Thrombospondin Modulates Human Breast Adenocarcinoma Cell Adhesion to Human Vascular Endothelial Cells

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Abstract

Thrombospondin (TSP), a Mr 450,000 cytoadhesive glycoprotein, has been shown to potentiate tumor cell metastasis in mice by a mechanism that involves the hematopoietic system of the host. In this study, the potential involvement of TSP in the interaction of human mammary adenocarcinoma MCF-7 cells with human umbilical vein endothelial cells (HUVECs) in culture was investigated. Using an ELISA, preconfluent HUVECs synthesized 100-fold more TSP than did MCF-7 cells during 24 h of culture (20 versus 0.2 µg/ml). Confocal microscopy localized TSP within intercellular junctions between aggregated MCF-7 cells in suspension. On adherent cells, TSP exhibited a patchy distribution both on the cell surface and in the cytosol. In HUVECs, TSP strongly stained the perinuclear space and was also found in association with cytoskeletal microfilaments. Flow cytometric analysis indicated the presence of a large number of unoccupied receptors for TSP on MCF-7 cells. Binding studies using [125I]TTPS demonstrated the presence of a 1.6 × 10^5 sites/cell with an apparent Kd of 28 nM. Attachment of radiolabeled MCF-7 cells to TSP-coated substrate and to HUVEC monolayers was inhibited in the presence of a polyclonal antibody to TSP (10 µg/ml) or increasing concentrations (1-10 µg/ml) of soluble TSP. Neither nonimmune IgG nor the TSP-coated substrate and to HUVEC monolayers was inhibited in the apparent presence of an excess of soluble TSP or anti-TSP antibody, MCF-7 cells did not form aggregates in suspension and preformed aggregates were readily dissociated by the addition of soluble TSP. These results indicate that mammary adenocarcinoma cells use TSP to form aggregates and to attach to human endothelial cells. These interactions may have physiological implications during the hematogenous spread of tumor cells.

Introduction

The metastatic cascade is a complex sequence of events involving multiple cell-cell and cell-matrix interactions (1, 2). During this process, tumor cells may alternatively demonstrate increased or decreased adhesive properties depending on the metastatic stage they have reached (3, 4). As a first step, neoplastic cells escape from a primary tumor, migrate through interstitial matrices, and adhere to the endothelium so as to penetrate into the vascular or lymphatic circulatory. Thereafter, at distant sites, tumor cells again adhere to the endothelium to enter into the extravascular compartment where they may metastases (5-7). Several cell surface adhesion molecules, including integrins, lectins, and members of the immunoglobulin superfamily, have been shown to be involved in mediating tumor cell interactions with the endothelium (3, 8-10). In addition, the ability of tumor cells to form emboli may facilitate their attachment to the endothelium (6, 7).

Recently, it was shown that the cytosol of malignant breast tissues contained significantly higher levels of TSP, a Mr 450,000, adhesive glycoprotein, than that of nonmalignant breast tissue (11). In addition, strong immunohistochemical labeling of TSP was seen in the demoplastic stroma or at the basement membrane associated with malignant ductal epithelium, as well as in the cytosol of tumor cells abutting these tissues (12, 13). TSP is synthesized by a wide range of cells in culture, including tumor cells, and is considered to be an important mediator of the adhesion and motility of several tumor cell lines (14-17), as well as a potential mediator of tumor cell metastasis in vivo (18, 19).

In the blood, platelets constitute an important source of TSP, which is secreted during platelet interaction with tumor cells (20). In addition, both endothelial cells and the human breast adenocarcinoma cell line MDA-MB-231 were shown to synthesize and secrete substantial amounts of TSP and to express specific TSP-binding sites that may modulate their adhesive properties (21-25). These findings prompted us to investigate whether TSP could be involved in the attachment of human breast adenocarcinoma cells to endothelial cells. In this report, we present biological, biochemical, and morphological evidence that TSP may function as an adhesive factor with the ability to promote both tumor cell-tumor cell and tumor cell-endothelial cell interactions.

