Thrombomodulin Is Found on Endothelium of Arteries, Veins, Capillaries, and Lymphatics, and on Syncytiotrophoblast of Human Placenta

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ABSTRACT We have used antibodies to human thrombomodulin isolated from placenta to investigate the distribution of this cofactor for protein C activation in human tissues. Thrombomodulin was found on endothelial cells of arteries, veins, capillaries, and lymphatics by immunocytochemical staining using an avidin–biotin peroxidase method. Thrombomodulin was not detected on sinusoidal lining cells of liver or on postcapillary high-endothelial venules of lymph node, although the latter contained another endothelial antigen, von Willebrand factor. Other cells noted to contain thrombomodulin antigen are those of the syncytiotrophoblast in placenta. The thrombomodulin in syncytiotrophoblast was primarily on the plasma membrane surface that forms the maternal blood sinus. Syncytiotrophoblast also stained with antibodies to von Willebrand factor, which implies that these cells have multiple endothelial functions. Thrombomodulin antigen was found in all organs studied, with the notable exception of brain.

Endothelium has the important function of maintaining blood fluidity. This is accomplished in part by the production of the icosanoid mediator PGI_2 in response to endothelial injury (2), thrombogenic stimuli such as thrombin (24), or inflammatory mediators such as histamine (1) and bradykinin (17). PGI_2 acts as a vasodilator and inhibits platelet activation (3). Recently, other endothelial factors that inhibit coagulation reactions have been described. These include heparin-like molecules on the cell surface that act as a cofactor for anti-thrombin III in inhibiting several coagulation factor proteases (13), and the cell surface protein, thrombomodulin, that acts as a cofactor for thrombin-catalyzed activation of protein C. Activated protein C inhibits coagulation by inactivating factors Va and VIIIa. Thrombomodulin was first isolated from detergent extracts of rabbit lung by affinity chromatography on thrombin–Sepharose (5–8). The protein was postulated to be of endothelial origin since perfusion of thrombin and protein C through rabbit vessels led to accelerated rates of protein C activation (6). A similar protein was thought to be present in humans since accelerated rates of thrombin-catalyzed protein C activation could also be demonstrated in the presence of intact human umbilical vein endothelial cells in culture (6), and antibodies directed against rabbit thrombomodulin partially inhibited protein C activation on human cells (15). The existence of thrombomodulin in human tissues has been recently established by the isolation of the protein from extracts of human placenta. Human thrombomodulin is similar but not identical to the rabbit protein (14, 20). Thus, human thrombomodulin activity is stimulated by coagulation factor Va and its isolated light chain. We have recently prepared antibodies against human thrombomodulin. Using these antibodies, we have studied the tissue distribution of thrombomodulin. Using an avidin–biotin immunoperoxidase method, we now report that thrombomodulin is found in most human endothelium including that of arteries, veins, capillaries, and lymphatics. Another cell type found to contain thrombomodulin is the placental syncytiotrophoblast. This cell has also been found to contain von Willebrand factor antigen, another marker of endothelium.

MATERIALS AND METHODS

Except when indicated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Human thrombin (16), human protein C (22), and anti-thrombin III (18) were isolated from human plasma as described. Anti-human von Willebrand factor rabbit IgG was purchased from Dako Corp. (Santa Barbara, CA). Nitrocellulose sheets were purchased from Bio-Rad Laboratories (Richmond, CA).

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Preparation of Anti-thrombomodulin IgG: Two male rabbits were immunized intradermally at 300 sites with 60 μg each of thrombomodulin in complete Freund's adjuvant by the method of Vaitukaitis (23). After 2 mo, potent antiserum was obtained. Anti-thrombomodulin IgG or control IgG was isolated using protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as follows: 5 ml of the anti-thrombomodulin serum or control serum was applied on a protein A-Sepharose column (1 × 5 cm) equilibrated with phosphate-buffered saline (PBS; 10 mM phosphate, 0.15 M NaCl, pH 7.4). After the column was washed with 50 ml of PBS, IgG was eluted with 0.1 M glycine, pH 3, and immediately dialyzed against the buffer with 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4. 0.05 pmol human thrombomodulin isolated from placenta was incubated with 0–10 μg of anti-thrombomodulin IgG for 15 min at 37°C, and then cofactor activity of the thrombomodulin was assayed in reaction mixtures containing 1.0 μM protein C and 40 nM thrombin in a total volume of 30 μl 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM $CaCl_2$, and 5 mg/ml bovine serum albumin (BSA). Protein C activation was terminated by addition of 40 U/ml hirudin and 350 μg/ml anti-thrombin III. The amount of activated protein C was assayed by measuring the rate of hydrolysis of 0.2 mM D-Phe-pipecolyl-Arg-*p*-nitroanilide (S2238, Kabi Diagnostica, Sweden).

Immunoblot of Thrombomodulin: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed by the method of Laemmli (11) using 4% acrylamide in the stacking and 10% acrylamide in the running gel. Samples of placental thrombomodulin were electrophoresed with 2-mercaptoethanol. Immunoblotting was performed by the method of Burnette (4). Anti-thrombomodulin serum was used at a 1:250 dilution, and detection was made using ^{125}I-protein A and autoradiography.

Cell Cultures and the Functional Assay of Thrombomodulin Activity: Primary cultures of human umbilical vein endothelial cells were prepared as previously described (10). One of the human choriocarcinoma cell lines, JAr, was provided by Dr. Irving Boime, Washington University, and the other, BeWo, was from American Type Culture Collection. These cells were cultured in plastic 30–100-mm petri dishes or 6–16-mm multi-well plates. Protein C activation was examined on these cells as previously described (15). The cultured cells were also used for immunochemical study.

Tissue Preparation: Tissues (excluding placenta) fixed in 10% formalin buffered with 0.1 M calcium acetate, pH 7.4, for a minimum of 24 h, were obtained from the autopsy pathology service. Placenta fixed in 10% formalin buffered with 0.15 M sodium acetate, pH 7.0, for a minimum of 24 h, was obtained from the surgical pathology service. Tissues were embedded in paraffin, and 6-μm-thick sections were cut and placed on microscope slides that had been cleaned with 10% potassium dichromate, 1% sulfuric acid solution, and coated with 0.25% gelatin containing 0.05% chrome alum as an adhesive (19). Sections were then dried at 60°C and stored at room temperature.

