Interactions of Thrombospondin with Endothelial Cells: Receptor-mediated Binding and Degradation

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Abstract. We studied binding and degradation of labeled platelet thrombospondin (TSP) by normal and variant bovine aorta endothelial (BAE) cells. [125I]-labeled TSP bound to cells at 37°C in a specific, saturable, and time-dependent fashion. Incubation of cell monolayers with fluoresceinated TSP resulted in punctate cellular staining, but no staining of the extracellular matrix. Heparin, fucoidan, chondroitin sulfate, platelet factor 4, beta-thromboglobulin, unlabeled TSP, and serum derived from whole blood all competed for binding of [125I]TSP. [125I]TSP was degraded to TCA-soluble radioactivity, which appeared in the medium after a 60-90-min lag. Degradation was inhibited to the same extent as binding by increasing concentrations of heparin, fucoidan, platelet factor 4, or whole blood serum. Normal BAE cells bound and degraded less [125I]TSP than variant BAE cells. The dissociation constants (Kd) for binding and the constants for degradation (Km) for degradation by the two cell strains, however, were similar (30-50 nM). The inhibitory effects of heparin and platelet factor 4 were lost when the two inhibitors were present in a 1:1 (wt/wt) ratio. Treatment of suspended cells with trypsin or heparitinase caused less binding of TSP. These results indicate that there is a specific receptor for TSP on endothelial cells which mediates binding and degradation. This receptor may be a heparan sulfate proteoglycan.

Thrombospondin (TSP) is a high molecular weight (450,000) glycoprotein which is composed of three identical, disulfide-linked, polypeptide chains (12, 19, 31, 32, 33, 39). TSP interacts with a variety of macromolecules, including heparin (17), thrombin (13), fibrinogen (6, 16, 29, 34), fibronectin (29, 30, 48), histidine-rich glycoprotein (35), plasminogen (59), calcium ion (15, 32), type V collagen (48), and sulfatides (56, 57). TSP is a major component of platelet alpha-granules and thus is secreted when platelets are stimulated with thrombin or other agents (4). This TSP becomes associated with platelet surfaces and the meshwork of the fibrin clot (5, 18, 20, 21, 41, 49, 53, 62). High and low affinity interactions of TSP with unstimulated and stimulated platelets have recently been reported (64).

TSP is synthesized by and deposited into the extracellular matrices of cultured endothelial cells (25, 42, 45), fibroblasts (26), glial cells (2), and smooth muscle cells (55), among others. Immunofluorescence microscopy revealed more TSP in injured tissues than in stable normal adult tissues (63). Isolated matrices derived from cultured microvascular endothelial cells of adult dermis contained less TSP than matrices of neonatal foreskin microvascular endothelial cells (27). The regulation of TSP synthesis by cells in culture is modulated by factors affecting the cell cycle, as evidenced by observations that synthesis of TSP was greater in cultures of subconfluent cells than in cultures of density-arrested cells (47) and was transiently enhanced when platelet-derived growth factor was given to serum-starved cultures (2, 36). TSP amplified the mitogenic effect of epidermal growth factor towards vascular smooth muscle cells (37). Thus, TSP appears to have important effects on cells, especially under conditions in which there is active tissue modeling or healing. The mechanisms by which TSP has its effects on cells are unknown.

A previous paper from our laboratory described binding of TSP to fibroblast monolayers and to isolated matrices derived from fibroblast cultures (40). Binding to the intact cell layers was transient, and bound TSP was rapidly degraded to a TCA-soluble form (40). Binding of radiolabeled TSP to the cell layers was not saturable because of extensive binding of TSP to extracellular matrix at higher TSP concentrations. However, degradation of radiolabeled TSP was saturable and exhibited characteristics of well-studied examples of receptor-mediated endocytosis (40). In subsequent experiments, we found that bovine aorta endothelial (BAE) cells could be used to study cellular binding and degradation of TSP without the complication of binding of TSP to extracellular matrix. The matrix formed by BAE cells is located primarily...
on the basal surfaces of the cells and therefore is not as accessible to exogenous molecules as the matrix of cultured fibroblasts. The BAE culture system is of physiologic and pathophysiologic relevance because vascular endothelium would be exposed to high concentrations of TSP during hemostatic and thrombotic events and perhaps also during neovascularization. We hypothesized, on the basis of previous studies (40), that heparin-like molecules functioned as thrombomodulin receptors. The heparan sulfate proteoglycans of BAE cells have been well characterized (38, among others). Therefore, we studied binding and degradation of TSP by BAE cells in considerable detail. Both events seem to be mediated by a cell surface molecule which has the characteristics of a heparan sulfate proteoglycan.

