Synthesis and Secretion of Thrombospondin by Cultured Human Endothelial Cells

DEANE F. MOSHER, MARY JEAN DOYLE, and ERIC A. JAFFE
*Department of Medicine, University of Wicsonsin-Madison, Madison, Wisconsin 53706; and
Department of Medicine, Cornell University Medical College, New York, New York 10021*

ABSTRACT   Thombospondin, the major glycoprotein released from α-granules of thrombin-stimulated platelets, is a disulfide-bonded trimer of 160 kilodalton subunits and apparently functions as a platelet lectin. Because cultured human umbilical vein endothelial cells synthesize and secrete a glycoprotein (GP-160) which is a disulfide-bonded multimer of 160 kdalton subunits, the possibility that GP-160 is thrombospondin was investigated. Tritiated GP-160 could be immunoisolated from [^{3}H]leucine-labeled endothelial cell postculture medium using a rabbit antiserum to human platelet thrombospondin. Thrombospondin and GP-160 comigrated in two different two-dimensional electrophoretic systems. Both proteins are disulfide-bonded trimers of acidic 160-kdalton subunits. A competitive radioimmunoassay for binding of ^{125}I-thrombospondin to the rabbit antibodies indicated that 49 μg of thrombospondin antigen per 10^6 confluent endothelial cells accumulated in postculture medium over 24 h. Thus, endothelial cells secrete large amounts of a glycoprotein that is identical or very similar to platelet thrombospondin.

Thrombospondin, also known as thrombin-sensitive protein (2, 3) or glycoprotein G (4), 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 (5-9). Studies by Lawler and co-workers indicate that thrombospondin is a 450-kdalton filamentous protein of dimensions 7 × 65 nm, has a pI of 4.7, and is composed of three large disulfide-linked subunits (10, 11). During the process of aggregation, thrombin-stimulated platelets develop a membrane-bound lectin activity (12-14) that originates from α-granules and appears to play an important role in mediating platelet aggregation by binding to a specific receptor on other platelets (15, 16). We recently found that purified human platelet thrombospondin has lectin activity (i.e., agglutinates fixed trypsinized sheep erythrocytes) and blocks the agglutination of thrombin-treated platelets. Therefore, we suggested that thrombospondin is the endogenous lectin of human platelets (17). This may explain why platelets from patients with the gray platelet syndrome, which lack α-granules and α-granule constituents, including thrombospondin, aggregate poorly in response to thrombin (18).

Cultured endothelial cells synthesize and secrete a glycoprotein, GP-160, which is a disulfide-bonded multimer of 160-kdalton subunits and resists digestion with bacterial collagenase (19, 20). A protein with the same properties is produced by cultured HT-1080 human sarcoma cells (21). Because of the similarities between thrombospondin and GP-160, we undertook the present experiments which indicate that GP-160 is identical or nearly identical to thrombospondin.

MATERIALS AND METHODS

Materials

Human plasma fibronectin was purified and iodinated as described previously (22). Human thrombin (23) and *Escherichia coli* RNA polymerase were generous gifts from Dr. John Fenton, II, New York State Department of Health, Albany, NY, and Dr. Richard Burgess, University of Wisconsin-Madison, Madison, WI, respectively. The following were purchased: Type I collagenase, Worthington Biochemicals, Freehold, NJ; L-[3,4,5-^{3}H]leucine (110 Ci/mmol), carrier-free Na^{125}I, and Enhance, New England Nuclear, Boston, MA; X-Omat XR-2 Film, Eastman Kodak, Co., Rochester, NY; protein A-Sepharose CL-4B, Pharmacia Fine Chemicals, Piscataway, NJ; hirudin, molecular size markers, bovine albumin (type V), swine skin type I gelatin, and phenylmethylsulfonyl fluoride (PMSF), Sigma Chemical Co., St. Louis, MO; Bio-Gel P-300, Bio-Gel A-15M, insolubilized goat anti-rabbit immunoglobulin (Immunobeads), and reagents for SDS PAGE, Bio-Rad Laboratories, Richmond, CA; heparin-agarose, Pierce Chemical Co., Rockford, IL; and protein A-bearing Staphylococci (IgGsorb), The Enzyme Center, Inc., Boston, MA.

A portion of this work was presented to the American Society for Cell Biology in Cincinnati, OH, November 14-18, 1980 (1).

Culture of Endothelial Cells

Human endothelial cells were derived from umbilical cord veins and cultured in medium 199 and 20% human serum using methods and materials previously described (19, 24).

Radioactively labeled proteins synthesized and secreted by confluent endothelial cells were prepared by incubating washed 75-cm² cell monolayers for 24 h at 37°C in leucine-free minimal essential medium, 10 ml per flask containing 200 μCi of L-[3,4,5-³H]leucine. In experiments in which [³H]leucine-labeled fibronectin and GP-160 were isolated by gelatin-agarose chromatography, the cells were labeled in medium containing 20% pooled human serum. In experiments in which [³H]leucine-labeled GP-160 was immunoisolated using rabbit antithrombospondin, the cells were labeled in medium containing either 20% rabbit serum or 0.5% tryptose phosphate broth.

In experiments in which the accumulation of thrombospondin antigen was quantitated by radioimmunoassay, endothelial cells were cultured in medium 199 containing 20% human serum in 2-cm² wells of multiwell plates. When the cells were confluent, the cell layer was washed, and medium containing 20% rabbit serum was placed over the cells. After a 24-h incubation, the cell layer was washed, and the cells were placed in 1 ml of fresh medium 199 containing 20% rabbit serum. At various times after the second medium change, postculture medium was removed, clarified by centrifugation at 8,000 g for 2 min in a microfuge, and frozen for further analysis. Cells were dispersed with 0.02% (wt/vol) collagenase and 0.01% (wt/vol) EDTA in 10 mM HEPES, 140 mM sodium chloride, pH 7.4, and enumerated with a Coulter electronic cell counter. To rule out the possibility that human thrombospondin might bind to endothelial cell layer during subculture and be released slowly into the culture medium during the 24-h preincubation and the experimental incubation in rabbit serum, we added ¹²⁵I-thrombospondin (see below) to serum-containing medium of confluent cells. After 24 h, <1% was associated with the cell layer. Human serum contained ~65 μg/ml thrombospondin (see below), and medium plus 20% serum would contain ~13 μg/ml. Thus, <130 ng would be in the cell layer at the beginning of the experimental incubation.

