

Thrombin Adhesive Properties: Induction by Plasmin and Heparan Sulfate

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Abstract. We have previously demonstrated that chemically modified thrombin preparations induce endothelial cell (EC) adhesion, spreading and cytoskeletal reorganization via an Arg-Gly-Asp (RGD) sequence and the $\alpha_v\beta_3$ integrin. Native thrombin, however, did not exhibit adhesive properties, consistent with crystal structure analysis, showing that Gly-Asp residues of the RGD epitope are buried within the molecule. We have now identified a possible physiological mean of converting thrombin to an adhesive protein. Plasmin, the major end product of the fibrinolytic system, converted thrombin to an adhesive protein for EC in a time and dose-dependent manner. EC adhesion and spreading was also induced by a low molecular weight ($\sim 3,000$ D) cleavage fragment generated upon incubation of thrombin with plasmin. Cell adhesion mediated by this fragment was completely inhibited by the synthetic peptide GRGDSP. Conversion of thrombin to an adhesive molecule was significantly enhanced in the presence of heparin or heparan sulfate, while other glycosaminoglycans (GAGs) (e.g., dermatan sulfate, keratan sulfate, chondroitin sulfate) had no effect. The role of cell surface heparan sulfate in thrombin conversion to EC adhesive protein was investigated using

CHO cell mutants defective in various aspects of GAG synthesis. Incubation of both thrombin and a suboptimal amount of plasmin on the surface of formaldehyde fixed wild-type CHO-KI cells resulted in an efficient conversion of thrombin to an adhesive molecule, as indicated by subsequent induction of EC attachment. In contrast, there was no effect to incubation of thrombin and plasmin with fixed CHO mutant cells lacking both heparan sulfate and chondroitin sulfate, or with cells expressing no heparan sulfate and a three-fold increase in chondroitin sulfate. A similar gain of adhesive properties was obtained upon incubation of thrombin and plasmin in contact with native, but not heparinase-treated extracellular matrix (ECM) produced by cultured ECs. It appears that cell surface and ECM-associated heparan sulfate modulate thrombin adhesive properties through its heparin binding site in a manner that enables suboptimal amounts of plasmin to expose the RGD domain. Our results demonstrate, for the first time, a significant modulation of thrombin molecule by heparin, resulting in its conversion to a potent adhesive protein for ECs. This conversion is most effective in contact with cell surfaces, basement membranes and ECM.

CELL interactions with the extracellular matrix (ECM)¹ play important roles in determining cellular morphology, growth, and differentiation (1, 31). When cells adhere to a substratum, contacts are established mainly via members of the integrin superfamily (37, 48). During this process, integrins become localized on the ventral surface of the cells in focal contacts, providing a transmembrane

link between the ECM and the cytoskeleton cell machinery (11, 40).

The vascular endothelium has emerged as a highly dynamic environment maintaining its integrity by factors that mediate the attachment and growth of endothelial cells (ECs). The integrity of the vascular endothelium is an essential requirement governing the vascular tone and permeability, as well as preventing the vessel wall from platelet deposition and thrombus formation (19, 36). Most EC receptors for ECM proteins belong to the integrin superfamily recognizing an Arg-Gly-Asp (RGD) containing sequence (1, 31). We have previously shown that thrombin, a serine protease with major roles in hemostasis, may function as a matrix adhesive protein (4, 5). Thrombin preparations modified either at the procoagulant or catalytic sites, induced EC adhesion medi-

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1. *Abbreviations used in this paper:* ATIII, anti-thrombin III; PN-1, protease nexin-1; EC, endothelial cells; ECM, extracellular matrix; GAG, glycosaminoglycan; HS, heparin sulfate; RGD, Arg-Gly-Asp.

ated by interaction of its RGD sequence (residues 187–189 of thrombin B-chain) with the $\alpha_v\beta_3$ integrin (4). Native thrombin, however, did not exhibit adhesive properties, consistent with crystal structure analysis showing that of the RGD sequence in thrombin, only Arg 187 is surface exposed (9).

During tissue repair and wound healing the hemostatic plug is dissolved by active fibrinolysis, where plasmin is generated to degrade the fibrin mesh, releasing functionally intact and active thrombin (8). The plasminogen activation system has been implicated in a variety of physiological and pathophysiological events, such as cell migration, fibrinolysis, tissue remodeling, inflammation, and tumor metastasis (13). The presence of plasminogen in appreciable quantities in extracellular fluids and extravascular compartments is a prerequisite for generation of plasmin through the concerted action of plasminogen activators and their inhibitors (41, 46). Moreover, cell surface receptors for these activators and plasminogen (7, 38), as well as the fibrin clot, may serve to localize plasmin formation during pericellular proteolysis and fibrinolysis.