Materials and Methods

Materials. DMEM, RPMI 1640, penicillin, streptomycin, HEPES, and PBS were obtained from GIBCO (Cergy-Pontoise, France). Heat-inactivated FCS, endothelial cell growth factor from bovine brain, and ABTS substrate were from Boehringer Mannheim (Meylan, France). BSA, gelatin, and heparin (porcine intestinal mucosa, grade I) were used for endothelial cell culture, and DABCO antifading agent was purchased from Sigma Chemical Co. (St. Louis, MO). Heparin used in the inhibition tests was supplied by Laboratoires LEO S. A. (Montigny-Le-Brotonneux, France). [125I]Indium chloride and sodium 125I were purchased from CIS Biointernational (Gif-Sur-Yvette, France). The peptides GRGDS and Gly-Arg-Gly-Glu-Ser were from Bachem Feinchemikalien AG (Budendorf, Switzerland). Human serum was prepared in our laboratory from the blood of healthy volunteers.

TSP. TSP was purified from the supernatant of thrombin-activated human platelets by heparin-Sepharose-4B chromatography followed by gel filtration, as described (26). The purified protein gave a single band (Mr 180,000) on electrophoresis in the presence of SDS and β-mercaptoethanol, and was stored at -80°C in Tris-buffered saline (pH 7.4) containing 1 mM CaCl2 and 20% sucrose (26).

Antibodies. The rabbit polyclonal anti-TSP antibody (R1) used in this study (a kind gift of Dr. J. Lawler, Harvard Medical School, Boston, MA) was prepared against purified platelet TSP. The antibody recognizes a single protein band with a molecular mass identical to that of TSP when tested by electrophoresis in the presence of SDS and β-mercaptoethanol, and was stored at -80°C in Tris-buffered saline (pH 7.4) containing 1 mM CaCl2 and 20% sucrose (26).

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The abbreviations used are: TSP, thrombospondin; GRGDS, Gly-Arg-Gly-Glu-Ser; HUVEC, human umbilical vein endothelial cell; ABTS, 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)]; GRGES, Gly-Arg-Gly-Glu-Ser.
TSP antibody was kindly donated by Dr. Jean Amiral (Serbio, Gennevilliens, France). Fluorescein-conjugated swine anti-rabbit IgG was from Dako S.A. (Trappes, France). Nonimmune rabbit IgG was prepared in our laboratory from rabbit serum by affinity chromatography on protein A-Sepharose. The monoclonal anti-α-actinin antibody was purchased from Sigma.

Cell Cultures. HUVECs were obtained as described by Jaffe et al. (28). The cells were grown on gelatin-coated plates in RPMI 1640 supplemented with 5% FCS-5% pooled human serum-20 μg/ml endothelial cell growth factor-50 μg/ml heparin-10 nm HEPES-50 IU/ml penicillin-50 IU/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. They were positive for von Willebrand factor antigen and exhibited their typical cobblestone morphology when confluent. Cells were harvested after a brief incubation with 0.05% trypsin-0.02% EDTA and used only after the first passage.

The human breast adenocarcinoma cell line MCF-7, originally established by Soule et al. (29), was kindly provided by Dr. G. Leclercq (Institut Bordet, Brussels, Belgium). The cells were routinely grown in DMEM supplemented with 10% FCS and were harvested with a brief incubation with 1 mm EDTA.

Quantitation of TSP Synthesized by MCF-7 and HUVECs. Tumor cells (5 × 10⁵) were seeded onto 6-well plates in DMEM culture medium supplemented with 10% FCS until they reached 80% confluence. They were then incubated in fresh DMEM medium for 24 h, and the conditioned medium was removed and clarified by low speed centrifugation. The adherent cells were washed, detached with 1 mm EDTA in PBS (pH 7.4) containing 0.1% glucose, and harvested. The cell number was determined using a hemocytometer, and cells were sonicated. Both the supernatants and the sonicated cells were aliquoted and stored at -20°C prior to being assayed.