Purification and Iodination of Thrombospondin

8 U of 1- to 3-d-old platelet concentrate was obtained from Badger Red Cross, Madison, WI. Platelets were washed and reacted with thrombin as described by Lawler et al. (10, 11); thrombin was inhibited with hirudin after 2 min of reaction. The supernatant, 15–30 ml, was frozen in a dry-ice ethanol bath and thawed in a 37°C bath. A small fibrin clot formed in the thawing solution; it was gently removed with a stir rod. The supernatant was applied to a 5 × 35-cm column of Bio-Gel P-300, equilibrated and eluted with TBS-EDTA. The first protein peak, which typically had a peak absorbance ($A^{280\ nm,\ 1\ cm}$) of 0.2, was applied to a 1.2 × 6-cm column of heparin-agarose equilibrated in TBS-EDTA. The heparin-agarose column was washed with 10 mM Tris, 140 mM sodium chloride, and 1 mM EDTA, pH 7.4 (TBS-EDTA), and thrombospondin was eluted with 0.55 M sodium chloride in 10 mM Tris, 1 mM EDTA, pH 7.4. Peak fractions from the heparin-agarose column were pooled, portions were frozen in a dry-ice ethanol bath, and the frozen solutions were stored at −70°C. The amount of thrombospondin in the initial platelet releasate, determined by radioimmunoassay (see below), was typically 7.5 mg, and the yield of purified thrombospondin, assuming that a 1 mg/ml solution has an $A^{280\ nm,\ 1\ cm}$ of 1.09 (11), was typically 1.5 mg (20% yield). The purity of thrombospondin, estimated by densitometry after SDS PAGE, was 97% (Fig. 1).

Thrombospondin was iodinated by the chloramine-T technique (25). Free iodine was removed by chromatography on Bio-Gel P-300 or extensive dialysis. The labeled protein was mixed with PMSF-treated albumin, 1 mg/ml, snap frozen in a dry-ice ethanol bath, and stored in portions at −70°C. Specific activity was ~0.5 mCi/mg. Radiochemical purity was assessed by autoradiography after SDS PAGE and found to be >95%.

Production and Characterization of Rabbit Antithrombospondin

Thrombospondin was further purified by preparative electrophoresis after reduction on SDS PAGE slab gels (26). The final product, 10–20 μg in 0.3 ml of 0.1% SDS, was emulsified with an equal volume of complete Freund's adjuvant and injected into a rabbit at multiple subcutaneous sites. The injections in complete adjuvant were repeated twice at monthly intervals. 2 wk later, 1 mg of protein from the heparin-affinity column was given without adjuvant. Antiserum was collected over the next 2 mo. Immunoglobulin from the antiserum, when loaded onto protein A-bearing Staphylococci (27), bound ¹²⁵I-thrombospondin and not ¹²⁵I-fibronectin. In experiments in which immune complexes were collected with beads coated with goat anti-rabbit immunoglobulin or with protein
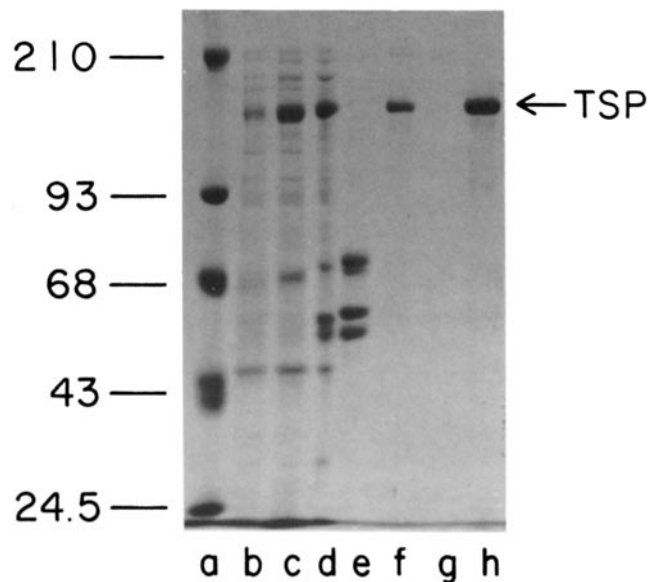

FIGURE 1 Purification of human platelet thrombospondin as monitored by SDS PAGE on 8% gels. Samples were reduced before analysis. The position of thrombospondin (TSP) is indicated. Lanes: (a) Molecular size standards: plasma fibronectin (210 kdaltons), phosphorylase (93 kdaltons), albumin (68 kdaltons), ovalbumin (43 kdaltons), and chymotrypsinogen (24.5 kdaltons). (b) Releasate from platelets (9 × 10⁹/ml) stimulated with thrombin (3 U/ml). The release reaction was performed in 0.1 M HEPES, 0.15 M sodium chloride, pH 7.6, containing 5 mM glucose and 2.5 mM EDTA for 2 min at 37°C. (c) Releasate from thrombin-stimulated platelets after freezing and thawing. (d) Clot that was recovered after freezing and thawing of platelet releasate. (e) Purified human plasma fibrinogen. (f) First protein peak from Bio-Gel P-300 column. (g) Unbound fraction from heparin-agarose affinity column. (h) Protein eluted from heparin-agarose affinity column by 0.55 M sodium chloride. Protein loads: lane a, 5 μg per band; lane b, 7 μg; lane c, 9.5 μg; lane e, 5 μg; lane f, 3 μg; lane g, 0.4 μg; and lane h, 6.5 μg. In other experiments, the β′ subunit of E. coli RNA polymerase, 150 kdaltons, was used to assign a molecular size of 160 kdaltons to thrombospondin. The α and β chains of platelet fibrin are slightly smaller than the Aα and Bβ chains of fibrinogen because of loss of the A and B fibrinopeptides.

