

# Thrombospondin: Synthesis and Secretion by Cells in Culture

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**ABSTRACT** Thrombospondin, a high molecular weight glycoprotein secreted by platelets in response to activation by thrombin, has been identified by immunofluorescence in bovine aortic endothelial cells, human foreskin fibroblasts, and human aortic smooth muscle cells. Immunofluorescence patterns were found to be similar using antisera raised to thrombospondins purified either from bovine aortic endothelial cells or from human platelets. Radioimmune precipitation of pulse-labeled cellular proteins confirmed the presence of thrombospondin in positively stained cells. A sensitive quantitative enzyme-linked immunosorbent assay (ELISA) was developed and used to determine that the accumulation of secreted thrombospondin was similar for endothelial cells and fibroblasts but was higher for smooth muscle cells. The presence of thrombospondin in a variety of cells suggests that its function may not be limited to an involvement in platelet interactions.

Thrombospondin (TS), a high molecular weight glycoprotein, is released from  $\alpha$ -granules after activation of platelets by thrombin (1, 2). After release, the protein binds to the activated platelet surface in a calcium-dependent fashion (3) and may participate in platelet-platelet interactions (4). Earlier work in this laboratory (5) identified a high molecular weight glycoprotein secreted by endothelial cells in culture that represented a substantial portion of the noncollagenous protein synthesized and secreted by these cells. This glycoprotein was subsequently shown to be indistinguishable from TS by a variety of criteria, including co-purification, molecular weight, amino acid composition, immunological cross-reactivity, and peptide maps (6).

Although TS has been shown to have lectinlike activity in platelet-platelet interactions (4), its function in endothelial cells is not known. This led us to examine other cells in culture for the synthesis and secretion of TS. We have now identified TS in a variety of mesenchymal cells. Since TS is secreted and deposited in the cell layer of these cells in culture, we postulate that it may function as a matrix protein *in vivo*.

## MATERIALS AND METHODS

### Cell Culture

Bovine aortic endothelial (BAE) cells were provided by Dr. S. Schwartz (University of Washington). They were maintained in Waymouth's medium supplemented with 10% fetal calf serum (FCS) (Reheis Chemical Company, Phoenix, AZ), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). Human dermal fibroblasts were isolated from foreskin explants and maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% FCS and antibiotics. Smooth muscle cells were obtained from human aortae and were provided by Dr. Russell Ross (University of Washington). They were maintained in DME supplemented with 20% FCS and antibiotics. Cells were grown at 37°C in an atmosphere of 5% CO<sub>2</sub>.

### Purification of Thrombospondins

BAE cell TS was isolated according to the method of McPherson et al. (6).

Human platelet TS was isolated from 1-d-old blood bank platelets according to the procedure of Lawler and Slayter (7), with modifications. 4 U of washed platelets were harvested by centrifugation and suspended in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM KCl, 5 mM  $\alpha$ -D-glucose, and 2.5 mM EDTA, pH 7.5. Alpha granule contents were released after activation by human thrombin (3.5 National Institutes of Health (NIH) U/ml) for 15 min at room temperature with stirring. Thrombin was inactivated by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 0.2 mM. Platelets were removed by centrifuging at 27,000 *g* for 20 min at 4°C. The supernatant fraction was applied to a Sepharose CL-4B column (2.5  $\times$  100 cm) equilibrated in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 2.5 mM EDTA, pH 7.5. Fractions were monitored by absorbance at 280 nm; fractions containing TS were pooled and gently shaken for 2 h at 4°C with heparin-Sepharose 6B. A column was poured with the gel, washed with a buffer containing 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, and the TS eluted with a buffer containing 20 mM Tris-HCl, 600 mM NaCl, pH 7.5. Typical yields were ~3 mg with peak concentrations of 1 mg/ml. Bovine platelet TS was prepared essentially as described above.

### Preparation of Antisera

Rabbit antisera were raised to BAE cell TS and human platelet TS. Rabbit IgG was purified by repeated ammonium sulfate precipitation and was assayed by an ELISA method. Human von Willebrand's factor and  $\alpha_2$ -macroglobulin were provided by Drs. Michael Chopek and Mark Lively (University of Washington), respectively; human fibronectin was purchased from Collaborative Research (Waltham, MA); mouse laminin was provided by Dr. George Martin (National Institutes of Health) and Dr. Atsuhiko Oohira (University of Washington). Collagens and procollagens from human or bovine tissue were prepared by standard methods as described by Sage and Bornstein (8). Contaminating activities were removed by affinity chromatography with Sepharose 4B-coupled ligands.

