

# Binding of Thrombin to Subendothelial Extracellular Matrix

## Protection and Expression of Functional Properties

Rachel Bar-Shavit, Amiram Eldor, and Israel Vlodavsky

Departments of Oncology and Hematology, Hadassah University Medical Center, Jerusalem, 91120 Israel

### Abstract

We have analyzed the binding of thrombin, a serine protease with central roles in hemostasis, to the subendothelial extracellular matrix (ECM) produced by cultured endothelial cells. This substrate provides a thrombogenic surface where hemostasis is initiated. Binding was saturable and equilibrium was achieved after 3 h incubation with  $^{125}\text{I}$ - $\alpha$ -thrombin. Scatchard analysis of thrombin binding revealed the presence of  $5.1 \times 10^9$  binding sites per squared millimeter ECM, with an apparent  $K_d$  of 13 nM. The catalytically blocked enzyme, diisofluorophosphate (DIP)- $\alpha$ -thrombin competed efficiently with  $^{125}\text{I}$ - $\alpha$ -thrombin, indicating that the binding was independent of its catalytic site. Moreover, high concentrations of the synthetic tetradecapeptide, representing residues 367–380 of thrombin B chain (the macrophage mitogenic domain of thrombin), competed with thrombin binding to ECM, indicating that the binding site may reside in the vicinity of "loop B" region. Thrombin binds to dermatan sulfate in the ECM, as demonstrated by the inhibition of  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM pretreated with chondroitinase ABC, but not with heparitinase or chondroitinase AC. This stands in contrast to  $^{125}\text{I}$ -FGF (fibroblast growth factor) binding to ECM, which was inhibited by heparitinase but not by chondroitinase ABC. ECM-bound thrombin exhibits an exposed proteolytic site as monitored by the Chromozyme TH assay and by its ability to convert fibrinogen to a fibrin clot and to induce platelet activation as indicated by  $^{14}\text{C}$ -serotonin release. ECM-bound thrombin failed to form a complex with its major circulating inhibitor-antithrombin III (AT III), compared with rapid complex formation with soluble thrombin. We propose that thrombin binds to subendothelial ECM where it remains functionally active, localized, and protected from inactivation by circulating inhibitors.

### Introduction

The serine protease thrombin, although elaborated by activation of the clotting cascade, possesses diverse bioregulatory functions in a variety of physiological processes. In addition to its central role in hemostasis, thrombin has been shown to stimulate proliferation and chemotaxis of inflammatory cells (1–4), making it a potentially active mediator of wound healing. Thrombin has also been shown to degrade various constit-

uents of basement membranes and thus may facilitate cell invasion and increase the metastatic potential of neoplastic cells (5).

Traditionally, the response to vascular injury is considered to begin when rapid activation of the hemostatic process is initiated after exposure of the subendothelium. This would then lead to thrombin generation, platelet activation and fibrin clot formation to establish a hemostatic plug (6). It has also been demonstrated that under certain circumstances, the vascular endothelium can actively bind various coagulation factors, resulting in thrombin production on the endothelial surface (7). Furthermore, thrombin has been shown to interact specifically with cell surface receptors on the endothelium (8, 9). Thrombin interaction with these or other binding sites on endothelial cells promotes the production and release of diverse cellular mediators and proteins, such as: prostacyclin ( $\text{PGI}_2$ ) (10), adenine nucleotides (11), plasminogen activator (12, 13), plasminogen activator inhibitor (14), vWF (15), fibronectin (16), platelet-activating factor (17, 18), and platelet-derived growth factor (19).

Under normal conditions, however, where the integrity of the endothelium is kept, thrombin has been shown to induce gap formation between adjacent endothelial cells via a rapid, noncytotoxic and reversible manner (20–22). Thus, thrombin may pass through the endothelial cell layer and reach subendothelial structures. Indeed, exposure of endothelial cells to either thrombin or phorbol myristate acetate (PMA) has been recently shown to enhance the thrombogenic properties of their underlying extracellular cell matrix (ECM)<sup>1</sup> (23). Moreover, thrombin may also be accessible to the vascular subendothelium even at postclotting events because upon fibrinolysis, it can be released from a fibrin clot, intact and functionally active (24, 25).

In view of thrombin accessibility to the subendothelial basement membrane, we have addressed the possibility that thrombin is capable of interacting with the subendothelium and thus may contribute to its thrombogenic properties. For this purpose we used the subendothelial ECM deposited by cultured endothelial cells. This ECM resembles the vascular basement membrane in composition and structural array of organization (26, 27) and is used as a model system to study the interaction of platelets, tumor cells, lymphocytes, neutrophils, and macrophages with the subendothelium (28–33). ECMs secreted by endothelial cells consist mainly of collagens type I, III and IV, fibronectin, laminin, thrombospondin, and the proteoglycans: heparan sulfate, dermatan sulfate, and chondroitin sulfate, forming a barrier to penetration of cells and particles through the vessel wall (34).

In this study, we show that thrombin binds specifically to

Address reprint requests to Dr. Rachel Bar-Shavit, Department of Oncology, Hadassah Medical Center, POB 12000, Jerusalem 91120, Israel.

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1. *Abbreviations used in this paper:* AT-III, antithrombin III; DIP- $\alpha$ -thrombin, diisofluorophosphate- $\alpha$ -thrombin; ECM, extracellular matrix; FGF, fibroblast growth factor; GAGs, glycosaminoglycans.

the subendothelial ECM via an anchorage binding domain, leaving the enzyme catalytic site intact and available of further interactions. In addition, ECM-bound thrombin is protected from being inactivated due to its inability to form complexes with the circulating plasma inhibitor antithrombin III (AT III).