A-bearing Staphylococci, maximum binding of 75 ng of ¹²⁵I-thrombospondin was obtained with 1 ml of a 1:1,000 dilution of antiserum.

Radioimmunoassay of Thrombospondin

Protein A-bearing Staphylococci were armed with antithrombospondin as described by O'Keefe and Bennett (27). Approximately 90% of ¹²⁵I-thrombospondin bound when mixed with an equal volume of a 10% (wt/vol) suspension of armed Staphylococci (Fig. 2A). Standard or unknown in physiologic saline solution, 100 μl, was mixed with ¹²⁵I-thrombospondin, ~2 × 10⁴ cpm in 100 μl, and then with 100 μl of a suspension that consisted of one part 10% armed Staphylococci and nine parts 10% "unarmed" carrier Staphylococci. After 3 h at 4°C, the mixture was centrifuged for 1 min at 10,000 g in a microfuge, the supernatant was discarded, and the pellet was analyzed in a gamma counter. The assay was sensitive to 0.7 μg/ml thrombospondin (Fig. 2B). Fibronectin, 200 μg/ml, did not inhibit in the assay. By the assay, the concentration of thrombospondin in serum (formed by allowing human blood in glass tubes to clot at 37°C for 1 h) was 65 ± 23 μg/ml (x̄ ± SD, n = 6).

Immunoisolation and Analysis of [³H]Leucine-labeled Endothelial Cell Proteins

Tritiated leucine-labeled endothelial cell medium was made 3% in SDS and 6 M in urea, heated in a boiling water bath for 5 min, dialyzed against two changes of 3% SDS in phosphate-buffered saline (PBS), diluted 10-fold with 0.12
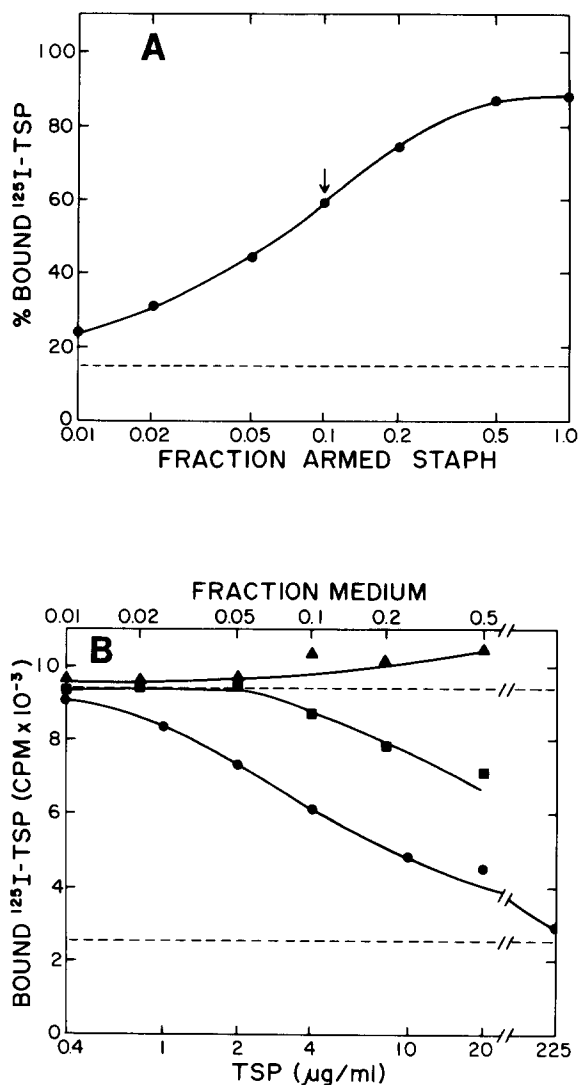
Partial Purification of [^{3}H]leucine-labeled GP-160 and Fibronectin on Gelatin-Agarose

Purified plasma fibronectin, 12 mg, was added to 25 ml of [^{3}H]leucine-labeled endothelial cell postculture medium, and the mixture was applied to a 1.5 × 12-cm column of gelatin-agarose. The column support consisted of gelatin coupled to cyanogen bromide-activated Bio-Gel A-15M by the method of March et al. (29). Unlabeled fibronectin, tritiated fibronectin, and tritiated GP-160 bound to the column. The column was washed with PBS-EDTA, and bound proteins were eluted with 1M sodium bromide, 20 mM sodium acetate, pH 5.0. The eluted proteins were dialyzed against TBS, snap frozen, and kept at −70°C until further studies were performed.

Polyacrylamide Slab Gel Electrophoresis

One-dimensional SDS PAGE was performed using the discontinuous slab gel system of Ames (30). Size markers included reduced fibronectin, 210 kdaltons; β' subunit of RNA polymerase, 150 kdaltons; β-galactosidase, 116 kdaltons; phosphorylase, 93 kdaltons; albumin, 68 kdaltons; ovalbumin, 43 kdaltons; and chymotrypsinogen, 24.5 kdaltons. Two-dimensional gel electrophoresis, in which reduced proteins were separated by isoelectric focusing in 8 M urea in the first dimension and SDS PAGE in the second dimension, was performed as described by Anderson and Anderson (31). Nonreduced-reduced two-dimensional SDS PAGE was performed by the method of Phillips and Poh Agin (32) with slight modifications. The first-dimension discontinuous gel was cast in 0.3 (i.d.) × 12-cm tubes. After electrophoresis without reduction, the first-dimension gel was incubated in sample buffer containing 1% β-mercaptoethanol. The gel was bound to the top of the second-dimension discontinuous slab gel by 1% agarose made up in sample buffer. After polymerization of the agarose, a small slot was made to the right (bottom) of the cylindrical gel, and size markers were analyzed along side the proteins in the cylindrical gel. Nonreduced plasma fibronectin was assumed to have a size of 420 kdaltons. Slab gels were stained with Coomassie Brilliant Blue, prepared for fluorography by incubation in Enhance, and dried onto filter paper. The corners of the paper were marked with ink that contained [^{14}C]glucose before the gel was placed against film. After the fluorogram was developed, alignment of the radioactive ink lines allowed exact comparisons of radioactive spots and protein-staining spots.

