Binding of thrombin to monolayer cultures of human umbilical vein endothelium is studied. Binding is measured as inhibition by unlabeled ligand of the binding of 131I-thrombin to the cells. Radioactivity bound to cultures at equilibrium is measured after draining but not washing the cells. To correct for unremoved supernatant, 131I-albumin is included as a second label in the medium. Equilibrium between bound and free thrombin is attained within 1 min, and Scatchard analysis indicates a population of approximately 3 x 10^5 sites/cell with a dissociation constant of 10^{-10} M, and a larger population with a dissociation constant greater than 10^{-9} M. The two populations of sites are also indicated by a biphasic dissociation of bound label. Thrombin inactivated with diisopropyl fluorophosphate binds to the same receptor, with an affinity similar to that of active thrombin. Binding is unaffected by albumin (an acidic protein) and cytochrome c (a basic protein). Cultures of umbilical cord smooth muscle and fibroblasts bind thrombin at least 100 times more weakly than endothelium, and no binding to erythrocytes or a monolayer culture of mouse neuroblastoma is detected.

Primary, spontaneous hemostasis results from a complex series of biological events which occur in immediate response to blood vessel injury. This response involves a triad of components: the damaged blood vessel, the blood platelets, and the plasma proteins involved in the blood coagulation process. It is generally accepted that the formation of thrombin represents a key development during normal hemostasis, and in thrombosis. In addition to its roles as a zymogen activator and regulator in the coagulation cascade, thrombin binds to the surface of platelets (1-7), and has the capacity to cause platelets to aggregate and to release some of their internal components including serotonin and adenosine nucleotides. Although binding of catalytically active thrombin to the platelet membrane is required for activation of the platelet by thrombin, binding per se does not require an active catalytic center and thus is physically discrete from subsequent events. Also of interest is the possibility that the vascular endothelium may interact with thrombin or other activated coagulation components. Such interactions might be components of a metabolic or protective mechanism, or conversely, might act to localize thrombin at the blood interface where it could exert a direct enzymatic effect. The present study addresses these questions, with standard approaches for the detection of receptor sites for human thrombin on monolayer cultures of human endothelial cells obtained from umbilical veins. The data presented demonstrate that human thrombin is bound reversibly and specifically by the plasma membrane of human endothelial cells.

MATERIALS AND METHODS

Sulfopropyl Sephadex, bovine serum albumin (essentially fatty acid-free), cytochrome c, and sodium dodecyl sulfate, were obtained from Sigma Chemical Co., St. Louis, MO. Female New Zealand White rabbits were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. Carrier-free 131I (as KI) was obtained from Amersham-Searle and 125I-albumin (Albunolclot 131) was the product of Squibb, Inc. Antibodies to human α2-macroglobulin was obtained from Miles Laboratories, Elkhart, Ind., and an antiserum to human von Willebrand Factor (Factor VIII related antigen) was prepared by the method of Zimmerman (8). Heparin (Panheparin) was obtained from Abbott Laboratories, North Chicago. III. Crude collagenase (sterile) was obtained from Worthington Biochemical Corp., Freehold, N. J.

Human thrombin was prepared as described previously (9). The product was homogeneous by gel electrophoresis in dodecyl sulfate. For some experiments, thrombin was inactivated at pH 7.5 with 1.0 mM DFP. Thrombin was labeled with 131I by the lactoperoxidase method of Thorell and Johansson (10). The labeled protein was separated from excess 131I and lactoperoxidase reagents by chromatography on SP-Sephadex. For each experiment, 10 to 15 µg of thrombin were labeled. The reaction was quenched and the mixture was diluted with a single step of 0.6 M NaCl, the 131I-thrombin was eluted in a peak with a thrombin concentration sufficient to quantitate clotting activity. The product had a specific radioactivity of 9 x 10^9 cpm/mg of protein. The final yield of clotting activity averaged 20%, whether or not the protein was subjected to radiiodination, with virtually all of the loss occurring during chromatography. When analyzed by gel electrophoresis in dodecyl sulfate, 95% of the 131I appeared in a peak with a migration distance identical with that of unlabeled thrombin. A small (<5%) peak of β-thrombin was detected, and no radioactivity was detected in the region of the tracking dye. The enzymatic activity of the product had a half-life in the frozen state of 19 to 34 h, a time during which unlabeled thrombin was stable. Such instability, presumably due to chemical damage, was reported also by Tolleson et al. (11). Therefore, thrombin was labeled routinely within 2 h of the beginning of an experiment.

