Binding and Degradation of Platelet Thrombospondin by Cultured Fibroblasts

PAULA J. McKEOWN-LONGO, ROXANN HANNING, and DEANE F. MOSHER
Department of Medicine, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Thrombospondin was purified from human platelets and labeled with ^{125}I, and its metabolism was quantified in cell cultures of human embryonic lung fibroblasts. ^{125}I-Thrombospondin bound to the cell layer. The binding reached an apparent steady state within 45 min. Trichloroacetic acid–soluble radioactivity was detected in the medium after 30 min of incubation; the rate of degradation of ^{125}I-thrombospondin was linear for several hours thereafter. Degradation of ^{125}I-thrombospondin was saturable. The apparent K_m and V_{max} for degradation at 37°C were 6×10^{-8} M and 1.4×10^5 molecules per cell per minute, respectively. Degradation was inhibited by chloroquine or by lowering the temperature to 4°C. Experiments in which cultures were incubated with thrombospondin for 45 min and then incubated in medium containing no thrombospondin revealed two fractions of bound thrombospondin. One fraction was localized by indirect immunofluorescence to punctate structures; these structures were lost coincident with the rapid degradation of 50–80% of bound ^{125}I-thrombospondin. The second fraction was localized to a trypsin-sensitive, fibrillar, extracellular matrix. ^{125}I-Thrombospondin in the matrix was slowly degraded over a period of hours. Binding of ^{125}I-thrombospondin to the extracellular matrix was not saturable and indeed was enhanced at thrombospondin concentrations $>3 \times 10^{-8}$ M. The ability of ^{125}I-thrombospondin to bind to extracellular matrix was diminished tenfold by limited proteolytic cleavage with trypsin. Degradation of trypsinized ^{125}I-thrombospondin was also diminished, although to a lesser extent than matrix binding. Heparin inhibited both degradation and matrix binding. These results suggest that thrombospondin may play a transitory role in matrix formation and/or organization and that specific receptors on the cell surface are responsible for the selective removal of thrombospondin from the extracellular fluid and matrix.

Thrombospondin is a component of platelet alpha granules and is released upon activation of platelets with thrombin (1). Platelet thrombospondin is a 450,000-mol-wt protein composed of three 160,000-mol-wt disulfide-bonded subunits (2, 3). After release from the alpha granules, thrombospondin becomes bound to the platelet membrane where it may mediate interactions with other platelets (4) or the endothelial substratum (5). Thrombospondin has recently been shown to be synthesized by cultured endothelial cells (6–8), smooth muscle cells (9), and fibroblasts (9, 10). In culture, thrombospondin is found in culture medium and the extracellular matrix (9, 10). Cell-synthesized thrombospondin is structurally and immunologically similar to thrombospondin purified from platelets (6, 8). Cultured fibroblasts synthesize and secrete several other molecules that become incorporated into the extracellular matrix, including fibronectin (11, 12), collagen (11, 13), and proteoglycans (13). Thrombospondin binds to heparin (2) and has been shown recently to interact with fibronectin (5, 14) and collagen (5).

In the present study, we quantified the metabolism of ^{125}I-labeled platelet thrombospondin by fibroblast cell layers. Thrombospondin bound in two fractions that were distinguished by their relative rates of turnover. One fraction of thrombospondin was degraded within minutes by a saturable, endocytic process. The remainder was localized to the extracellular matrix and was degraded more slowly over several hours.

MATERIALS AND METHODS

Materials: Ham's nutrient medium (F-12) and Hanks' balanced salt solution were obtained from Grand Island Biological Co. (Grand Island, NY). Fetal calf serum was from Sterile Systems (Logan, UT). Bio-Gel P-300, heparin-

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agarose and electrophoresis materials were from Bio-Rad Laboratories (Richmond, CA). Na^{125}I was from New England Nuclear (Boston, MA). Fluorescein-conjugated rabbit anti-mouse IgG was from Cappel Laboratories (Cochranville, PA). *N*-Ethylmaleimide was from Pierce Chemical Co. (Rockford, IL). Materials for autoradiography were from Kodak (Rochester, NY). Heparin and other biochemicals were from Sigma Chemical Co. (St. Louis, MO). Human plasma fibronectin was purified from a by-product of Factor VIII production (15). Type I collagen from fetal bovine skin (16) and fibrinogen (17) and α_2-macroglobulin (18) from human plasma were purified according to published procedures.

Cell Culture: Human embryonic lung fibroblast cells were derived from a locally established strain (Dr. Catherine Reznikoff, University of Wisconsin). Cells were grown in F-12 nutrient medium containing 10% fetal calf serum, 100 U/ml penicillin, 50 μg/ml streptomycin and 2 μg/ml fungezone. Cells were customarily split 1:10 every 7 d, and experiments were done on cells between passages 4 and 15. All experiments were done using confluent cell layers in 60-mm tissue culture plates. At confluence, cultures contained ~5 × 10^5 cells, as determined after trypsinization by enumeration in a hemocytometer.