FIGURE 2 Radioimmunoassay of thrombospondin. Panel *A* shows the binding of ^{125}I-thrombospondin to various ratios of armed and carrier protein A-bearing Staphylococci. Nonspecific binding to Staphylococci armed with normal rabbit serum was 15% (dashed line). The arrow points to the ratio of armed and "unarmed" Staphylococci that was used in the radioimmunoassay. Panel *B* shows typical inhibition curves in the radioimmunoassay: ●, purified human platelet thrombospondin standard; ■, supernatant from confluent human umbilical vein endothelial cells after 24 h in culture medium containing 20% rabbit serum; and ▲, medium after 24 h in culture plates that contained no cells. The lower dashed line represents nonspecific binding to Staphylococci armed with normal rabbit serum and the upper dashed line represents binding to antibody-armed and carrier Staphylococci in the absence of unlabeled thrombospondin. Total ^{125}I-thrombospondin was 1.9 × 10^4 cpm per assay.

M sodium chloride, 33 mM Tris acetate, pH 8.5, and made 1.7% in Triton X-100 (28). The mixed micellar solution, 30 ml, was incubated with 5 μl of rabbit antithrombospondin, preimmunization serum from the antithrombospondin rabbit, rabbit antifibronectin (19), or rabbit antiovalbumin (19) previously coupled to 10 μl of protein A-Sepharose. After overnight incubation with end-over-end rotation at 20°C, the beads were centrifuged, washed eight times with 2 M urea, 0.1 M glycine, 1% Triton X-100 (28), and eluted by boiling in 2% SDS, 6 M urea. Immunoisolated labeled proteins were analyzed by electrophoresis in SDS in continuous 3% acrylamide-0.5% agarose gels with and without reduction (19). Unlabeled purified fibronectin and thrombospondin were analyzed in simultaneously run gels. Unlabeled proteins were visualized by staining with Coomassie

RESULTS

Cultured human endothelial cells were incubated with [^{3}H]-leucine, and the metabolically labeled proteins that were secreted into the medium were analyzed by SDS PAGE with and without reduction (Fig. 3*A*). Without reduction, 31% of the [^{3}H]leucine migrated in a peak that had an apparent size of 450 kdaltons. With reduction, the peak at 450 kdaltons disappeared and was replaced by peaks with apparent sizes of 220 and 160 kdaltons. The 220-kdalton peak, which accounted for 17% of the labeled proteins, has been shown previously to be fibronectin (19, 33, 34). The 160-kdalton peak accounted for 14% of the labeled proteins.

When rabbit antithrombospondin serum was reacted with [^{3}H]leucine-labeled postculture medium, antithrombospondin specifically immunoisolated ^{3}H-labeled protein with apparent sizes of 450 kdaltons when analyzed without reduction (Fig. 3*B*) and 160 kdaltons when analyzed with reduction (Fig. 3 *C*). Purified human platelet thrombospondin, electrophoresed as a marker, migrated in SDS PAGE with the immunoisolated 160-kdalton [^{3}H]leucine-labeled peak when analyzed after reduction and with the immunoisolated 450-kdalton [^{3}H]leucine-labeled peak when analyzed without prior reduction (Fig. 3, arrows). No labeled proteins were specifically isolated in control experiments with antiovalbumin (Figs. 3 *B* and *C*) or with preimmune serum from the antithrombospondin rabbit. Immunoisolation of the 160-kdalton protein was not blocked by
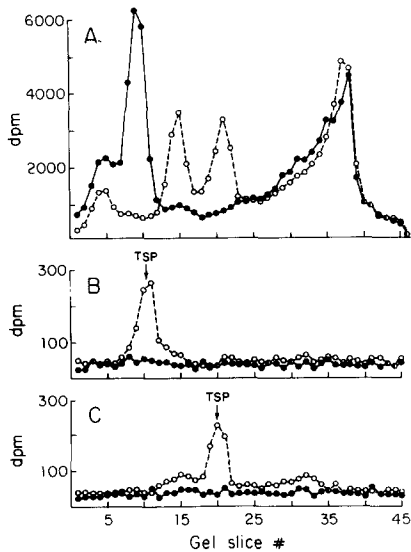
FIGURE 3 Analysis of [³H]leucine-labeled postculture medium and immunoisolated [³H]leucine-labeled GP-160. The following samples were analyzed by SDS PAGE: A, unfractionated postculture medium without (solid line) or with (dashed line) prior reduction; B, labeled protein that was immunoisolated with antithrombospondin (dashed line) or antiovalbumin (solid line) and analyzed without reduction; and C, labeled protein isolated with antithrombospondin (dashed line) or antiovalbumin (solid line) and analyzed with reduction. Arrows point to the positions of migration of nonreduced (450 kdaltons) and reduced (160 kdaltons) marker thrombospondin. In parallel gels, we found that [³H]fibronectin bound to antifibronectin and migrated at slice 10 without reduction and slices 14 and 15 with reduction and that there was only background binding to preimmune serum. Size standards are described in Fig. 1.

preadsorption of the antithrombospondin antiserum (5 µl) with purified fibronectin (100 µg).