Preparation of Cells—Primary cultures of human endothelial cells were prepared from human umbilical veins according to a slight modification of the method of Jeffrey et al. (11). Umbilical cords were collected in Medium M-199 with 10% fetal calf serum. The umbilical vein was dilated and rinsed with 60 to 100 ml of phosphate-buffered saline. After clamping one end with a hemostat, the vein was filled with 0.2 to 0.3% collagenase. After 6 min, the cell suspension was collected in Medium M-199 with 10% fetal calf serum. The cell suspension was resuspended in fresh Medium M-199 with 20% fetal calf serum, and the vessel was rinsed with an additional 60 ml of Medium M-199. The cell pellet resulting from centrifugation of the combined suspension was resuspended in fresh Medium M-199 with 20% fetal calf serum and seeded in Petri dishes (35 x 10 mm) at a density of 900,000 cells/dish. Cultures were incubated in a 5% CO2 atmosphere at 37°C. After 24 h, the monolayers were first washed with fresh...
Medium in order to remove excess red blood cells and were layered with a fresh 2-ml portion of Medium M-199 with 20% fetal calf serum. Confluent primary human endothelial cell monolayers of 7 to 8 x 10^4 cells were used for the binding studies 3 to 5 days after the Petri dishes were seeded. Confluent cultures of human umbilical vein fibroblasts and smooth muscle cells were prepared as described previously (12). Endothelial, fibroblasts, and smooth muscle cells were differentiated by phase-contrast microscopy and their ultrastructure was monitored by electron microscopy. Cell identification was consistent with published criteria for these types of cells (13–15).

Approximately 15 min before the beginning of the experiment, the cultures were washed three times with 1 ml/wash of Hank's Balanced Salt Solution (16), modified by substituting 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, for NaHCO₃. All subsequent steps were performed with the modified Hank's solution.

**Binding of Thrombin to Endothelial Cells**—Binding was evaluated by two methods.

For preliminary experiments, 1 ml of modified Hanks' solution containing 0.005 NIH unit (1.6 ng) of 125I-thrombin was placed upon the washed endothelial cell cultures for 30 to 45 s at room temperature. A 100-μl sample of the incubation medium was then taken to determine free thrombin concentration. The remaining medium was suctioned off and the cell layer was rinsed twice, as rapidly as possible, with 1 ml/wash of modified Hanks' solution. The cells were then solubilized with 1 ml of 1% sodium deoxycholate, 0.01 M NaOH, 0.01 M EDTA. The cells were allowed to solubilize for 1 h, at which time the solution was transferred to capped plastic test tubes and the radioactivity was determined.

To study binding under equilibrium conditions, bound thrombin was determined after removal of the medium, but without washing the cells. Such measurements are complicated by a small, but unavoidable trace of medium remaining on the cell monolayer, even with the most thorough aspiration. The volume of unremoved medium, and accordingly the quantity of unbound thrombin which remained on the cells was determined by including 125I-albumin as a second label in the incubation medium. Because of a 20% spill of 125I into the 125I channel of the scintillation spectrometer, 125I-albumin concentration was adjusted so that counts per min per ml in the 125I channel was approximately that in the 131I channel. One milliliter of the 125I-thrombin-125I-albumin incubation medium was placed on the washed endothelial cells for 30 to 45 s. A 100-μl sample was then taken to determine free thrombin concentration. Remaining medium was suctioned off and without washing, the cells were solubilized.

Bound thrombin was calculated as:

\[
\frac{[^{125}I \text{ cpm, cells}]}{[^{125}I \text{ cpm, medium}]} = \frac{[^{125}I \text{ cpm, cells}]}{[^{125}I \text{ cpm, medium}]} - \frac{[^{131}I \text{ cpm, medium}]}{[^{125}I \text{ cpm, medium}]}
\]

The 125I counts per min were corrected for 131I spill.

Gel electrophoresis in deoxycholate was performed by the method of Laemmli (17). 125I-Labeled products were determined by dividing the gels after electrophoresis into 2-mm slices.

**RESULTS**

Maximum binding of thrombin by the cells occurred within 30 s, in the range of thrombin concentrations used for these experiments (Fig. 1). With up to 10 min of additional incubation, there was no increase or decrease in bound radioactivity. Label bound in the presence of 1 unit/ml of unlabeled thrombin averaged 30% of the total tracer bound, at either 30 s or at 10 min. Since maximum uptake was reached by 30 s, with no significant change in total uptake for 10 min, all binding experiments were terminated after 30 to 45 s of incubation. When endothelial cells were incubated for 30 s to 5 min with the labeled thrombin, washed, dissolved, and then analyzed by gel electrophoresis in deoxycholate, a single peak of radioactivity with a migration rate indistinguishable from that of unlabeled thrombin, was detected.

Use of the double label to measure thrombin binding at equilibrium requires that the binding of albumin to the cells is insignificant compared to the binding of thrombin, and that albumin does not interfere with thrombin binding. If the medium is simply removed after exposure of the cells to both labels and cells are counted without washing, the proportion of thrombin counts remaining is about twice the proportion of albumin counts (Fig. 2); a single wash removes virtually all of the labeled albumin. The decrease in the difference with each wash reflects removal of bound thrombin from the membrane, as it is only with unwashed cells that bound and free thrombin are in equilibrium. Subsequent experiments determined that albumin does not affect thrombin binding. These data also demonstrated that less than 30% of bound thrombin is eluted with two washes, where virtually all of the albumin is removed.

To insure that possible differences in binding between unlabeled and labeled thrombin were not being measured, binding was measured at a constant, tracer concentration of labeled thrombin. Other details are described under "Materials and Methods." Open circles are uncorrected data and closed circles represent data corrected by subtraction of lower affinity binding.
were incubated for 30 s with 0.005 unit/ml of \( ^{125}\text{I}-\text{thrombin} \). Medium was removed with suction and then replaced with buffer. After the indicated intervals, buffer was removed and the cells were solubilized.