Materials and Methods

Materials

The following were purchased: DME and Ham's F12 medium (Gibco Laboratories, Grand Island, NY); PBS (Hyclone Laboratories, Sterile Systems, Inc., Logan, U T); vitrornectin (Calbiochem-Behring Corp., San Diego, CA); trypsin, BSA, fucoidan, heparin (porcine intestinal mucosa), chondroitin sulfate, hyaluronic acid, and dextran sulfate (mol wt 500,000) (Sigma Chemical Co., St. Louis, MO); hyaluronidase, chondroitinase ABC, and heparitinase (Miles Laboratories, Elkhart, IN); FITC (Cappell Laboratories, Cochranville, PA); rabbit antibodies to human factor VIII-related antigen (Dako Corp., Santa Barbara, CA, or Calbiochem-Behring Corp.); and rhodamine- or FITC-labeled secondary antibodies (Jackson Laboratories, Ayondale, PA). Monoclonal antibodies to muscle-specific and smooth muscle-specific actins (22) were provided by Dr. Allen Gown (University of Washington, Seattle, WA). Carrier-free sodium 125I was obtained from New England Nuclear (Boston, MA).

Purification of TSP and Other Proteins

TSP was purified from the releaseate of thrombin-stimulated human platelets by affinity chromatography on heparin-agarose and gel filtration in the presence of 0.1 mM calcium chloride, according to a previously published procedure (49). The purification also resulted in good yields of platelet factor 4 (obtained by eluting the heparin-agarose with 2 M sodium chloride and beta-thromboglobulin precursor (separated from TSP on the P-300 gel filtration column (49). The amino terminal sequence of the beta-thromboglobulin, kindly determined by Dr. Karma Skortensgaard, was predominantly (795%) Asn-Leu-Ala-Lys-Gly-Lys-Glu-Glu; i.e., it contained four residues at the amino terminus which are largely missing from beta-thromboglobulin obtained from outdated platelets (21). Fibrinectin was purified from a plasma protein side fraction by heat precipitation and chromatography on DEAE-cellulose (43). BAE cells appear to differ from the "sprouters" described by Sehgartz (58) in that the elongated cells in the cords were present in preconfluent and newly confluent cultures, whereas sprout cells appear in cultures maintained after confluence. When the normal BAE strain was kept past confluence, cells exhibiting typical sprouting morphology were observed.

When doing binding or degradation assays (see below), cells in replicate plates were enumerated in a microscopic counting chamber after trypanstaination.

Fluorescence Microscopy

Cells were grown to confluence on glass coverslips and fixed with 100% methanol for 5 min at -20°C. Cells were stained for the presence of factor VIII-related antigen using rabbit polyclonal antibodies to human factor VIII-related antigen at either a 1:50 (Dako Corp.) or 1:100 (Calbiochem-Behring Corp.) dilution in the presence of 0.1% ovalbumin. Cells were washed three times with 0.1 M Tris-buffered saline, pH 7.2, and stained with FITC-labeled goat anti-rabbit IgG antibody. Murine monoclonal antibodies to muscle-specific and smooth muscle-specific actins were used at the dilutions suggested by Dr. Gown and detected with a secondary FITC-labeled antibody against mouse IgG. Coverslips were mounted onto glass slides using 50% glycerol and examined by epifluorescence using a Nikon Optiphot microscope equipped with a UFX-II camera.

Cells were incubated with Dil-Ac-LDL at 10 µg/ml for 1-4 h at 37°C in a 5% CO2 atmosphere, rinsed, and fixed according to the protocol of Voyta et al. (61). Coverslips were mounted and examined by fluorescence microscopy using excitation and barrier filters appropriate for rhodamine fluorescence. Cells on coverslips were also incubated with FITC-labeled TSP at 10 µg/ml at 37°C in 5% CO2 under various conditions. Coverslips were washed with DME with 0.2% BSA before fixation with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

Binding Assays

Binding assays were performed on confluent monolayers in 35-mm tissue culture dishes as follows. Monolayers were rinsed twice with DME containing 0.2% BSA, 1 ml of a binding mixture containing [125I]TSP and unlabeled TSP in DME containing 0.2% BSA was added, and cells were incubated at either 37 or 4°C for the appropriate time. At the end of the
incubation, cell layers were washed 4 times with ice-cold DME containing 0.2% BSA and solubilized overnight with 1 ml 1 N sodium hydroxide. Solubilized material was removed, and plates were washed with an additional 1 ml of 1 N sodium hydroxide. The radioactivity in the pooled extracts was then determined using a gamma counter.