## Methods

**Materials.** Purified preparations of human  $\alpha$ -thrombin and DIP- $\alpha$ -thrombin were kindly provided by Dr. J. W. Fenton II (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY). Synthetic peptides of various length corresponding to residues 367–380 of human thrombin B-chain were a generous gift of Dr. George D. Wilner (Washington University, St. Louis, MO). Recombinant human basic fibroblast growth factor (FGF) was kindly provided by Takeda Chemical Industries (Osaka, Japan). Glycosaminoglycans (heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate), bacterial heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) (*Flavobacterium heparinum*) were obtained from Seikagaku Kogyo Co. (Tokyo). Chondroitinase ABC and chondroitinase AC were purchased from Sigma Chemical Co. (St. Louis, MO). AT III was a generous gift from Behring Institute (Marburg, FRG). Heparin (156.9 U/mg) was obtained from Diosynth (Oss, The Netherlands), and hirudin from American Diagnostica (New York, NY). DME (1 g glucose/liter), calf serum, and FCS were obtained from Gibco Laboratories, (Grand Island, NY). Saline containing 0.05% trypsin, 0.01 M sodium phosphate pH 7.2 and 0.02% EDTA (STV) was obtained from Biological Industries (Beit Haemek, Israel). Tissue-culture dishes and 96-well plates were obtained from Falcon Labware Division, Becton Dickinson & Co. (Oxnard, CA). Four-well tissue culture plates were from Nunc (Roskilde, Denmark). Chemicals were of reagent grade, purchased from Sigma Chemical Co.

**Cells.** Cultures of bovine corneal endothelial cells were established from steer eyes, as previously described (35). Stock cultures were maintained in DME (1 g glucose/liter) supplemented with 10% bovine calf serum, 5% FCS, penicillin (50 U/ml) and streptomycin (50  $\mu$ g/ml) at 37°C in 10% CO<sub>2</sub>-humidified incubators. Partially purified bovine brain basic FGF (100 ng/ml) was added every other day during the phase of active cell growth.

**Preparation of dishes coated with ECM.** Bovine corneal endothelial cells were dissociated from stock cultures (second to fifth passage) with STV and plated into 4- and 96-well plates at an initial density of  $5 \times 10^4$  cells/ml. Cells were maintained as described above, except that 5% dextran T-40 (Pharmacia Fine Chemicals, Uppsala, Sweden) was included in the growth medium to obtain a thicker ECM layer. ECM produced in the presence or absence of dextran T-40 exhibited a similar interaction with thrombin. 6–8 d after the cells reached confluency, the subendothelial ECM was exposed by dissolving (3 min, 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM NH<sub>4</sub>OH in PBS, followed by four washes in PBS (36). The ECM remained intact, firmly attached to the entire area of the tissue culture dish and free of nuclear or cellular debris.

**Human thrombin preparations.** Preparation and characterization of active human  $\alpha$ -thrombin and esterolytically inactive human DIP- $\alpha$ -thrombin have been described (37). The preparation of various synthetic peptides corresponding to residues 367–380, 370–380, and 375–380 of human thrombin B chain was described elsewhere (38–40).

**Iodination of thrombin.** Human thrombin was iodinated by the Iodogen procedure according to Glenn et al. (41). Briefly, Iodogen was dissolved at 1 mg/ml in CH<sub>2</sub>Cl<sub>2</sub>; 100  $\mu$ l of this solution was placed in a glass tube (10  $\times$  75 mm) and the Iodogen was coated on the surface by drying under stream of N<sub>2</sub>. Approximately 100  $\mu$ g of  $\alpha$ -thrombin and 1.0 mCi of Na<sup>125</sup>I (100 mCi/ml, carrier free; Amersham International, Amersham, UK) were added to 0.1 M NaCl containing 50 mM sodium phosphate, pH 7.0, and 160  $\mu$ M benzamidine. The mixture was added to the Iodogen-coated tube and incubated for 10 min at 4°C. Iodination was terminated by removing the reaction mixture from the Io-

dogen-coated tube. Free iodine and benzamidine were removed by gel filtration (Bio-Gel P-2; Bio-Rad Laboratories, Richmond, CA) and dialysis against 0.75 M NaCl buffered with 50 mM sodium phosphate, pH 7.0 at 4°C. The <sup>125</sup>I-labeled preparations had a specific activity of 6.0–12.2  $\times 10^6$  cpm/ $\mu$ g and comigrated as a single band with unlabeled thrombin on SDS polyacrylamide gels. <sup>125</sup>I- $\alpha$ -thrombin retained nearly 100% of its catalytic (measured by the Chromozyme-TH assay) and mitogenic activities.

**Binding studies.** ECM-coated 96-well tissue culture plates were used for binding assay. ECM was incubated with 1 mg/ml of BSA for 2 h at 37°C before binding to saturate nonspecific protein binding sites. The ECM was washed three times and incubated with iodinated human thrombin for 3 h at 37°C in PBS containing 1% BSA. Specifically bound thrombin was determined by subtracting nonspecific binding obtained in the presence of a 100-fold excess unlabeled thrombin. Experiments using bovine <sup>125</sup>I- $\alpha$ -thrombin, gave similar results.

**[<sup>14</sup>C]Serotonin release from platelets.** Platelets were collected from fresh human blood, prepared by centrifugation of the blood at 150 g for 10 min and labeled with [<sup>14</sup>C]serotonin (Amersham International) in a manner that 95% uptake of radioactivity was achieved ( $4 \times 10^8$  platelets/0.4  $\mu$ Ci). The platelets were washed twice with a solution containing 136 mM NaCl, 25 mM Tris-HCl, 1 mM EDTA, 0.8 mM sodium citrate, pH 7.4 and resuspended in the same buffer containing 5 mM glucose, as described (42). 1 ml of washed platelets ( $4 \times 10^8$  platelets), was layered over 35-mm plates coated with either ECM alone or with ECM that was initially incubated (4 h, 37°C) with thrombin at various concentrations ( $10^{-10}$ – $10^{-6}$  M). 0.1-ml aliquots of washed platelets were removed from the plates at various time intervals (either 15 or 60 min), centrifuged in an Eppendorf microcentrifuge (model 5414; Brinkmann Instruments Co., Westbury, NY) for 30 s and the radioactivity of the supernatants was determined. Nonspecific release of [<sup>14</sup>C]serotonin was estimated after centrifugation of washed platelets that were not exposed to ECM.