HUVECs harvested from monolayers were seeded (7 × 10⁶ cells) onto gelatin-coated 6-well plates in RPMI 1640 supplemented with 20% FCS until they reached 80% confluence. They were then processed as described above for tumor cells.

TSP was measured on triplicate samples by ELISA as described previously (30). Briefly, the plate was coated overnight with an anti-TSP monoclonal antibody (10 μg/ml); the remaining free sites were blocked with 0.5% gelatin. Samples to be assayed were incubated for 1 h at 37°C and the bound TSP was measured using the peroxidase-conjugated rabbit anti-TSP antibody and ABTS as the substrate. Purified platelet TSP, diluted in the same buffer as that used in the assays, was used as the standard. The plates were read against appropriate controls in a titertek Twinerader using a 405-nm filter.

TSP Immunolocalization in MCF-7 and HUVECs by Confocal Microscopy. The analysis was performed on confluent MCF-7 cells or HUVEC monolayers and on MCF-7 cell aggregates in suspension. Tumor cell aggregates were allowed to form by incubating cells harvested from the monolayers in DMEM supplemented with 0.2% BSA for 90 min at 37°C under gentle rotation. Cells were incubated in DMEM containing 10 μg/ml of rabbit anti-TSP or nonimmune rabbit IgG for 1 h at 37°C. Cells were washed once with DMEM containing 0.2% BSA, incubated with 200 μl of fluorescein-conjugated swine anti-rabbit IgG, washed again, and finally resuspended in 200 μl of PBS containing 1% paraformaldehyde and mounted in 50% glycerol containing 2% antifading agent on glass coverslips before observation using a confocal microscope.

For analysis of adherent cells, MCF-7 cells or HUVECs were seeded on 8-chamber tissue-culture slides (Lab-Tek; Nunc, Kampstrup, Denmark) in DMEM supplemented with 10% FCS or in RPMI supplemented with 20% FCS, respectively. After 16 h of incubation, cells were washed with the appropriate culture medium containing 0.2% BSA, fixed in PBS containing 1% paraformaldehyde, and permeabilized with ice-cold methanol for 3 min. After washing, cells were processed as described above. Confocal microscopy was performed with an INSIGHT IQ system (MERIDIAN Instruments) equipped with an argon-ion laser mounted on an Olympus IMT-2 inverted microscope. Most observations were recorded using an Olympus 100 x 1.25 N.A. oil-immersion objective. Image analysis was performed using the INSIGHT IQ 1.0 software.

Analysis of Cell Surface-associated TSP by Flow Cytometry. These assays were performed on cells in suspension as described previously (25). Briefly, HUVECs or tumor cells harvested from monolayers were aliquoted (1.5 × 10⁵/sample) in Eppendorf tubes and centrifuged at 400 × g for 4 min. Each pellet was resuspended in 200 μl of DMEM containing 10 μg/ml of rabbit anti-TSP or nonimmune rabbit IgG and incubated for 1 h at 4°C. In parallel samples, cells were preincubated for 1 h at 4°C with 20 μg/ml of TSP before incubation with the antibody for an additional 1 h. Cells were washed once with DMEM containing 0.2% BSA, incubated with 200 μl of fluorescein-conjugated swine anti-rabbit IgG, washed again, and finally resuspended in 200 μl of PBS containing 1% paraformaldehyde. Staining was analyzed by a Becton Dickinson flow cytometer using FACScan research software.

TSP Radiolabeling. Purified TSP was labeled with carrier-free sodium ¹²⁵I to a specific activity of 0.2–0.5 μCi/μg, using the chloramine-T method under mild conditions as reported previously (26). The labeled protein was more than 97% precipitable by 20% trichloroacetic acid and appeared intact when analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (26).