Binding assays were performed on cells in suspension in some experiments. Monolayer cells at confluence were harvested with either 2.2 mM EDTA or EDTA and various concentrations of trypsin as described below, pelleted by centrifugation, and resuspended in DME containing 0.2% BSA. After resuspension, cells were greater than 97% viable as determined by dye exclusion with Trypan blue. Cells (0.5-1 x 10^6/100 μl) were added to microfuge tubes containing a 1-ml mixture of DME, 0.2% BSA, 125I-labeled TSP, and the appropriate amount of unlabeled TSP. The microfuge tubes were precoated with 2% BSA to minimize nonspecific binding. The suspensions were incubated at 37°C in 5% CO₂ with frequent inversion. At the end of the incubation period, cells were pelleted by centrifugation for 2 min in a microfuge (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY). Medium was removed, and the cell pellets were put on ice and washed three times with ice-cold DME containing 0.2% BSA with minimal disturbance of the pellets. Radioactivity remaining in the cell pellet was measured.

Nonspecific binding was routinely determined in the presence of 1 μg/ml heparin rather than with an excess of unlabeled TSP (see Results). In no instance did binding of radioactivity to the tissue culture plates in the absence of heparin and cells exceed the nonspecific binding in the presence of heparin and cells. All samples were run in duplicate. Each experiment was performed a minimum of two times.

Enzymatic Pretreatment of Cells

Cells to be treated with enzymes before suspension binding assays were released from confluent monolayers with 2.2 mM EDTA and resuspended in DME containing 0.2% BSA. Aliquots of cells in 1-ml vol were then treated at 37°C for either 5 min with increasing concentrations of trypsin or for 3 h with heparitinase, chondroitinase ABC, or hyaluronidase diluted in DME containing 0.2% BSA. The reaction was stopped by pelleting the cells in a microfuge, removing the enzyme in the supernatant, and washing the pellet once with DME containing 0.2% BSA. When trypsin was used, a fivefold excess (wt/wt) of soybean trypsin inhibitor was added before centrifugation. Cells were gently resuspended in DME containing 0.2% BSA and used in the binding assays described above. Aliquots of cells were

Figure 1. Microscopy of cultured BAE cells. (a) The normal strain of endothelial cells at confluence with typical cobblestone morphology. (b) The variant strain of endothelial cells at confluence: central foci of cobblestone cells are surrounded by cells in multiple layers of cords (arrows). (c) Binding of FITC-TSP to monolayers of normal endothelial cells after incubation for 4 h at 37°C, showing punctate distribution of labeled protein. Monolayers of variant BAE cells showed similar, but more intense, punctate cellular staining. Cells incubated with FITC-TSP in the presence of 10 μg/ml heparin had no staining (not shown). (d) BAE cells incubated with 10 μg/ml FITC-TSP for 60 min, followed by further incubation for 3 h in binding medium without labeled TSP. Bars, 100 μm.
Figure 2. Time course of binding of TSP to endothelial cells in monolayer. Normal (A) and variant (B) endothelial cells were grown to confluence in 35-mm culture dishes and incubated for varying times with 1 μg/ml 125I-labeled TSP. At various time points, the plates were washed, and bound TSP was extracted with 1 N sodium hydroxide as described in Materials and Methods. Binding in the presence of 1 μg/ml heparin (○) was subtracted from the total binding in the absence of heparin (●) to determine the specific binding of [125I]TSP to the cell layers (▲).

checked for viability by the Trypan blue exclusion method. Control cells were treated by the same protocols, except that the enzymes were omitted.

Assay of Degradation

Degradation of [125I]TSP was determined from the amount of radioactivity in the medium which became soluble in 10% TCA after incubation with cells (40). Duplicates of each experimental point were done. Degradation was monitored during a time period when the degradation was linear.