**Chromozyme-TH assay.** 50  $\mu$ l of Chromozyme-TH (1–10  $\mu$ M) was added to increasing concentrations of  $\alpha$ -thrombin, either in solution or when bound to ECM, at a final volume of 600  $\mu$ l PBS. Incubations were carried out at 37°C for 30 min, and the reaction was terminated by adding an equal volume of acetic acid. Samples were read for color development at 405 nm versus appropriate blanks.

**Thrombin-AT III complex formation.** Complex formation between thrombin and AT III in presence of heparin was studied as described before (43). Radiolabeled thrombin (1.37 nM) was incubated with AT III (13.7 nM) at 37°C in the presence of heparin (0.3 U/ml). Reaction was stopped at various time intervals by the addition of sample buffer (without mercaptoethanol) and boiling for 3 min before electrophoresis on 5–15% SDS-PAGE. The gels were stained and dried and <sup>125</sup>I- $\alpha$ -thrombin was detected by autoradiography.

## Results

**<sup>125</sup>I- $\alpha$ -thrombin binding to subendothelial ECM.** Binding of <sup>125</sup>I- $\alpha$ -thrombin to ECM-coated plates was analyzed as a function of thrombin concentration as described in Methods. As shown in Fig. 1, apparent saturation of binding was achieved with a half-maximal binding at 7.5 nM <sup>125</sup>I- $\alpha$ -thrombin. Scatchard analysis of the data indicated a single class of binding sites (Fig. 1). The apparent dissociation constant of thrombin interaction with ECM was  $13 \times 10^{-9}$  M, with an estimated  $5.1 \times 10^9$  binding sites per square millimeter of ECM, corresponding to the area occupied by  $\sim 10^3$  confluent bovine aortic endothelial cells. Time course of thrombin binding to ECM is shown in Fig. 2 A. Apparent equilibrium was reached after 2 h at 37°C. Up to 70% of the bound thrombin remained associated with the ECM after being rinsed and incubated for 24 h with PBS at 37°C (Fig. 2 B). It should be emphasized that the above binding parameters are given to permit comparison

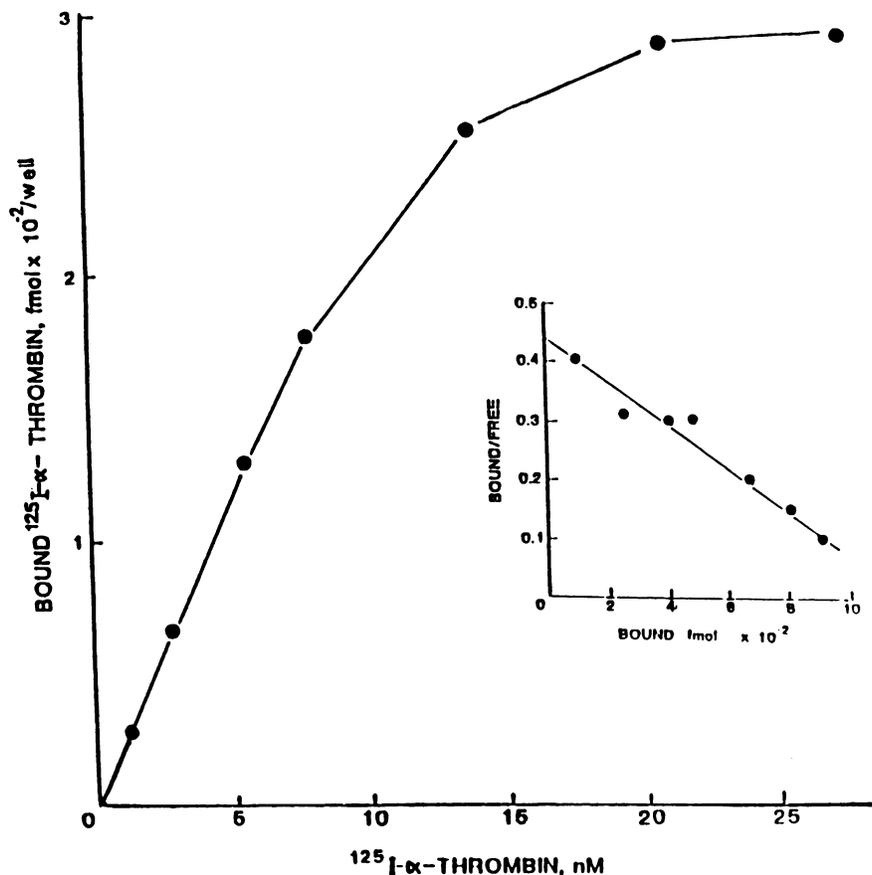


Figure 1. Binding of <sup>125</sup>I-α-thrombin to the subendothelial ECM. ECM-coated 96-well plates were incubated with 0.1% BSA for 1 h at 37°C before binding. Increasing amounts of <sup>125</sup>I-α-thrombin were then added to the wells and incubated for 4 h at 37°C. Specific binding was determined by subtracting the nonspecific binding obtained in the presence of 100-fold excess unlabeled thrombin. Similar results were obtained by using <sup>125</sup>I-bovine α-thrombin for binding. (Inset) Scatchard plot analysis.

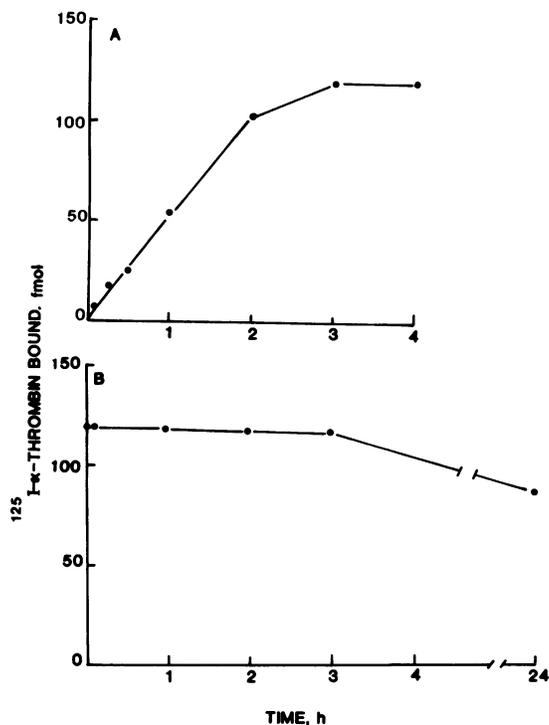
with other binding systems, bearing in mind that Scatchard analysis assumes reversible binding conditions (unlike our data [Fig. 2 B]) (44). Note also that <sup>125</sup>I-α-thrombin does not bind covalently to the ECM, as > 75% of the ECM-bound thrombin was released upon a mild treatment with acetic acid (0.2 M glacial acetic acid, 0.5 M NaCl, 5 min) (45), (data not shown).