In the presence of purified fibronectin (0.5 mg/ml in PBS), [³H]leucine-labeled endothelial cell fibronectin and GP-160 bound to gelatin-agarose and could be eluted with acetate-buffered 1 M NaBr, pH 5 (Fig. 4). SDS PAGE of unbound and bound fractions (Fig. 4) and calculations of yields of labeled proteins indicated that affinity chromatography on gelatin-agarose in the presence of unlabeled fibronectin resulted in nearly quantitative recovery of [³H]leucine-labeled fibronectin and [³H]leucine-labeled GP-160. To make further comparisons between thrombospondin and [³H]leucine-labeled GP-160, thrombospondin was added to the mixture of unlabeled fibronectin, [³H]leucine-labeled endothelial cell fibronectin, and [³H]leucine-labeled endothelial cell GP-160. The four proteins were then analyzed by two different two-dimensional electrophoretic systems: isoelectric focusing of reduced proteins in 8 M urea followed by SDS PAGE (Fig. 5A) and SDS PAGE without reduction followed by SDS PAGE after reduction (Fig. 5B). GP-160 was acidic (Fig. 5A, left), had an apparent size of 450 kdaltons in its unreduced form (Fig. 5B, left), and was apparently a disulfide-bonded trimer of 160-kdalton subunits (Fig. 5B, left). GP-160 migrated at exactly the same positions as thrombospondin in both two-dimensional systems. GP-160 and thrombospondin exhibited the same limited isoelectric heterogeneity (Fig. 5A). In contrast, labeled endothelial cell fibronectin was slightly larger than plasma fibronectin in its reduced (220 vs. 210 kdaltons) and unreduced forms and thus migrated slightly higher and more to the left in the nonreduced-reduced system (Fig. 5B). There were also slight differences in isoelectric heterogeneity between endothelial cell and plasma fibronectins (Fig. 5A).

Thrombospondin antigen was first detectable by radioimmunoassay in the postculture medium of confluent endothelial cells after 8 h of culture; after 24 h the postculture medium
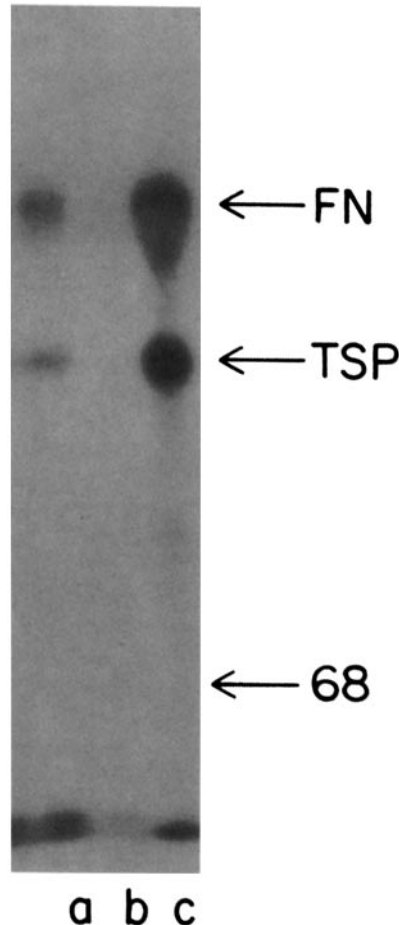

FIGURE 4 Chromatography of a mixture of purified plasma fibronectin and [³H]leucine-labeled postculture medium on gelatin-agarose as monitored by SDS PAGE on 6% gels. Samples were reduced before analysis, and radioactive bands were detected by fluorography. Lanes are (a) unfractionated proteins, (b) proteins not bound to gelatin-agarose, and (c) proteins bound to gelatin agarose and eluted with 1 M sodium bromide, pH 5.0. Of the many proteins in the serum-containing growth medium that could be detected with Coomassie Brilliant Blue, only fibronectin bound to gelatin-agarose. Unlabeled fibronectin disturbed the banding pattern of [³H]leucine-labeled fibronectin and caused the rarefaction on the fluorogram in the fibronectin (FN) region. The positions of thrombospondin (TSP, electrophoresed as a marker) and of human albumin (68 kdaltons, detected by protein staining in lanes a and b) are indicated. Labeled proteins < 60 kdaltons in size migrated at the dye front.

contained 49 µg/10⁶ cells (Table I).[1] The curves for inhibition of ¹²⁵I-thrombospondin binding to rabbit antibodies were similar for purified platelet thrombospondin and endothelial cell-conditioned medium, although we could not construct a full inhibition curve for conditioned medium because of its limited antigen concentration (Fig. 2B). The value of 4.4 µg thrombospondin/ml of 24-h conditioned medium is comparable to the value of 5.2 µg fibronectin/ml of 72-h conditioned medium that we determined earlier (19).

DISCUSSION

Several different comparisons of GP-160 synthesized and secreted by cultured endothelial cells and thrombospondin secreted by thrombin-stimulated platelets indicate that these two proteins are the same. GP-160 and thrombospondin migrated identically in two different two-dimensional gel systems. Therefore, it seems likely that the differences between GP-160 and thrombospondin, if present, are minor.