### Immunofluorescence Microscopy

Cells were grown to confluence on glass microscope slides or cover slips. They were fixed in neutral buffered paraformaldehyde (3%) and permeabilized in absolute ethanol at -70°C for 15 s when appropriate. Cells were then exposed to antiserum (1:10) for 30 min at 37°C, washed, and exposed to fluorescein-conjugated goat antirabbit IgG (Miles-Yeda Ltd., Rehovot, Israel) at 1:20 dilution for 30 min at 37°C.

## Radioimmune Precipitation

Cells grown in standard medium were washed twice in serum-free Waymouth's medium minus cysteine and incubated at 37°C in the presence of 100  $\mu$ Ci/ml  $^{35}$ S-cysteine (New England Nuclear, Boston, MA; 937 Ci/mmol). The medium was discarded and the cells were washed three times with ice-cold PBS and harvested in PBS containing 0.5% deoxycholate, 0.5% Triton X-100, 0.2 mM PMSF, and 10 mM *N*-ethylmaleimide (NEM). After vortexing, the insoluble debris was removed by a brief centrifugation. Aliquots of the supernatant fraction (100–200  $\mu$ l) were transferred to microfuge tubes precoated with a solution of 0.1% bovine serum albumin (BSA), and 10  $\mu$ l of antiserum was added. The mixture was allowed to stand for 1–2 h at room temperature, then 100  $\mu$ l sheep anti-rabbit IgG was added, and the mixture was allowed to stand for another 2 h. The precipitate was washed three times in PBS containing detergents and protease inhibitors and once in PBS before being dissolved in 0.01 N HCl and neutralized with NaOH. The sample was then boiled in SDS electrophoresis sample buffer with reducing agent and subjected to SDS PAGE.

## Quantitative ELISA

Human TS, 1  $\mu$ g/ml of PBS, was used to coat Immulon I 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). All dilutions of antiserum and TS were made in a buffer containing 150 mM NaCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 10.8 mM  $\text{Na}_2\text{HPO}_4$ , 2.7 mM KCl, 0.05% Tween 20, 0.02% NaN<sub>3</sub>, and 1 mg/ml BSA. Antiserum was diluted 314-fold; TS was diluted to concentrations in the range of 0–1,000 ng/ml. Absorption of antiserum was accomplished by incubating 110  $\mu$ l of diluted antiserum with 110  $\mu$ l of TS at different concentrations in uncoated microtiter wells for 1 h at 37°C. 200  $\mu$ l of each solution was withdrawn, added to appropriate wells of coated microtiter plates, and incubated for 30 min at 37°C. The plates were then washed with a solution containing 0.15 M NaCl and 0.05% Tween 20. Alkaline phosphatase-conjugated goat anti-rabbit IgG (200  $\mu$ l of 1:1,000 dilution) (Miles Laboratories, Inc., Elkhart, IN) was added to each well and incubated for 4–6 h at room temperature. The plates were washed as before, drained, and 200  $\mu$ l of *p*-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 9.8) was added. After a 15-min incubation at room temperature, the reaction was stopped by adding 50  $\mu$ l of 1 M NaOH. Optical density in each well was measured with a Dynatech plate scanner at 400 nm. Samples containing unknown amounts of TS were diluted with the above buffer into the concentration range measurable by the assay, preincubated with antibody in parallel with TS standards, and then incubated with TS-coated plates. Each sample was assayed in duplicate at two or more concentrations.

## Quantification of Thrombospondin in Culture Media

Cells were grown to confluence in 12-well Costar tissue culture plates (Costar, Data Packaging, Cambridge, MA) containing 1 ml of 5% heat-inactivated porcine serum in Waymouth's medium. At the start of the measurement, the medium was replaced with 0.6 ml of fresh medium containing 1% serum; after 18 h, 0.5 ml of the medium was transferred to a 1.5-ml microfuge tube containing 0.05 ml of an inhibitor solution (250 mM EDTA, 100 mM NEM, and 2 mM PMSF) at 4°C. The sample was centrifuged to pellet any cells, and the supernatant fraction was diluted in buffer for quantification of TS by the ELISA method. The cell layer was trypsinized and cell number was determined by electronic counting.

