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Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix

(monoclonal anti-thrombospondin/enzyme-linked immunosorbent assay/immunofluorescence/immunological isolation technique)

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ABSTRACT **Thrombospondin, a major glycoprotein released from α granules of thrombin-stimulated platelets, is a disulfide-bonded trimer of 160-kilodalton subunits. Cultured human foreskin and fetal lung fibroblasts secreted thrombospondin (determined by enzyme-linked immunosorbent assay) into the culture medium in a time-dependent manner (15.7 and 5.8 μg per 10^6 cells per 24 hr, respectively); secretion was blocked by cycloheximide. [^{3}H]Thrombospondin was isolated from [^{3}H]leucine-labeled fibroblast postculture medium and from cell layers with rabbit polyclonal or mouse monoclonal anti-thrombospondin coupled to staphylococcal protein A-Sepharose. The immunologically isolated [^{3}H]thrombospondin migrated in NaDodSO$_4$/polyacrylamide gels with purified marker platelet thrombospondin both with and without reduction. Immunofluorescence microscopy using rabbit polyclonal and mouse monoclonal anti-thrombospondin antibodies localized thrombospondin to the fibrillar extracellular matrix surrounding the cells. Thus, cultured human fibroblasts secrete thrombospondin and incorporate it into the extracellular matrix.**

Thrombospondin is a major platelet α granule glycoprotein that is secreted and then partially bound to platelet membranes when human platelets aggregate in response to thrombin (1–8). Thrombospondin is a 450-kilodalton (kDal) protein and is composed of three large disulfide-linked subunits (9, 10). During platelet aggregation, thrombin-stimulated platelets develop a membrane-bound lectin-like activity (11–13), which originates from α granules and appears to play a role in mediating platelet aggregation by binding to a specific receptor on other platelets (14, 15). Recent evidence suggests that thrombospondin is the "endogenous lectin" of human platelets (16). Thrombospondin preadsorbed to *ex vivo* polymeric shunts causes rapid binding and activation of platelets on the polymeric surface (17).

Cultured human and bovine endothelial cells have recently been shown to synthesize and secrete thrombospondin (18–20). This finding raised the question of whether thrombospondin, like factor VIII/von Willebrand factor, is specific for platelets and endothelial cells or, like fibronectin, is synthesized by a wide variety of cell types. Fibroblasts are known to synthesize a multimeric, disulfide-bonded protein composed of 170-kDal subunits that has a rapid turnover, is protease sensitive, and is found in the extracellular matrix of confluent cells (21–24). Because this protein has the properties of thrombospondin, we investigated whether fibroblasts also synthesize thrombospondin and incorporate it into extracellular matrix.

MATERIALS AND METHODS

Culture of Fibroblasts. Human foreskin fibroblasts were the generous gift of Daniel Rifkin (Dept. of Cell Biology, New York University). Human fetal skin (GM1381) and fetal lung (GM1604) fibroblasts were obtained from the Institute of Medical Research (Camden, NJ). Fetal lung fibroblasts were also obtained from Catherine Reznikoff (Dept. of Human Oncology, University of Wisconsin). Fibroblasts were cultured in minimal essential medium containing 20% fetal calf serum. Fibroblasts cultured on glass coverslips for immunofluorescence microscopy were cultured in minimal essential medium containing either 20% rabbit serum or 10% or 20% fetal calf serum. Human endothelial cells were derived from umbilical veins and cultured in medium 199 and 20% human serum by using methods and materials previously described (25, 26).

Radioactively labeled proteins synthesized by confluent fibroblasts were prepared by incubating washed 75-cm^2 cell monolayers for 24 hr at 37°C in 10 ml of leucine-free minimal essential medium containing 20% rabbit serum, Trasylol at 50 units/ml, and 200 μCi of L-[3,4,5-^{3}H]leucine (110 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} Bq). The radioactive postculture medium was removed, centrifuged at 8,000 × *g* for 2 min to remove cells and debris, and frozen at −35°C. The radioactive cell layers were washed twice, removed with a rubber policeman, pelleted by centrifugation, dissolved by boiling for 5 min in 2% NaDodSO$_4$ containing protease inhibitors as described (20), and frozen until analyzed.

