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Effect of Cell Density on Thrombin Binding to a Specific Site on Bovine Vascular Endothelial Cells

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ABSTRACT We studied thrombin binding to proliferating and confluent endothelial cells derived from bovine vascular endothelium. [^{125}I]thrombin was incubated with nonconfluent or confluent endothelial cells and both the total amount bound and the amount linked in a 77,000-dalton thrombin-cell complex were determined. Approximately 230,000 molecules of thrombin bound per cell in nonconfluent cultures compared to 12,800 molecules per cell in confluent cultures. Approximately 67,000 thrombin molecules were bound in an apparently covalent complex, $M_r = 77,000$, with each cell in sparse cultures, whereas only 4,600 thrombin molecules per cell were bound in this complex with confluent cultures. Similar studies with [^{125}I]thrombin and endothelial cells derived from bovine cornea revealed no difference either in the total amount of thrombin bound or in the amount bound in the 77,000-dalton complex using sparse or confluent cultures. When confluent vascular endothelial cultures were wounded, additional cellular binding sites for the 77,000-dalton complex with thrombin appeared within 24 h. A 237% increase in the amount of thrombin bound to these sites was induced by a wound which resulted in a 20% decrease in cell number in the monolayer. There was no significant increase in thrombin binding to other cellular sites at 24 h. These experiments provide evidence that the first change in thrombin binding after injury is an increase in the cellular sites involved in the 77,000-dalton complex, and suggest that thrombin binding to endothelial cells may be important in the vascular response to injury.

The endothelium serves as an interface between blood and the vessels in which it circulates. Previous studies have emphasized the importance of the endothelium in preventing activation of the coagulation system (25). The endothelium appears to be unreactive toward clotting enzymes which have not been activated; however, at least two reactions occur between thrombin, an activated coagulation factor, and endothelial cells in tissue culture. Thrombin stimulates prostaglandin synthesis which results in the formation and release of prostacyclin by umbilical vein endothelial cells (28). Thrombin also inhibits activation of plasminogen by endothelial cells derived from a variety of tissues (8, 21). Although it is not mitogenic for endothelial cells, thrombin enhances the mitogenic effect of fibroblast growth factor (FGF) on human aortic endothelial cells (14). This observation should be interpreted with caution, however, because thrombin does not have this effect on bovine aortic endothelial cells.

To date, little information has been published on the nature of the interaction between thrombin and vascular endothelium. Specific and saturable binding of thrombin to umbilical vein endothelium has been described (1). Like the binding reaction to platelets (26), binding to vascular endothelium is reversible, and both native and active site-inhibited thrombin bind identically to endothelial cells. Because inhibited thrombin has no known physiological effect (5), the significance of this reaction is unclear. Moreover, excess unlabeled thrombin inhibited at its active site blocks binding of native thrombin to platelets, but does not prevent thrombin-induced secretion. These binding sites are specific for thrombin because neither prothrombin or prethrombin intermediates bind to them (27).

A second type of thrombin binding to fibroblasts (2) and endothelial cells derived from the bovine cornea (BCE) (15) and umbilical vein (20) has been reported. Thrombin binds to specific cellular sites with the formation of an apparent covalent bond. Thrombin inhibited at its active serine site does not appear to bind covalently to these sites, however. Moreover, urokinase (3) and trypsin (18) appear to bind covalently to the same site.

The purpose of this study was to determine the nature of the interaction between thrombin and the aortic endothelium, the

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first cells to come into contact with thrombin in the interaction between blood and the vessel wall. We have discovered that covalent thrombin binding is markedly influenced by whether or not the cells are contact-inhibited, and that covalent binding sites rapidly appear on adult bovine aortic endothelial cells (ABAE) after injury to confluent endothelium. We believe that these results provide evidence of a role for the covalent binding sites in the vascular response to injury.

MATERIALS AND METHODS

Materials

Crystalline bovine serum albumen, Fraction V (BSA), lactoperoxidase, and glucose oxidase (type 5) were obtained from Sigma Chemical Co. (St. Louis, Mo.); Dulbecco's modified Eagles medium (DMEM) and calf serum were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Gentamycin was obtained from Schering (Kenilworth, N. J.). Tissue-culture dishes were purchased from Falcon Plastics, Inc. (Oxnard, Calif.). Carrier-free, sodium [^{125}I]iodide was purchased from Amersham Corp. (Arlington Heights, Ill). A molecular weight standards kit was obtained from Bio-Rad Laboratories (Richmond, Calif.). Diisopropylfluorophosphate (DFP) was purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

Preparation of Thrombin and FGF

Human α thrombin was a generous gift from J. W. Fenton, II (9). The specific activities ranged from 2,400–4,000 U/mg. Fibroblast growth factor (FGF) was purified from bovine brain as previously described (13).