Results

Localization of FITC-TSP Bound to BAE Cells

BAE cells were incubated with 10 μg of FITC-labeled TSP at 37°C for different periods of time, washed, fixed, and examined by fluorescence microscopy to determine the localization of TSP bound to cells in monolayer. Staining was primarily in a punctate pattern with no detectable binding to fibrillar matrix (Fig. 1 c). Addition of 10 μg/ml heparin simultaneously with FITC-TSP blocked binding. Little FITC-TSP was seen when cells were incubated for 1 h with FITC-TSP, washed, and incubated an additional 3 h in the absence of FITC-TSP (Fig. 1 d).

Time Course of Binding of TSP to Cells

Binding of [125I]TSP to normal BAE cells at 37°C rapidly increased over the first 15 min and remained fairly constant for up to 4 h (Fig. 2 A). Binding to variant BAE cells reached a maximum between 45 and 60 min and decreased thereafter (Fig. 2 B). Degradation of [125I]TSP by normal BAE cells, as monitored by the appearance of TCA-soluble radioactivity in the medium, was first detected at 60 min. The amount of TCA-soluble radioactivity increased linearly over the next 3 h (not shown). Degradation of labeled TSP by variant BAE cells was apparent by 45 min of incubation at 37°C and increased linearly for the next 3 h (not shown). Binding to both cell types at 4°C was maximal at 120 min; no degradation was evident at this temperature (not shown).

Inhibition of TSP Binding by Various Macromolecules

Increasing amounts of unlabeled TSP competitively inhibited binding of [125I]TSP to cells in monolayer (Table I) or

Table I. Inhibitors of [125I]TSP Binding to Endothelial Cell Monolayers

| Competitor                | ED50* (μg/ml) | Range of concentrations tested (μg/ml) |
|---------------------------|--------------|---------------------------------------|
| Thrombospondin            | 25-50        | 0.1-100                               |
| Fucoidan                  | 0.05-0.1     | 0.01-1                                 |
| Heparin                   | 0.1          | 0.001-1                                |
| Chondroitin sulfate       | 50           | 0.1-100                                |
| Dextran sulfate           | >10          | 0.1-10                                 |
| Hyaluronic acid           | >100         | 0.1-100                                |
| Beta-thromboglobulin      | 1            | 0.1-50                                 |
| Platelet factor 4         | 1-5          | 0.1-50                                 |
| Fibronectin               | >50          | 0.1-50                                 |
| Vimentin                  | >20          | 0.1-20                                 |
| Fibrinogen                | >100         | 0.1-100                                |
| Whole blood serum         | 2            | 0.2-10                                 |
| Plasma-derived serum      | >10          | 0.2-10                                 |

* Concentration of competitor giving 50% inhibition of total binding of 0.1 μg/ml [125I]TSP to variant endothelial cell monolayers (1 x 10⁶ cells) after a 60-min incubation at 37°C. The experiment with beta-thromboglobulin was done with 1.0 μg/ml [125I]TSP.

No inhibition was seen at these concentrations.
Table II. Scatchard Analysis of Binding of [125I]TSP by Cells in Monolayer

| Cell type     | n   | Kd (nM) | Binding sites/cell |
|--------------|-----|---------|-------------------|
| BAE          | 3   | 43 ± 30 | 5.1 ± 3.8         |
| BAE variant  | 4   | 45 ± 12 | 30 ± 14           |
| Fibroblasts  | 2   | Not saturable | Not saturable    |

n, number of separate experiments. The SD is given for experiments done more than twice.

thromboglobulin and heparin did not neutralize one another as inhibitors in the TSP-binding assay (not shown).

Whole blood serum was more effective than serum derived from platelet-poor plasma (plasma serum) in blocking TSP binding to cells (Table I). As determined by ELISA, plasma serum had a TSP concentration 25-fold lower than whole blood serum. The differences in platelet factor 4 and beta-thromboglobulin concentrations between plasma serum and whole blood serum, determined by commercial radioimmunoassay kits, were even greater (data not shown). Fibrinogen and fibronectin, plasma proteins that are known to interact with TSP, did not compete for binding of TSP to cells (Table I). Vitronecet also did not compete with TSP for binding to cells (Table I).