Binding of [¹²⁵I]TSP to MCF-7 Cells in Suspension. Cells were harvested from monolayers by 1 mm EDTA, washed 3 times, and aliquoted in Eppendorf tubes (1.2 × 10⁵ cells). After centrifugation at 400 × g for 2 min, cells were resuspended in DMEM containing 0.2% BSA with [¹²⁵I]TSP at various concentrations ranging from 1 to 20 μg/ml. Non-specific binding was routinely determined in the presence of 10 μg/ml heparin rather than in the presence of a 100-fold excess of unlabeled TSP as identical results were obtained (25). After 2 h of incubation at 4°C, 50-μl triplicate aliquots were centrifuged (400 × g for 2 min) through 450 μl of 20% sucrose in DMEM supplemented with 0.2% BSA. The supernatants were removed by aspiration and the radioactivity associated with the cell pellets was determined in a γ-counter (Beckman, San Monam, CA). Specific binding was calculated for each point by subtracting nonspecific binding from the total radioactivity, and the results were analyzed by Scatchard plot (31).

Radiolabeling of MCF-7 Cells with ¹¹¹Indium. MCF-7 cells harvested from monolayers with EDTA were radiolabeled with ¹¹¹Indium essentially as reported previously (25). Briefly, cells (3 × 10⁶/ml) were incubated for 15 min at room temperature with 10 μCi of ¹¹¹Indium tropolonate in DMEM supplemented with 10% FCS, washed 3 times, and resuspended in DMEM supplemented with 0.2% BSA.

Tumor Cell Adhesion to a TSP Substrate. The adhesion assay was performed as described (25). Separable wells of 96-well plates (EIA/RIA, 8-well strips; Costar, Cambridge, MA) were coated with TSP (10 μg/ml) in acetate-buffered saline (pH 4.0) overnight at 4°C. The wells were then washed with PBS containing 0.1% BSA and saturated with 2% BSA for 2 h at 37°C. After washing, the wells were overlaid with 100 μl of ¹¹¹Indium-radiolabeled tumor cells (10⁴ cells) in DMEM containing 0.2% BSA and the plates were incubated at 37°C in a 5% CO₂ atmosphere. At various time intervals, non-adherent cells were discarded and the wells were washed 3 times with PBS supplemented with 0.1% BSA. Each well was separated, and the radioactivity associated with cells in each individual well was directly counted in a γ-counter.

Tumor Cell Attachment to HUVEC Monolayers. HUVECs harvested from monolayers were seeded on 0.1% gelatin-coated separable wells of 8-well strips (40,000 cells/well) and maintained in RPMI supplemented with 20% FCS for 16 h to allow the formation of a confluent monolayer. The monolayers were rinsed twice with RPMI supplemented with 0.2% BSA and overlaid with 100 μl of ¹¹¹Indium-radiolabeled tumor cells (10⁴ cells) in DMEM supplemented with 0.2% BSA. The plates were incubated at 37°C in a 5% CO₂ atmosphere for various periods of time. Nonattached cells were eliminated by washing once with DMEM supplemented with 0.2% BSA. The wells were separated, and the radioactivity of the adherent cells in each individual well was counted.

Modulation of Tumor Cell Aggregates by TSP. MCF-7 cells were detached from monolayers with 1 mm EDTA, rinsed twice, and incubated for 90 min at 37°C in DMEM containing 0.2% BSA in the absence or the presence of 10 μg/ml of TSP or rabbit anti-TSP antibody. In another set of experiments, cells were first allowed to aggregate as described above before being incubated with 20 μg/ml of soluble TSP for an additional 30-min period. At the end of these incubations, cells were observed under an inverted microscope and photographed.