Immunocytochemistry: Sections were de-waxed in 100% xylene for 10 min with one change, and rehydrated in 100% isopropyl alcohol for 10 min with one change, 70% isopropyl alcohol for 5 min, and PBS for 15 min with two changes. Rabbit anti-thrombomodulin serum and control rabbit serum were diluted 1:1,000; rabbit anti-thrombomodulin IgG and normal control rabbit IgG were diluted to 10 μg/ml; and rabbit anti-von Willebrand factor IgG and its control rabbit IgG were diluted to 20 μg/ml. All dilutions were made in 10% normal human serum in PBS. Diluted reagents were centrifuged at 20,000 *g* for 20 min to remove any large aggregates. Normal human serum provided optimal blocking of nonspecific staining compared with normal goat serum or no serum, with no impairment of specific staining. For anti-von Willebrand factor staining, sections were preincubated with 0.025% trypsin (KC Biological Inc., Lenexa, KS) in 0.1% $CaCl_2$, 0.05 M Tris-HCl, pH 7.6 for 30 min at 37°C. This was necessary in order to expose von Willebrand factor antigenic determinants in formalin-fixed tissue. Thrombomodulin staining was unaffected by the trypsin preincubation. These sections were then washed in PBS for 15 min with two changes. All sections were incubated with either antiserum, immune IgG, or control preparations for 16–20 h at 4°C in a humid atmosphere, gently washed in PBS for 15 min with two changes, and then incubated with biotinylated affinity-purified goat anti-rabbit immunoglobulin (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature. The latter reagent was diluted to the recommended concentration (45 μl in 10 ml) in 10% normal human serum in PBS. Sections were again gently washed in PBS for 15 min with two changes, incubated with avidin-biotinylated horseradish peroxidase complex (ABC reagent, Vector Laboratories, Inc.) at the recommended concentration (90 μl avidin and 90 μl biotinylated horseradish peroxidase in 10 ml PBS, allowed to incubate for 30 min before use) for 45 min, washed in PBS for 5 min and 100 mM Tris-HCl buffer, pH 7.2, for 10 min with one change. After incubation in 0.05% diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in Tris-HCl buffer, pH 7.2, for 5 min, sections were washed in the Tris-HCl buffer for 10 min with one change and in deionized water for 5 min. Sections were then counter-stained in 0.5% Harris hematoxylin (21) for 2 min, differentiated by quick dipping in acid alcohol (1% HCl, 70% ethanol), and blued in gently running tap water for 5 min. Sections were finally dehydrated in 70% isopropyl alcohol for 5 min, 100% isopropyl alcohol for 10 min with one change, and 100% xylene for 10 min with one change, and mounted in Permount. Cultured cells of monolayers were also fixed and stained as described above.

Specific Absorption of Antibodies: The specificity of anti-thrombomodulin IgG and anti-von Willebrand factor IgG was established by absorption with the respective purified antigens. Anti-thrombomodulin IgG (20 μg/ml) was incubated with 30 μg thrombomodulin/ml (threefold excess) isolated from human placenta for 24 h at 4°C and then centrifuged at 20,000 *g* for 30 min and the supernatant collected and used for immunohistochemistry. Anti-von Willebrand factor IgG (20 μg/ml) was incubated with 25 μg von Willebrand factor/ml as described above. The concentration of von Willebrand factor was five times that required to neutralize the antibody. The protein was homogeneous as determined by SDS PAGE and was a gift from Drs. Evan Sadler (Washington University) and Earl Davie (University of Washington). The respective antisera were also absorbed with human serum albumin as a control. Sections of human placenta were stained using these absorbed IgG solutions as described above.

RESULTS

Characterization of Polyclonal Anti-Thrombomodulin IgG

The polyclonal rabbit anti-thrombomodulin IgG used in these experiments binds thrombomodulin as shown by immunoblotting (Fig. 1). We assayed the affinity of the antibody for thrombomodulin by measuring its ability to block protein C activating cofactor activity as described in Materials and Methods. The apparent K_d of the IgG was 22 nM with complete inactivation of thrombomodulin at concentrations of 33 μg/ml (320 nM).

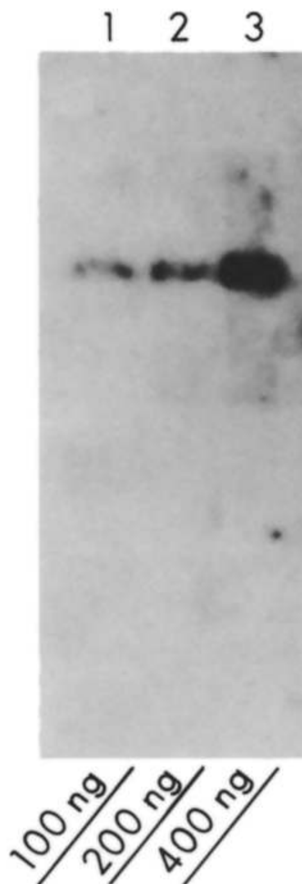


FIGURE 1 Immunoblot of thrombomodulin from placenta. The immunoblot was performed as described in Materials and Methods. Lane *1*, 100 ng; lane *2*, 200 ng; lane *3*, 400 ng of thrombomodulin.

Immunohistochemical Localization of Thrombomodulin

In all the studies reported here, the anti–thrombomodulin IgG was incubated with sections at 10–20 μg/ml that gave maximal staining as determined in preliminary experiments using sections of placenta. The IgG absorbed with purified placental thrombomodulin, as described in Materials and Methods, gave no staining of sections from placenta, indicating that the antigen detected was thrombomodulin.

We examined a variety of human tissues for the presence of thrombomodulin antigen by an avidin-biotin peroxidase method. We found that endothelial cells were positive for thrombomodulin in most tissues examined as summarized in Table I. Endothelium stained in a thin continuous line suggesting a surface localization, although we cannot be sure of the intracellular distribution of the antigen with the current techniques. Endothelium in veins, arteries, and capillaries all stained. Cultured endothelial cells from umbilical cord vein also stained (data not shown). Other cells that contained thrombomodulin antigen were those of the syncytiotrophoblast in placenta (see below). The pattern of thrombomodulin staining of endothelium is illustrated in Fig. 2. Coronary artery, and an arteriole and vein from lung are depicted. Staining controls using nonimmune rabbit IgG were negative (Fig. 2, *a*, *c*, and *e*). The stain observed in erythrocytes and granulocytes is due to endogenous peroxidase activity. In several tissues, including the gastrointestinal tract and lymph nodes, we noted that lymphatic endothelium contains thrombomodulin. This is apparent in the section of lymph node shown in Fig. 3, *a* and *b*. There was prominent staining of the subcapsular lymphatic endothelium (Fig. 3*b*). Some types of endothelium apparently do not contain thrombomodulin. These include hepatic sinusoids, and most strikingly, brain. We examined multiple sections from two brains and found only scant thrombomodulin staining. The high-endothelial venules of lymph node also appear to lack thrombomodulin antigen as shown in Fig. 4. Adjacent sections were stained after exposure to either anti–thrombomodulin (Fig. 4*c*) or anti–von Willebrand factor IgG (Fig. 4*b*). It is apparent that lymphatic endothelium (solid arrows, Fig. 4*a*, clear arrows, Fig. 4*b*) contains thrombomodulin but not von Willebrand factor. The high-endothelial venules (clear arrows, Fig. 4*a*, solid arrows, Fig. 4*b*) show the opposite pattern of staining; that is, thrombomodulin is absent and von Willebrand factor is present.