To characterize the ECM binding domain of thrombin, we tested catalytically blocked thrombin (DIP-α-thrombin) and various thrombin fragments (corresponding to the region of "loop B" macrophage mitogenic peptide) (40) for their ability to compete with thrombin binding to the ECM. The results demonstrated that DIP-α-thrombin competed with <sup>125</sup>I-α-thrombin binding to ECM with the same efficiency as native α-thrombin (Fig. 3), indicating that the enzyme proteolytic site was not involved in thrombin binding to ECM. When competition studies were performed with the synthetic tetradecapeptide corresponding to residues 367–380 of thrombin B-chain, 50% inhibition was obtained at ~ 10<sup>-5</sup> M, compared with 10<sup>-9</sup> M of native thrombin (Fig. 3). This inhibition was specific, as when only one amino acid was substituted (Trp for Tyr) at position 370, the inhibitory effect on thrombin binding was abolished (Fig. 3). There was also no inhibition in the presence of a shorter synthetic peptide of 10 residues (designated as peptide II) and representing residues 371–380 of thrombin B-chain (Fig. 3).

*Effect of glycosaminoglycans (GAGs) on thrombin binding to ECM.* Thrombin possesses highly cationic residues that bind effectively to the negatively charged sulfated polysaccharide heparin. Interestingly, such a positively charged cluster

is located in the vicinity of the thrombin loop B insertion site (46). Therefore, we have analyzed the effect of heparin on thrombin binding to ECM. Increasing concentrations of heparin were preincubated at 24°C for 1 h with ECM-coated plates, followed by <sup>125</sup>I-α-thrombin binding. The results indicated that heparin inhibited effectively thrombin binding to ECM with 50% inhibition at 0.5 μg/ml heparin (Fig. 4). Similar studies indicated that heparan sulfate and dermatan sulfate competed effectively with thrombin binding, whereas, chondroitin sulfate, keratan sulfate, and hyaluronic acid at concentrations as high as 100 μg/ml failed to affect thrombin binding to ECM (Fig. 4). Two other heparin binding proteins, protamine sulfate (47) and lipoprotein lipase (48), had opposing effects on thrombin binding to ECM. Although PS had no effect, LPL effectively inhibited thrombin binding to ECM, suggesting, perhaps, competition over the same binding site in the matrix.

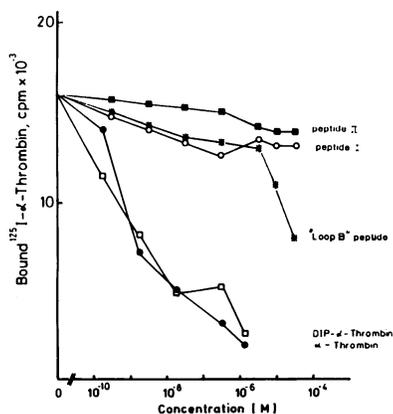
*ECM-degrading enzymes and their effect on thrombin binding to ECM.* The possibility that thrombin binds to a specific GAG in the ECM was addressed by using different degradative enzymes that cleave certain GAGs while leaving others intact. For this purpose, ECM was treated with either heparitinase, heparinase, chondroitinase AC, or chondroitinase ABC, washed extensively, and tested for its ability to bind thrombin. Treatment with chondroitinase ABC resulted in a significant inhibition of thrombin binding to ECM (Fig. 5), while treatment with heparitinase (which cleaves heparan sulfate side chains) or chondroitinase AC (which cleaves all GAGs except of heparan sulfate and dermatan sulfate) were ineffective. Based on the inhibitory effect of chondroitinase



**Figure 2.** Time dependence and reversibility of  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM. ECM-coated 96-well plates were incubated with 0.1% BSA for 1 h at 37°C. (A) Time dependence of  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM. At the indicated time points, wells were extensively washed and processed by the addition of 1 M NaOH. (B) Reversibility of  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM. ECM-coated wells were incubated with  $^{125}\text{I}$ - $\alpha$ -thrombin ( $1 \times 10^5$  cpm/well; 5 nM) for 4 h at 37°C, at a final volume of 0.2 ml. The ECM was washed free of unbound ligand, PBS was added and aliquots were removed at each time point for  $\gamma$  counting.

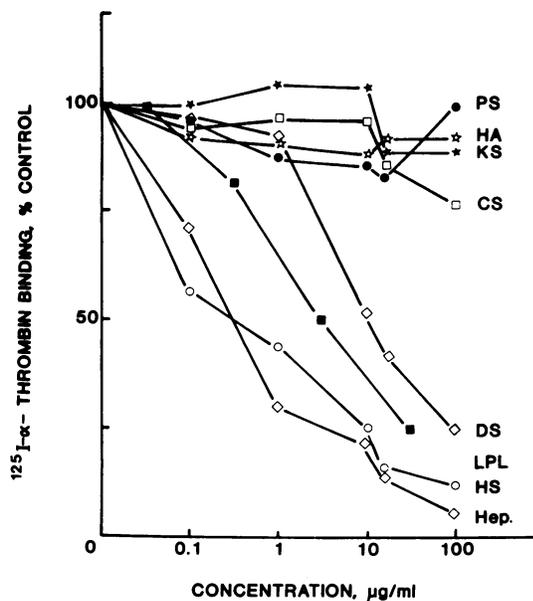
ABC (which degrades all GAGs except heparan sulfate) (Fig. 5), we concluded that thrombin is bound through a dermatan sulfate moiety in the ECM.