We investigated whether activation of the fibrinolytic system may act on the procoagulant protein thrombin in a manner that exposes its RGD domain. Our results indicate that plasmin converts thrombin to a potent adhesive molecule in a process that is significantly enhanced by soluble heparin and by heparan sulfate on cell surfaces and in the ECM.

Materials and Methods

Materials

Plasmin was purchased from American Diagnostica Inc. (New York, NY). Anti-prothrombin antibodies were obtained from Dakopatts Inc. (Glostrup, Denmark) and heparin was from Kabi-Pharmacia (Uppsala, Sweden). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson & Co. (Oxnard, CA), 4-well plates were from Nunc (Roskilde, Denmark) and 96-well plates from Costar Co. (Cambridge, MA). DME (1 g glucose/liter or 4.5 g glucose/liter, calf serum, FCS, penicillin, streptomycin, and saline containing 0.05% trypsin, 0.01 M sodium phosphate, and 0.02% EDTA (STV), were obtained from Biological Industries (Beit Haemek, Israel). Bacterial (flavobacterium heparinum) heparinase I (EC 4.2.2.7, IBEX 1011) was kindly provided by Dr. J. Zimmermann (IBEX Technologies, Montreal, Canada). The synthetic hexapeptides (GRGDSP, GRGESP) were obtained from Peninsula Laboratories, Inc. (Belmont, CA).

Cells

Cloned populations of adult bovine aortic endothelial cells (ABEC) and early passage cultures of bovine corneal endothelial cells were established as previously described (23, 24). Cells were cultured in DME (1 g glucose/liter) containing 10% bovine calf serum, penicillin (50 U/ml), and streptomycin (50 μ g/ml) (GIBCO-BRL, Gaithersburg, MD) at 37°C in 10% CO₂ humidified incubators. Partially purified, brain-derived bFGF (100 ng/ml) was added every other day during the phase of active cell growth. Cells were dissociated with 0.05% trypsin/0.02% EDTA, 0.01 M sodium phosphate (pH 7.4) (STV) solution and subcultured at a split ratio of 1:5. ECs were characterized by indirect immunofluorescence using rabbit anti-human factor VIII antigen (Behringwerke Ag, Mailburg, Germany). The CHO parental line (KI) and mutant lines (pgs A-745, pgs D-803, and pgs D-677) selected for deficiencies in glycosaminoglycans (GAGs) were prepared as described (16, 17). Mutant 745 cells are deficient in xylosyltransferase, which catalyzes the first sugar transfer step in GAG biosynthesis. The total sulfated GAGs produced by these cells is <5% of the amount made by the wild type. Mutant 803 cells produce about 16% of the sulfated GAGs found in wild type cells due to a defect in glycosaminoglycan elongation, with decreased heparan sulfate and somewhat decreased content of chondroitin sulfate proteoglycans. Mutant 677 does not make any heparan sulfate, but synthesizes threefold more chondroitin sulfate proteoglycans

compared to the wild type (15–17, 34). These cells were cultured in F12 medium supplemented with 10% FCS.

Preparation of Dishes Coated with ECM

Bovine corneal endothelial cells were dissociated from stock cultures (2nd–5th passage) with STV and plated into 4-well plates at an initial density of 5×10^4 cells/ml. Cells were maintained as described above, except that 5% dextran T-40 was included in the growth medium. For preparation of sulfate-labeled ECM, Na₂(³⁵S)O₄ was added (40 μ Ci/ml) 3 and 7 d after seeding the cells and the cultures were incubated with the label with no medium change. After the cells reached confluence (6–8 d), 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 NH₄OH in PBS, followed by four washes in PBS (4, 25, 50). The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish. The presence of nuclei, cytoskeletal elements and serum proteins could not be detected in the denuded ECM (25).