Thrombomodulin and von Willebrand Factor Antigen in Placenta

We previously observed that placenta has a high content of thrombomodulin based upon the protein C activating cofactor activity of crude extracts (20). The immunohistochemistry of this organ gave a surprising result. The most prominent staining was observed in syncytiotrophoblast as shown in Fig. 5. There was also staining of capillary endothelium. In some areas it appeared that stromal cells stained with anti-thrombomodulin. We are uncertain as to the significance of this finding; possibly these are endothelial precursors. The staining of the syncytiotrophoblast was primarily linear along the cell surface exposed to maternal blood. While these cells are known to be hormone producing, it is not surprising that they also contain thrombomodulin since, like endothelium, they are exposed to blood. In view of this finding, we also stained sections of placenta using an anti–human von Willebrand factor rabbit IgG. This antigen is a marker of endothelium and is also present on syncytiotrophoblast as shown in Fig. 6. However, two cell lines of human chorionic carcinoma, BeWo and JAr, had neither thrombomodulin activity nor von Willebrand factor antigen. That staining with the anti-von Willebrand factor IgG was specific for this antigen was established by showing that sections of human placenta stained with von Willebrand factor–absorbed IgG showed no endothelial or syncytiotrophoblast staining.

TABLE I. *Immunoperoxidase Localization of Thrombomodulin*

	Other findings
Organs displaying endothelial thrombomodulin in arteries, veins, and capillaries	
Lung	
Heart	Endocardium (+)
Liver	Sinusoids (−)