Preparation of Isolated Matrices: Isolated cell matrices were prepared from confluent embryonic lung fibroblast cultures by extraction with 1% deoxycholate in 20 mM Tris-HCl buffer (pH 8.3) containing 2 mM phenylmethylsulfonylfluoride, 2 mM ethylene diamine tetraacetic acid, 2 mM methyl maleimide, and 2 mM iodoacetic acid. Extractions were carried out for 10 min at room temperature. Matrices were rinsed three times with Hanks' balanced salt solution before use.

Purification and Iodination of Thrombospondin: Thrombospondin was purified from thrombin-activated human platelets by gel filtration chromatography on Bio-Gel P-300 and affinity chromatography on heparin-agarose as described previously (8). Thrombospondin concentration was calculated on the basis of a published extinction coefficient (3). Purified thrombospondin, ~200 μg, was iodinated with 1 mCi Na^{125}I by the chloramine-T method (19). The iodinated protein was repurified on heparin agarose and eluted with 0.55 M sodium chloride in 10 mM Tris-HCl, pH 7.4. The specific activity of the labeled molecule was generally 0.5 mCi/mg protein. ^{125}I-Thrombospondin was mixed with phenylmethylsulfonylfluoride-treated bovine serum albumin, 1 mg/ml, dialyzed against 10 mM Tris-HCl containing 150 mM sodium chloride, and frozen at −70°C until use. The integrity of the labeled molecule was assessed by PAGE in SDS with and without reduction (Fig. 1). Reduced thrombospondin (Fig. 1, lane *c*) migrated primarily as a 160,000-mol-wt dalton polypeptide. There were minor bands of 145,000, 103,000, and 80,000 mol wt which were seen in reduced but not unreduced thrombospondin (in Fig. 1, compare lane *c* to lane *a*).

Monoclonal Antibody to Thrombospondin: Preparation of mouse monoclonal antibody to human platelet thrombospondin has been described previously (10). The IgG fraction from the mouse ascites fluid was purified by chromatography on DEAE-cellulose. The antibody was used in immunofluorescence experiments (see below) and to quantify human and bovine thrombospondin by enzyme-linked immunoabsorbent assay (10).

Binding and Degradation of Thrombospondin by Cell Layers: Confluent cell layers were incubated with ^{125}I-thrombospondin in F-12 containing 0.1% albumin (binding medium). To quantify binding and degradation, we usually mixed the iodinated protein with unlabeled thrombospondin as described in the figure or table legends. Proteolytic degradation of thrombospondin by the cultures was monitored by the appearance of radioactivity in the medium which was soluble in 10% trichloroacetic acid. To determine the amount of thrombospondin bound in the cell layer, we removed medium containing ^{125}I-thrombospondin, and the cell layer was rinsed three times in Hanks' buffered salt solution, and then scraped into 1 M sodium hydroxide. Radioactivity was determined in a gamma counter. To estimate background binding and degradation, we preincubated blank culture dishes with complete medium (F-12; 10% fetal calf serum) for one h at 37°C, rinsed, and subjected to the same procedures described above.

Immunofluorescence: For indirect immunofluorescent staining, cells were grown on glass coverslips until confluent. Cultures were then incubated with purified thrombospondin (1 or 20 μg/ml) in binding medium for 45 min at 37°C. At 45 min, the thrombospondin-containing medium was removed, the cell layers were rinsed, and one set of cultures was fixed and stained. A duplicate set was "chased" in binding medium containing no thrombospondin for 3 h before fixation and staining. Fixation was done for 10 min at room temperature with 1% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. Coverslips were cleared with acetone for 10 min at −20°C. Coverslips were stained with a purified IgG fraction (100 μg/ml in Hanks' buffered salt solution) of mouse monoclonal antibody to thrombospondin. Staining was carried out for 1 h at room temperature. Coverslips were washed for 30 min with three changes of Hanks' balanced salt solution and stained with fluoroscein-conjugated rabbit anti-mouse IgG diluted 1:100 in Hanks' buffered salt solution. Coverslips were put onto glass slides in Tris-buffered saline, pH 7.4, containing 1 mg/ml phenylenediamine. Photography was performed with a Leitz microscope equipped with epifluorescence and phase contrast.