RESULTS

Quantitation of TSP Synthesis and Secretion by MCF-7 Cells and HUVECs. The amount of TSP synthesized and secreted into the culture medium by preconfluent MCF-7 cells and HUVECs over a ...
24-h period was determined by ELISA using purified platelet TSP as the standard. HUVECs synthesized a high level of TSP, the major part of which was secreted into the supernatant (Fig. 1A). The amount of TSP in the conditioned medium of HUVECs at 24 h was 21.5 ± 2 μg/10⁶ cells. In comparison, MCF-7 cells synthesized approximately 100-fold less TSP and only 0.2 μg TSP/10⁶ cells were measured in the conditioned medium after 24 h of culture (Fig. 1B).

**TSP Immunolocalization in MCF-7 Cells and HUVECs Using a Confocal Microscope.** Because MCF-7 cells have the ability to spontaneously form aggregates when they are resuspended in culture medium, we examined the distribution of TSP in tumor cell aggregates by confocal microscopy. Cells were aggregated and then incubated with the anti-TSP antibody (10 μg/ml) and the second fluorescent antibody as described in "Materials and Methods." The analysis of a laser optical view indicated that TSP was essentially localized within intercellular junctions of adjacent cells (Fig. 2A). Adherent MCF-7 cells and HUVECs cultured in tissue chamber culture slides exhibited clusters of TSP on their apical surface (Fig. 2, C and D, respectively), whereas cells incubated with a rabbit nonimmune IgG were not labeled as illustrated for HUVECs (Fig. 2B). The analysis of a laser optical view through the center of the cells indicated weak labeling of the MCF-7 cell cytosol (Fig. 2E) as compared to strong staining of the HUVEC perinuclear region (Fig. 2, F and G). In both MCF-7 cells and HUVECs, intracellular TSP displayed a fibrillar pattern that looked very similar to that produced by an anti-α-actinin antibody (Fig. 2H).

**Analysis of Cell Surface-associated TSP by Flow Cytometry.** To determine whether TSP could be a mediator of the interaction between MCF-7 cells and endothelial cells, we analyzed TSP expression on the surface of these two cell types by flow cytometry. Cells harvested with EDTA were reacted with a polyclonal rabbit anti-TSP antibody or a nonimmune rabbit IgG. Fluorescence-activated cell sorting analysis showed that the anti-TSP antibody produced a shift to the right in the cell fluorescence intensity as compared with the nonimmune IgG (Fig. 3A, curve b versus a). No additional shift was observed when HUVECs were preincubated with soluble TSP (20 μg/ml) before incubation with the anti-TSP antibody (Fig. 3A, curve c). Analysis of cell surface-associated TSP on MCF-7 cells under similar experimental conditions showed a lower fluorescence intensity compared to HUVECs (Fig. 3B, curve b). However, the preincubation of MCF-7 cells with soluble TSP (20 μg/ml) resulted in a 20-fold increase in the mean fluorescence intensity, indicating the presence of many unoccupied receptors on these cells (Fig. 3B, curve c).

**Binding of [125I]TSP to MCF-7 Cells.** The number of free receptors for TSP on MCF-7 cells was quantified by incubating the cells with increasing concentrations of [125I]TSP for 2 h at 4°C. TSP was shown to bind to MCF-7 cells in a dose-dependent manner over the range 1–20 μg/ml and was saturable (Fig. 4A). Analysis of the binding data by the Scatchard method indicated the presence of a single class of binding sites with a dissociation constant of 27.9 ± 3.6 nm. The number of binding sites measured at saturation was 1.59 ± 0.22 × 10⁶/cell (Fig. 4B).

**MCF-7 Cell Adhesion to a TSP Substrate.** Adhesion of MCF-7 tumor cells to TSP was studied by measuring the attachment of ¹¹¹Indium-radioabeled cells to wells precoated with TSP as compared to wells precoated with BSA. As shown in Fig. 5A, attachment of MCF-7 cells to a TSP substrate was observed as early as 15 min after plating and was maximal within 30 min. A maximum of 43% of cells attached to TSP under our experimental conditions. Preincubation of the cells with either polyclonal rabbit anti-TSP antibody (10 μg/ml) or soluble TSP (20 μg/ml) inhibited the interaction of MCF-7 cells with TSP by about 50 and 30%, respectively (Fig. 5B). Heparin (10 μg/ml) also inhibited MCF-7 cell attachment by 64%, whereas the synthetic peptide GRGDS (100 μM) had only a minor effect. Increasing the dose of the peptide (1 mM) did not enhance the inhibitory effect (not shown).