Kidney	Glomerular capillary (+)
Pancreas	
Thyroid	
Colon	Lymphatics (+)
Spleen	
Adrenal	
Lymph node	Lymphatic (+) Postcapillary venule (−)
Aorta	Endothelium (+)
Vena cava	Endothelium (+)
Spleen	
Skeletal muscle	
Duodenum	Lymphatics (+)
Stomach	Lymphatics (+)
Esophagus	
Placenta	Syncytiotrophoblast (+)
Organ with trace or absent thrombomodulin in endothelium	
Brain	Occasional positive vein or capillary

DISCUSSION

In the present investigation, we have established that thrombomodulin is widely distributed on vascular endothelium. This localization well serves its putative function as a cofactor for protein C activation. Activated protein C is a physiological anticoagulant that inactivates factors Va and VIIIa and thereby maintains blood fluidity. That lymphatic endothelium also contains thrombomodulin may also be of physiological importance. Lymph contains coagulation factors and can clot (12). Therefore, thrombomodulin in lymph may serve to ensure its fluidity. Similarly, the syncytiotrophoblast of placenta is exposed to relatively stagnant blood, and thrombomodulin in this site may be important in blocking coagulation in maternal blood sinuses. The finding that these placental cells also contain another endothelial-specific antigen, von Willebrand factor, is further evidence that these cells are endothelial despite their well known endocrine function. If thrombomodulin is important in the placenta, disorders in its function might lead to diseases such as recurrent abortion. We plan to screen the plasma of patients with recurrent abortions for inhibitors of thrombomodulin. Alternatively,

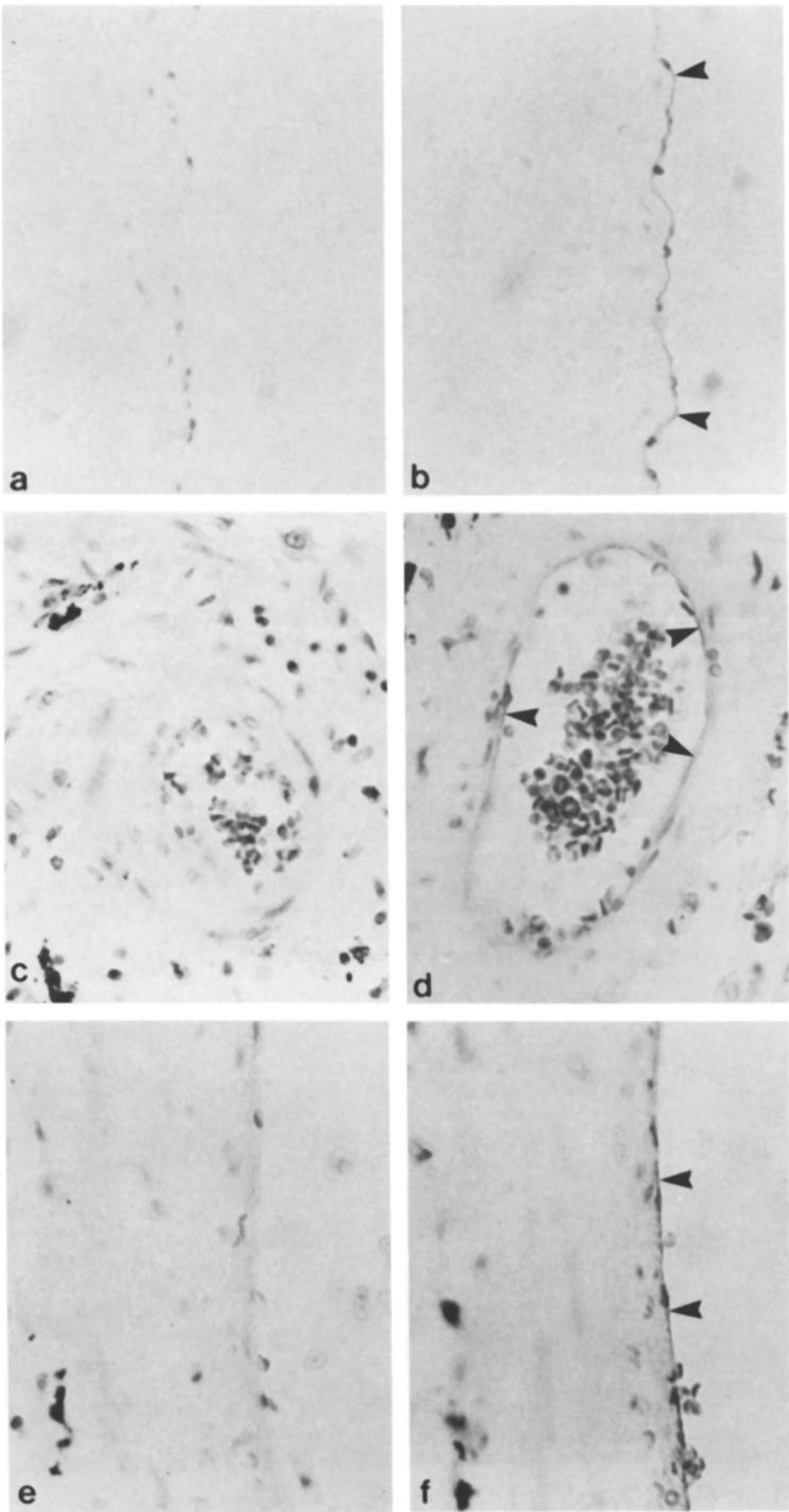
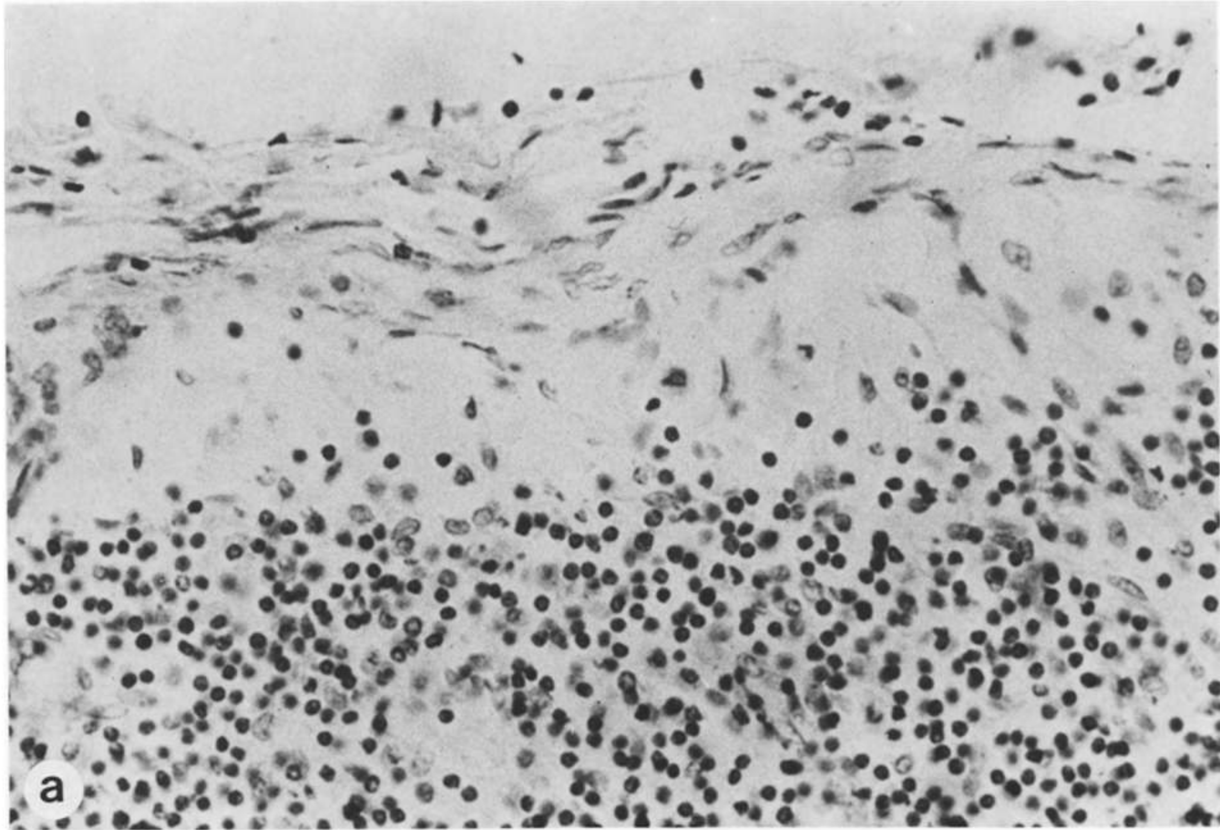