In experiments in which the accumulation of thrombospondin antigen was measured by enzyme-linked immunosorbent assay (ELISA), fibroblasts were cultured in minimal essential medium containing 20% rabbit serum in 2-cm^2 wells of multiwell plates. When the cells were confluent, the cells were washed, and the medium was replaced with 1 ml of fresh minimal essential medium containing 20% rabbit serum. At various times after the medium change, the postculture medium was removed, centrifuged at 8,000 × *g* for 2 min, and frozen until assayed. The fibroblasts were dispersed with 0.05% trypsin/0.02% EDTA and counted with a Coulter Counter. Endothelial cell postculture medium studied in parallel experiments was obtained from endothelial cells grown in medium 199 and 20% rabbit serum.

Immunofluorescence Microscopy. Confluent cell layers of human foreskin or fetal lung fibroblasts grown on glass coverslips were washed with Hanks' balanced salt solution (HBSS) and fixed with 3.5% (wt/vol) formaldehyde for 20 min at 20°C. The cells were then washed with HBSS and incubated with monoclonal antibodies (see below) to thrombospondin (20) or human plasma fibronectin for 1 hr at 20°C. Purified IgG to thrombospondin was prepared from mouse ascites fluid as described below and used at a concentration of 4.8 μg/ml. Mouse ascites fluid containing anti-fibronectin was diluted 1:2,000.

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Abbreviations: kDal, kilodalton(s); ELISA, enzyme-linked immunosorbent assay; HBSS, Hanks' balanced salt solution.

After washing with HBSS, the cells were stained with fluorescein-conjugated rabbit anti-mouse IgG (1:100, Cappel Laboratories, Cochranville, PA) for 1 hr at 20°C. The cells were again washed with HBSS and mounted on glass slides in 50% glycerol/50% phosphate-buffered saline (vol/vol). Control experiments were done by first absorbing anti-thrombospondin or anti-fibronectin with either purified fibronectin (50 μg/ml) or thrombospondin (50 μg/ml). Fluorescence observed with anti-thrombospondin was blocked only by absorption with thrombospondin and fluorescence observed with anti-fibronectin was blocked only by fibronectin.

Preparation of Thrombospondin and Rabbit Polyclonal Anti-Thrombospondin Antibodies. Human platelet thrombospondin and rabbit polyclonal anti-thrombospondin were prepared as described (20).

Production of Monoclonal Mouse Hybridoma Antibodies to Platelet Thrombospondin. Details of antibody production and characterization will be described in more detail elsewhere. Briefly, a BALB/c × C mouse was immunized intraperitoneally with human platelet thrombospondin over a 3-month period, splenocytes were fused with P3-Nsl-AG4 mouse myeloma cells, hybrids were cultured in selective medium, and cells producing anti-thrombospondin (as determined by an ELISA, see below) were subcloned twice (27). A clone that produces an IgG with the mobility of a gamma globulin was selected for antibody production in mice. The antibody was purified from ascites fluid by ammonium sulfate precipitation and chromatography on DEAE-cellulose. Specificity of the antibody was determined by ELISA and by immunoblotting of platelet extracts, plasma, serum, and native and trypsinized purified thrombospondin. The antibody recognized an antigen in the disulfide-bonded core (9, 10) remaining after trypsin digestion of human thrombospondin. It did not react with any serum or platelet proteins except thrombospondin. The antibody recognized bovine thrombospondin but not the thrombospondin present in rabbit serum (Fig. 1). A monoclonal antibody to fibronectin was produced in a similar fashion.