Endothelial Cell Preparation

BCE and ABAE cells were isolated as previously described (11, 12). Cells in their second to twelfth passage were grown to confluence at 37°C in 10% CO_2 in DMEM containing 10% calf serum. 100 ng/ml FGF were added every other day to the cultures.

Iodination of Thrombin

Human thrombin (150 U) was radioiodinated at room temperature using 0.05 U lactoperoxidase, 0.01 U glucose oxidase, 0.2% β-d-glucose, 4 mCi sodium [^{125}I]iodide, 0.01 M benzamidine, and buffer containing 0.2 M NaCl–0.05 M sodium phosphate (pH 6.7) for 1 min in a total volume of 0.2 ml. [^{125}I]thrombin was separated from free sodium [^{125}I]iodide by gel filtration.

Preparation of Diisopropyl Fluorophosphate (DFP)-inhibited Thrombin (DIP-Thrombin)

Thrombin, 4 mg in 1 ml of 0.75 M NaCl, 0.05 M imidazole, 0.025 M sodium phosphate, pH 7.50, was incubated with 0.01 M DFP for 50 min at room temperature (~22°C). The thrombin was then dialyzed against 250 ml of 0.75 M NaCl, 0.05 M sodium phosphate, pH 6.65, for 16 h at 4°C. [^{125}I]thrombin was treated similarly, except that excess DFP was removed by gel filtration.

Binding of [^{125}I]Thrombin

Confluent monolayers containing 9.0×10^5–1.2×10^6 cells or nonconfluent (sparse) 0.18–0.50 $\times 10^6$ cells were grown on 35-mm culture dishes. The cells were washed three times with DMEM supplemented with 0.5% bovine serum albumin (BSA). To initiate binding, radiolabeled ligand was added to the cells in 1.0 ml of the same medium. After incubation at 37°C, the cell monolayers were washed rapidly ten times with 1–2 ml of cold phosphate-buffered saline (PBS) containing 0.1% BSA. The washed monolayers were lysed in 0.1 ml of buffer containing 15% glycerol, 2% SDS, 75 mM Tris-HCl (pH 6.8), 2 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA. 1 mM N-ethylmaleimide and 1.0 mM iodoacetic acid were added to block free sulfhydryl groups (henceforth referred to as SDS-lysis buffer). Specific binding was determined by measuring the difference in cell-bound radioactivity in the presence and absence of a 100-fold excess of unlabeled thrombin. The cell-bound radioactivity in the presence of an excess amount of unlabeled thrombin was considered to be nonspecific and was subtracted from the results.

PAGE

We applied aliquots (0.033 ml) containing one-third of the total solubilized cellular material to 10% polyacrylamide slab gels with 3% stacking gel, as described by Laemmli (19). After electrophoresis at 20 mA, the slab gel was stained with Coomassie Blue (0.1%) in 50% trichloracetic acid and subsequently destained overnight. The gels were dried and subjected to autoradiography on Kodak NS-2T x-ray film. The areas of the gel corresponding to the band of radioactivity on the autoradiogram were sliced and counted in a Beckman gamma-300 spectrometer (Beckman Instruments, Inc., Irvine, Calif.).

Calculation of thrombin binding to cells was corrected for quenching of the gamma radioactivity by the polyacrylamide gel using [^{125}I]Na standards.

Cell Monolayer Wounding

Confluent ABAE cultures in 35-mm dishes were washed once with fresh DMEM supplemented with 10% calf serum. A wound running from side to side (3–4 mm in width) was made in the center of the monolayer with a rubber policeman. Cultures were then washed three times to remove cell aggregates made by wounding. Cultures were examined by phase-contrast microscopy to ensure that all cells released from the culture dish had been removed. Medium supplemented with 10% calf serum and FGF, 100 ng/ml, was then added to the cultures. After 24 h, the cells were washed three times with DMEM-0.5% BSA and [^{125}I]thrombin binding was measured. Triplicates of wounded and control confluent cultures were trypsinized and cell number was determined in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

RESULTS

Measurement of Binding of [^{125}I]Thrombin to ABAE Cells

Confluent and nonconfluent ABAE cells were incubated with [^{125}I]thrombin, then washed and solubilized, and the radioactivity associated with the cells measured by gamma scintillation counting. As shown in Fig. 1, [^{125}I]thrombin bound to both confluent and nonconfluent cells. In nonconfluent