## RESULTS

### Purification and Characterization of Antisera

Rabbit anti-bovine endothelial cell TS contained activity against bovine and human TS, human  $\alpha_2$ -macroglobulin and mouse laminin. The contaminating activities were removed by absorption with  $\alpha_2$ -macroglobulin-Sepharose 4B and laminin-Sepharose 4B. By analysis using the direct ELISA method, the purified antiserum reacted with thrombospondin but not with human type I, type III, or type V collagen, bovine type III procollagen, or type IV collagen, von Willebrand's factor, fibronectin, or fibrinogen (data not shown). Rabbit anti-human platelet TS had activity against human and bovine TS and fibronectin. The contaminating activity was removed by absorption with fibronectin-Sepharose 4B; the purified antiserum reacted only with TS and not with any of the other antigens mentioned above, as analyzed by ELISA.

Since the antisera were raised to thrombospondins of different species, it was of interest to know whether antiserum raised against bovine endothelial cell TS reacted with human TS, and vice versa. Fig. 1 shows the results of quantitative assays using the bovine and human proteins. The anti-bovine endothelial cell TS reacted equally well with human or bovine TS (Fig. 1A). However, a greater amount of bovine compared to human TS was required to achieve the same degree of inhibition of the reaction of anti-human TS with its antigen (Fig. 1B). Thus the anti-human TS serum shows some species specificity and reacts better with the human than with the bovine antigen.

## Immunofluorescence Microscopy

Representative fluorescence micrographs of bovine aortic endothelial cells, human foreskin fibroblasts, and human aortic smooth muscle cells are shown in Fig. 2. Cells were permeabilized and stained with either anti-BAE cell TS or anti-human platelet TS, followed by staining with fluorescein-conjugated goat anti-rabbit IgG. A prominent pattern of granules of intracellular fluorescence in a perinuclear distribution was present in all three cell types using either antiserum. In addition, some extracellular matrix staining of a fibrillar nature was observed. Only the fibrillar pattern was seen in cells that were not permeabilized. In other experiments we showed that the extracellular fluorescence was not due to trapping or adsorption of TS from serum contained in culture medium, because the same pattern of extracellular fluorescence was seen when cells were grown in medium containing pig serum; porcine TS was not detected by anti-human or anti-bovine TS (see below).

The pattern of intracellular staining for TS was the same as that for fibronectin but the extracellular staining was clearly different from the pattern for fibronectin (9). Parallel experiments using affinity-purified rabbit anti-human fibronectin antibodies showed that absorption of anti-TS sera with an amount of fibronectin sufficient to prevent the appearance of fluorescence due to fibronectin in endothelial cells and fibroblasts had no effect on the fluorescence due to TS. On the other hand, preincubation of anti-TS serum with human platelet TS inhibited the appearance of fluorescence in endothelial cells and fibroblasts. No fluorescence was seen when a preim-

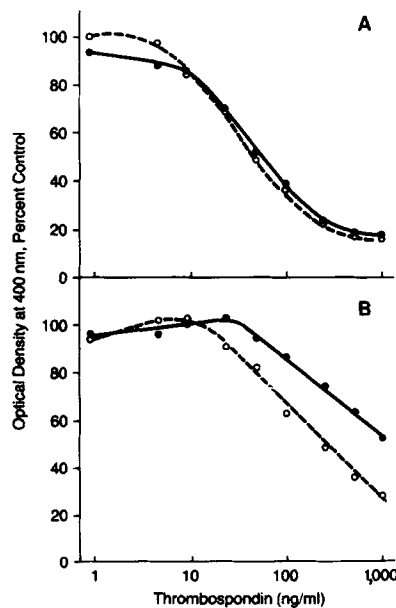


FIGURE 1 Characterization of antisera to thrombospondins by ELISA. (A) Standard curves developed using anti-bovine aortic endothelial cell TS reacting with microtiter plates coated with human TS. The competing antigen was: (●) Bovine platelet TS. (○) Human platelet TS. (B) Standard curves developed using anti-human platelet TS reacting with microtiter plates coated with human TS. The competing antigen was: (●) Bovine platelet TS. (○) Human platelet TS.