ELISA of Thrombospondin. The supernatant of thrombin-stimulated washed platelets (20) was diluted 1:30 to 1:100 in 10 mM Tris·HCl, 140 mM sodium chloride, pH 7.4 (Tris/NaCl), containing 0.1% bovine serum albumin, and 200 μl, containing approximately 0.5 μg of thrombospondin, was placed in each well of a 96-well polystyrene culture plate (model no. 3596, Costar, Cambridge, MA). In a second 96-well plate, dilutions of thrombospondin standard (thrombospondin, 20 μg/ml in 0.5% bovine albumin, stored in portions at −70°C) and unknowns were made in 10 mM sodium phosphate/140 mM sodium chloride/1 mM EDTA, pH 7.4, containing 3% bovine albumin. The dilutions were made so that 100 μl of antigen solution was left in each well. An equal volume (i.e., 100 μl) of Tris/NaCl containing mouse monoclonal anti-thrombospondin, 0.38 μg/ml, and 0.3% bovine albumin was added. Both plates were left overnight at 4°C. The plate containing platelet "releasate" was washed three times with Tris/NaCl containing 0.05% Tween 20. The mixtures of antigen and antibody, 175 μl, were then transferred to the releasate-coated plate. After incubation at 24°C for 30 min, the mixtures were removed, the plate was washed three times in Tris/NaCl containing 0.05% Tween 20, and 200 μl of alkaline phosphatase-conjugated goat anti-mouse IgG (Kirkegaard and Perry, Gaithersburg, MD), 2 μg/ml in Tris/NaCl containing 0.25% gelatin, 0.05% Nonidet P-40, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, and 10% fetal calf serum, was added to each well. After incubation for 2 hr at 24°C, the plate was washed three times with the gelatin-containing buffer, and 200 μl of sodium *p*-nitrophenyl phosphate, 1 mg/ml in Tris/NaCl, pH 9.0, was added. The appearance of *p*-nitrophenol was quantitated by monitoring A_{400} with a micro ELISA plate reader (Dynatech, Alexandria, VA).

Isolation and Analysis of Radioactively Labeled Fibroblast Proteins. ^{3}H-Labeled thrombospondin was isolated from radiolabeled cells and postculture medium by batch immunoadsorption with rabbit anti-thrombospondin as described ("immunoisolation") (20). When these techniques were used with the mouse anti-thrombospondin ascites fluid, the mouse anti-thrombospondin was coupled to staphylococcal protein A-Sepharose 4B beads that had previously reacted with rabbit anti-mouse IgG. Immunoisolated labeled proteins with and without reduction were analyzed by polyacrylamide gel electrophoresis in $NaDodSO_4$ in tube gels containing 3% acrylamide and 0.5% agarose (20). Molecular weights were estimated by using factor VIII (200,000), myosin (200,000), β-galactosidase (116,300), phosphorylase a (92,500), human serum albumin (65,000), and ovalbumin (45,000) as size markers. Anti-ovalbumin used as a control and rabbit anti-mouse IgG used to couple the mouse anti-thrombospondin to protein A-Sepharose were obtained from Cappel Laboratories.

RESULTS

Time Course of Secretion of Thrombospondin. An ELISA using a mouse monoclonal anti-thrombospondin antibody was developed to quantitate secreted thrombospondin. The ELISA did not recognize the rabbit thrombospondin present in the rabbit serum in the preculture medium but did recognize thrombospondin secreted by both human foreskin fibroblasts and human endothelial cells (Fig. 1). The inhibition curves for purified platelet thrombospondin, fibroblast postculture medium, and endothelial cell postculture medium were all parallel, suggesting that the antigenic sites recognized by the mouse monoclonal anti-thrombospondin were the same in all three samples.

Human foreskin fibroblasts secreted thrombospondin into the postculture medium in a time-dependent manner; synthesis and secretion were blocked by cycloheximide (Table 1). Human

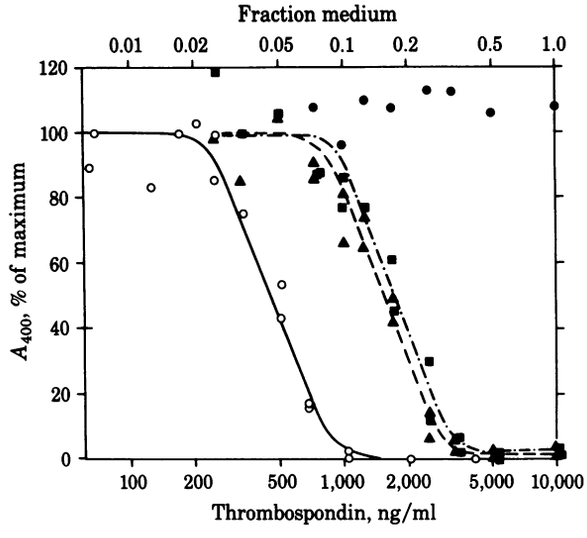

FIG. 1. ELISA of thrombospondin in conditioned medium of human foreskin fibroblasts and umbilical vein endothelial cells cultured in medium containing 20% rabbit serum. Dilutions of four different samples were analyzed: ○, purified platelet thrombospondin standard; ●, preculture medium; ■, 24-hr conditioned medium from fibroblasts; and ▲, 24-hr conditioned medium from human endothelial cells. Values are pooled from experiments performed on two different days. A_{400} is expressed as a percentage of the A_{400} obtained without competing thrombospondin; maximal A_{400} after 3 hr of incubation with substrate at 24°C was 0.7–1.0.

foreskin fibroblasts secreted thrombospondin at the rate of 15.7 ± 2.6 μg per 10^6 cells per 24 hr (mean ± SEM, $n = 3$). In similar experiments, human fetal lung fibroblasts and umbilical vein endothelial cells secreted thrombospondin at rates of 5.8 and 21 μg per 10^6 cells per 24 hr, respectively.