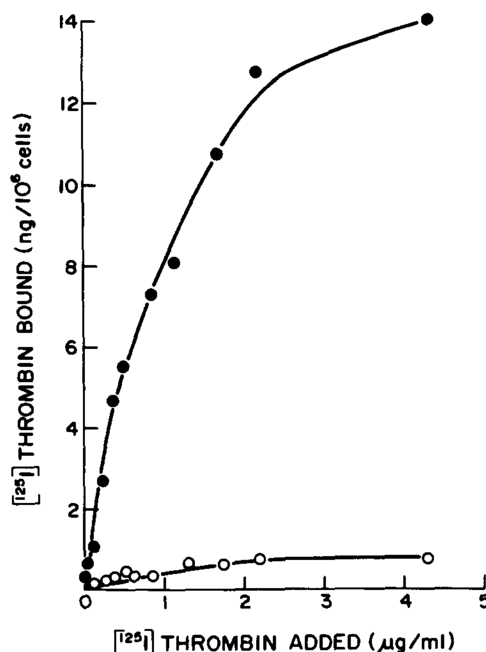

FIGURE 1 Binding of [^{125}I]thrombin to ABAE cells. Confluent (1,179,000 cells/plate) or nonconfluent (182,550 cells/plate) were incubated with [^{125}I]thrombin (10.0×10^6 cpm/U) for 30 min at 37°C in DMEM. The incubation was terminated by rapidly washing the plates; then the cells were solubilized in SDS buffer (see Materials and Methods). The amount of [^{125}I]thrombin bound to the cells was determined in a gamma scintillation counter. Specific binding represented [^{125}I]thrombin associated with the cells which could be blocked by simultaneous incubation of a 100-fold excess of unlabeled thrombin. This represented $\leq$10% of the total amount bound. ●—●, nonconfluent. ○—○, confluent.

cultures (1.8×10^5 cells/35-mm dish), thrombin binding was linear up to a concentration of ~0.5 μg/ml. The amount bound to the cells appeared to reach a plateau at 4 μg/ml of added enzyme. Considerably less [^{125}I]thrombin bound to confluent ABAE cells (1.2×10^6 cells/35-mm dish). Saturation was reached at ~2 μg/ml of added thrombin. Approximately 230,000 molecules of thrombin bound per cell in the nonconfluent culture and 12,800 molecules/cell in the confluent culture.

Nonconfluent cells were incubated with [^{125}I]thrombin for varying intervals, and the amount bound was determined. As shown in Fig. 2, binding could be detected after only 1 min of incubation. Thrombin binding was relatively rapid for the initial 30–60 min, then continued at a slower rate for the next 2.5 h. In previous studies of thrombin binding to BCE cells, we showed that the slower rate of thrombin binding is largely the result of cellular internalization of the thrombin-binding site complex (23). It is possible that the same process occurs with the ABAE cells. When a time-course of thrombin binding to confluent ABAE cells was carried out, results similar to Fig. 2 were obtained (data not shown).

Thrombin Binding to a Specific Site on ABAE Cells

Nonconfluent ABAE cells were incubated with 2.15 μg/ml of [^{125}I]thrombin for 60 min, and after washing they were solubilized in SDS and electrophoresed in SDS–10% polyacrylamide gels. An autoradiogram of the gel showed a [^{125}I]thrombin-complex, M_r ~77,000 daltons (Fig. 3). Formation of [^{125}I]thrombin-cell complex was completely blocked by addition of a 100-fold excess of unlabeled thrombin (Fig. 3). In addition to the 77,000 dalton complex and free [^{125}I]thrombin, other radioactive bands were occasionally noted on autoradiography. The most prominent of these bands were M_r 45,000 and 32,000 daltons. The latter band which is smaller than thrombin (36,500 daltons) was also noted in the presence of excess unlabeled thrombin. Relatively more 45,000 dalton complex was seen with confluent than nonconfluent cells.