FIGURE 2 Immunological localization of thrombomodulin by an avidin-biotin immunoperoxidase method. (*a*) Coronary artery with control IgG; (*b*) coronary artery with anti-thrombomodulin IgG; (*c*) lung arteriole with control IgG; (*d*) lung arteriole with anti-thrombomodulin IgG; (*e*) lung vein with control IgG; (*f*) lung vein with anti-thrombomodulin IgG. Positive staining of endothelium is shown by arrows. × 500.



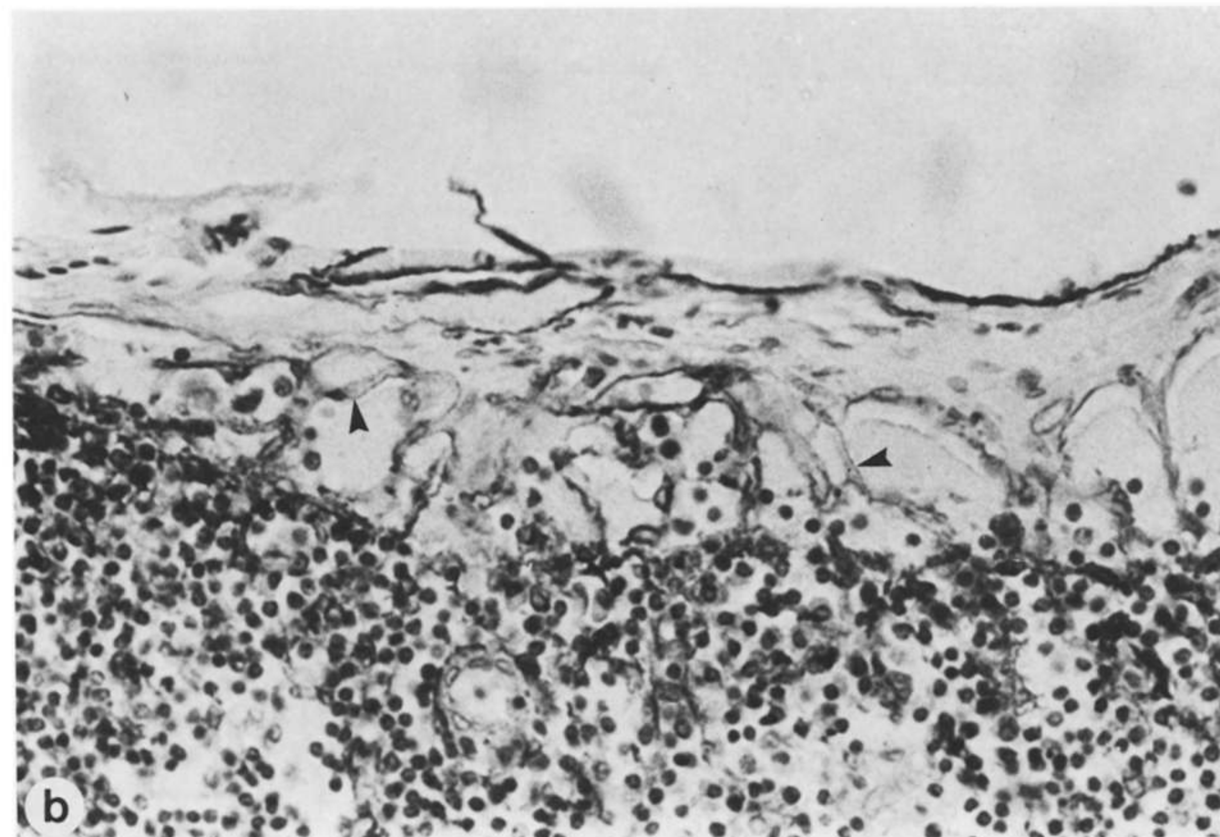
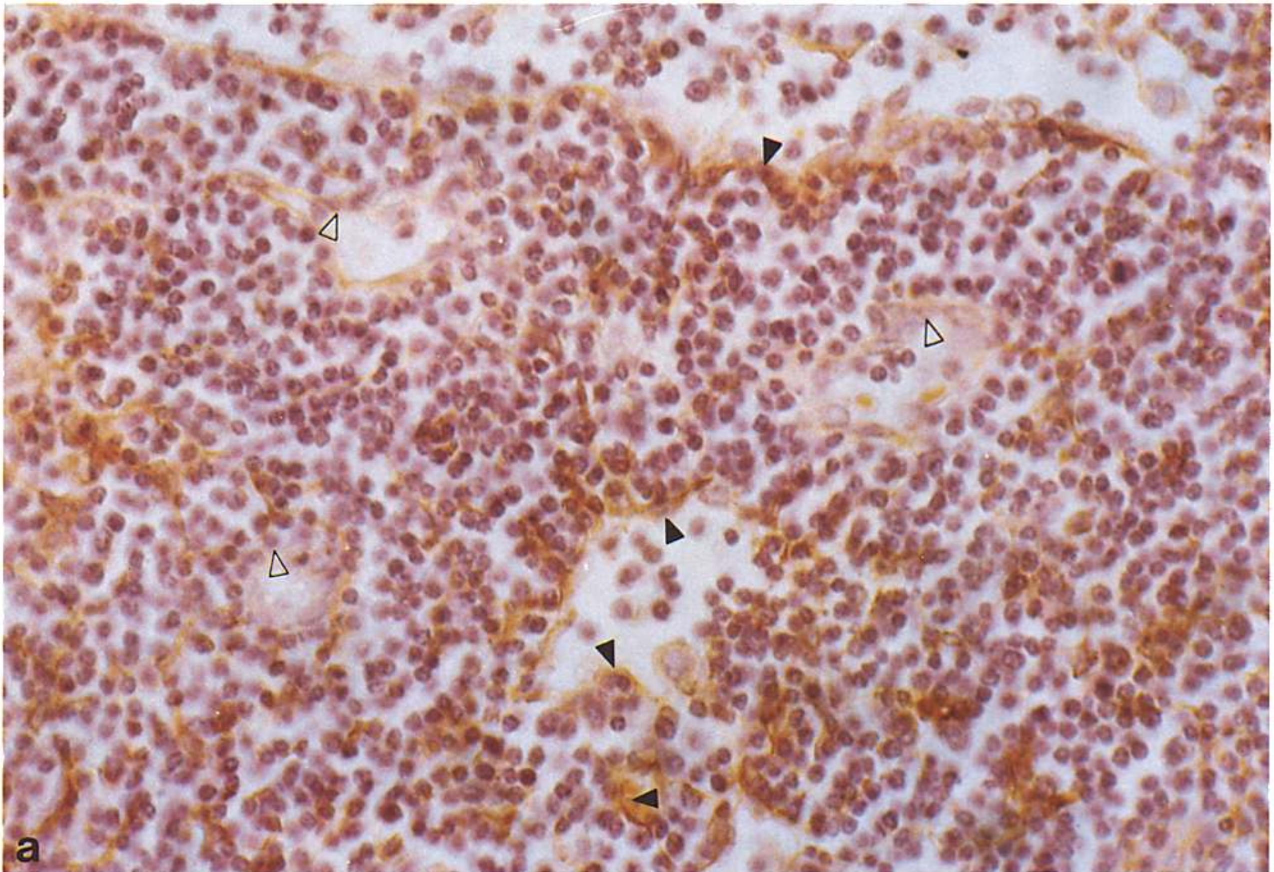


FIGURE 3 Immunoperoxidase staining of subcapsular lymphatic sinus of lymph node. (*a*) Control IgG; (*b*) anti-thrombomodulin IgG. The endothelium of the lymphatic sinus and of its trabecular meshwork (arrows) is stained. × 500.