**MCF-7 Cell Interaction with HUVEC Monolayers.** The potential role of TSP in the interaction between MCF-7 cells and endothelial cells was assessed by measuring the binding of radiolabeled tumor cells to HUVEC monolayers. The percentage of MCF-7 cells attached to endothelial cells increased sharply during the first 15 min of incubation and then progressively up to 120 min to reach approximately 87% (Fig. 6A). The attachment was inhibited by 36% when the assay was performed in the presence of a polyclonal rabbit anti-TSP antibody (10 μg/ml), whereas a nonimmune IgG had no significant effect (Fig. 6B). Heparin (10 μg/ml) also inhibited tumor cell attachment to HUVECs by 30%, whereas GRGDS (100 μM) had no significant effect. Preincubation of MCF-7 cells with soluble TSP resulted in a dose-dependent inhibition of cell attachment to HUVECs, with a maximum of 31% inhibition at 10 μg/ml TSP. Interestingly, no inhibition was observed in parallel experiments where HUVECs were preincubated with soluble TSP and then washed before the addition of MCF-7 cells.

**Modulation of MCF-7 Cell Aggregate Formation by TSP.** MCF-7 cells, incubated for 90 min in DMEM supplemented with 0.2% BSA, spontaneously formed aggregates that were constituted by more than 50 cells (Fig. 7A). When the incubation was performed in the presence of either soluble TSP (10 μg/ml; Fig. 7B) or polyclonal rabbit anti-TSP antibody (10 μg/ml; Fig. 7C), the size of the cell aggregates was markedly reduced, as only small aggregates comprised of no more than 5 cells were observed. Moreover, incubation of preformed cell aggregates with soluble TSP (10 μg/ml) resulted in a rapid disassociation of the aggregates (Fig. 7D).

**DISCUSSION**

During metastatic spread, tumor cells circulate in the blood or lymph vessels and stop at distant sites to penetrate into the host tissue. Adhesion of the circulating tumor cells to the endothelial lining of vessels is the initial critical step in the invasion process, which is followed by local proteolysis and migration through the vascular wall.
The mechanism by which tumor cells attach to the endothelium has been studied in vitro in animal models and in a model of ex vivo perfusion of the human umbilical vein (2–10, 32–35). These studies showed that tumor cells attach to the surface of the endothelium and induce endothelial cell retraction. Gaps are thus formed that facilitate the penetration of tumor cells into the vessel wall and their subsequent adhesion to the subendothelial matrix. Adhesion molecules are likely to play a major role in these events by modulating the adhesive properties of both tumor and endothelial cells (2–4). Indeed, several cell surface-associated molecules, including lectins, integrins, and members of the IgG superfamily, have been shown to participate in the interactions of tumor cells with the endothelium and may modulate tumor progression and metastasis (3–10). However, other secreted proteins such as laminin and fibronectin may also play significant roles in the metastatic process (36).

Recently, particular interest has been given to TSP, a $M_r$ 450,000 multidomain adhesive glycoprotein that was shown to promote the adhesion and motility of a number of tumor cell lines in vitro (14–17) and to potentiate the rate of lung metastasis formation in mice (18, 19). TSP is synthesized by both breast cancer cells and stromal cells and accumulates in the center of tumor masses, as well as in the desmoplastic stroma and epithelial basement membrane surrounding the tumor (12, 13). The potential role of TSP in the hematogenous spread of breast cancer is suggested by the following observations: (a) breast cancer cells express TSP both in vivo and in vitro (13, 25); (b) breast cancer cell lines exhibit specific cell surface receptors for TSP.
TSP MEDIATES MCF-7-ENDOTHELIUM INTERACTIONS