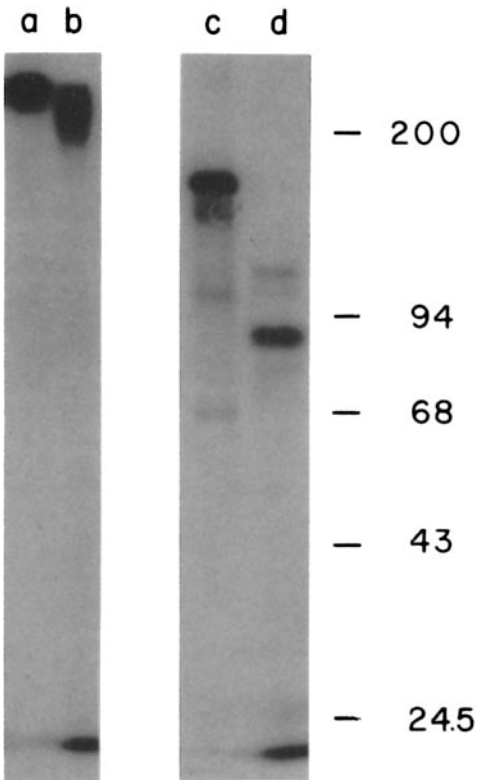

FIGURE 1 Autoradiography of intact (lane *a* and *c*) and trypsinized (lanes *b* and *d*) ^{125}I-thrombospondin. ^{125}I-Thrombospondin was trypsinized for 10 min at 37°C with 2 μg/ml trypsin. The reaction was stopped with a 10-fold (wt/wt) excess of soybean trypsin inhibitor. Each well contained 100,000 cpm of radioactivity, and lanes *c* and *d* were reduced with 2-mercaptoethanol before electrophoresis on an 8% polyacrylamide gel.

Gel Electrophoresis: SDS PAGE was performed on slabs of 8% separating and 3.3% stacking gels using a discontinuous buffer system (20). Marker proteins (see reference 8) were visualized by staining with Coomassie Brilliant Blue. For visualizing ^{125}I-thrombospondin, slabs were dried and autoradiographed with XAR-2 film.

RESULTS

Time Course of Binding and Turnover of Thrombospondin by Fibroblast Cell Cultures

Confluent cell layers of human embryonic lung fibroblasts were incubated with ^{125}I-thrombospondin for 4 h, and binding and degradation of thrombospondin by the cell layers were quantified (Fig. 2). Thrombospondin became rapidly associated with the cell layer, and maximum binding was achieved within 45 min. Degradation of thrombospondin by the cell layers was first detected at 30 min and proceeded at a linear rate between 45 and 150 min. The amount of thrombospondin degraded after 4 h represented 12% of the added radioactivity. The remaining 88% was precipitable in trichloroacetic acid and migrated as intact thrombospondin in polyacrylamide gels (data not shown). When ^{125}I-thrombospondin was allowed to bind to cell layers for 45 min and then "chased" (Fig. 2, arrow), the bound fraction had two fates. After 3 h of "chase," 60% of the material bound at 45 min had been

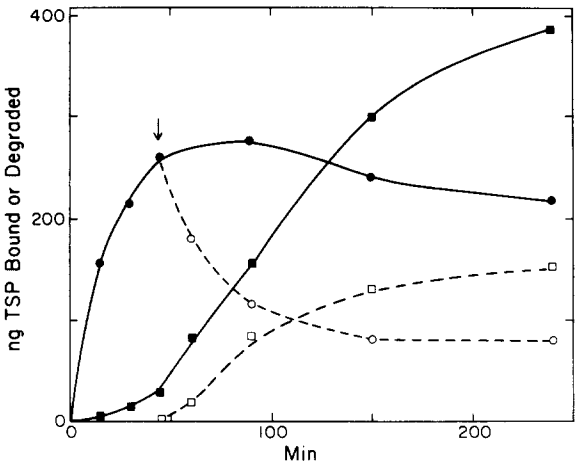

FIGURE 2 Time course of binding and degradation of thrombospondin by confluent human embryonic lung fibroblasts. Cultures were incubated with 3 ml of binding medium containing 1 μg/ml thrombospondin (300,000 cpm/ml) for the designated times. Cell layers were either incubated continuously with ^{125}I-thrombospondin (●, ■) or incubated with ^{125}I-thrombospondin for 45 min and then "chased" (arrow) with fresh binding medium containing no thrombospondin (○, □). Degradation was monitored by the appearance of trichloroacetic acid-soluble radioactivity (■, □) in the medium. Cell layers were solubilized in sodium hydroxide to determine bound thrombospondin (●, ○).

degraded. The rate of degradation decreased during the "chase" period, and the remaining ^{125}I-thrombospondin remained bound in the cell layer. Bound ^{125}I-thrombospondin was judged to be intact thrombospondin when analyzed by SDS PAGE (data not shown). Intermediate degradation products of thrombospondin were never detected, either in the medium or in the cell layer, indicating that thrombospondin was rapidly degraded into small fragments.

