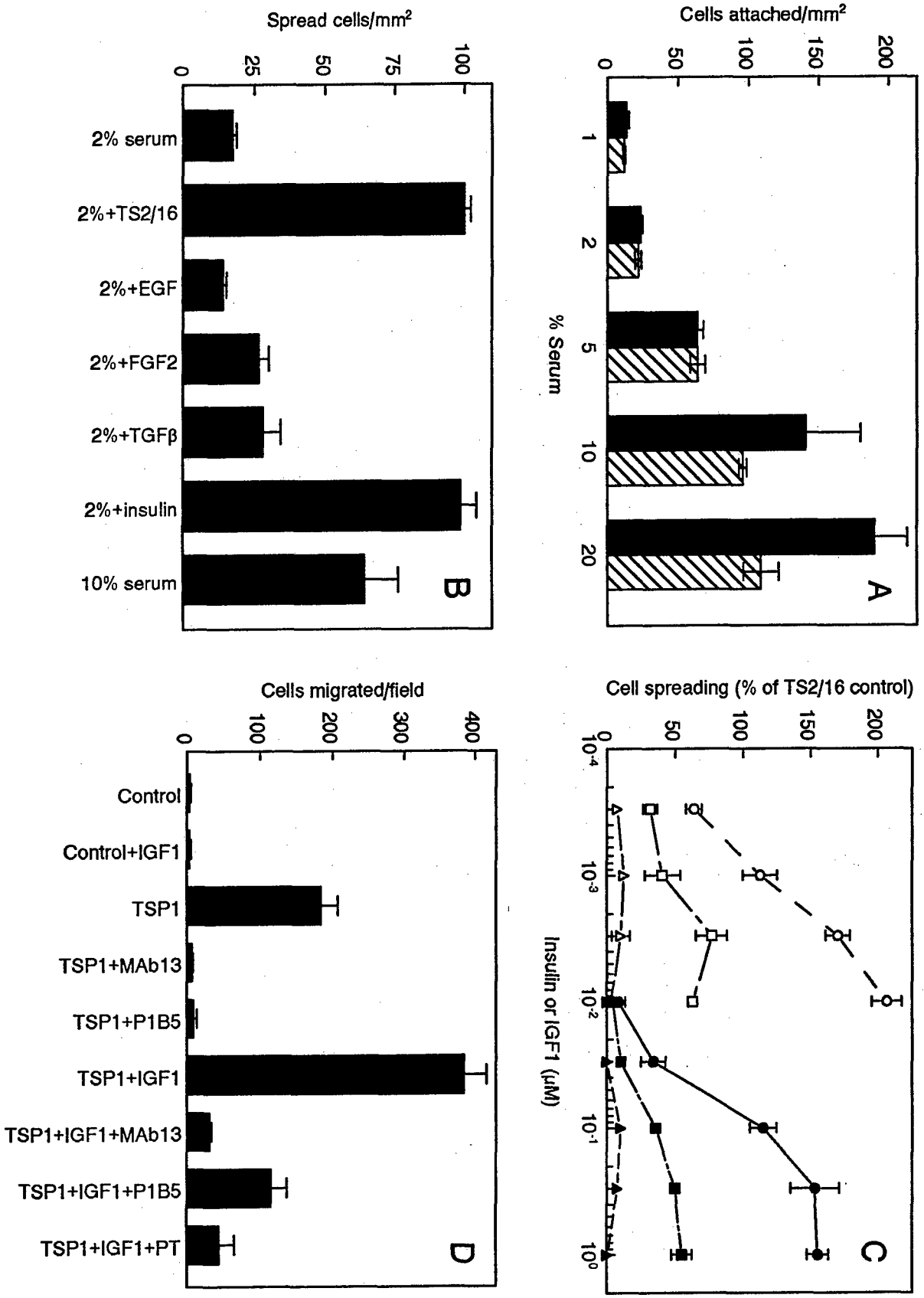
PT-B oligomer
TS2/16
B
Spread cells/mm²
125
100
75
50
25
0
control
PT
PT-B oligomer
TS2/16
TS2/16+PT
TS2/16+PT-B