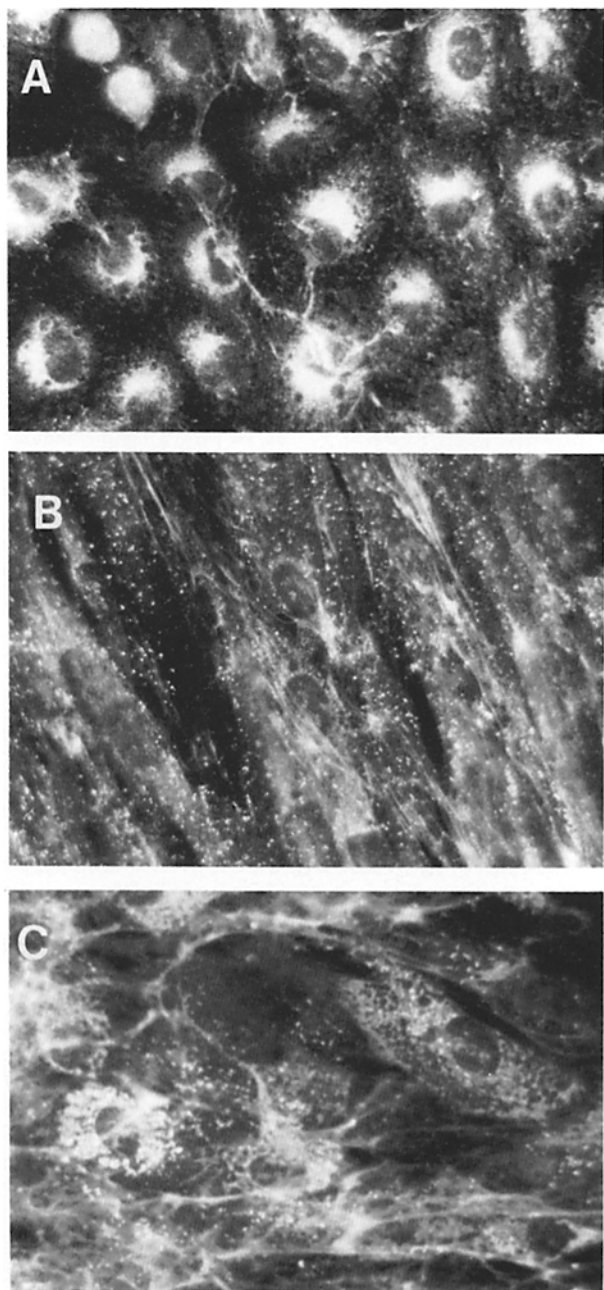


FIGURE 2 Fluorescence microscopy. Cells were cultured on glass slides, fixed and permeabilized, exposed to anti-TS antiserum followed by staining with fluorescein-conjugated goat anti-rabbit IgG. (A) Bovine endothelial cells treated with anti-BAE cell TS. (B) Human foreskin fibroblasts treated with anti-human platelet TS. (C) Human aortic smooth muscle cells treated with anti-human platelet TS.  $\times 400$ .

mune antiserum was used or when the first antibody was omitted and only the goat anti-rabbit IgG was used. Human epidermoid carcinoma cells (A431) and a human leukemia cell line (K562) were negative for TS by immunofluorescence (data not shown).

#### Radio-immune Precipitation

To confirm the presence of TS in cultured cells, precipitation of radio-labeled cultures with anti-BAE cell or human platelet TS was performed. As shown in Fig. 3, the predominant protein precipitated by the antiserum in lysates of endothelial

cells, fibroblasts, and smooth muscle cells comigrated on SDS slab gels with a human platelet TS standard. The band below TS in lanes *c* and *f* (Fig. 3) may correspond to the derivative of TS noted by McPherson et al. (6).