Since submission of this manuscript, McPherson et al. (35) have described the isolation and characterization of a glyco-

---

[1] Recently, we developed an enzyme-linked immunoabsorbent assay (ELISA) for human thrombospondin based on a mouse monoclonal IgG antibody to thrombospondin isolated from platelets. Analyses of human thrombospondin in the 24-h tissue culture medium by ELISA agree within 40% of the values obtained by radioimmunoassay.
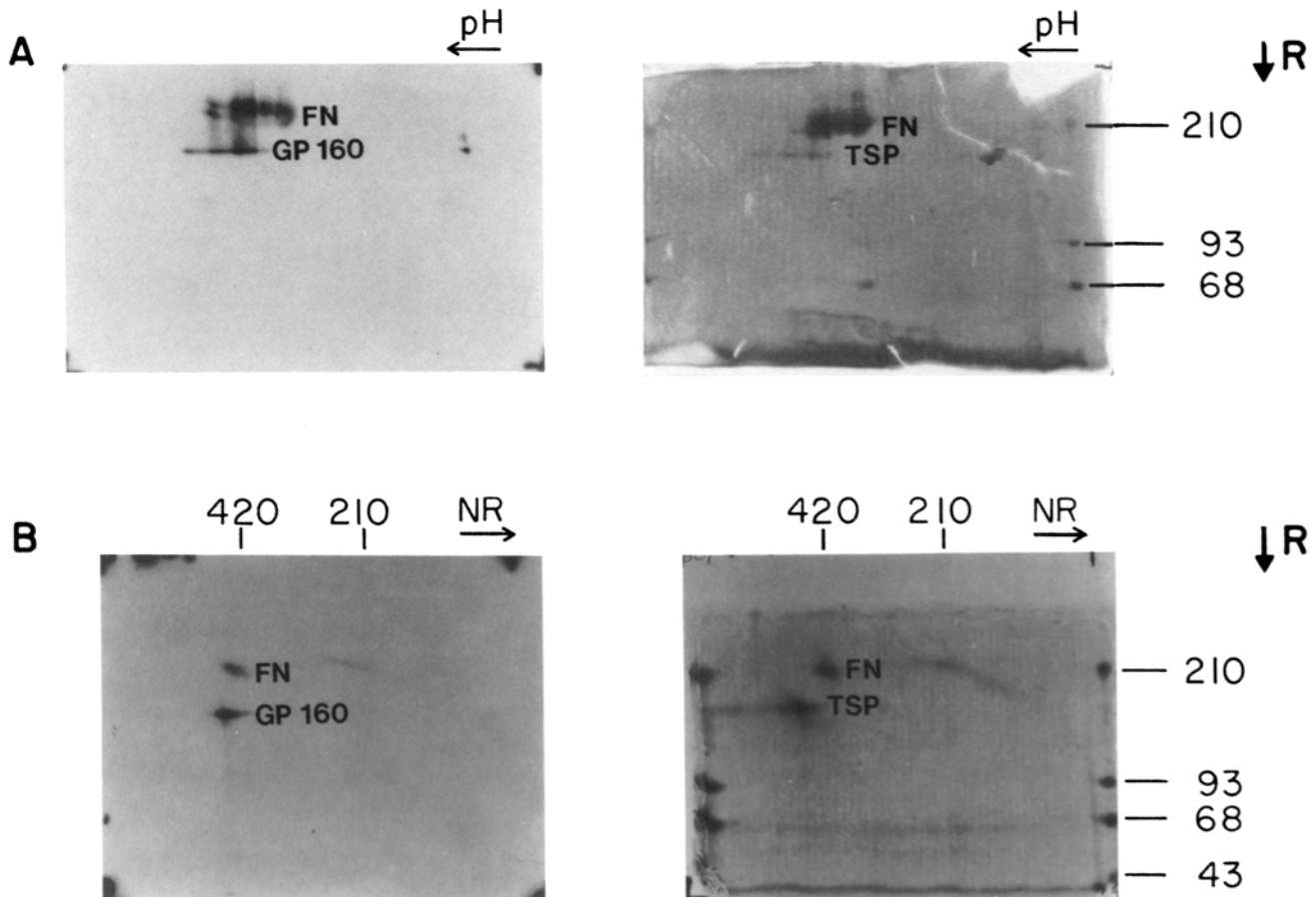
FIGURE 5 Two-dimensional analyses of fibronectin (*FN*), GP-160, and thrombospondin (*TSP*). The following mixture of proteins was analyzed: plasma fibronectin, platelet thrombospondin, and [^{3}H]leucine-labeled endothelial cell fibronectin and GP-160. Two systems were used: *A*, isoelectric focusing (pH, acid side to the left) in 8 M urea followed by electrophoresis through 6% polyacrylamide in SDS after reduction (*R*); and *B*, electrophoresis through 4% polyacrylamide in SDS without reduction (*NR*) followed by electrophoresis through 6% polyacrylamide in SDS after reduction (*R*). The slabs were stained for protein and dried onto filter paper (right) and analyzed by fluorography (left). Apparent size in kilodaltons is indicated on the right. Standards are described in Fig. 1. The figure has been made to allow one-for-one comparisons of fluorographic and protein-staining patterns. We could make a more critical evaluation by overlaying the dried stained gel with the developed fluorography film.

TABLE I

Accumulation of Thrombospondin Antigen in Culture Medium of Human Umbilical Vein Endothelial Cells as Ascertained by Radioimmunoassay

Culture	Thrombospondin antigen*	
h	µg/ml	µg/10⁶ cells
0	<0.7	<7
4	<0.7	<7
8	0.8 ± 0.1	10 ± 1
14	3.4 ± 0.8	36 ± 6
24	4.4 ± 0.2	49 ± 4

* $\bar{x}$ ± SD, n = 3.

protein from serum-free conditioned media of bovine aortic endothelial cells which is apparently homologous to human endothelial cell GP-160. Following our lead (1), McPherson et al. (35) compared the glycoprotein to thrombospondin. The amino acid composition of the glycoprotein was similar to that published for human platelet thrombospondin, antibodies to the glycoprotein reacted with bovine thrombospondin after electrophoretic transfer from SDS polyacrylamide gels onto nitrocellulose paper, and two-dimensional peptide maps of the iodinated glycoprotein and iodinated thrombospondin were

similar. Thus, thrombospondin synthesis by endothelial cells in culture must be a general phenomenon.