Immunofluorescent Localization of Bound Thrombospondin

To investigate further the two fractions of cell-associated thrombospondin, we incubated cell layers with purified platelet thrombospondin, and thrombospondin was then localized by indirect immunofluorescence using a mouse monoclonal antibody prepared against platelet thrombospondin. The monoclonal antibody recognizes human thrombospondin synthesized by cultured cells and the bovine thrombospondin present in fetal calf serum (10). Therefore, cell layers were incubated with two concentrations of purified human platelet thrombospondin, either 1 or 20 μg/ml. The increased fluorescent staining seen in cultures labeled with 20 μg/ml thrombospondin was assumed to represent the exogenous thrombospondin added during the experiment. Cultures were incubated with thrombospondin for 45 min. At 45 min, one set of cultures was processed for immunofluorescence, and the other set was "chased" for 3 h. Cultures incubated for 45 min showed both a punctate and a fibrillar type fluorescence when fixed with paraformaldehyde and acetone (Fig. 3, *a* and *c*). This pattern was similar to that detected when cell layers grown in fetal calf serum are analyzed by immunofluorescence with antithrombospondin (9, 10). Punctate fluorescence was much less prominent in cells fixed only with paraformaldehyde (data not shown), suggesting that some of the punctate structures were intracellular. "Chased" cell layers exhibited fibrillar fluorescence patterns (Fig. 3, *b* and *d*). The fibrillar fluorescence patterns were similar in cell layers fixed only in paraformaldehyde, indicating that these fibrils were extracellular (data not shown). These data in combination with Fig. 2 suggest that the punctate fluorescence represents thrombospondin in endocytic vesicles destined for degradation in lysosomes. These structures were not present after a 3-h "chase" and therefore do not represent synthetic pools. Rather, the diffuse perinuclear staining pointed out by arrows in Fig. 3, *c* and *d* probably represents recently synthesized thrombospondin in the rough endoplasmic reticulum and Golgi complex.

Effect of Concentration on Degradation and Matrix Binding of Thrombospondin

Cell layers were incubated with increasing concentrations of ^{125}I-thrombospondin, and both the rate of thrombospondin degradation and the amount of thrombospondin bound in the matrix were determined (Fig. 4*a*). The rate of thrombospondin degradation approached saturation at concentrations >20 μg/ml. Thrombospondin binding in the extracellular matrix, however, did not saturate over the concentration range studied. Instead, the proportion of thrombospondin bound into the matrix increased with increasing thrombospondin concentrations, suggesting that deposition of throm-

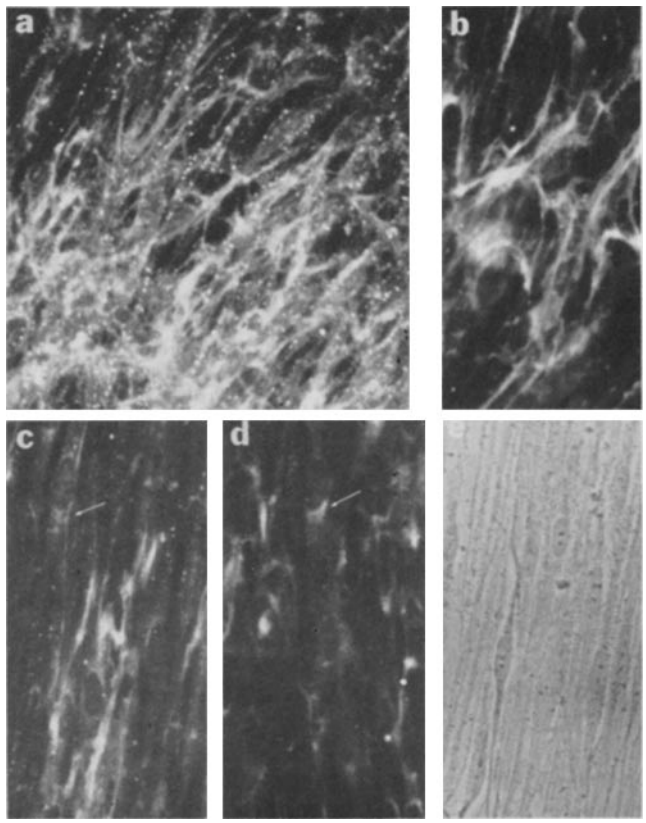

FIGURE 3 Localization of thrombospondin in cell layers by indirect immunofluorescence. Confluent cell layers of lung fibroblasts were incubated with either 20 μg/ml (*a* and *b*) or 1 μg/ml (*c* and *d*) of purified platelet thrombospondin for 45 min at 37°C. After 45 min, one set of cultures (*a* and *c*) were fixed and stained for thrombospondin. A second set of cultures (*b* and *d*) were "chased" for 3 h in binding medium containing no thrombospondin before fixation and staining. e is a phase contrast of c. × 200.