Immunoisolation of Thrombospondin from Fibroblast Postculture Medium and Cell Layers. When rabbit polyclonal anti-thrombospondin serum coupled to protein A-Sepharose was allowed to react with [^{3}H]leucine-labeled postculture medium from human foreskin fibroblasts, the anti-thrombospondin specifically immunoisolated a ^{3}H-labeled protein that migrated in $NaDodSO_4$/polyacrylamide gel electrophoresis with purified platelet thrombospondin both with and without reduction (Fig. 2 *a* and *b*). No labeled proteins were specifically isolated in control experiments with anti-ovalbumin. The ^{3}H-labeled peak immunoisolated from human foreskin fibroblast postculture medium had a molecular weight of 158,000 ± 4,100 (mean ± SEM, $n = 7$) when analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction. When mouse monoclonal anti-thrombospondin coupled to protein A-Sepharose with rabbit anti-mouse IgG was allowed to react with [^{3}H]leucine-labeled human foreskin fibroblast postculture medium, similar results were obtained (Fig. 2*c*).

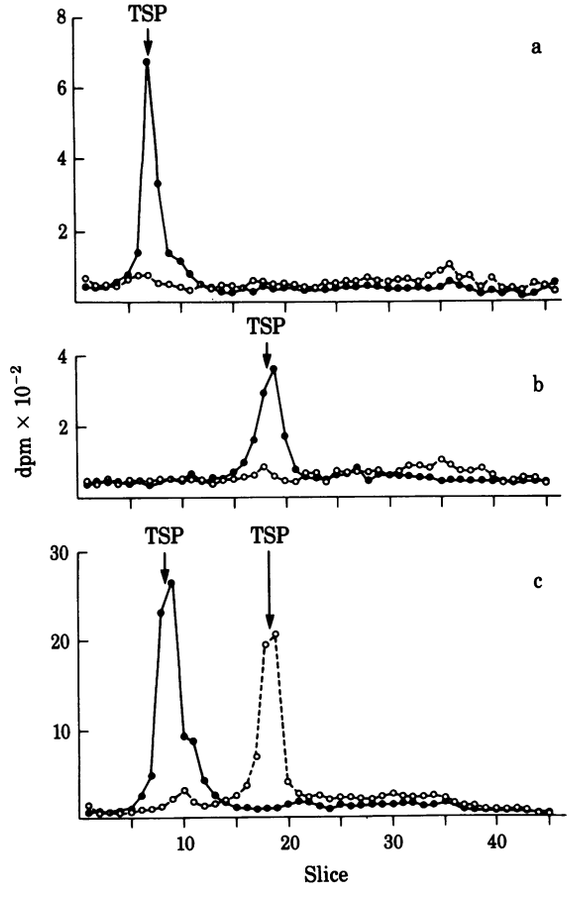

FIG. 2. Immunoisolation of [^{3}H]thrombospondin synthesized and secreted into the culture medium by cultured human foreskin fibroblasts. Distribution of radioactivity in $NaDodSO_4$/polyacrylamide tube gels of: (*a*) labeled protein immunoisolated with rabbit anti-thrombospondin (●) or anti-ovalbumin (○) and analyzed without reduction; (*b*) labeled protein isolated with rabbit anti-thrombospondin (●) or anti-ovalbumin (○) and analyzed with reduction; and (*c*) labeled protein immunoisolated with monoclonal mouse anti-thrombospondin and analyzed with (○) and without (●) reduction. Arrows point to the positions of migration of nonreduced (450 kDal) and reduced (160 kDal) purified platelet thrombospondin (TSP) run as a marker in parallel gels.