Although specific binding of [^{125}I]DIP-thrombin to ABAE cells was observed, no 77,000-dalton complex was demonstrable on the autoradiogram (Fig. 3, Lanes *4* and *5*). A 40-fold excess of unlabeled DIP-thrombin blocked both covalent and noncovalent binding of [^{125}I]thrombin to ABAE cells (Lane *3*). These results indicate that although the active site of thrombin

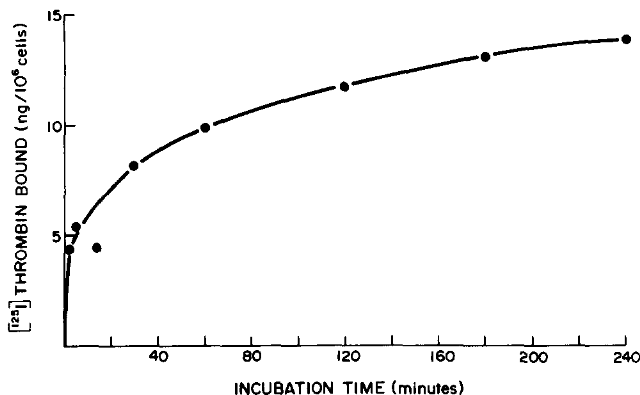

FIGURE 2 Time-course of binding of [^{125}I]thrombin to ABAE cells. Nonconfluent cells were incubated with 2.17 μg/ml of [^{125}I]thrombin at 37°C for varying intervals and the amount of thrombin bound determined as described in Fig. 1.

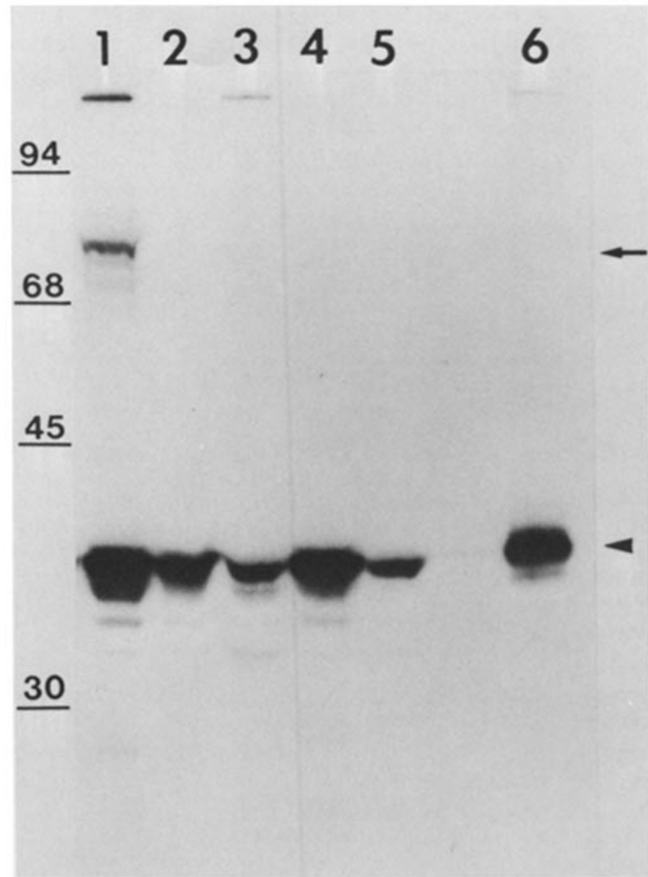

FIGURE 3 Binding of [^{125}I]thrombin to a specific site on ABAE cells. [^{125}I]thrombin or [^{125}I]DIP-thrombin, 2 μg/ml, was incubated with nonconfluent cells (5×10^5/35-mm dish) for 60 min at 37°C as described in Fig. 1. After washing and solubilization, the cells were electrophoresed in an SDS-10% polyacrylamide gel. An autoradiogram was then made of the gel. Lane *1*, [^{125}I]thrombin and ABAE cells; lane *2*, [^{125}I]thrombin, ABAE cells, and 100-fold excess of thrombin; lane *3*, [^{125}I]thrombin, ABAE cells, and 40-fold excess of DIP-thrombin; lane *4*, [^{125}I]DIP-thrombin, and ABAE cells; lane *5*, [^{125}I]DIP-thrombin, ABAE cells, and 40-fold excess DIP-thrombin; lane *6*, [^{125}I]thrombin.

is necessary for covalent complex formation with ABAE cell binding sites, active site inhibited thrombin binds reversibly to these same ABAE cellular sites.