Although thrombospondin and GP-160 are disulfide-bonded trimers of 160-kdalton subunits, there is no evidence that thrombospondin and GP-160 are collagenous. We have been able to degrade human endothelial cell GP-160 using purified bacterial collagenase (unpublished experiments carried out in both of our laboratories). Similarly, Sage et al. (20) were unable to degrade bovine endothelial GP-160 by purified bacterial collagenase under conditions in which type III procollagen was readily degraded. By amino acid analysis, thrombospondin is not rich in proline or glycine (3, 10).

We are unsure of the determinants of binding of [^{3}H]leucine-labeled GP-160 to gelatin-agarose in the presence of unlabeled fibronectin. In preliminary studies, we found that >60% of ^{125}I-thrombospondin bound to gelatin-agarose, underived agarose, or Bio-Gel P-300. The presence of fibronectin had little influence on this apparently nonspecific binding. Thus, while chromatography on gelatin-agarose in the presence of fibronectin allowed us to partially purify GP-160, a better experimental system is needed to allow investigation of specific binding.

We can only speculate on the function(s) of endothelial cell thrombospondin. As described above, thrombospondin is secreted and bound by thrombin-activated platelets (2-9) and

apparently mediates thrombin-induced aggregation (12–17). Two proteins, von Willebrand factor and fibronectin, are also found in platelet α-granules (36–39), bind to the surface of thrombin-stimulated platelets (40–42), and are synthesized by endothelial cells (19, 33, 34, 43, 44). Von Willebrand factor and fibronectin are found in plasma and in the subendothelium (45, 46). Von Willebrand factor mediates platelet adhesion to subendothelium (47, 48) whereas the role of fibronectin in platelet function is unknown. Although plasma thrombospondin levels as measured by radioimmunoassay are quite low (~1 μg/ml, our unpublished data), endothelial cells may, when appropriately stimulated, release thrombospondin into local microenvironments and thus support platelet interactions with endothelial cells and the subendothelium. Thrombospondin, because of its lectin activity, may also play important roles in the interaction of endothelial cells with each other and with the underlying extracellular matrix. Experiments to test these hypotheses are currently underway.