To demonstrate specificity of the enzymatic degradations, basic FGF, which has been recently shown to bind to heparan



**Figure 3.** Inhibition of  $^{125}\text{I}$ - $\alpha$ -thrombin binding in the presence of  $\alpha$ -thrombin, DIP- $\alpha$ -thrombin, and synthetic thrombin fragment analogues. ECM-coated plates were incubated (1 h, 37°C) with  $^{125}\text{I}$ - $\alpha$ -thrombin ( $1 \times 10^5$  cpm/well; 5 nM) in the presence of increasing concentrations of either  $\alpha$ -thrombin (●), DIP- $\alpha$ -thrombin (□), a tetradecapeptide loop B representing residues 367–380 of thrombin B-chain (\*), loop B peptide substituted with Tyr for Trp; peptide I (○), or a decapeptide representing residues 367–376 of thrombin B-chain; peptide II (■). Incubations were carried out as detailed in Methods, followed by determination of ECM-bound  $^{125}\text{I}$ - $\alpha$ -thrombin.

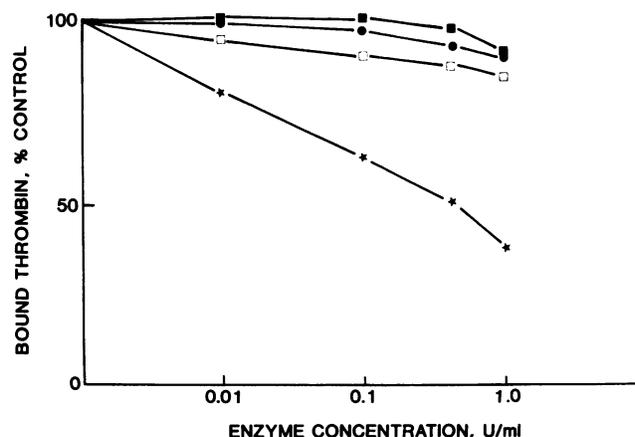
representing residues 367–380 of thrombin B-chain (\*), loop B peptide substituted with Tyr for Trp; peptide I (○), or a decapeptide representing residues 367–376 of thrombin B-chain; peptide II (■). Incubations were carried out as detailed in Methods, followed by determination of ECM-bound  $^{125}\text{I}$ - $\alpha$ -thrombin.



**Figure 4.** Effect of various GAGs and GAG-binding proteins on  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM. ECM-coated plates were incubated (1 h, 37°C) with  $^{125}\text{I}$ - $\alpha$ -thrombin ( $1 \times 10^5$  cpm/well; 5 nM) in the presence of increasing concentrations of heparin (◇), heparan sulfate (○), dermatan sulfate (◇), chondroitin sulfate (□), keratan sulfate (\*), hyaluronic acid (\*), protamine sulfate (●), or lipoprotein lipase (■). Binding conditions were as described in Methods.

sulfate moieties in the ECM (49, 50), was tested for binding under similar conditions. As demonstrated in Fig. 6, treatment with heparitinase inhibited almost completely FGF binding to ECM, whereas thrombin binding was unaffected. Treatment of the ECM with chondroitinase ABC, on the other hand, inhibited effectively thrombin binding, but had no effect on the binding of FGF to ECM.

**Functional activities of ECM-bound thrombin.** The ability of DIP- $\alpha$ -thrombin to compete effectively with the native enzyme for binding to ECM (Fig. 3) indicated that thrombin



**Figure 5.** Effect of GAG-degrading enzymes on thrombin binding to ECM. ECM plates were treated (1 h, 37°C) with increasing concentrations of heparitinase (■), heparinase (□), chondroitinase AC (●), or chondroitinase ABC (\*). The ECM was washed four times and tested for its ability to bind  $^{125}\text{I}$ - $\alpha$ -thrombin (5 nM). Results are expressed as percent of thrombin binding in the absence of enzyme treatment. 100% = 5 pmol thrombin per 16-mm ECM-coated well.

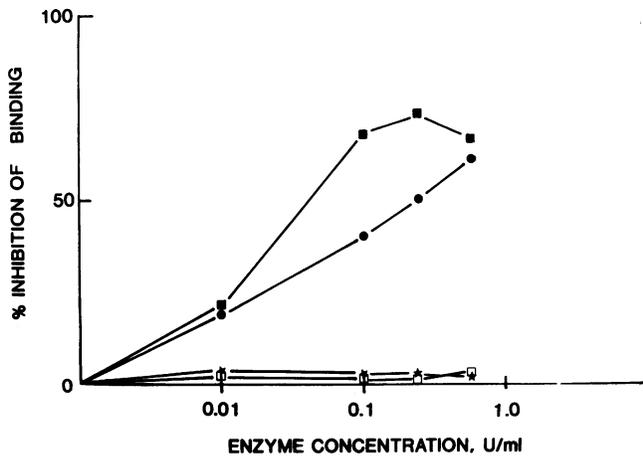


Figure 6. Effect of GAG degrading enzymes on  $^{125}\text{I}$ - $\alpha$ -thrombin and  $^{125}\text{I}$ -bFGF to ECM treated plates. ECM-coated plates were treated (1 h,  $37^\circ\text{C}$ ) with increasing concentrations of either heparitinase ( $\blacksquare$ ,  $\square$ ) or chondroitinase ABC ( $\bullet$ ,  $\star$ ). The ECM was washed four times and tested for its ability to bind  $^{125}\text{I}$ - $\alpha$ -thrombin ( $1 \times 10^5$  cpm/well; 5 nM) ( $\bullet$ ,  $\square$ ) and  $^{125}\text{I}$ -bFGF ( $1 \times 10^5$  cpm/well; 0.4 nM) ( $\blacksquare$ ,  $\star$ ) as described in Methods.

binding is not mediated through its catalytic site. Indeed, when citrated platelet-poor plasma (PPP) was added to an ECM plate that was preincubated with thrombin, clot formation was observed within 10–30 s, whereas ECM plates alone did not induce clot formation.