Preparation of Coated Surfaces

Thrombin and various modified thrombin preparations were diluted to 10 μ g/ml in PBS containing 0.1% BSA and adsorbed onto the surface of 16-mm wells of 4-well plates for 2 h at 4°C. Unbound thrombin was removed and the dishes were washed three times with PBS. To determine the amount of thrombin adsorbed to the surface of the dish, thrombin was iodinated as described (3) and the amount of protein adsorbed to the tissue culture plastic determined by γ counting of radioactive material solubilized with 1 N NaOH. In representative experiments, [¹²⁵I]thrombin was included in the incubation medium with plastic, cells or ECM, together with unlabeled thrombin at a final concentration of 10 μ g/ml. The concentration of plasmin was 2 μ g/ml when incubated with thrombin alone or 0.2 μ g/ml when incubated in the presence of CHO cells or ECM. These measurements revealed that regardless of the amount of cpm added (up to 1×10^6 cpm) ~1% of the thrombin was bound to both plastic and each of the CHO cell types, as compared to ~7% that was bound to ECM.

Attachment Assay

Confluent endothelial cells were dissociated with trypsin-EDTA solution, washed once in growth medium, and resuspended in DME containing 0.2% BSA. Cells (1.3×10^5 cells/well) were added to each protein-coated well and incubated at 37°C for 2 h. The plates were washed three times with PBS and the firmly attached cells were fixed with 3% paraformaldehyde. Fixed cells were rinsed with 0.1 M borate buffer (pH 8.5), stained (10 min at 22°C) with 0.1 ml/well methylene blue (1% in 0.1 M borate buffer, pH 8.5), and washed four times in borate buffer. This procedure removed practically all noncell-bound dye. Specific cell incorporated methylene blue was dissolved with 0.1 N HCl (0.2 ml/well, 40 min, 37°C) and determined by its absorbance at 600 nm. Uptake of methylene blue is linearly correlated to the number of viable cells (22).

Results

Effect of Plasmin on Thrombin-induced Endothelial Cell Attachment

Native α -thrombin does not exhibit adhesive properties although it possesses an RGDA sequence at residues 187–190 of thrombin B-chain (5). We have previously demonstrated that chemical modification of the molecule gives rise to species of thrombin that are highly adhesive to ECs. This points to the possibility that chemical modifications alter the molecule and expose its RGD domain. We investigated whether plasmin, the major enzyme active during fibrinolysis, may interact with thrombin in a manner that reveals its RGD domain. For this purpose, soluble thrombin was incubated with increasing concentrations of plasmin and then analyzed for its ability to promote EC attachment. As shown in Fig. 1 *a*, incubation of thrombin with plasmin (0.2–3.5 μ g/ml, 25°C, pH 6.5) followed by coating of plastic surfaces, rendered

thrombin potent adhesive properties. There was no effect to coating with plasmin alone. When thrombin (10 $\mu\text{g/ml}$) was first used to coat the plastic surface and then treated with plasmin (1 $\mu\text{g/ml}$, pH 6.5, 25°C), little or no attachment activity was observed (not shown), indicating that only soluble thrombin can be modulated by plasmin. In support of this observation are experiments subjecting [^{125}I]thrombin and (5×10^5 cpm) to plasmin (2 $\mu\text{g/ml}$, 2 h, 25°C) followed by SDS/PAGE and autoradiography, showing little or no degradation of immobilized thrombin as compared to low relative molecular weight fragments generated in solution (not shown). This does not exclude the possibility that a limited degradation and/or modulation of the bound thrombin may occur. Time course studies showed that a 30-min exposure of thrombin to plasmin was sufficient to obtain a maximal attachment of EC to surface immobilized thrombin (Fig. 1 b).

In other experiments thrombin (50 $\mu\text{g/ml}$) was preincubated (4 h, 25°C) with plasmin (10 $\mu\text{g/ml}$) and cleavage fragments were subjected to gel filtration analysis on FPLC Superdex 75 column (Fig. 2 a). Four distinct cleavage products were obtained and the pooled fractions of each peak used to coat the surfaces of 4-well plates. EC adhesion was induced by a low relative molecular weight ($\sim 3,000$ D) cleavage product (peak IV) and was completely inhibited in the presence of the synthetic peptide GRGDSP (Fig. 2 b). A very low residual attachment activity was induced by cleavage products other than those eluted in pooled fraction IV. This background activity was similar to that obtained by native thrombin and may be mediated by RGD of other adhesive proteins, mainly fibronectin, possibly secreted by endothelial cells during the attachment assay. In an attempt to identify and sequence the RGD-containing fragment, the active peak (#IV, Fig. 2 a) was subjected to reverse phase HPLC (C₁₈ column). Several peptides (5–7, in different experiments) were obtained and tested for adhesion promoting activity. For this purpose the acetonitrile was evaporated and the samples lyophilized, with little or no effect on the adhesion promoting activity. Both the active and inactive peptides were subjected to partial sequence analysis. Among the peptides that were clearly identified was a disulfide-linked peptide (retention time 40.7 min) immediately adjacent to Arg of the RGD sequence, having Lys₁₈₆ followed by GEDPKYGAC in its COOH terminus and Gly₁₅₀ in its NH₂ terminus (Fig. 2 c). The disulfide linkage is between Cys₁₈₂ and Cys₁₆₉). We also identified a peptide (retention time 29.39 min) starting from Lys₂₂₃ to Arg₂₃₂. From these results we concluded that exposure of thrombin to plasmin generated a 31-amino acid peptide, containing the RGD sequence (Arg₁₈₇–Arg₂₂₁; Fig. 2 c). This is due to formation of a disulfide bond (Cys₁₉₁ and Cys₂₁₉) between two fragments, taking into account that a 6-amino acid peptide (Lys₂₀₂–Arg₂₀₆) is cleaved away (Fig. 2 c). We have thus sequenced two peptides adjacent to the 31-amino acid fragment of thrombin which contains the active RGD. Our results also indicate that plasmin preferentially cleaves next to lysine₁₈₆, leaving the Arg₁₈₇ intact.