Table 1. Time course of thrombospondin secretion by human foreskin fibroblasts

Material tested*	Thrombospondin,† μg/ml
Preculture medium	<0.75
Postculture media	
1 hr	<0.75
4 hr	<0.75
8 hr	0.88 ± 0.11
10 hr	1.28 ± 0.05
14 hr	1.85 ± 0.05
16 hr	2.00 ± 0.1
24 hr	2.23 ± 0.37
24 hr + cycloheximide (1 μg/ml)	<0.75
24 hr + cycloheximide (10 μg/ml)	<0.75

* Triplicate wells of confluent human foreskin fibroblasts were incubated in minimal essential medium and 20% rabbit serum for the specified time, and the postculture media were assayed for thrombospondin by ELISA. Cell counts on all wells tested varied <15%.

† Although the minimal amount of thrombospondin detectable by our ELISA technique is 0.25 μg/ml (Fig. 1), the minimal amount of thrombospondin detectable in this particular study was 0.75 μg/ml because the samples were diluted 1:3. The values represent the mean ± SEM of triplicate wells assayed at each time point.

Thrombospondin could also be immunoisolated from the solubilized cell layer of cultures of human foreskin fibroblasts labeled with [^{3}H]leucine (Fig. 3). When cultures were labeled

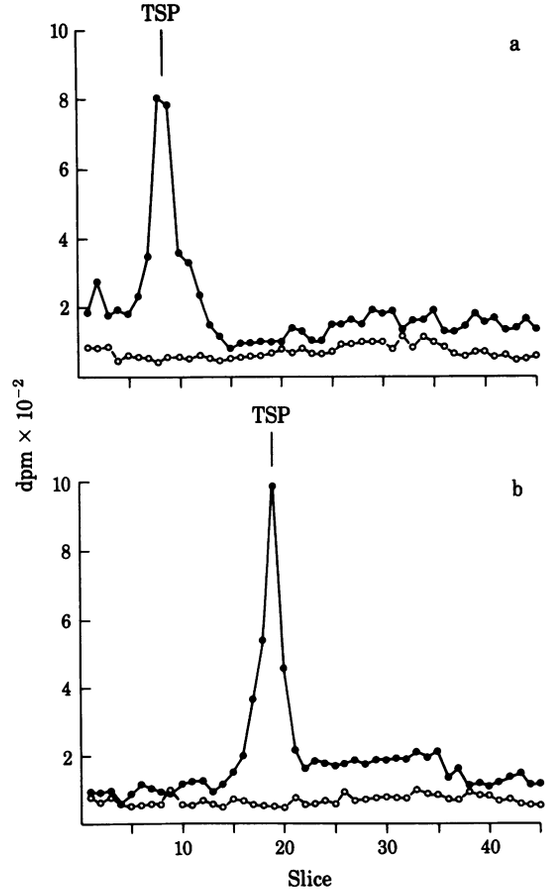

FIG. 3. Immunoisolation of [^{3}H]thrombospondin synthesized by cultured human foreskin fibroblasts and incorporated into the cell layer. Distribution of radioactivity in $NaDodSO_4$/polyacrylamide tube gels of labeled protein immunoisolated with rabbit anti-thrombospondin (●) or anti-ovalbumin (○) and analyzed without (*a*) or with (*b*) reduction with dithiothreitol. TSP, thrombospondin markers.