#### Quantification of Thrombospondin

TS levels were measured by ELISA in the sera of seven individuals aged 22–53. Blood was collected and placed in glass tubes at room temperature for 2 h and then at 4°C overnight. The value obtained was  $15.3 \pm 4.0 \mu\text{g/ml}$ . There was no obvious sex or age dependence of the serum content of TS. There was no interference from other serum proteins, since TS was accurately measured in bovine plasma-derived serum (which contains  $<1 \mu\text{g/ml}$  of TS) when the purified protein was deliberately added to it. These findings are in agreement with those in a recently published paper (10). A sample of fetal calf serum was analyzed for TS on nine different days and a value of  $31.6 \pm 4.6 \mu\text{g/ml}$  was obtained. No immunodetectable TS ( $<0.02 \mu\text{g/ml}$ ) was found in sera of swine, horses, chickens, or mice. This finding almost certainly reflects the species specificity of the antiserum used.

Quantification of TS levels in the media of cultured cells (Table I) indicated that both fibroblasts and smooth muscle

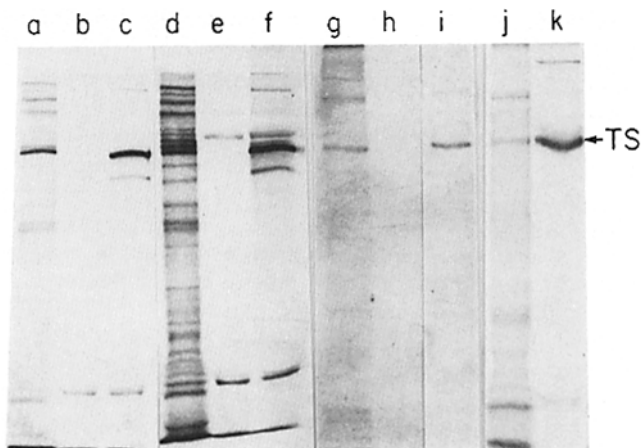


FIGURE 3 Autoradiograms of SDS slab gels. Anti-TS serum was used to precipitate proteins from lysates of cells labeled with  $^{35}\text{S}$ -cysteine. (Lanes *a*–*c*) Human fibroblasts labeled for 1 h: *a*, cell lysate; *b*, precipitate with preimmune serum; *c*, precipitate with anti-BAE cell TS serum. (Lanes *d*–*f*) Human fibroblasts labeled for 4 h: *d*, cell lysate; *e*, precipitate with preimmune serum; *f*, precipitate with anti-BAE cell TS serum. (Lanes *g*–*i*) Human smooth muscle cells labeled for 30 min: *g*, cell lysate; *h*, precipitate with preimmune serum; *i*, precipitate with anti-human platelet TS serum. (Lanes *j* and *k*) BAE cells labeled for 45 min: *j*, cell lysate; *k*, precipitate with anti-BAE cell TS serum. TS indicates the position of migration of a human platelet TS standard.

TABLE I  
Thrombospondin Levels in Cell Culture Medium\*

Cell type	Thrombospondin; ng/ $10^4$ cells	Cell density; cells/cm $^2 \times 10^{-4}$
Bovine aortic endothelial cells	$10 \pm 3.2 \dagger$	$8.9 \pm 0.6 \dagger$
Human fetal fibroblasts	$25 \pm 3.8$	$4.7 \pm 0.1$
Human smooth muscle cells	$71 \pm 13$	$4.1 \pm 0.5$

\* The results represent quantities accumulated during an 18-h period as measured by ELISA. Standard curves were generated using an anti-human platelet TS serum and either bovine or human TS.

$\dagger \pm$  SD ( $n = 3$ ).

cells secrete TS at higher levels than do endothelial cells. Preliminary experiments indicate that the concentration of secreted TS for all three cell types varies inversely with cell density.

## DISCUSSION

Thrombospondin, a high molecular weight glycoprotein of unknown function, was first identified in platelets (1-3) and a very similar and possibly identical protein was then shown to be synthesized by bovine aortic (5, 6) and human umbilical vein (11) endothelial cells. A variety of other endothelial cells also synthesize the protein (12). The presence of thrombospondin in both endothelial cells and platelets suggested the possibility that the protein might function in activities of a vascular nature (6, 11). Indeed, evidence has recently been presented in support of a role for TS as an endogenous lectin in the aggregation of human platelets (4), possibly by interaction with surface membrane-bound fibrinogen (4, 13). The findings reported here, which show that fibroblasts and smooth muscle cells also synthesize and secrete TS, oblige us to consider a broader function for this protein. This is also suggested by the presence of the immunoreactive TS in the extracellular matrix of cultured cells (Fig. 2).