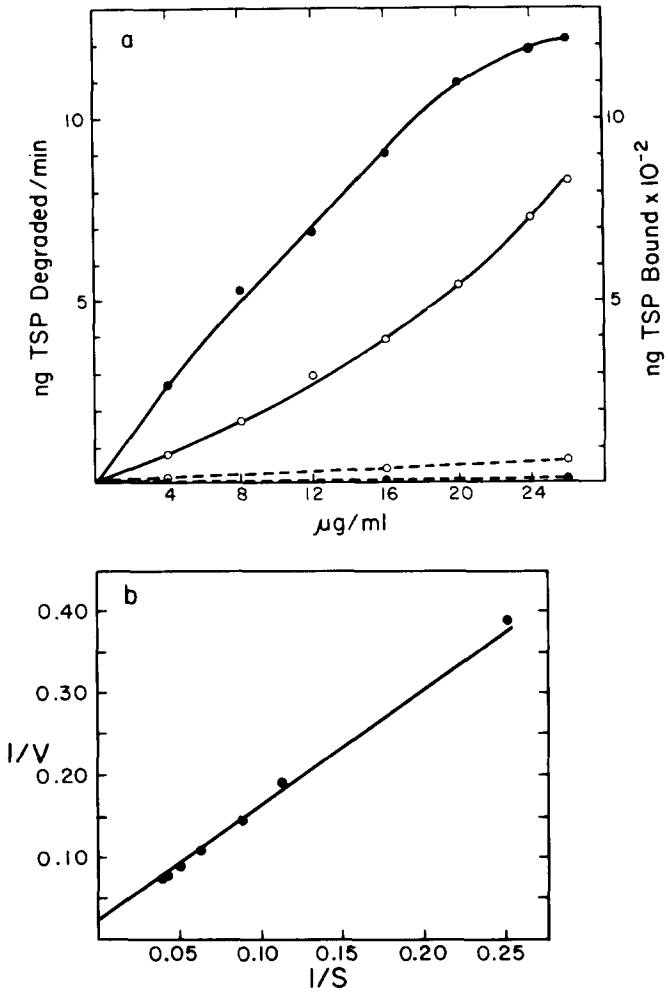

FIGURE 4 Effect of concentration on binding and degradation of thrombospondin by fibroblast cell layers. Confluent cell layers were incubated with 2 ml of binding medium containing increasing concentrations of a mixture of ^{125}I-thrombospondin and unlabeled thrombospondin. The specific activity of this mixture was 4 μCi/mg. Degradation rates of ^{125}I-thrombospondin (●—●) were determined between 45 and 180 min of incubation. For determination of bound thrombospondin (○—○), cultures were incubated with ^{125}I-thrombospondin for 45 min, "chased" for 3 h in unlabeled medium, and solubilized in 1 M sodium hydroxide. Binding (○- -○) and degradation (●- -●) on blank plates were also monitored. The turnover data were replotted by the Lineweaver-Burke method for calculation of kinetic constants (*b*).

bospondin into the extracellular matrix is enhanced by higher thrombospondin concentration. Background binding and turnover of thrombospondin on blank tissue culture plates were also determined over a range of thrombospondin concentrations (Fig. 4*a*). There was no detectable degradation of thrombospondin, and only a small amount of binding (<10% of the amount bound in the cell layer) was observed.

The turnover data in Fig. 4*a* were replotted on a double reciprocal plot for determination of kinetic constants (Fig. 4*b*). The data were fitted to a straight line by linear regression with a correlation coefficient (r) = 0.998. The apparent Michaelis-Menten constant (K_m) and maximum rate of turnover (V_{max}) in four experiments were 29 ± 17 μg/ml and 55 ± 25 ng/min ($\bar{x}$ ± SD), respectively. Thus, K_m for degradation was ~6×10^{-8} M, and V_{max} was approximately 1.4×10^5 molecules degraded per cell per minute.

Inhibition of Degradation and Matrix Binding of Thrombospondin

Inasmuch as various amines have been shown to block receptor-mediated protein degradation, probably by raising the pH of endocytic vesicles (21) or lysosomes (22), the effects of such amines on thrombospondin metabolism were tested. Chloroquine at >1×10^{-4} M completely blocked thrombospondin degradation (Fig. 5). In similar experiments, both methylamine and ammonium chloride were also effective inhibitors of thrombospondin turnover at concentrations of 1×10^{-2} M (data not shown).

Degradation of ^{125}I-thrombospondin was inhibited by low temperature, i.e., there was no detectable increase in trichloroacetic acid–soluble radioactivity in the medium of cultures incubated at 4°C even after 4 h of incubation (data not shown).

The ability of fragments of trypsinized thrombospondin to be bound and degraded by fibroblasts was also tested (Table I). Mild trypsinization of ^{125}I-thrombospondin resulted in two labeled fragments, one having a nonreduced molecular weight of ~300,000 and a second of <30,000 which moved at the dye front (Fig. 1). The large fragment was composed of disulfide-bonded subunits with individual sizes of 90,000 mol wt. This fragment has been designated the tryptic-resistant core of thrombospondin (2, 3, 23). The <30,000-mol-wt

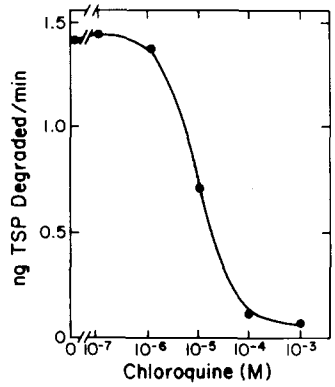

FIGURE 5 Effect of chloroquine on thrombospondin degradation. Cell layers were incubated with 2 ml of binding medium containing 0.5 μg/ml ^{125}I-thrombospondin (40,000 cpm/ml) for 180 min in the presence of increasing concentrations of chloroquine. Degradation rates were calculated between 45 and 180 min of incubation.