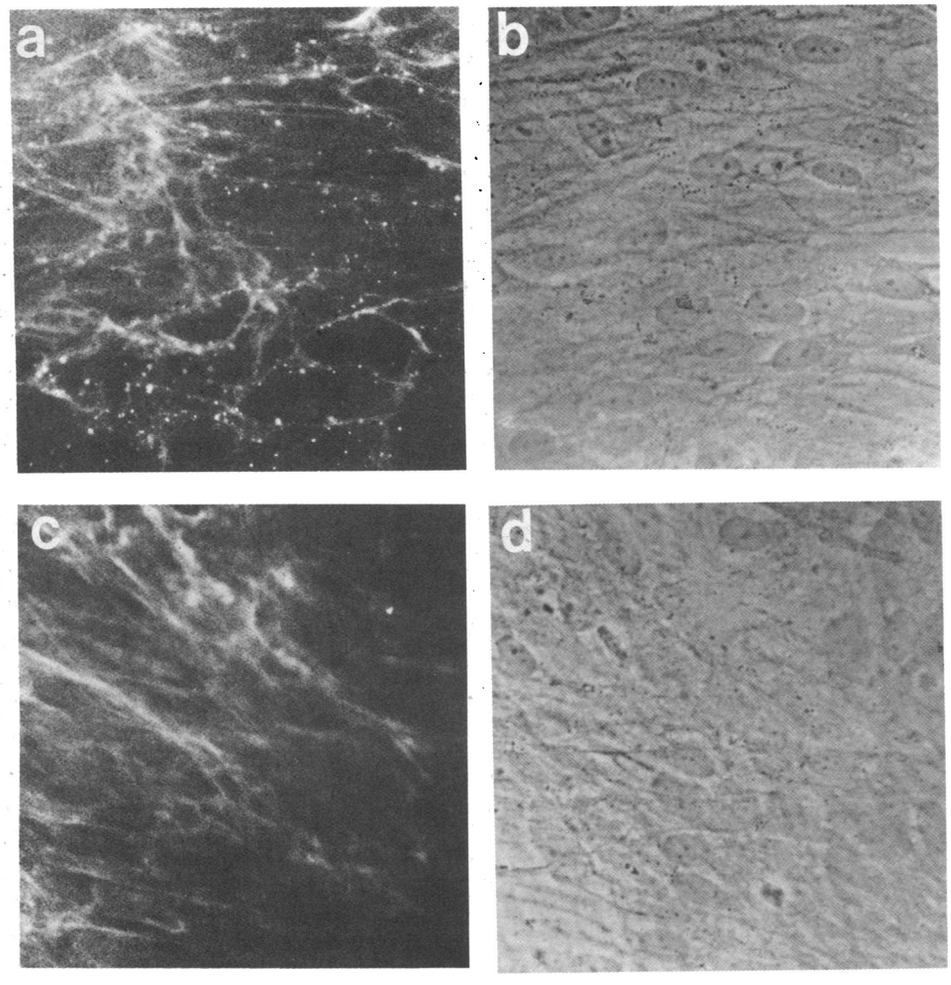

FIG. 4. Immunofluorescence study of cultured human fetal lung fibroblasts. Cells were treated with either mouse monoclonal anti-thrombospondin IgG (*a* and *b*) or mouse monoclonal anti-fibronectin ascites (*c* and *d*) and then with fluorescein-conjugated rabbit anti-mouse IgG. With both treatments a meshwork of extracellular fibrils is brightly stained (*a* and *c*); the corresponding phase-contrast pictures are shown in *b* and *d*. (×500).

for 24 hr with [^{3}H]leucine and then chased with unlabeled leucine (100 μM), labeled thrombospondin could be immunoisolated in decreasing amounts from the cell layer for up to 24 hr after the beginning of the chase.

In addition, by using the same techniques, ^{3}H-labeled peaks migrating with marker thrombospondin both with and without reduction were specifically isolated from radiolabeled postculture media and cell layers obtained from human fetal skin and fetal lung fibroblasts (data not shown).

Immunofluorescence Studies. In order to morphologically localize the thrombospondin in the cell layer, immunofluorescence studies were performed on human foreskin and fetal lung fibroblasts at various times after subculture. When confluent cultures of human fetal lung fibroblasts cultured in medium containing fetal calf serum were fixed in formaldehyde and stained with mouse monoclonal anti-thrombospondin, fibrillar meshworks of extracellular fibrils surrounding the cells were brightly stained (Fig. 4 *a* and *b*). In addition, a punctate staining pattern was also observed. A similar fibrillar staining pattern was seen with mouse monoclonal anti-fibronectin (Fig. 4 *c* and *d*). When fibroblasts were fixed with formaldehyde and extracted with acetone, more of the brightly fluorescent punctate staining was present in cells stained with anti-thrombospondin. Isolated matrices prepared by extraction of cell layers with 1% deoxycholate (23) prior to fixation with formaldehyde lacked punctate anti-thrombospondin immunofluorescent staining, though the fibrillar staining pattern seen with anti-thrombospondin and anti-fibronectin was not diminished. No staining was seen when fetal lung fibroblasts were stained with mouse monoclonal anti-thrombospondin that had been previously absorbed with thrombospondin, though staining was not decreased when the mouse monoclonal anti-thrombospondin was absorbed with fibronectin. Similarly, no staining was seen when fetal lung fibroblasts were stained with mouse monoclonal anti-fibronectin that had been previously absorbed with fibronectin, though staining was not decreased when the antibody was absorbed with thrombospondin. The fluorescence staining with mouse monoclonal anti-thrombospondin or anti-fibronectin was seen in fibroblasts cultured in either rabbit or fetal calf serum.