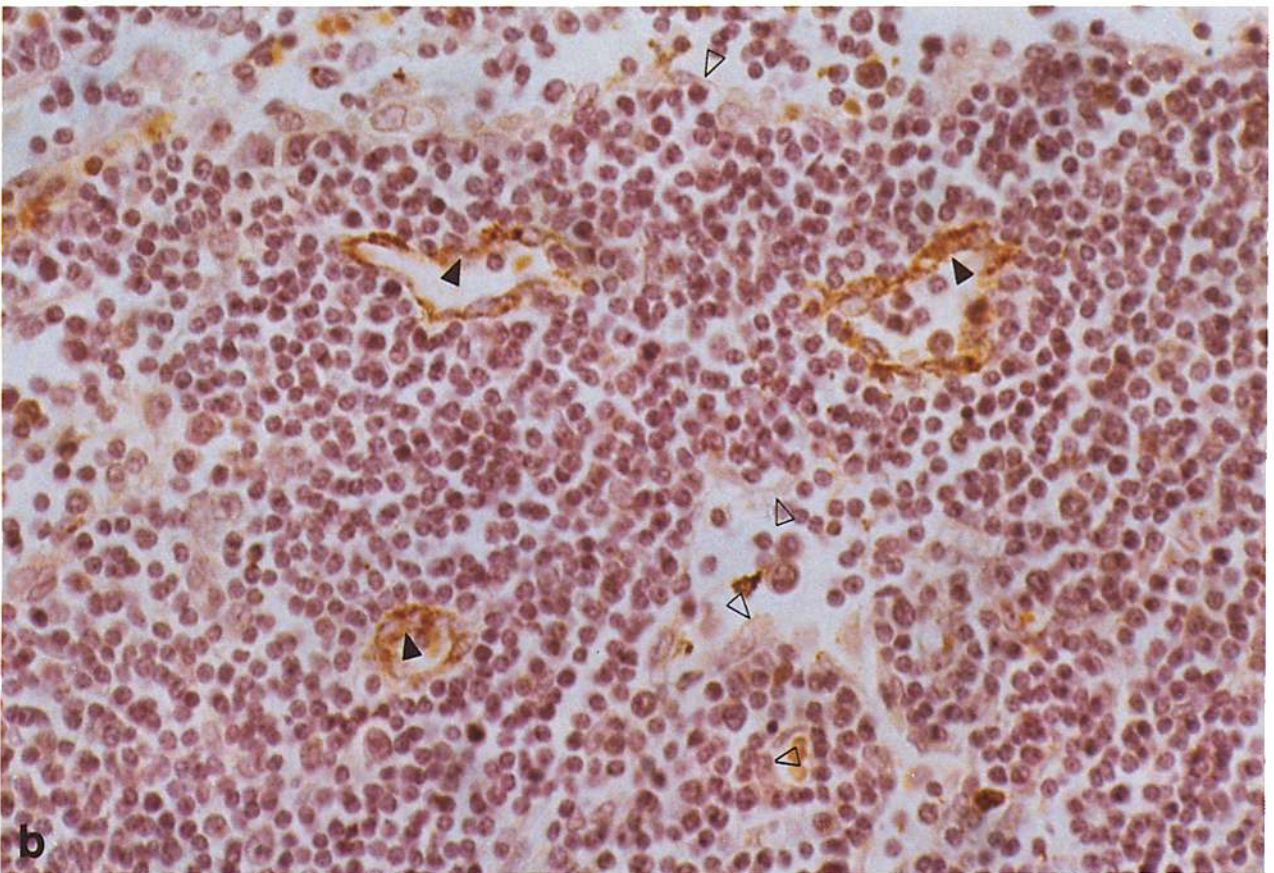
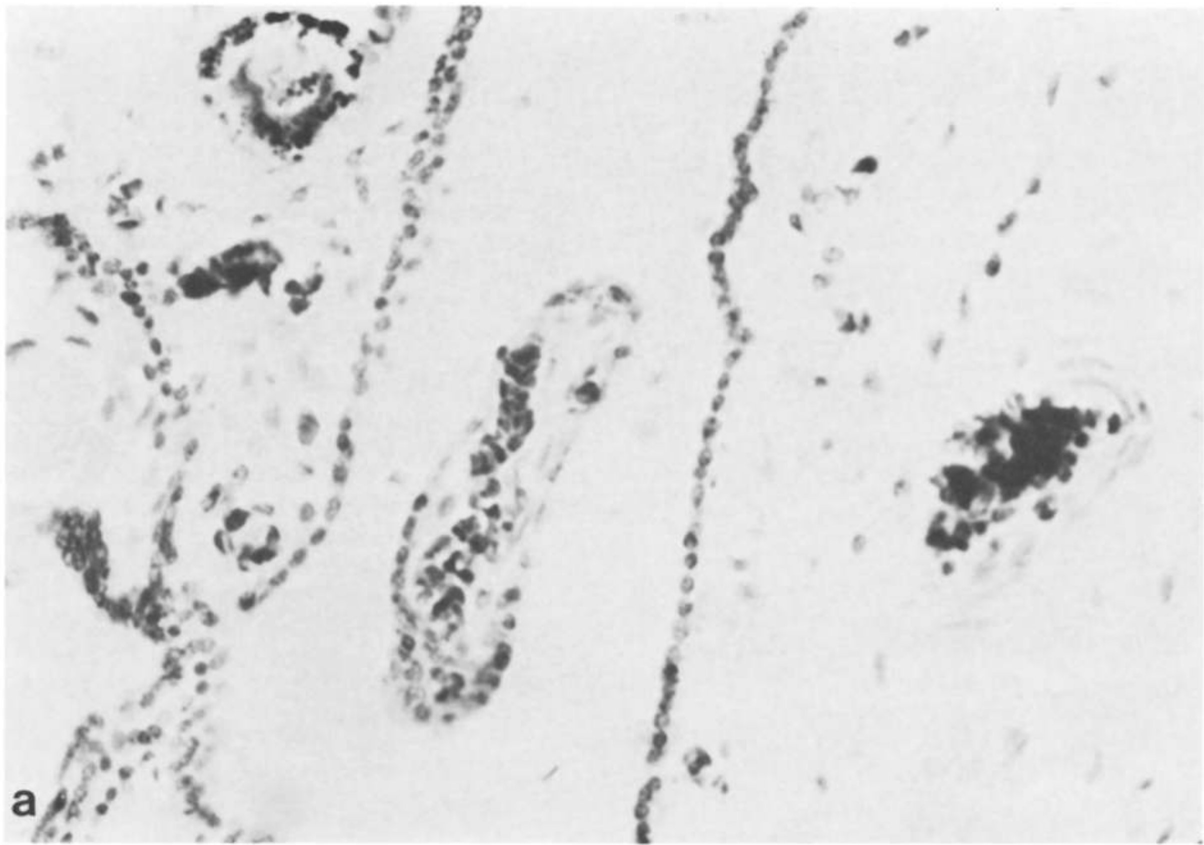


FIGURE 4 Immunoperoxidase staining of postcapillary high-endothelial venules of lymph node. (*a*) Anti-thrombomodulin IgG; (*b*) anti-von Willebrand factor IgG. With anti-thrombomodulin IgG (*a*) there is a positive staining of endothelium (solid arrows) but not of endothelium from postcapillary high-endothelial venules (open arrows). With anti-von Willebrand factor IgG (*b*) there is positive staining of postcapillary high endothelial venules (solid arrows) but not of lymphatic endothelium (open arrows). × 500.