Figure 10


Cells attached/mm²
0
50
100
150
200
1
2
5
10
20
% Serum
A
Spread cells/mm²
0
25
50
75
100
2% serum
2%+TS2/16
2%+EGF
2%+FGF2
2%+TGFβ
2%+insulin
10% serum
B
Cell spreading (% of TS2/16 control)
0
50
100
150
200
10⁻⁴
10⁻³
10⁻²
10⁻¹
10⁰
Insulin or IGF1 (μM)
C
Cells migrated/field
0
100
200
300
400
Control
Control+IGF1
TSP1
TSP1+MAb13
TSP1+P1B5
TSP1+IGF1
TSP1+IGF1+MAb13
TSP1+IGF1+P1B5
TSP1+IGF1+PT
D

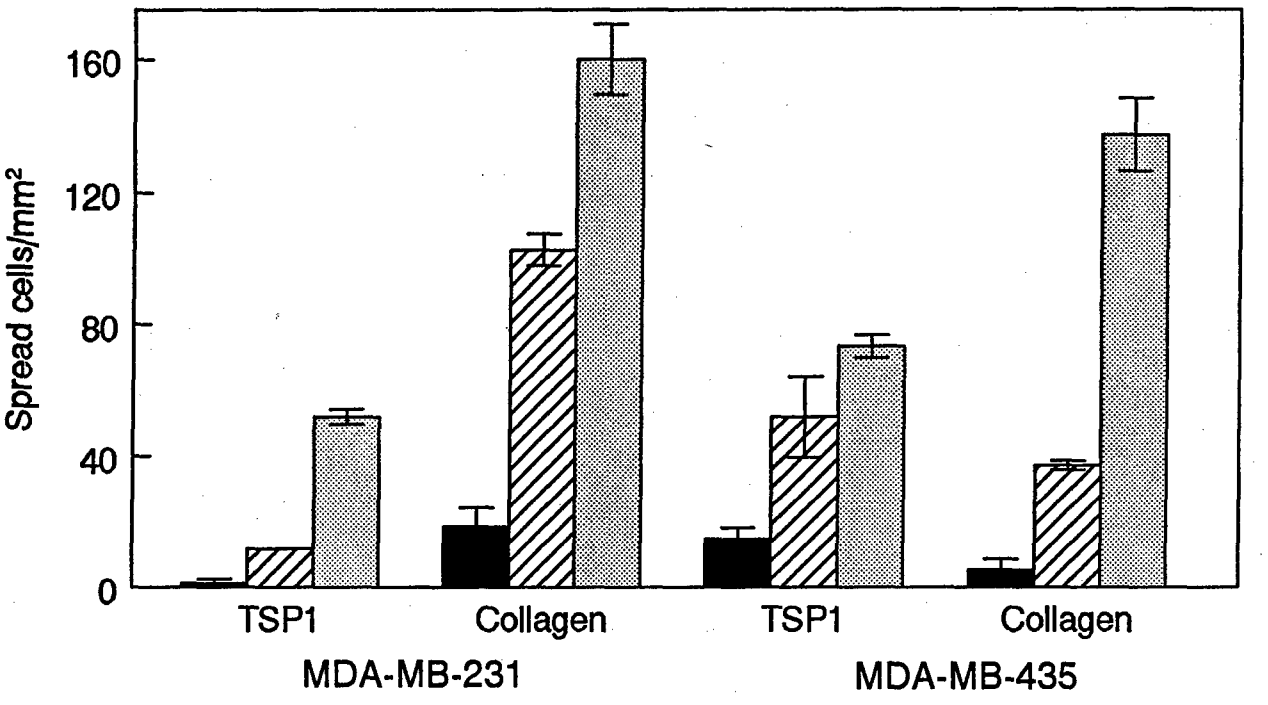

Spread cells/mm²
160
120
80
40
0
TSP1
Collagen
TSP1
Collagen
MDA-MB-231
MDA-MB-435

Figure 11

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

PHYLLIS 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
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DAMD17-95-C-5078	ADB232057
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DAMD17-95-C-5078	ADB253038
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DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
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DAMD17-94-J-4413	ADB232293
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