Saturation of Binding of TSP to Cells in Monolayer

[125I]TSP bound to both normal (Fig. 5) and variant (data not shown) BAE cells in a saturable manner. Binding to fibroblast monolayers, done in parallel assays, was not saturable. The two BAE cell strains had similar dissociation constants (Kd) as determined by Scatchard analysis, but the variant strain cells had sixfold more binding sites (Table II). These estimations of binding kinetics assume that one molecule of TSP binds to one binding site, assume that heparin will compete for all specific binding, and ignore the contribution of internalized TSP. Inasmuch as >80% of cell-associated TSP was promptly released upon addition of heparin, the contribution of internalized TSP to the calculation of the number of binding sites/cell was small.

Degradation of TSP

The degradation of [125I]TSP by cells at 37°C was estimated by the increase in TCA-soluble radioactivity in the medium at 240 min as compared with 60 min. The velocity of degrada-

Figure 4. Effect of platelet factor 4 on the binding and degradation of TSP. (A) Variant endothelial cells were incubated for 60 min at 37°C with labeled 0.1 μg/ml TSP; platelet factor 4 at concentrations ranging from 0.1 to 50 μg/ml, and no heparin (●, - - -), or 1 μg/ml heparin (○, - - -). Cell layers were then washed, and bound [125I]TSP was extracted and quantified. (B) Degradation of [125I]TSP in the medium to TCA-soluble products was determined as described for Fig. 3. The medium contained labeled TSP, increasing amounts of platelet factor 4, and no heparin (△, - - -), or heparin (○, - - -) as in A.
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Figure 6. Degradation of TSP by normal endothelial cells in monolayer. (A) Degradation of increasing concentrations of TSP (25 cpm/ng sp act) by cells in monolayer was monitored between 60 and 240 min as described for Fig. 3. Specific degradation of TSP (a) was calculated by subtraction of the amount of [125I]TSP degraded in the presence of 1 µg/ml heparin (c) from the amount degraded in the absence of heparin (b). (B) Data replotted to determine the kinetics of turnover by Lineweaver-Burke analysis.

Discussion

[125I]TSP bound to monolayers of endothelial cells in a specific and saturable manner indicative of a receptor-mediated process. The binding was primarily to the endothelial cells themselves, as assessed by fluorescence microscopy of bound FITC-TSP, with very little, if any, binding to matrix. The difference between fibroblasts and endothelial cells in monolayer is most likely due to differences in the types and location of extracellular matrix. BAE cultures have less extensive matrix formation than fibroblast cultures, and the location of extracellular matrix. BAE cultures have less extensive matrix formation than fibroblast cultures, and the bulk of the endothelial cell matrix is not accessible to exogenous proteins because it is located on the basal side of the cells. We were able to demonstrate saturability of [125I]TSP binding to suspended human foreskin fibroblasts or normal BAE cells stripped of extracellular matrix by trypsinization (Table IV), whereas binding to monolayer fibroblasts or to normal BAE cells suspended with EDTA in the absence of

| Cell type      | n  | Ks  | Vmax |
|----------------|----|-----|------|
|                |    | nM  | (molecules/cell per min × 10^-3) |
| BAE            | 2  | 30  | 3.3  |
| BAE variant    | 2  | 48  | 45   |
| Fibroblasts    | 2  | 68  | 16   |

n, number of separate experiments.
Table IV. Scatchard Analyses of Binding of $[^{25}I]TSP$

| Cell type | $n$ | Temperature | Method of harvest | $K_d$ (nM) | Binding sites/cell |
|-----------|-----|-------------|------------------|------------|------------------|
| BAE       | 2   | 37          | T-EDTA           | 10.8       | 1.0               |
| BAE variant 3 | 3   | 37          | T-EDTA           | 4.6 ± 0.7  | 2.5 ± 0.5         |
| BAE variant 2 | 2   | 4           | T-EDTA           | 4.4        | 2.9               |
| BAE variant 1 | 1   | 37          | EDTA             | 15.5       | 32                |
| Fibroblasts | 3   | 37          | T-EDTA           | 4.7 ± 3.1  | 2.6 ± 0.2         |

$n$, number of separate experiments. The SD is given for experiments done more than twice.

* Cells were harvested from monolayers with 0.05% trypsin and 0.5 mM EDTA (T-EDTA) or with 2.2 mM EDTA and no trypsin (EDTA) and resuspended in medium for suspension assay as described in Materials and Methods.

tryptin was not saturable. As discussed below, however, TSP may bind to molecules on trypsinized cells in suspension which are different from the molecules which mediate binding and degradation of TSP by cells in monolayer.