TABLE I

Binding and Degradation of Trypsinized Thrombospondin

	% Total trichloroacetic acid precipitable cpm added		
	Degraded	Bound	
	135–45 min	At 45 min	After 180-min chase
Control ^{125}I-thrombospondin	5.7	5.1	1.7
"Mock-trypsinized" ^{125}I-thrombospondin	6.1	5.5	1.5
Trypsinized ^{125}I-thrombospondin	4.0	2.1	0.17

^{125}I-Thrombospondin (400,000 cpm) was treated with trypsin (2 μg/ml in Tris-buffered saline), inactive or "mock" trypsin (2 μg/ml Trypsin, 20 μg/ml soybean trypsin inhibitor), or Tris-buffered saline alone for 10 min at 37°C. Soybean trypsin inhibitor, 20 μg/ml, was then added to trypsin-treated and control incubations. ^{125}I-Thrombospondin in binding medium (100,000 cpm/ml) was incubated with cell layers. Degradation was measured between 45 and 135 min of incubation as appearance of trichloroacetic acid-soluble radioactivity in the medium. For determination of binding, cell layers were labeled for 45 min and then scraped directly into 1 M sodium hydroxide or incubated for 180 min in binding medium containing no thrombospondin before solubilization in sodium hydroxide. Determinations represent the average from duplicate plates which did not differ by >3%.

labeled fragment had a size of 22,000-mol-wt when analyzed on a more concentrated gel (data not shown). The 30,000-mol-wt heparin-binding fragment described by Lawler and Slayter (23) labeled very poorly with ^{125}I and was barely visualized by autoradiography. Trypsinized thrombospondin was bound in the cell layer at ~30% of control levels. This amount represented thrombospondin present in the matrix and in the endocytic pool. Binding to the extracellular matrix was most affected by trypsinization; trypsinized thrombospondin bound at <10% of control levels. Degradation of trypsinized thrombospondin over the 90-min time course was reduced by 33%.

Thrombospondin has been reported to interact with collagen (5), fibrinogen (14), fibronectin (5, 14), and heparin (2, 23). These molecules, as well as α_2-macroglobulin, a glycoprotein with a size and pI similar to those of fibrinogen and fibronectin, were tested for possible effects on degradation and matrix incorporation of thrombospondin. Like thrombospondin, α_2-macroglobulin has been shown to be specifically bound and degraded by cultured fibroblasts (24–26). The results in Table II indicate that collagen, fibronectin, fibrinogen, and α_2-macroglobulin had little effect on thrombospondin binding or turnover, whereas heparin was a potent inhibitor of both processes.

Thrombospondin Metabolism in Cell Matrices

To determine whether thrombospondin could bind directly to the extracellular matrix, we prepared isolated matrices by extraction of confluent cell layers with 1% deoxycholate. Indirect immunofluorescence of the deoxycholate insoluble matrix indicated that these matrices contained fibronectin, collagen, and thrombospondin (data not shown). Similarly prepared matrices (12, 13, 27) have also been shown to contain proteoglycans, hyaluronic acid, and some actin. Isolated matrices and confluent cell cultures were incubated with ^{125}I-thrombospondin for up to 4 h. ^{125}I-Thrombospondin was degraded in intact cultures (Fig. 6*a*) but not by isolated matrices (Fig. 6*b*). Initial binding to be the matrix proceeded at a slower rate than to intact cultures, and binding was linear over 4 h. Matrices that were "chased" after 45 min (Fig. 6*b*, arrow) lost little thrombospondin over the subsequent 3 h. That exogenous thrombospondin was bound to the matrix in deoxycholate-extracted cell layers was demonstrated by indirect immunofluorescent localization of thrombospondin to matrix fibrils (data not shown).

Thrombospondin turnover in the matrices of intact cell layers and cell-free matrices was also compared over a longer timespan (Fig. 7). In intact cell layers, 75% of bound ^{125}I-thrombospondin was lost in a biphasic manner from the cell layer over a period of 44 h. Loss of ^{125}I-thrombospondin from the cell layers was accompanied by a corresponding increase in trichloroacetic acid–soluble radioactivity in the culture medium. In contrast, only 20% of bound thrombospondin was lost from the isolated matrices after 44 h. None of the radioactivity in culture medium of isolated matrices became soluble in trichloroacetic acid, suggesting that the small proportion of intact thrombospondin that was lost from the matrices was lost by diffusion rather than by degradation. Degradation of matrix-bound thrombospondin in intact cell layers was inhibited by chloroquine, even in the presence of medium conditioned by cells not treated with chloroquine (data not shown). This finding suggests that the turnover of matrix-bound thrombospondin occurs intracellularly and not by the action of a secreted protease.

^{125}I-Thrombospondin bound to the matrix of intact cell layers was treated with trypsin and various extractants (Table III). Of the agents tested, trypsin (88% release) and 3 M guanidine hydrochloride (64% release) were the most effective. Although heparin blocked thrombospondin incorporation into the matrix (Table II), heparin, 100 μg/ml, extracted

TABLE II

Effect of Various Macromolecules on Thrombospondin Metabolism

Macromolecule	Degradation	Matrix Incorporation
	(% *of control*)	
Collagen		
25 μg/ml	92	118
Fibronectin		
25 μg/ml	95	112
125 μg/ml	85	106
Fibrinogen		
25 μg/ml	100	108
125 μg/ml	92	96
α_2-Macroglobulin		
25 μg/ml	94	102
125 μg/ml	94	104
Heparin		
5 μg/ml	27	12
25 μg/ml	30	10

Cell layers were incubated with ^{125}I-thrombospondin (1 μg/ml; 250,000 cpm/ml) in binding medium containing the above macromolecules at the indicated concentrations. Degradation was measured as trichloracetic acid-soluble radioactivity appearing in the medium between 45 and 180 min of incubation. Matrix incorporation was measured as the amount of radioactivity remaining in the cell layer after labeling cultures for 45 min and "chasing" for 3 h in binding medium containing no thrombospondin. The data are expressed as percent of control levels, and determinations represent averages of duplicates that varied by <5%.