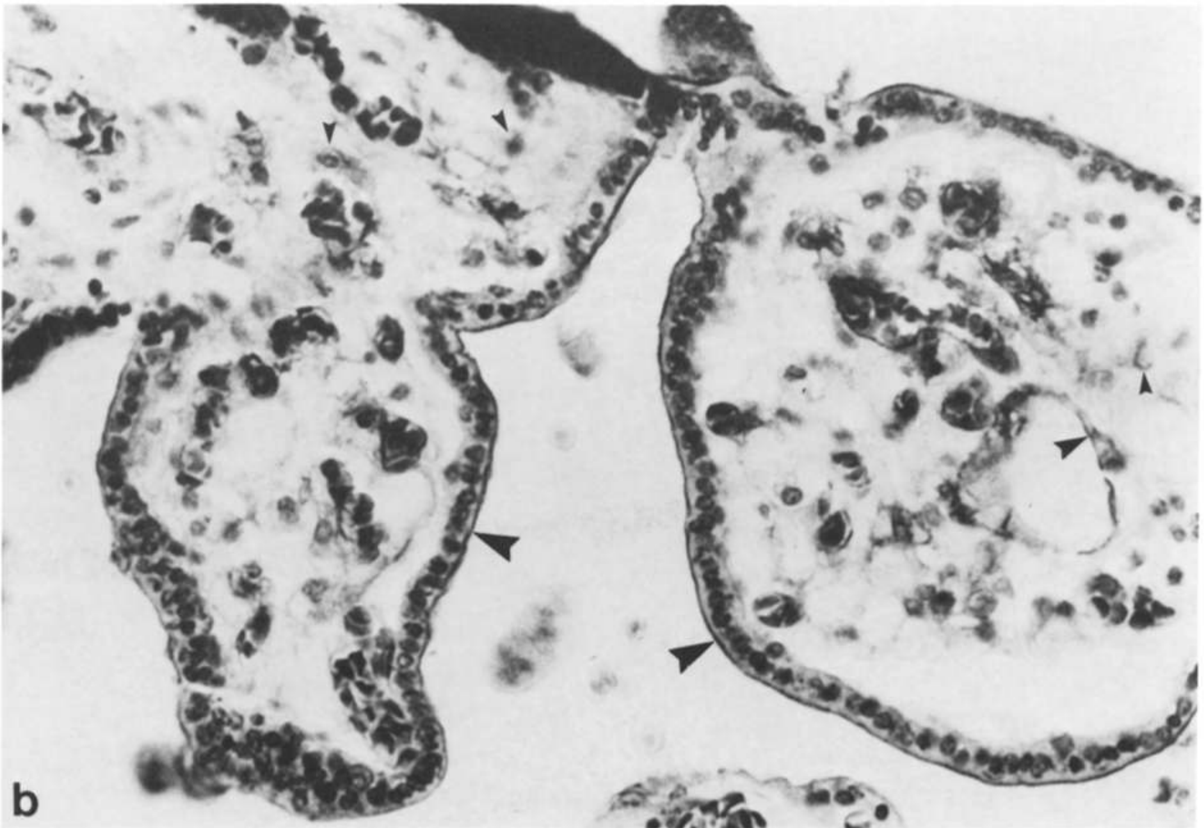
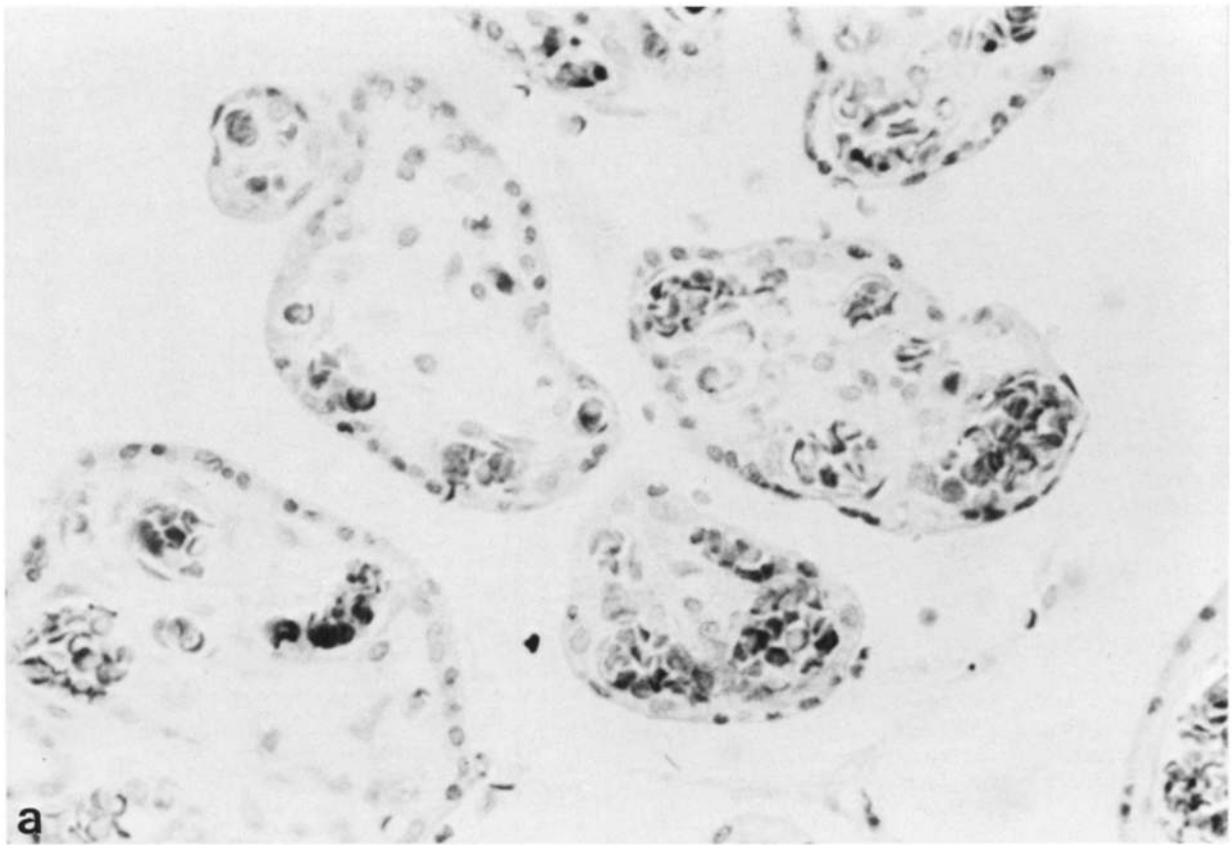


FIGURE 5 Immunoperoxidase staining of syncytiotrophoblast in placenta. (*a*) Control IgG; (*b*) anti-thrombomodulin IgG. The staining is prominent on syncytiotrophoblast as shown by the large arrows in *b*. Capillary endothelium (mid-size arrows) and stromal cells (small arrows) stained (*b*). × 500.