Degradation of TSP by endothelial cells was saturable and inhibited by chloroquine or incubation of cells in the cold, as has been previously described for degradation of TSP by fibroblasts (40). The following observations indicate that the cellular moiety which mediates binding of TSP by endothelial cells in monolayer at 37°C also mediates degradation of TSP: $K_{ds}$ of binding were the same as the $K_{ds}$; the variant BAE cells both bound more TSP and degraded more TSP as compared to the normal cells; and binding and degradation of TSP were suppressed in parallel by a number of experimental conditions. Binding constants were estimated under less than ideal conditions. We were limited in the amount of TSP we could purify, and some of our preparations of TSP may bind to molecules on trypsinized cells in suspension which are different from the molecules which mediate binding and degradation of TSP by cells in monolayer.

In particular, the $K_d$ of binding (45 nM, Table II) agreed well with the concentrations of unlabeled TSP that caused 50% inhibition of binding of labeled TSP (55–110 nM, Table I).

Heparin, fucoidan, platelet factor 4, and beta-thromboglobulin all caused less binding and degradation of TSP by cells. Chondroitin sulfate also inhibited binding and degradation of TSP, although higher concentrations were required. Dextran sulfate and hyaluronic acid did not inhibit. These results differ from experiments reported by Roberts et al. (56), in which heparin, fucoidan, and dextran sulfate, but not chondroitin sulfate, inhibited binding of TSP to sulfatides. Three adhesive proteins known to bind to TSP and/or to cells, (i.e., fibrinogen, fibronectin, and vitronectin) did not inhibit binding of TSP. Thus, the interaction of TSP with Cells in Suspension. The inhibitory effect of whole blood on binding and degradation of TSP is likely a sum of the inhibitory effects of platelet factor 4 and beta-thromboglobulin, inasmuch as the combined concentration of these 2 proteins in whole serum is ~20 µg/ml (46).

The major TSP-binding site on endothelial cells was sensitive to digestion by trypsin or heparitinase. Chondroitinase ABC and hyaluronidase had no effect. A decrease in binding of platelet factor 4 to endothelial cells after heparitinase treatment has also been reported (10). No additive decrease in TSP binding was observed in cells which were treated with both trypsin and heparitinase. This result suggests that the trypsin-sensitive binding site is the same as the heparitinase-sensitive binding site. The higher affinity, lower capacity sites that were unmasked when suspended cells were treated with trypsin and/or heparitinase may involve molecules, such as sulfatides, distinct from those that mediate binding to cells in monolayer.

The inhibitory effects of heparin, heparin-binding proteins, and heparitinase all point to a heparin-like molecule on the endothelial cell surface as a receptor for TSP. Recently, we had the opportunity to study the binding and degradation of TSP by Chinese hamster ovary (CHO) cell mutants defective in glycosaminoglycan biosynthesis and found that mutants lacking heparan sulfate were unable to bind or degrade TSP (50 and Murphy-Ullrich, J. E., L. G. Westrick, J. D. Esko, and D. F. Mosher, manuscript submitted for publication). Thus, we think that the TSP receptor on endothelial cells to bind TSP. Variant endothelial cells were released from monolayer with 2.2 mM EDTA and resuspended in DME containing 0.2% BSA. Aliquots of the suspension were treated at 37°C for 5 min with increasing concentrations of trypsin ($\Delta$) or for 3 h with increasing concentrations of chondroitinase ABC (■) or heparitinase (●) according to the protocol described in Materials and Methods. In addition, cells were released from monolayer with 50 µg/ml trypsin and 0.5 mM EDTA before treatment with increasing concentrations of heparitinase (○). Enzyme-treated cells and controls were incubated in suspension with labeled 1 µg/ml TSP for 60 min at 37°C, and the bound fraction was separated as described in Materials and Methods. Results are expressed as % of $[^{25}I]TSP$ bound to cells not treated with an enzyme.