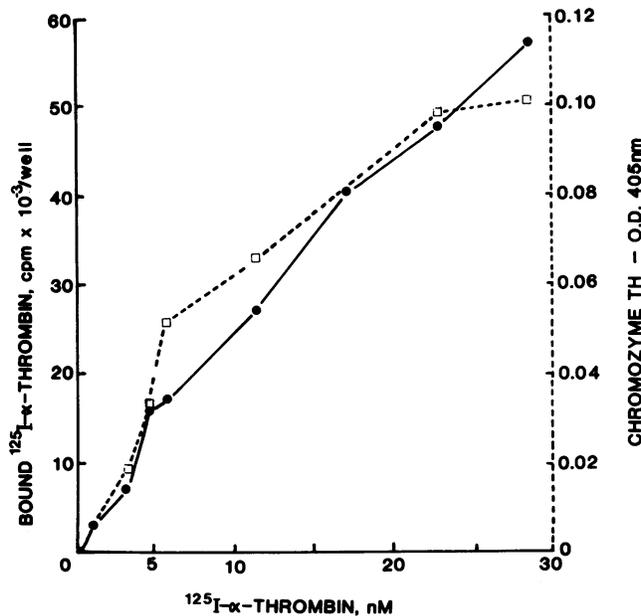


Figure 7. Amidolytic activity of ECM-bound thrombin. ECM coated wells were incubated (4 h,  $37^\circ\text{C}$ ) with increasing concentrations of  $^{125}\text{I}$ - $\alpha$ -thrombin. The ECM was washed free of unbound ligand and tested for the amount of bound thrombin ( $\bullet$ ) and amidolytic activity ( $\square$ ). Amidolytic activity was determined by adding 50  $\mu\text{l}$  of Chromozyme TH to each well containing 500  $\mu\text{l}$  PBS, pH 7.4. Plates were then incubated for 30 min at  $37^\circ\text{C}$  and the reaction was stopped by the addition of acetic acid. The degree of color development was determined by a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at a wavelength of 405 nm.

Quantitative analysis was further performed using the Chromozyme-TH assay to monitor for the amidolytic activity of the enzyme. The results indicated increased amidolytic activity in thrombin-bound ECM plates in a linear fashion parallel to the amount of bound thrombin (Fig. 7), whereas ECM-coated plates alone did not exhibit a detectable amidolytic activity. Analysis of comparable thrombin concentrations in solution indicated similar levels of Chromozyme-TH activity. As shown in Fig. 7, 0.45 pmol thrombin in solution, generated 0.1 OD of amidolytic activity, as was observed with the same amount of ECM-bound thrombin. Thus, ECM-bound thrombin contains fully functional and exposed catalytic sites of the molecule.

The ability of ECM-bound thrombin to activate platelets was investigated by the addition of [ $^{14}\text{C}$ ]serotonin-labeled platelets (washed, free of plasma constituents) to ECM-coated plates that were preincubated with increasing amounts of thrombin. [ $^{14}\text{C}$ ]Serotonin release was determined 15 and 60 min after addition of the labeled platelets. As shown in Fig. 8, ECM that was preincubated with  $10^{-8}$  M  $\alpha$ -thrombin, stimulated an almost maximal release of [ $^{14}\text{C}$ ]serotonin. Approximately 55% release was obtained after 15 min, which increased to 84% after 1 h incubation. These data correlate with thrombin binding, reaching saturation at  $2 \times 10^{-8}$  M (Fig. 1). ECM alone induced only basal levels of serotonin release during the time interval tested, similar to that obtained by incubation of labeled platelets with ECM-bound DIP- $\alpha$ -thrombin (which by itself does not induce platelet activation) (Fig. 8). These results indicate that thrombin when bound to subendothelial ECM is capable of facilitating platelet activation and fibrin clot formation.

*Formation of thrombin-AT III complex in a fluid phase vs. ECM.* Circulating thrombin is known to be rapidly inactivated by plasma inhibitors, mainly AT III, as well as three other minor inhibitors (51). We have investigated the ability of ECM-bound thrombin to form complexes with AT III and compared it with the extent of complex formation with

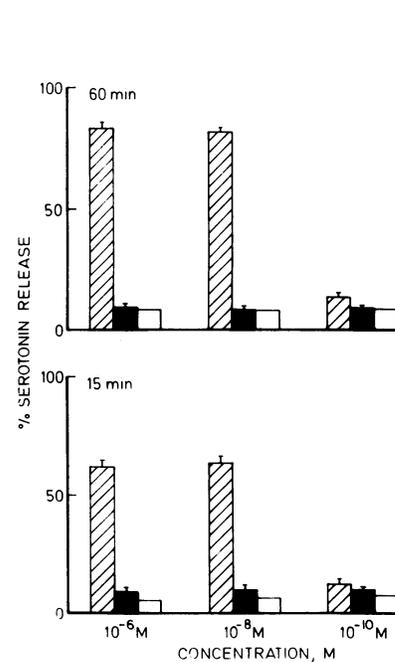
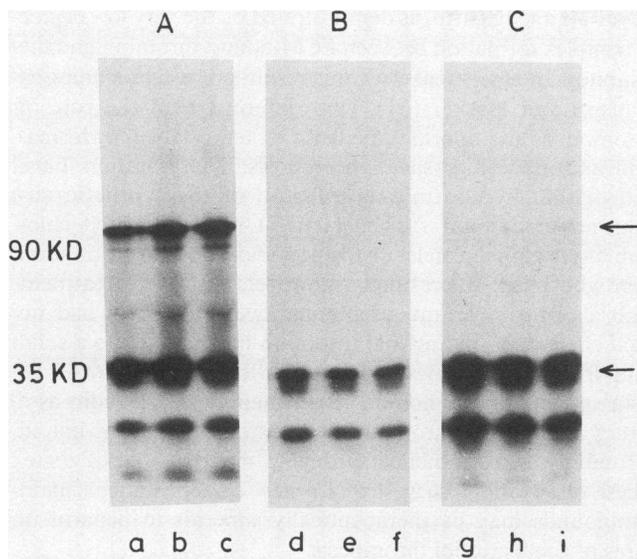