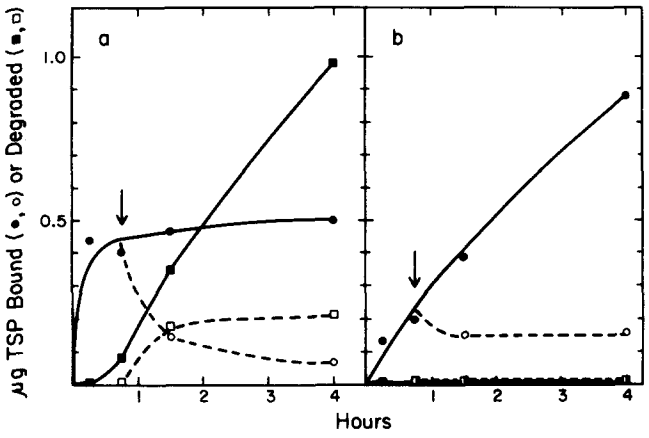

FIGURE 6 Thrombospondin binding and degradation by isolated cell matrices. ^{125}I-Thrombospondin (5 μg/ml, 400,000 cpm/ml) was incubated with intact (*a*) and 1% deoxycholate-extracted (*b*) fibroblast cell layers. Cultures were either incubated continuously with ^{125}I-thrombospondin (●—●, ■—■) or incubated with ^{125}I-thrombospondin for 45 min and then "chased" (arrow) for 3 h with fresh binding medium containing no thrombospondin (○- -○, □- -□). Degradation was monitored by the appearance of trichloroacetic acid-soluble radioactivity (■, □) in the medium. Cell layers were solubilized in 1 M sodium hydroxide to determine bound thrombospondin (●, ○). Background binding (30–120 ng) and degradation (1–20 ng) on blank plates have been subtracted from each time point.

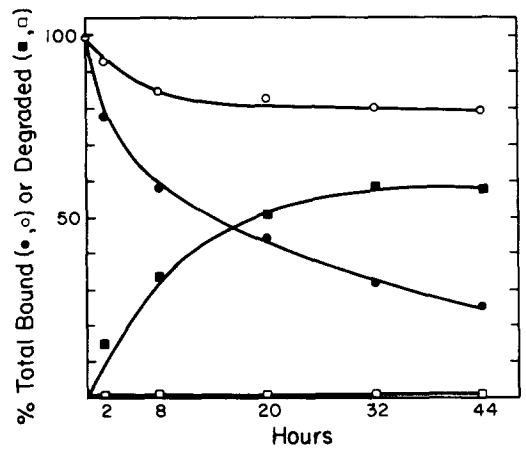

FIGURE 7 Persistence of thrombospondin in the extracellular matrix. ^{125}I-Thrombospondin (5.5 μg/ml, 400,000 cpm/ml) was incubated with intact cell layers (●, ■) and deoxycholate-extracted matrices (○, □) for 45 min in binding medium and then "chased" for 3 h in binding medium containing no thrombospondin. At this time (0 time in the graph), there were 177 ng of thrombospondin bound in the intact cell layer and 354 ng bound in the isolated matrix. Less than 25 ng of thrombospondin bound to serum-coated plates handled in a similar manner. Plates were incubated further in F-12 containing 10% fetal calf serum and monitored for degradation (■, □) and binding (●, ○) as described in the legend to Fig. 6.

TABLE III

Extraction of Matrix Bound Thrombospondin

Enzyme or extractant	% Extracted
Trypsin, 100 μg/ml	88
Trypsin, 10 μg/ml	88
Guanidine, 3 M	64
Deoxycholate, 1%	34
Sodium chloride, 1 M	34
Heparin, 100 μg/ml	23
Urea, 3 M	22
Triton, 1%	20
Hanks' buffered salt solution	15

^{125}I-Thrombospondin (5 μg/ml; 170,000 cpm/ml) was incubated with cell layers for 45 min in binding medium. Labeled medium was removed, and cell layers were "chased" in binding medium for 3 h. Cell layers, containing 168 ng of thrombospondin, were scraped into 1 ml of Hanks' buffered salt solution, transferred to centrifuge tubes, and treated with extractants for 10 min at room temperature. Trypsinization was stopped with a 10-fold (wt/wt) excess of soybean trypsin inhibitor. Insoluble material was removed by centrifugation, and radioactivity in the supernatant was determined. Determinations represent average of duplicates that varied <5%.

little thrombospondin from the matrix when compared to Hanks' buffered salt solution alone. Sodium chloride, 1 M, and 1% deoxycholate were moderately effective extractants.