Figure 7. Effects of enzymatic treatment on the ability of endothelial cells to bind TSP. Variant endothelial cells were released from monolayer with 2.2 mM EDTA and resuspended in DME containing 0.2% BSA. Aliquots of the suspension were treated at 37°C for 5 min with increasing concentrations of trypsin ($\Delta$) or for 3 h with increasing concentrations of chondroitinase ABC (■) or heparitinase (●) according to the protocol described in Materials and Methods. In addition, cells were released from monolayer with 50 µg/ml trypsin and 0.5 mM EDTA before treatment with increasing concentrations of heparitinase (○). Enzyme-treated cells and controls were incubated in suspension with labeled 1 µg/ml TSP for 60 min at 37°C, and the bound fraction was separated as described in Materials and Methods. Results are expressed as % of $[^{25}I]TSP$ bound to cells not treated with an enzyme.
a number of cell types may be a heparan sulfate proteoglycan, probably an integral membrane molecule such as that described by Rapraeger et al. (54). At present, we do not know how to relate our findings to those of Asch et al. (1). These authors presented several kinds of evidence that the 88,000-mol-wt glycoprotein recognized by a commercially available monoclonal antibody, OKM5, mediated the binding of TSP to human platelets, C52 melanoma cells, and HT1080 fibroscroma cells.

We fortuitously found a stable morphologic variant of BAE cells which also was variant for the metabolism of TSP. Immunofluorescence studies of factor VIII-related antigen, Dil-Ac-LDL, and muscle specific actin and electron microscopy all indicated that the variants were endothelial cells. The variant strain arose spontaneously in the laboratory of Dr. Robert Auerbach. It is not known if these cells represent a satellite population of aortic endothelial cells which is present normally in vivo.

Endothelial cells have been shown to have receptors for many different molecules, including heparin (23), thrombin (3), platelet factor 4 (10), antithrombin III (38), fibrinogen (14), beta-thromboglobulin (24), multiplication stimulating factor (insulin-like growth factors 1 and 2) (8), and insulin (7), among others. In the present studies, we provide evidence for the existence of a receptor on endothelial cells for TSP. The binding of TSP to this receptor must be short-lived, because bound molecules were rapidly degraded. At the very least, the receptor would be expected to mediate efficient clearance of TSP in vivo. Mumby et al. (47) have shown that the synthesis of TSP is affected by cell density and is increased in subconfluent cells as compared to confluent cells in monolayer. Other investigators have reported that TSP synthesis by smooth muscle and glial cells is inducible by platelet-derived growth factor (2, 36). Preliminary experiments from our laboratory suggest that binding and degradation of TSP, like TSP synthesis, is significantly elevated in subconfluent cultures as compared to confluent monolayers (data not shown). Thus, TSP may interact with the cellular receptor in a temporally specific manner and function as an effector or regulatory molecule to control cell division. The interaction, furthermore, may be subject to complex modulation inasmuch as molecules packaged alongside TSP in platelet alpha granules (platelet factor 4, beta-thromboglobulin, and platelet heparitinase [52]) decreased the binding of TSP to endothelial cells. Thus, binding of TSP to endothelial cells immediately after platelet release may be transiently blocked.

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References

1. Asch, A. S. J., J. Barnwell, R. L. Silverstein, and R. L. Nachman. 1987. Isolation of the thrombospondin membrane receptor. J. Clin. Invest. 79:1054–1061.

2. Asch, A. S., L. L. K. Leung, J. Shapiro, and R. L. Nachman. 1986. Human brain glial cells synthesize thrombospondin. Proc. Natl. Acad. Sci. USA. 83:2904–2908.

3. Awbrey, B. J., J. C. Hoak, and W. G. Owen. 1979. Binding of human thrombin to cultured human endothelial cells. J. Biol. Chem. 254:4092–4095.

4. Bannenger, N. L., G. N. Brodie, and P. W. Majerus. 1972. Isolation and properties of a thrombin-sensitive protein of human platelets. J. Biol. Chem. 248:2723–2731.

5. Bale, M. D., and D. F. Mosher. 1986. Effects of thrombospondin on fibrin polymerization and structure. J. Biol. Chem. 261:862–868.

6. Bale, M. D., L. Westrick, and D. F. Mosher. 1985. Incorporation of thrombospondin into fibrin clots. J. Biol. Chem. 260:7502–7508.

7. Bar, R. S., J. C. Hoak, and M. L. Peacock. 1978. Insulin receptors in human endothelial cells: identification and characterization. J. Clin. Endocrinol. Metab. 47:999–702.