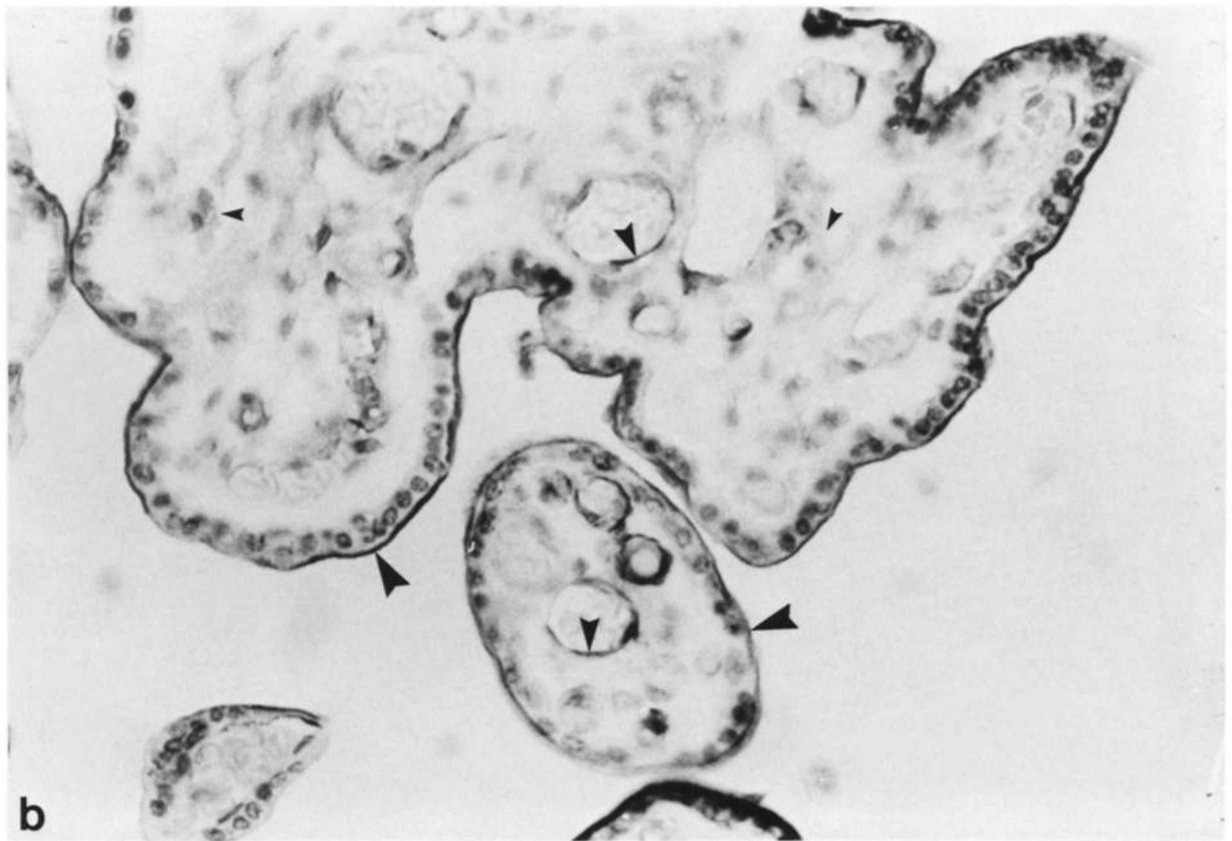


FIGURE 6 Presence of von Willebrand factor antigen on syncytiotrophoblast. (*a*) Control IgG; (*b*) anti-von Willebrand factor IgG. × 500. The staining pattern is similar to that with anti-thrombomodulin IgG. × 500.

we can use a radioimmunoassay to measure thrombomodulin antigen in tissue from spontaneous abortions.

We also demonstrated that thrombomodulin is not present on the sinusoidal lining cells of liver or on postcapillary high-endothelial venules of lymph node. In contrast, von Willebrand factor antigen is on postcapillary venules but not lymphatic endothelium of lymph node. This striking observation is difficult to explain, but may somehow relate to the function of the high endothelium. Lymphocytes migrate from the blood into lymph nodes through these specialized endothelial cells (9).

We have no explanation for the finding that brain appears deficient in thrombomodulin. We do not believe that the absence of histochemical identification of thrombomodulin in brain results from an artifact of our procedure. In experiments where we used a radioimmunoassay of crude tissue homogenates of brain and other organs to estimate total thrombomodulin content, we found no thrombomodulin in brain tissue, whereas it was present in many other organs assayed. Thrombomodulin was found in homogenates of large arteries that supply the brain (carotid, basilar; unpublished observation).

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REFERENCES

1. Baenziger, N. L., L. E. Force, and P. R. Becherer. 1980. Histamine stimulates prostacyclin synthesis in cultured human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* 92:1435–1440.
2. Baenziger, N. L., M. J. Dillender, and P. W. Majerus. 1977. Cultured human skin fibroblasts and arterial cells produce a labile platelet-inhibitory prostaglandin. *Biochem. Biophys. Res. Commun.* 78:294–301.
3. Bunting, S., R. Gryglewski, S. Moncada, and J. R. Vane. 1976. Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coelic arteries and inhibits platelet aggregation. *Prostaglandins.* 12:897–913.
4. Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.
5. Esmon, C. T., N. L. Esmon, and K. W. Harris. 1982. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J. Biol. Chem.* 257:7944–7947.
6. Esmon, C. T., and W. G. Owen. 1981. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. 1981. *Proc. Natl. Acad. Sci. USA.* 78:2249–2252.
7. Esmon, N. L., W. G. Owen, and C. T. Esmon. 1982. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. *J. Biol. Chem.* 257:859–864.
8. Esmon, N. L., R. C. Carroll, and C. T. Esmon. 1983. Thrombomodulin blocks the ability of thrombin to activate platelets. *J. Biol. Chem.* 258:12238–12242.
9. Farr, A. G., and P. P. H. DeBruyn. 1975. The mode of lymphocyte migration through postcapillary venule endothelium in lymph node. *Am. J. Anat.* 143:59–92.
10. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745–2756.
11. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
12. Lindena, J., and I. Trautschold. 1983. Enzymes in lymph: a review. *J. Clin. Chem. Clin. Biochem.* 21:327–346.
13. Marcum, J. A., L. Fritze, S. J. Galli, G. Karp., and R. D. Rosenberg. 1983. Microvascular heparin-like species with anticoagulant activity. *Am. J. Physiol.* 245:H725–H733.
14. Maruyama, I., H. H. Salem, H. Ishii, and P. W. Majerus. 1985. Human thrombomodulin is not an efficient inhibitor of the procoagulant activity of thrombin. *J. Clin. Invest.* 75:987–991.
15. Maruyama, I., H. H. Salem, and P. W. Majerus. 1984. Coagulation factor Va binds to human umbilical vein endothelial cells and accelerates protein C activation. *J. Clin. Invest.* 74:224–230.
16. Miletich, J. P., C. M. Jackson, and P. W. Majerus. 1978. Properties of the factor Xa binding site on human platelets. *J. Biol. Chem.* 253:6908–6916.
17. Needleman, P., S. D. Bronson, A. Wyche, M. Sivakoff, and K. C. Nicokou. 1978. Cardiac and renal prostaglandin I_2. *J. Clin. Invest.* 61:839–849.
18. Owen, W. G. 1975. Evidence for the formation of an ester between thrombin and heparin cofactor. *Biochem. Biophys. Acta.* 405:380–387.
19. Pappas, P. W. 1971. The use of chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. *Stain Technol.* 46:121–124.
20. Salem, H. H., I. Maruyama, H. Ishii, and P. W. Majerus. 1984. Isolation and characterization of thrombomodulin from human placenta. *J. Biol. Chem.* 259:12246–12251.
21. Stevens, A. 1982. The haematoxylins. *In* Theory and Practice of Histological Techniques. J. D. Bancroft and A. Stevens, editors. Churchill Livingston Inc., New York. Second ed. 109–121.
22. Suzuki, K., J. Stenflo, B. Dahlback, and G. Teodorsson. 1983. Inactivation of human coagulation factor V by activated protein C. *J. Biol. Chem.* 258:1914–1920.
23. Vaitukaitis, J. L. 1981. Production of antisera with small doses of immunogen: multiple intradermal injection. *Methods Enzymol.* 73:46–52.
24. Weksler, B. B., C. W. Ley, and E. A. Jaffe. 1978. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and ionophore A23187. *J. Clin. Invest.* 62:923–930.