REFERENCES

1. Doyle, M. J., D. F. Mosher, and E. A. Jaffe. 1980. Endothelial cells synthesize and secrete thrombospondin. *J. Cell Biol.* 87 (2, Pt. 2): 306*a* (Abstr.).
2. Baenziger, N. L., G.N. Brodie, and P. W. Majerus. 1971. A thrombin-sensitive protein of human platelet membranes. *Proc. Natl. Acad. Sci. U. S. A.* 68:240–243.
3. Baenziger, N. L., G. N. Brodie, and P. W. Majerus. 1972. Isolation and properties of a thrombin-sensitive protein of human platelets. *J. Biol. Chem.* 247:2723–2731.
4. George, J. N. 1978. Studies on platelet plasma membranes. Quantitative analysis of platelet membrane glycoproteins by (¹²⁵I)-diazotized diiodosulfanilic acid labeling and SDS-polyacrylamide gel electrophoresis. *J. Lab. Clin. Med.* 92:430–446.
5. Hagen, I. 1975. Effects of thrombin on washed, human platelets: changes in subcellular fractions. *Biochim. Biophys. Acta.* 392:242–254.
6. Hagen, I., T. Olsen, and N. O. Solum. 1976. Studies on subcellular fractions of human platelets by the lactoperoxidase-iodination technique. *Biochim. Biophys. Acta.* 455:214–225.
7. Lawler, J.W., F. C. Chao, and P.-H. Fang. 1977. Observation of a high molecular weight platelet protein released by thrombin. *Thromb. Haemostasis.* 37:355–357.
8. George, J. N., R. M. Lyons, and R. K. Morgan. 1980. Membrane changes associated with platelet activation. Exposure of actin on the platelet surface after thrombin-induced secretion. *J. Clin. Invest.* 66:1–9.
9. Phillips, D. R., L. K. Jennings, and H. R. Prasanna. 1980. Ca²⁺-mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. *J. Biol. Chem.* 255:11629–11632.
10. Lawler, J. W., H. S. Slayter, and J. E. Coligan. 1978. Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. *J. Biol. Chem.* 253:8609–8616.
11. Margossian, S. S., J. W. Lawler, and H. S. Slayter. 1981. Physical characterization of platelet thrombospondin. *J. Biol. Chem.* 256:7495–7500.
12. Gartner, T. K., D. C. Williams, and D. R. Phillips. 1977. Platelet plasma membrane lectin activity. *Biochem. Biophys. Res. Commun.* 79:592–599.
13. Gartner, T. K., D. C. Williams, F. C. Minion, and D. R. Phillips. 1978. Thrombin-induced platelet aggregation is mediated by a platelet plasma membrane-bound lectin. *Science (Wash. D. C.).* 200:1281–1283.
14. Gartner, T. K., D. R. Phillips, and D. C. Williams. 1980. Expression of thrombin-enhanced platelet lectin activity is controlled by secretion. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 113:196–200.
15. Gartner, T. K., J. M. Gerrard, J. G. White, and D. C. Williams. 1981. Fibrinogen is the receptor for the endogenous lectin of human platelets. *Nature (Lond.).* 289:688–700.
16. Gartner, T. K., J. M. Gerrard, J. G. White, and D. C. Williams. 1981. The endogenous lectin of human platelets in an α-granule component. *Blood.* 58:153–157.
17. Jaffe, E. A., L. L. K., Leung, R. L. Nachman, R. 1. Levin, and D. F. Mosher. 1981. Thrombospondin is the endogenous lectin of human platelets. *Nature (Lond.).* In press.
18. Gerrard, J. M., D. R. Phillips, G. H. R. Rao, E. F. Plow, D. A. Walz, R. Ross, L. A. Harker, and J. G. White. 1980. Biochemical studies of two patients with the gray platelet syndrome. Selective deficiency of platelet alpha granules. *J. Clin. Invest.* 66:102–109.
19. Jaffe, E. A., and D. F. Mosher. 1978. Synthesis of fibronectin by cultured human endothelial cell. *J. Exp. Med.* 147:1779–1791.
20. Sage, H., E. Crouch, and P. Bornstein. 1979. Collagen synthesis by bovine aortic endothelial cells in culture. *Biochemistry.* 18:5433–5442.
21. Alitalo, K., A. Vaheri, T. Krieg, and R. Timpl. 1980. Biosynthesis of two subunits of type IV procollagen and of other basement membrane proteins by a human tumor cell line. *Eur. J. Biochem.* 109:247–255.
22. Mosher, D. F., P. E. Schad, and J. M. Vann. 1980. Cross-linking of collagen and fibronectin by Factor XIIIₐ: localization of participating glutaminyl residues to a tryptic fragment of fibronectin. *J. Biol. Chem.* 255:1181–1188.
23. Fenton, J. W., II, M. J. Fasco, A. B. Stackrow, D. L. Aronson, A. M. Young, and J. S. Finlayson. 1977. Human thrombins. Production, evaluation, and properties of α-thrombin. *J. Biol. Chem.* 252:3587–3598.
24. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745–2756.
25. Mosher, D. F., and A. Vaheri. 1978. Thrombin stimulates the production and release of a major surface-associated glycoprotein (fibronectin) in cultures of human fibroblasts. *Exp. Cell Res.* 112:323–334.
26. Lazarides, E. 1976. Two general classes of cytoplasmic actin filaments in tissue culture cells: the role of tropomyosin. *J. Supramol. Struct.* 5:531–563.
27. O'Keefe, E., and V. Bennett. 1980. Use of immunoglobulin-loaded protein A-bearing Staphylococci as a primary solid phase immunoadsorbent in radioimmunoassay. *J. Biol. Chem.* 255:561–568.
28. Erickson, A. H., and G. Blobel. 1979. Early events in the biosynthesis of the lysosomal enzyme cathepsin D. *J. Biol. Chem.* 254:11771–11774.
29. March, S. C., I. Parikh, and P. Cuatrecasas. 1979. A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Anal. Biochem.* 60:149–152.
30. Ames, G. F.-L. 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. Membrane, soluble, and periplasmic fractions. *J. Biol. Chem.* 249:634–644.
31. Anderson, L., and N. G. Anderson. 1977. High resolution two-dimensional electrophoresis of human plasma proteins. *Proc. Natl. Acad. Sci. U. S. A.* 74:5421–5425.
32. Phillips, D. R., and P. Poh Agin. 1977. Platelet plasma membrane glycoproteins. Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two-dimensional gel electrophoresis. *J. Biol. Chem.* 252:2121–2126.
33. Birdwell, C. R., D. Gospodarowicz, and G. L. Nicolson. 1978. Identification, localization, and role of fibronectin in cultured bovine endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:3273–3277.
34. Macarak, E. J., E. Kirby, T. Kirk, and N. A. Kefalides. 1978. Synthesis of cold-insoluble globulin by cultured calf endothelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 75:2621–2625.
35. McPherson, J., H. Sage, and P. Bornstein. 1981. Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture. Apparent identity with platelet thrombospondin. *J. Biol. Chem.* 256:11330–11336.
36. Nachman, R. L., and E. A. Jaffe. 1975. Subcellular platelet Factor VIII antigen and von Willebrand factor. *J. Exp. Med.* 141:1101–1112.
37. Koutts, J., P. N. Walsh, E. F. Plow, J. W. Fenton, II, B. N. Bouma, and T. S. Zimmerman. 1978. Active release of human platelet factor VIII-related antigen by adenosine diphosphate, collagen, and thrombin. *J. Clin. Invest.* 62:1255–1263.
38. Plow, E. F., C. Birdwell, and M. H. Ginsberg. 1979. Identification and quantitation of platelet-associated fibronectin antigen. *J. Clin. Invest.* 63:540–543.
39. Zucker, M. B., M. W. Mosesson, M. J. Broekman, and K. L. Kaplan. 1979. Release of platelet fibronectin (cold-insoluble globulin) from alpha granules induced by thrombin or collagen: lack of requirement of plasma fibronectin in ADP-induced platelet aggregation. *Blood.* 54:8–12.
40. Ginsberg, M. H., R. G. Painter, J. Forsyth, C. Birdwell, and E. F. Plow. 1980. Thrombin increases expression of fibronectin antingen on the platelet surface. *Proc. Natl. Acad. Sci. U. S. A.* 77:1049–1053.
41. Plow, E. F., and M. H. Ginsberg. 1981. Specific and saturable binding of plasma fibronectin to thrombin-stimulated human platelets. *J. Biol. Chem.* 256:9477–9482.
42. Hawiger, J., T. Fujimoto, and S. Ohara. 1981. The contrasting effect of thrombin and prostacyclin on availability of the receptor for factor VIIIᵥwₜ on human platelets. *Clin. Res.* 29:571. (Abstr.).
43. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* 52:2757–2764.
44. Jaffe, E. A., and R. L. Nachman. 1975. Subunit structure of Factor VIII antigen synthesized by cultured human endothelial cells. *J. Clin. Invest.* 56:698–702.
45. Stenman, S., and A. Vaheri. 1978. Distribution of a major connective tissue protein, fibronectin, in normal human tissues. *J. Exp. Med.* 147:1054–1064.
46. Rand, J. H., I. I. Sussman, R. E. Gordon, S. V. Chu, and V. Solomon. 1980. Localization of factor-VIII-related antigen in human vascular subendothelium. *Blood.* 55:752–756.
47. Tschopp, T. B., H. J. Weiss, and H. R. Baumgartner. 1974. Decreased adhesion of platelets to subendothelium in von Willebrand's disease. *J. Lab. Clin. Med.* 83:296–300.
48. Weiss, H. F., H. R. Baumgartner, T. B. Tschopp, V. T. Turitto, and D. Cohen. 1978. Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. *Blood.* 51:267–279.