DISCUSSION

The studies reported here demonstrate that cultured human fibroblasts synthesize thrombospondin, secrete it into the culture medium, and incorporate it into the extracellular matrix. Proteins with similar characteristics have been noted by other workers but never specifically identified. Ruoslahti *et al.* showed that chicken skin fibroblasts possess a protease-sensitive surface antigen with a size after reduction of 145 kDal (21). Alitalo *et al.* (22) demonstrated that HT-1080 human sarcoma cells secrete a protein that is a disulfide-bonded multimer of 160-kDal subunits and resists digestion with bacterial collagenase. Carter and Hakamori (23) showed that human and guinea pig fibroblasts synthesize a 170-kDal glycoprotein (GP170) that forms disulfide-linked multimers and is present in the extracellular matrix. Carter has estimated that the half-life of GP170 in the extracellular matrix of fetal lung fibroblasts is 5 hr (24). Because thrombospondin is a glycoprotein and a disulfide-bonded trimer of 160-kDal subunits (9, 10), it is likely that some of these proteins are thrombospondin.

The incorporation by fibroblasts of thrombospondin into extracellular matrix is reminiscent of fibronectin matrix development (28). However, the material we visualized by immunofluorescence microscopy was definitely thrombospondin and

not fibronectin because absorption of the mouse monoclonal anti-thrombospondin with fibronectin did not diminish the immunofluorescence staining, whereas staining was abolished by absorption with thrombospondin. Serum fibronectin in culture media can be incorporated into extracellular matrix (29). Because the fibroblasts in some immunofluorescence experiments were cultured in media containing fetal calf serum and the mouse monoclonal anti-thrombospondin antibodies crossreact with bovine thrombospondin, some of the immunofluorescence seen in these experiments may have been due to bovine thrombospondin incorporated into extracellular matrix. However, we obtained similar results with fibroblasts cultured in media containing rabbit serum. Because mouse monoclonal anti-thrombospondin antibody does not crossreact with the thrombospondin present in rabbit serum (Fig. 1), at least some of the thrombospondin in the extracellular matrix must be derived from endogenously synthesized thrombospondin. The demonstration that fibroblasts incubated with [^{3}H]leucine for 24 hr and then chased for 1–24 hr with medium containing unlabeled leucine contain immunoisolatable cellular [^{3}H]thrombospondin supports our conclusion. In a recent abstract, Lawler *et al.* (30) reported that chicken embryo fibroblasts grown in fetal calf serum contained thrombospondin in a fibrillar network revealed by immunofluorescence microscopy with anti-human thrombospondin.

Although the functions of thrombospondin synthesized by fibroblasts are presently unclear, it seems worthwhile to compare thrombospondin to fibronectin and laminin, two other glycoproteins secreted by cultured fibroblasts and incorporated into extracellular matrix. Thrombospondin and fibronectin are both present in a fibrillar extracellular matrix (Fig. 4); thrombospondin is also present in punctate structures. Because fibronectin codistributes with laminin, collagen, and heparan sulfate proteoglycan when extracellular matrix is examined by immunofluorescence microscopy (31–34), thrombospondin may also codistribute with these proteins. Thrombospondin, laminin, and fibronectin all interact with heparin (9, 28, 35). In addition, the binding of fibronectin to collagen is enhanced by glycosaminoglycans, and the complexes formed by these interactions are insoluble (36–38). Also, fibronectin crosslinks to sulfated proteoglycans on cell surfaces (39) and thrombospondin interacts with fibronectin (40). It is thus likely that thrombospondin may interact with these components on the cell surface or extracellular matrix. Thrombospondin, like cellular fibronectin, has lectin-like activity (16, 41), and fibronectin and laminin both enhance cell adhesion and spreading (28, 42). Thrombospondin also causes rapid binding and spreading of platelets when it is adsorbed to biomaterials (17). Thus, thrombospondin is similar to fibronectin and laminin in several respects and, like these two proteins, may have a role in the formation and organization of the extracellular matrix and in cell–cell and cell–matrix interactions.

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