Fig. 3. Flow cytometric analysis of cell surface-associated TSP on HUVECs and MCF-7 cells. HUVECs (A) and MCF-7 cells (B) (1.5 X 10^5/200 μl) in suspensions were incubated with 10 μg/ml of either a nonimmune IgG (curve a) or an anti-TSP antibody (curve b) for 1 h at 4°C, followed by fluorescein-conjugated goat anti-rabbit IgG for 30 min at 4°C. In separate samples, cells were preincubated with soluble TSP (20 μg/ml) for 1 h at 4°C prior to their incubation with the anti-TSP antibody (curve c). Staining was analyzed on a Becton Dickinson flow cytometer using FACSscan research software program.

Fig. 4. Binding of [125I]TSP to MCF-7 cells in suspension. (A) Cells (1.2 X 10^5/sample) were harvested with EDTA, washed, and incubated in DMEM culture medium with increasing concentrations (1-20 μg/ml) of [125I]TSP for 2 h at 4°C. At this time, 50-μl triplicate aliquots were centrifuged (400 x g for 2 min) through a 450-μl cushion of 20% sucrose, and the radioactivity associated with the pellets was counted. Nonspecific binding was calculated by subtracting for each point nonspecific binding from the total radioactivity (O). Specific binding of TSP (θ) was calculated by subtracting each point nonspecific binding from the total radioactivity (Ο). (B) Analysis of the binding data by the Scatchard plot.

Comparison of the amounts of TSP synthesized by HUVECs and MCF-7 cells quantified by ELISA indicated that HUVECs synthesized about 100-fold more TSP than did MCF-7 cells. More than 90% of the TSP synthesized by both cell types was secreted into the culture medium. Confocal optical views through endothelial cells showed strong staining of TSP in the perinuclear region, which argued for a high synthetic activity. Clusters of TSP were also observed on both the luminal and basal endothelial cell plasma membranes and on the MCF-7 cell surface. The perinuclear region of the latter cells was only weakly stained, which reflected their low TSP synthesis compared to HUVECs. In addition, intracellular TSP in both cell types seemed to colocalize with cytoskeletal proteins as it exhibited a fibrillar pattern similar to that obtained with an anti-α-actinin antibody. The association of TSP with microfibrillar elements might play a role in the intracellular trafficking of TSP to the plasma membrane. Strong expression of TSP on the surface of HUVECs was also evidenced by flow cytometry. Interestingly, although MCF-7 cells showed only low levels of TSP on their surface, they exhibited a high number of unoccupied binding sites that could be saturated by the addition of soluble TSP. Binding studies performed with radiolabeled TSP have shown that TSP binds to about 1.6 X 10^6 sites/cell with an apparent Kd of 28 nM. This may provide a basis for the interaction of MCF-7 cells with endothelial cells, as free receptors on the former cells may recognize cell surface-associated TSP on the latter.

As also described for other cell types (14, 23-25, 37), MCF-7 cells adhered readily to TSP-coated coverslips, but they did not spread out on this substrate. A similar observation was made for the binding of MCF-7 cells to HUVEC monolayers. MCF-7 cell binding to the TSP
supporting the idea that it is preferentially involved in the attachment of endothelial cells to matrix-bound TSP (23, 40).