Figure 8. Release of [ $^{14}\text{C}$ ]serotonin from platelets by ECM-bound  $\alpha$ -thrombin. ECM was preincubated with increasing concentrations of  $\alpha$ -thrombin ( $\square$ ), or DIP- $\alpha$ -thrombin ( $\blacksquare$ ) and washed free of unbound thrombin, as described in Methods. [ $^{14}\text{C}$ ]Serotonin-labeled washed platelets (0.4  $\mu\text{Ci}/4 \times 10^8$  platelets), prepared as described in Methods, were added to either ECM alone ( $\square$ ), ECM-bound thrombin, or to ECM-bound DIP- $\alpha$ -thrombin. Release of [ $^{14}\text{C}$ ]serotonin was determined by counting the supernatants of samples taken after 15 and 60 min.



**Figure 9.** Thrombin-AT III complex formation by ECM-bound thrombin as compared with thrombin in solution. Time dependence of thrombin-AT III complex formation was determined by incubating a fixed amount of  $^{125}\text{I}$ - $\alpha$ -thrombin (0.685 pmol) either in solution or ECM-bound with AT-III (13.7 nM) in the presence of heparin (0.3 U/ml). Incubations were carried out at 37°C for 5 s (*a*, *d*, and *g*); 20 s (*b*, *e*, and *h*) and 5 min (*c*, *f*, and *i*), followed by SDS-PAGE. (*A*) Complex formation of AT III and thrombin (1.37 nM) in solution. (*B*) Complex formation of AT III and ECM-bound thrombin. AT III and heparin were added to ECM-bound  $^{125}\text{I}$ - $\alpha$ -thrombin (0.685 pmol). At the indicated time intervals, the reaction was stopped by addition of SDS-PAGE sample buffer. (*C*) Complex formation of AT III and an increased amount of ECM-bound thrombin. As shown in *B*, except that excess of  $^{125}\text{I}$ - $\alpha$ -thrombin (2 pmol) was initially bound to the ECM.

thrombin in solution. ECM-bound  $^{125}\text{I}$ - $\alpha$ -thrombin (0.68 pmol) was allowed to interact with AT III (13.7 nM) and heparin (0.3 U/ml). The reaction of complex formation was stopped at different time intervals by adding SDS-PAGE sample buffer. Similar experiments were carried out with the same amount of thrombin in solution. Samples were subjected to SDS-PAGE for identification of a band at  $\sim 93$  kD, corresponding to the combined molecular masses of thrombin (35 kD) and AT III (58 kD). Fig. 9 demonstrates that in a fluid phase the complex is formed within 5 s, reaching a maximum at 20 s. In contrast, thrombin-AT III complexes were not detected upon incubation of AT III with ECM-bound thrombin (Fig. 9 *B*). Moreover, even when a higher amount of thrombin was bound to ECM (2 pmol), only trace levels of complexes could be detected (Fig. 9 *C*). In addition, experiments were carried out to determine the amidolytic activity of equivalent amounts of ECM-bound thrombin and thrombin in solution that were incubated with increasing concentrations of AT III. The results (Table I) indicated that inhibition of soluble thrombin was detected at a ratio of 1:5 (thrombin/AT III) reaching complete inhibition at a ratio of 1:10. On the other hand, when ECM-bound thrombin was tested, no inhibition was obtained even at a thrombin/AT III ratio of 1:140 (Table I). In contrast, when hirudin (a leech-derived thrombin high-affinity specific inhibitor) was used, inhibition of thrombin amidolytic activity was observed with both ECM-bound thrombin and thrombin in solution (data not shown). This

**Table I.** AT III Inhibition of Soluble and ECM-bound Thrombin

	AT III (concentration)	% Inhibition of thrombin activity*
Soluble $\alpha$ -thrombin, $10^{-8}$ M (10 pmol)	$1 \times 10^{-6}$ M	100
	$1 \times 10^{-7}$ M	97
	$5 \times 10^{-8}$ M	45
	$1 \times 10^{-8}$ M	15
	$1 \times 10^{-9}$ M	0
ECM-bound $\alpha$ -thrombin (10 pmol) <sup>‡</sup>	$1.4 \times 10^{-6}$ M	12.5
	$1 \times 10^{-6}$ M	10
	$1 \times 10^{-7}$ M	8
	$1 \times 10^{-8}$ M	0
	$1 \times 10^{-9}$ M	0

\* 100% amidolytic activity of  $10^{-8}$  M  $\alpha$ -thrombin corresponds to 0.128OD at 405 nm. Thrombin-AT III complexes were formed in presence of 0.3 U/ml heparin and the amidolytic activity was determined as described in Methods.

<sup>‡</sup>  $\alpha$ -Thrombin was preincubated with the ECM in a manner that resulted in binding of 10 pmol thrombin per 35-mm dish.

result indicates that hirudin binds to a blocking site on thrombin which is not affected by the interaction with ECM.

## Discussion

The subendothelial basement membrane forms a scaffolding support for the vascular endothelium and provides a surface where hemostasis is actively initiated when injury occurs. Thus, whereas the luminal endothelial cell surface exhibits thromboresistant properties designed to maintain proper blood fluidity, the underlying subendothelium is thrombogenic as manifested by platelet adhesion and aggregation (44, 52, 53). In this study, we have examined the possibility that thrombin, the major end product of the clotting cascade, may interact with the subendothelial basement membrane and thus contribute to its thrombogenic properties. We have used the ECM secreted by cultured endothelial cells as a model providing a reproducible isolated system with which to study thrombin interactions with the subendothelium.

Thrombin binds to the subendothelial ECM in a saturable manner and remained bound over an extended period of incubation in a thrombin-free medium. Scatchard analysis revealed that thrombin binds to ECM with an apparent  $K_D$  of 13 nM, similar to that reported for thrombin low-affinity binding to endothelial cells (8, 54), macrophage-like cells (55), and fibroblasts (56). Comparison of the molecular mass of thrombin that binds to ECM with the actual biological activity of ECM-bound thrombin should, however, be done with caution because Scatchard analysis is not truly valid under the non-equilibrium conditions observed in this study.