DISCUSSION

The present experiments were modeled after our recent investigations of fibronectin (28). Like thrombospondin, fibronectin is synthesized by cells in culture and incorporated into the extracellular matrix (9, 10). In addition, plasma fibronectin in serum-containing growth medium becomes incorporated into the extracellular matrix of the cultured cells (28, 29). Plasma fibronectin also becomes localized in connective tissues in vivo (30). As described in the introduction, thrombospondin synthesized by cultured cells and platelet thrombospondin are very similar if not identical. Thrombospondin is present in plasma in only trace amounts (31, 32), and concentrations of exogenous thrombospondin in vivo are probably significant only in localized wound areas where there is degranulation of platelets. Fetal calf serum contains 30–40 μg/ml thrombospondin as assayed by our enzyme-linked immunoabsorbent assay (10; unpublished results). Therefore, the usual conditions of cell culture mimic a wound area in the sense that exogenous thrombospondin is present in serum-containing growth medium.

Using ^{125}I-thrombospondin purified from human platelets, we demonstrated that exogenous thrombospondin does bind to cell layers of cultured human embryonic lung fibroblasts. A portion of the bound thrombospondin was degraded to a form that is soluble in trichloroacetic acid. The remainder formed a more stable association with the cell layer. This stable fraction of thrombospondin could be localized to the extracellular matrix (Fig. 3) where it was present in fibrillar structures reminiscent of fibrils known to contain fibronectin, collagen, and proteoglycans (13). We also prepared thrombospondin in the presence of calcium to maintain calcium-sensitive structures within the molecule (33). Thrombospondin prepared in this manner was bound and degraded by cultured fibroblasts with kinetics similar to those shown in Fig. 2.

Immunofluorescent localization of thrombospondin in punctate structures after 45 min and the subsequent disappearance of thrombospondin in these structures after a 3-h "chase" (Fig. 3) coincident with the rapid appearance of trichloroacetic acid-soluble radioactivity in the medium (Fig. 2) suggest that the punctate fluorescence seen in cells stained with antithrombospondin (9, 10) represents thrombospondin in coated pits, endocytic vesicles, and lysosomes. Degradation of thrombospondin was a saturable process (Fig. 4*a*) that could be inhibited by weak bases (Fig. 5) and low temperature (4°C). Rapid turnover of thrombospondin, therefore, is very similar to the specific and saturable receptor-mediated endocytosis and degradation of α_2-macroglobulin (24–26) and low density lipoprotein (34). The fact that trypsinized fragments of thrombospondin were degraded less rapidly than intact thrombospondin (Table I) also indicates that turnover occurs by specific endocytosis rather than a more generalized process, i.e., fluid phase pinocytosis (35). Degradation of thrombospondin (Table II) and low density lipoprotein (36) is inhibited by heparin. Both proteins bind to heparin (23, 37). In the case of low density lipoprotein, heparin may compete with the cell surface receptor for ligand binding (36). The same may be true for thrombospondin.

Binding of thrombospondin to the extracellular matrix was not saturable. Indeed, increasing concentrations of thrombospondin resulted in higher proportions of thrombospondin remaining in the matrix (Fig. 4*a*). Thus, interactions among molecules at higher concentrations may alter the way in which thrombospondin associates with the matrix, and exogenous thrombospondin concentration may regulate the content of matrix-bound thrombospondin. Thrombospondin bound directly to isolated cell matrices prepared by deoxycholate extraction (Fig. 6*b*). Plasma fibronectin, in contrast, does not bind directly to the extracellular matrix, but to a cellular receptor that apparently mediates its assembly into the matrix (28).

Full release of thrombospondin from the matrix required proteolysis or strong denaturation (Table III), suggesting that thrombospondin is tightly bound to the matrix. Similar ex-

traction methods must be employed to remove ^{125}I-plasma fibronectin from the matrix (28; and unpublished observations). Matrix-bound thrombospondin was slowly degraded and, after 24 h, 60% of it was in a trichloroacetic acid–soluble form (Fig. 7). In contrast, there is no degradation of matrix-bound ^{125}I-fibronectin over the same time period (28).

Thrombospondin has been reported to interact with fibronectin and collagen when one of the molecules is adsorbed onto plastic (5, 14). However, collagen and fibronectin did not have significant effects on thrombospondin binding or degradation by fibroblast cell layers. The inhibition of thrombospondin binding to matrix by heparin suggests a possible role for heparan sulfate proteoglycans in the metabolism of thrombospondin by these cell cultures. Heparan sulfate proteoglycans are present in the extracellular matrix (13) and may be integral components of the plasma membrane (38, 39). Heparin and a concentration of sodium chloride considerably higher than that required to disrupt heparin-thrombospondin binding (3), however, were only slightly effective in releasing thrombospondin from the matrix. Thus, further studies are needed to sort out the interactions responsible for initial and long-term binding of thrombospondin to the matrix.

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