Taken together, our data indicate that endothelial cell-associated TSP may facilitate the arrest of breast cancer cells in the vasculature by interacting with heparin-like receptors present in high amounts on these tumor cells (this study and Ref. 25). In addition, our study emphasizes the controversial role attributed to TSP in the adhesive process (40–42). TSP has been described as an adhesive protein for a variety of cells including keratinocytes, melanoma cells, platelets, and endothelial cells (14, 23–25, 37–40). Nevertheless, it may also exert an antiadhesive function that leads to cell rounding and detachment from a substrate (41, 42). For example, TSP behaves as an adhesive substrate for endothelial cells (23, 24, 40), yet it reduces the number of focal adhesions when it is added to fully adherent cells (43, 44). In the present study, solid phase-adsorbed TSP was able to induce MCF-7 cell adhesion, and cell surface-associated TSP promoted the

Fig. 5. MCF-7 cell adhesion to a TSP substrate. (A) Separable wells of 8-well strips were coated with TSP (10 μg/ml) in acetate-buffered saline (pH 4.0) (C) or with 2% BSA in PBS (pH 7.4) (□) overnight at 4°C. The wells were saturated with 2% BSA in PBS (pH 7.4), washed, and overlaid with 111Indium-labeled cells (10⁶) in DMEM containing 0.1% BSA for various times at 37°C. At the end of the incubation period, unattached cells were removed by washing. The wells were separated and the radioactivity of the adherent cells in each independent well was directly counted in a γ-counter. Points, average of 3 experiments run in triplicate; bars, SD. (B) MCF-7 cells were seeded on TSP-coated wells in the presence of a polyclonal anti-TSP antibody (10 μg/ml), nonimmune (NI) IgG (10 μg/ml), heparin (10 μg/ml), GRGDS peptide (100 μM), Gly-Arg-Gly-Glu-Ser (GRGES) peptide (100 μM), or soluble TSP (10 μg/ml) as indicated. The number of adherent cells was measured after 30 min. The results are expressed as percentages of inhibition of cell adhesion considering the adhesion of cells in DMEM containing 0.1% BSA as 100%. Columns, average of 3 experiments run in triplicate; bars, SD.

Fig. 6. MCF-7 cell adhesion to HUVEC monolayers. (A) HUVEC monolayers established in 8-well strips were overlaid with 10⁶ radiolabeled tumor cells in DMEM containing 0.2% BSA at 37°C for various times. At the end of the incubation, unattached cells were removed by washing. The wells were separated and the radioactivity in the individual wells was measured. Points, average of 3 experiments run in triplicate; bars, SD. (B) Radiolabeled MCF-7 cells were seeded onto HUVEC monolayers in the presence of a polyclonal anti-TSP antibody (10 μg/ml), nonimmune (NI) IgG (10 μg/ml), heparin (10 μg/ml), GRGDS peptide (100 μM) or Gly-Arg-Gly-Glu-Ser (GRGES) peptide (100 μM). In these experiments, MCF-7 cells were also preincubated for 1 h with various concentrations of soluble TSP (1–10 μg/ml) prior to their incubation with HUVEC monolayers (MCF-7 + TSP). On the other hand, HUVEC monolayers were incubated for 1 h with soluble TSP (10 μg/ml) and washed prior to the addition of MCF-7 cells (HUVEC + TSP). The results are expressed as percentages of inhibition of cell adhesion considering the adhesion of cells in DMEM containing 0.1% BSA as 100%. Columns, average of 3 experiments run in triplicate; bars, SD.
attachment of these tumors to HUVEC monolayers. However, the addition of soluble TSP led to a reduced adhesion of tumor cells to both the TSP substrate and HUVEC monolayers. A subtle role for TSP in tumor cell–tumor cell interactions was also indicated by the observation that TSP could promote both the formation of MCF-7 cell aggregates or their disassociation depending on the experimental conditions. Thus, it seems likely that the adhesive or antiadhesive function of TSP depends on the local microenvironment to which tumor cells are exposed during metastatic dissemination. We currently hypothesize that the degree of saturation of specific TSP-binding sites on tumor cells may modulate their adhesion to the vessel wall and to the subendothelial matrix, and thereby influences their invasive behavior. The ability of soluble TSP to antagonize the effect of surface-associated TSP would provide a highly regulated mechanism for cell-cell and cell-matrix interactions during the multistep metastatic process.

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