8. Bar, R. S., M. L. Peacock, M. M. Rechler, and S. P. Nissley. 1981. Receptors for multiplication-stimulating activity on human arterial and venous endothelial cells. J. Clin. Endocrinol. Metab. 52:814–816.

9. Booyse, P. M., B. J. Sedlak, and M. E. Raffelson, Jr. 1975. Culture of arterial endothelial cells. Characterization and growth of bovine aortic endothelial cells. Thromb. Diath. Haemorrh. 34:825–839.

10. Busch, C., J. Dawes, D. S. Peppier, and A. Wateson. 1980. Binding of platelet factor 4 to cultured human umbilical vein endothelial cells. Thromb. Res. 19:129–137.

11. Castor, C. W., J. W. Miller, and D. A. Walz. 1983. Structural and biological characteristics of connective tissue activating factor (CTAP-III); a major platelet-derived growth factor. Proc. Natl. Acad. Sci. USA. 80:765–769.

12. Colligan, J. E., and H. S. Slayer. 1984. Structure of thrombospondin. J. Biol. Chem. 259:3944–3948.

13. Danishefsky, K. J., R. J. Alexander, and T. C. Detweiler. 1985. Formation of a stable complex of thrombin and the secreted platelet glycoprotein G (thrombin-sensitive protein, thrombospondin) by thiol-disulfide exchange. Biochemistry. 23:4984–4990.

14. Dixit, V. M., N. J. Galvin, K. M. O'Rourke, and W. A. Frazier. 1986. Monoclonal antibodies that recognize calcium-dependent structures of human thrombospandin. Characterization and mapping of their epitopes. J. Biol. Chem. 261:1962–1968.

15. Dixit, V. M., G. A. Grant, W. A. Frazier, and S. A. Santoro. 1984. Isolation of the fibrinogen-binding region of platelet thrombospondin. Biochem. Biophys. Res. Commun. 119:1075–1081.

16. Dixit, V. M., G. A. Grant, S. A. Santoro, and W. A. Frazier. 1984. Isolation and characterization of a heparin-binding domain from the amino terminus of platelet thrombospondin. J. Biol. Chem. 259:10010–10015.

17. Dixit, V. M., D. M. Haverstick, K. M. O'Rourke, S. W. Hennessy, G. A. Grant, S. A. Santoro, and W. A. Frazier. 1985. Effects of anti-thrombospondin monoclonal antibodies on the aggregation of erythrocytes and fixed, activated platelets by purified thrombospondin. Biochemistry. 24:4270–4275.

18. Galvin, N. J., V. M. Dixit, K. M. O'Rourke, S. A. Santoro, G. A. Grant, and W. A. Frazier. 1985. Mapping of epitopes for monoclonal antibodies against human platelet thrombospondin with electron microscopy and high sensitivity amino acid sequencing. J. Cell Biol. 101:1434–1441.

19. Gartner, T. K., and M. E. Doctor. 1983. Secreted platelet thrombospondin binds monovalently to platelets and erythrocytes in the absence of free Ca2+. Thromb. Res. 33:19–30.

20. Gartner, T. K., M. D. Doyle, and D. F. Mosher. 1984. Effect of anti-thrombospondin antibodies on the hemagglutination activities of the endogenous platelet lectin and thrombospondin. Thromb. Haemostasis. 52:354–357.

21. Gown, A. M., M. A. Vogel, D. Gordon, and P. L. Lu. 1985. A smooth muscle-specific monoclonal antibody recognizes smooth muscle actin iso-zymes. J. Cell Biol. 100:807–813.

22. Giemelius, B., C. Busch, and M. Höök. 1978. Binding of heparin on the surface of cultured human endothelial cells. Thromb. Res. 12:773–782.

23. Hawe, W. T., J. Martin, C. S. Chesterman, and P. J. Morgan. 1979. Human beta-thromboglobulin inhibits PG1 production and binds to a specific site in bovine aortic endothelial cells. Nature. (Lond.) 282:210–212.

24. Hunter, R. N., J. Dawes, I. R. MacGregor, and D. S. Pepper. 1984. Quantification by radiinmunooassay of thrombospondin synthesized and secreted by human endothelial cells. Thromb. Haemostasis. 52:288–291.

25. Jaffe, E. A., J. T. Ruggiero, L. L. K. Leung, M. D. Doyle, P. J. McKeown-Longo, and D. F. Mosher. 1983. Cultured human fibroblasts synthesize...