The amount of 77,000-dalton complex formed between [^{125}I]thrombin and ABAE cells was dependent on whether the cells were confluent or nonconfluent. To determine the amount of thrombin bound in the complex, the gels were sliced and the amount of radioactive thrombin in the complex measured. Only a small amount of thrombin formed a complex with confluent ABAE cells compared to nonconfluent cells (Fig. 4). (The graph is a representative result from five separate experiments.) In nonconfluent cultures, thrombin binding saturated at a concentration of 1.25–2.5 μg/ml. Of the total amount of thrombin specifically bound, 28% was bound in the 77,000-dalton complex; thus, ~70% of thrombin binding appeared to be noncovalent. As noted, considerably less thrombin bound to confluent cells; 37% of the total thrombin bound was in the 77,000-dalton complex. The amount of thrombin bound in the 77,000-dalton complex was 4,600 molecules/cell for confluent ABAE and 67,000 molecules/cell for nonconfluent ABAE. When similar binding studies were carried out with confluent and nonconfluent BCE cells, there was no difference in the

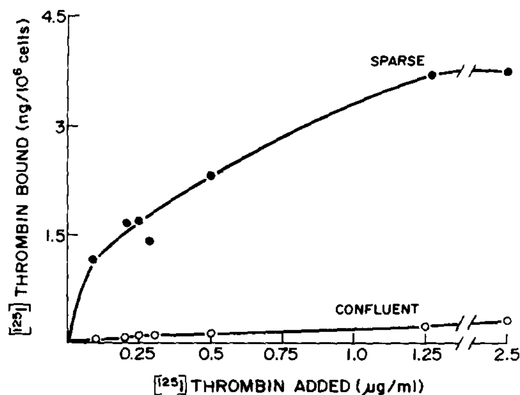

FIGURE 4 [^{125}I]thrombin binding to ABAE cells as a function of the thrombin concentration. [^{125}I]thrombin was incubated with sparse or confluent cells for 30 min at 37°C. After washing, the cells were solubilized, and subjected to SDS PAGE. After autoradiography to identify the 77,000-dalton complex, the gel was sliced and the amount of [^{125}I]thrombin in the complex determined by gamma scintillation counting. Calculation of thrombin bound in the 77,000-dalton complex was corrected for quenching of the radioactivity by the gel using [^{125}I]standards.

amount of thrombin bound in the 77,000-dalton complex: 90,000 molecules/cell. Thus, in contrast to the ABAE cells, contact inhibition does not appear to affect the number of these binding sites in BCE cells.

Appearance of Thrombin-binding Sites in Confluent Cells after Wounding

To obtain information which might clarify the role of the thrombin binding sites, ABAE cells were grown to confluence, then a wound was made across the culture plate. After 24 h, [^{125}I]thrombin was added to the wounded cell cultures and to confluent control cells. As shown in Fig. 5, release of the cells from contact-inhibition by wounding resulted in an increase in 77,000-dalton thrombin–cellular complexes. As indicated in Table I, there was a 237% increase in the amount of thrombin bound in the 77,000-dalton complex after wounding. This was associated with a 20% decrease in cell number in the plate containing the wounded monolayer compared to control confluent cultures. No increase in the total amount of noncovalent thrombin binding occurred in the wounded cultures (Table I). Thus there was a selective increase in those cellular binding sites with which thrombin forms the covalent 77,000-dalton complex. In parallel cultures which were wounded similarly to those in the thrombin binding studies, confluence was reached 3–4 d after injury.

DISCUSSION

Previous reports have described both noncovalent (1, 25) and covalent (2, 15, 24) binding of thrombin to platelets, endothelial cells, fibroblasts, and carcinoma cells. The significance of either type of binding is unknown. Spontaneous covalent binding of thrombin to a single type of site resulting in a complex of ~68,000–77,000 daltons has been reported with all of these cells (2, 3, 15, 16, 20, 24). The role of covalent-thrombin binding to these diverse types of cells is unknown. Saturation of covalent surface binding to fibroblasts and endothelial cells requires ~30–60 min (17, 18), while stimulation of platelets and endothelial cells by thrombin occurs within 1–2 min (4, 6). It has also been suggested that these binding sites are required for thrombin-induced mitogenesis of fibroblasts (10); however, there is no direct evidence of this.