Binding of thrombin to ECM does not require the catalytic site of the molecule. This was shown by the effective competition of DIP- $\alpha$ -thrombin with  $^{125}\text{I}$ - $\alpha$ -thrombin binding to ECM. The synthetic tetradecapeptide representing residues 367–380 of thrombin B-chain (loop B insertion site) inhibited thrombin binding by 50% at  $10^{-5}$  M, whereas 50% inhibition by DIP- $\alpha$ -thrombin was obtained at  $10^{-9}$  M. The inhibitory effect of loop B peptide was specific because it was lost by substitution of a single amino acid (Trp for Tyr at position 370) or when a

shorter synthetic peptide of 10 residues (370–380) was used. The loop B insertion sequence is located in a unique exosite region on thrombin B chain and was shown to possess a growth promoting activity specific for the mononuclear phagocytic cell lineage (40). Our data suggest that the ECM binding domain of thrombin might be in the vicinity of this region.

Thrombin is known to possess a heparin binding site whose exact localization has not yet been identified and that consists mainly of positively charged residues. Interestingly, a major cationic cluster exists near the loop B insertion site of thrombin (46). Binding of thrombin to ECM in the presence of different GAGs indicated a marked inhibition in the presence of heparin, heparan sulfate, or dermatan sulfate, whereas chondroitin sulfate, keratan sulfate, and hyaluronic acid had no effect on thrombin binding. Furthermore, various modified heparins such as *N*-desulfated or *N*-acetylated heparin and low-molecular weight oligosaccharides derived from depolymerized heparin, effectively inhibited thrombin binding (our unpublished observations). These modified heparins and heparin fragments were devoid of anticoagulant activity (57), suggesting that the inhibitory effect of heparin was independent of its anticoagulant activity.

The use of different GAG-degrading enzymes together with competition studies performed in presence of various GAG molecules, suggest that dermatan sulfate is the component through which thrombin binds to ECM. This is consistent with data reported by Hatton et al. (58), who demonstrated that thrombin binds to dermatan sulfate in subendothelial layers of mechanically deendothelialized vascular segments. Other constituents of the ECM may, however, participate in this interaction, forming high-affinity binding sites for thrombin. In support of this possibility are our preliminary studies showing specific binding of thrombin to laminin-coated plates (data not shown). This might, explain the inhibitory effect of heparin on thrombin binding to the ECM, indirectly, by interacting with various matrix constituents containing heparin binding sites (e.g., laminin, fibronectin, collagen) (34), thus masking potential thrombin binding sites.

Exposure of ECM-bound thrombin to citrated PPP, resulted in clot formation, whereas ECM alone did not exhibit any procoagulant activity. Indeed, ECM-bound thrombin was shown to retain functional catalytic sites, as monitored by the Chromozyme-TH assay. ECM-bound thrombin also facilitated platelet activation as demonstrated by induction of [<sup>14</sup>C]serotonin release. The release reaction was nearly maximal with ECM that was preincubated with  $10^{-8}$  M  $\alpha$ -thrombin. This stands in correlation with <sup>125</sup>I- $\alpha$ -thrombin binding to ECM that reached saturation at  $\sim 2 \times 10^{-8}$  M (Fig. 1). This activation did not occur upon incubation of platelets with DIP- $\alpha$ -thrombin, either bound to ECM or in solution. These results indicate that thrombin binds to the subendothelial ECM through a short anchorage binding region, leaving the catalytic site accessible to stimulate platelets. That this may be the case in vivo was suggested by Hatton et al., who demonstrated a specific and significant binding of thrombin to the subendothelial layers of mechanically deendothelialized vascular segments (58).

Thrombin, generated in vivo in the blood stream, is cleared rapidly from the circulation by specific plasma inhibitors, mainly AT III (59, 60). The ECM-immobilized enzyme, on the other hand, was found to be protected from such inactivation

by AT III and heparin, as demonstrated by the very low degree of complex formation between ECM-bound thrombin and the inhibitor. In agreement with this result, are recent studies by Hanson and Harker (61), who demonstrated the role of thrombin as an important mediator of hemostatic plug formation and acute high-shear thrombosis. These authors have shown that by continuous infusion of the synthetic antithrombin inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone, platelet deposition and thrombus formation were abolished. Interestingly, however, sustained treatment with a comparable anticoagulating level of heparin had no such effect. Our finding that thrombin immobilized to a solid support (ECM) becomes less accessible for inhibition by AT III is in support of this notion. Experiments using hirudin as a direct inhibitor of thrombin, demonstrate that ECM-bound thrombin can be inhibited through a different blocking site. These observations suggest that a new class of anticoagulant compounds may be therapeutically superior to heparin in cases of acute arteriol thrombosis.

Other plasma proteins participating in the hemostatic process were also shown to bind specifically to the ECM. These include vWF (62), mediating platelet adhesion to the vascular subendothelium, and plasminogen participating in the fibrinolytic system. Moreover, immobilized plasminogen was found to be a better substrate for tissue plasminogen activator than soluble plasminogen and was protected from its inhibitor  $\alpha_2$ -plasmin inhibitor (63). Therefore, a concept emerges where the ECM not only binds and localizes different hemostatic proteins, but also modulates their mode of action and provides a protective environment from the circulating plasma inhibitors.

Recently, we have identified bFGF in the subendothelial ECM, both in vitro and in vivo (49). This factor was released upon degradation of the ECM heparan sulfate (50). Platelet-derived growth factor was shown to bind to collagen while retaining its mitogenic activity (64). These observations, together with the present results, indicate that various biologically active molecules can be sequestered and stabilized by the ECM and participate in the induction of various cellular responses, so as to allow a more persistent and localized effects, compared with the same molecules in a fluid phase. In view of other biological activities of thrombin (chemotaxis, mitogenesis, etc.), its presence in an active form bound to the ECM may have a functional significance during inflammation and localized wound healing.

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