Although it was difficult to purify TS from either platelets or endothelial cells and to obtain monospecific antisera, the following evidence supports our contention that we have identified TS both by immunofluorescence and by immunoprecipitation. (a) Both the absorbed anti-BAE cell TS and the anti-human platelet TS sera reacted only with TS and not with any of many other possible antigens as tested by ELISA. (b) Immunofluorescence patterns were the same with antisera prepared to antigens derived from very different sources (endothelial cells and platelets) and therefore likely to be contaminated by very different proteins; specific immunofluorescence was inhibited by preabsorption of antisera with purified TS. (c) Immunoprecipitation of radio-labeled proteins from three different cell types with purified immune reagents yielded essentially a single band identified as TS by SDS PAGE.

The quantitative ELISA for TS that we have developed can detect the protein at levels as low as 10 ng/ml, is highly reproducible in the range of 25-500 ng/ml and is not interfered with by other proteins. These characteristics are very similar to those of the radioimmunoassay recently developed by Saglio and Slayter (10) and the human serum TS level reported by these workers,  $15.7 \pm 6.2 \mu\text{g/ml}$ , is essentially the same as that found by us. These values, however, differ from that found by Mosher et al. (11) who have developed a radioimmunoassay that uses protein A-bearing Staphylococci "armed" with anti-TS IgG. These workers reported a human serum level of  $65 \pm 23 \mu\text{g/ml}$  and measured levels of TS secreted by human

umbilical vein endothelial cells in the range of  $490 \pm 40 \text{ ng}/10^4 \text{ cells}/24 \text{ h}$ . These values are likely to be overestimates and may have resulted from the limited sensitivity of the method, which was reported to be 700 ng/ml (11).

The presence of TS in both endothelial cells and platelets was compatible with the possibility that endothelial cells represented the source of platelet TS. Uptake from plasma may be a source for platelet fibronectin, although the issue remains to be settled (9). However, the low plasma TS level, 20-300 ng/ml (10), makes uptake from plasma less likely for TS. We have recently demonstrated TS in megacaryocytes by immunofluorescence examination of human bone marrow spreads (unpublished observations). The synthesis of TS may therefore be shared by a wide variety of cells.

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## REFERENCES

1. 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.
2. 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.
3. Phillips, D. R., L. K. Jennings, and H. R. Prasanna. 1980.  $\text{Ca}^{++}$ -mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. *J. Biol. Chem.* 255:11629-11632.
4. Jaffe, E. A., L. K. Leung, R. L. Nachman, R. I. Levin, and D. E. Mosher. 1982. Thrombospondin is the endogenous lectin of human platelets. *Nature (Lond.)* 295:246-248.
5. Sage, H., E. Crouch, and P. Bornstein. 1979. Collagen synthesis by bovine aortic endothelial cells in culture. *Biochemistry* 18:5433-5442.
6. 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.
7. Lawler, J. W., and H. S. Slayter. 1981. The release of heparin-binding peptides from platelet thrombospondin by proteolytic action of thrombin, plasmin, and trypsin. *Thromb. Res.* 22:267-279.
8. Sage, H., and P. Bornstein. 1982. Preparation and characterization of procollagens and procollagen-collagen intermediates. *Methods Enzymol.* 82A:96-127.
9. Hynes, R. O. 1981. Fibronectin and its relation to cellular structure and behavior. *In Cell Biology of Extracellular Matrix*. E. D. Hay, editor. Plenum Press, New York. 295-334.
10. Saglio, S. D., and H. S. Slayter. 1982. Use of a radioimmunoassay to quantify thrombospondin. *Blood* 59:162-166.
11. Mosher, D. F., M. J. Doyle, and E. A. Jaffe. 1982. Synthesis and secretion of thrombospondin by cultured human endothelial cells. *J. Cell Biol.* 93:343-348.
12. Sage, H., P. Pritzl, and P. Bornstein. 1981. Secretory phenotypes of endothelial cells in culture: comparison of aortic, venous, capillary, and corneal endothelium. *Arteriosclerosis* 1:427-442.
13. 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.)* 289:688-690.