Competition with labeled tracer thrombin was used to evaluate the specificity of thrombin binding to the cells. In these experiments, unlabeled proteins were added with the labeled thrombin, and binding of label was determined. Fig. 4 shows the results obtained with unlabeled thrombin, cytochrome c, albumin, and thrombin which had been inactivated with DFP. Inactive thrombin competed equally with active thrombin for binding (Fig. 4, open circles). Conversely, no competition by either cytochrome c (Fig. 4, squares) or albumin (Fig. 4, triangles) was observed. Since thrombin binds to hydrophilic cation exchangers (9), cytochrome c, which also binds to cation exchangers, was evaluated to determine that thrombin does not bind by nonspecific but high affinity, saturable ion exchange. Albumin was evaluated both as a representative plasma protein and to validate its use as a second label. The finding that albumin does not compete for binding with thrombin is consistent with the finding (Fig. 2) that binding of albumin to the cells is insignificant compared to that of thrombin.

Dissociation of bound thrombin was biphasic (Fig. 5). The half-times for dissociation were approximately 1 min and 60 min for the fast and slow rates, respectively. The rates of dissociation were unaffected by addition of excess (3 \( \mu \text{g/ml} \)) unlabeled thrombin to the medium.

Of plasma proteins known to interact directly or indirectly with thrombin, two have been localized in endothelium: \( \alpha \)-macroglobulin (21) and von Willebrand Factor (22, 23). To test the possibility that either protein could function as a thrombin receptor, cells were incubated for 5 min with antiserum to \( \alpha \)-macroglobulin or von Willebrand Factor (Factor VIII-related antigen), with antibody concentrations sufficient to detect antigen in fixed cells by indirect immunofluorescence. Neither antiserum had an effect on binding of thrombin to the cells. Likewise, pretreatment of the cells with heparin and then washing the monolayer had no effect on thrombin binding.

When compared with endothelial cells (Fig. 6A), monolayer cultures of umbilical cord smooth muscle (Fig. 6B) and fibroblasts (Fig. 6C) bound little thrombin per unit cell surface area, and the binding was not saturable, that is, was not inhibited by excess unlabeled thrombin in the range of concentrations studied. Thrombin did not bind to a mouse neuroblastoma monolayer culture (Fig. 6D) or to human erythrocytes (Fig. 6E).

**DISCUSSION**

The specificity of thrombin binding to endothelium suggests the presence on the cell surface of a thrombin receptor, and thus communication between the humoral hemostasis system and the blood vessel wall. Analysis of the binding by the method of Scatchard (18) demonstrated a population of binding sites of approximately 3300 sites per endothelial cell with an apparent dissociation constant of \( 1 \times 10^{-10} \text{M} \), and a second, larger population of binding sites with an apparent dissociation constant of \( > 2 \times 10^{-9} \text{M} \). The latter were subtracted for analysis of high affinity sites, but there is no evidence that they are nonspecific. Although this analysis is performed with the assumptions of equilibrium and of absence of site-site interactions, the important conclusions, that binding is high affinity, saturable, and specific, stand independent of those assumptions.
Dissociation of more than 65% of the $^{125}$I-thrombin within 30 min, when the cells are incubated in thrombin-free buffer, suggests that binding observed within short incubation periods is unlikely to represent thrombin taken into the cells. In addition, the localization of radioactivity only in the thrombin band on sodium dodecyl sulfate gel electrophoresis after solubilization and lyophilization of labeled cells showed that the labeled thrombin remained intact during the period of study. However, internalization of a fraction of the bound thrombin cannot be excluded. These findings, together with the failure of an antiserum to $\alpha_{2}$-macroglobulin to affect thrombin binding, exclude $\alpha_{2}$-macroglobulin as a candidate for the receptor.

Labeled thrombin was competed for equally by DFP-thrombin and unlabeled thrombin. This indicates that the active site of the thrombin molecule is not involved in binding to endothelial cells. Human blood vessel smooth muscle cells and blood vessel fibroblasts, in contrast to endothelial cells, lacked high affinity thrombin receptors, and human red cells and a mouse neuroblastoma cell line monolayer bound essentially no thrombin. Lower affinity binding to these cells was not evaluated. These findings provide further evidence that the uptake of thrombin by endothelial cells is specific.

The recent reports (12, 24, 25) that thrombin can cause release of PG1$_2$ (prostacyclin) from endothelium are of particular relevance. PG1$_2$ is the most potent known inhibitor of platelet aggregation and adherence, and its release by a potentially thrombogenic substance, thrombin, suggests that intact endothelium may be involved in the control of hemostasis and thrombosis. Studies are in progress to delineate the importance of thrombin receptors in this phenomenon.

**Acknowledgments**—The endothelial cultures were prepared and maintained by Ms. Glenna Fry. We thank Dr. Lawrence DeBault for generously providing the mouse neuroblastoma cultures.

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