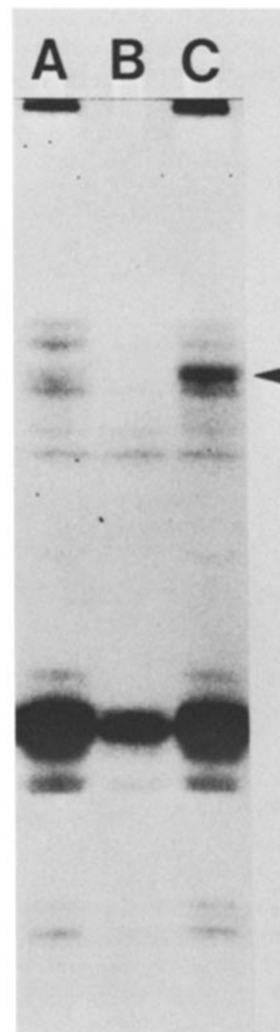

FIGURE 5 Appearance of 77,000-dalton complex between [^{125}I]-thrombin and ABAE cells in wounded confluent cultures. Confluent ABAE cells were wounded with a rubber policeman as described in Materials and Methods. After 24 h, the cells were incubated with [^{125}I]thrombin, 2 µg/ml, for 1 h, and then washed, solubilized, and subjected to slab gel electrophoresis and autoradiography as described in Fig. 3. The arrowhead indicates the 77,000-dalton complex. *A*, control confluent ABAE cells and [^{125}I]thrombin. *B*, confluent ABAE in which a wound was made 24 h previously, [^{125}I]thrombin and a 100-fold excess of unlabeled thrombin. *C*, confluent ABAE cell in which a wound was made 24 h previously and [^{125}I]thrombin.

In platelets, similar binding sites for thrombin appear only after secretion.[1] These binding sites have not been localized in other types of cells; however, human fibroblasts and bovine corneal endothelial cells release covalent binding sites for thrombin into the culture media (3, 23). Thus, it is possible that the binding sites are not an integral part of the plasma membrane. Furthermore, because these sites bind a variety of proteases, it may be that the binding of thrombin has no physiological importance, and that their primary function is to bind another protease. Thus, although the protease inhibitor α_2-plasmin inhibitor binds plasmin and thrombin covalently,

[1] T. Maerowitz, J. W. Fenton II, and M. A. Shuman. Manuscript submitted for publication.

TABLE I

Effect of Culture Wounding on Formation of the 77,000-dalton Complex between [^{125}I]Thrombin and Confluent ABAE Cells

	Total thrombin bound (% increase)	Total thrombin in 77,000-dalton complex (% increase)
	ng/10⁶ cells	*ng/10⁶ cells*
No treatment	3.14	0.35
Wounding of cell culture	3.61 (15%)	0.83 (237%)

Confluent ABAE cells were wounded with a rubber policeman as described in Materials and Methods. The cells were incubated for 24 h at 37°C, then [^{125}I]thrombin, 1 μg/ml, was added for 1 h, and the cells were washed and solubilized in SDS buffer. The total amount of [^{125}I]thrombin bound was measured as indicated in Fig. 1. The amount of thrombin bound in the 77,000-dalton complex was measured as indicated in Fig. 4.

binding of thrombin probably has no physiological consequences, whereas binding of plasmin is the major mechanism for inhibiting fibrinolysis (7, 22).

The importance of thrombin binding to fibroblasts is unclear because these cells have, at most, limited contact with the coagulation system. This study was undertaken to determine whether these covalent binding sites are present on endothelial cells derived from the aorta, a tissue in which there is an extensive interaction with thrombin when blood coagulation is activated. In demonstrating the presence of these binding sites, we discovered that the number of sites is modulated by the density of cells in culture. This finding is not a result of the larger size of nonconfluent cells. To account for a 15-fold greater number of binding sites, the nonconfluent cells would need to be 3.5-fold longer in diameter, and this is clearly not the case. Furthermore, one major cause for the overall increase in thrombin binding is an increase in the number of cellular sites to which thrombin binds and forms a 77,000-dalton complex. This increased binding, therefore, cannot be the result of adherence of thrombin to exposed areas of the culture dish. That the number of binding sites on confluent and nonconfluent BCE cells is the same, indicates a specific effect of cell density on thrombin binding to aortic endothelium.

Although the function of the covalent binding sites has not been demonstrated, our finding that additional sites rapidly appear after wounding of ABAE cells suggests their importance in the response to vascular injury. Appearance of these binding sites may be a response to a cellular requirement for thrombin in wound healing. Alternatively, they may serve to inactivate excess protease formed when the coagulation system is activated by damaged blood vessels. Because the active site serine is required for covalent enzyme binding, uncontrolled proteolysis by thrombin may be inhibited in this way. Further characterization of these binding sites in vascular endothelial cells will establish their role in cell function.

This material was presented in part at the annual meeting of the American Society of Hematology in December 1979, Phoenix, AZ, and the Contributions to Hemostasis Symposium, May, 1980, Detroit, MI.

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