Effect of GAGs on Plasmin-stimulated Thrombin-Induced EC Attachment

Thrombin possesses highly cationic residues that bind effectively to the negatively charged sulfated polysaccharide

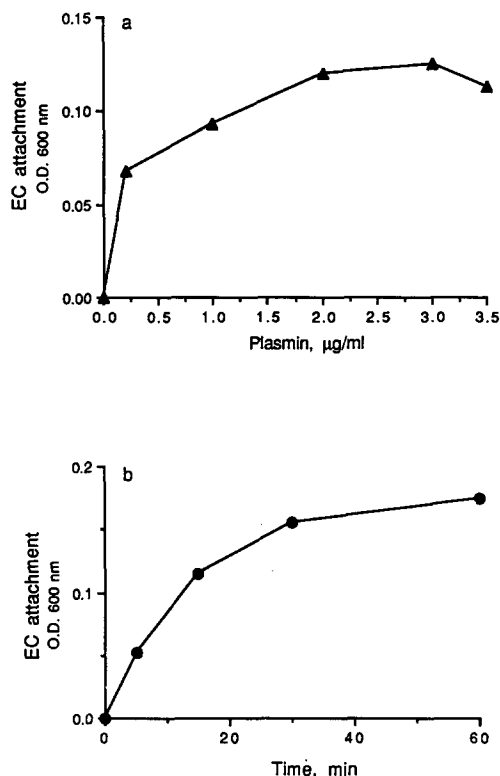


Figure 1. Effect of plasmin on thrombin adhesive properties. (a) Dose response. Thrombin (10 $\mu\text{g/ml}$ in 10 mM Tris-HCl, pH 6.5) was preincubated for 1 h with increasing concentrations of plasmin prior to coating plastic surfaces. ECs (0.5×10^6 cells/well) were seeded in medium containing 0.2% BSA and unattached cells removed after a 2-h incubation at 37°C. The number of attached cells was evaluated by methylene blue uptake. (b) Time course. 4-well plates were coated with thrombin (10 $\mu\text{g/ml}$) that was preincubated with 1 $\mu\text{g/ml}$ plasmin for varying periods of time, as indicated. Cell attachment obtained in the presence of thrombin alone (<0.03 OD) was subtracted. EC attachment was then measured as described in Materials and Methods. The variation between triplicate determinations did not exceed $\pm 15\%$ of the mean.

heparin. We have therefore analyzed the effect of heparin and other GAGs on plasmin modulation of thrombin, as monitored by the induction of cell attachment activity. EC attachment was markedly enhanced when thrombin was pretreated with plasmin in the presence of increasing concentrations of heparin, reaching a maximal effect at 30 $\mu\text{g/ml}$ heparin (not shown). This effect was best demonstrated at a suboptimal concentration of plasmin (0.2 $\mu\text{g/ml}$), capable of inducing a low level of EC attachment to thrombin. As shown in Fig. 3, a marked elevation in the attachment activity of thrombin was also observed following incubation of thrombin with plasmin in the presence of heparan sulfate (HS). The effect was specific to heparin and HS since other GAGs (e.g., dermatan sulfate, keratan sulfate, chondroitin sulfate) had no effect on the ability of thrombin to induce EC attachment (Fig. 3).

Effect of Cell Surface Heparan Sulfate on Plasmin-Stimulated Thrombin-induced EC Attachment

We investigated the potential involvement of cell membrane

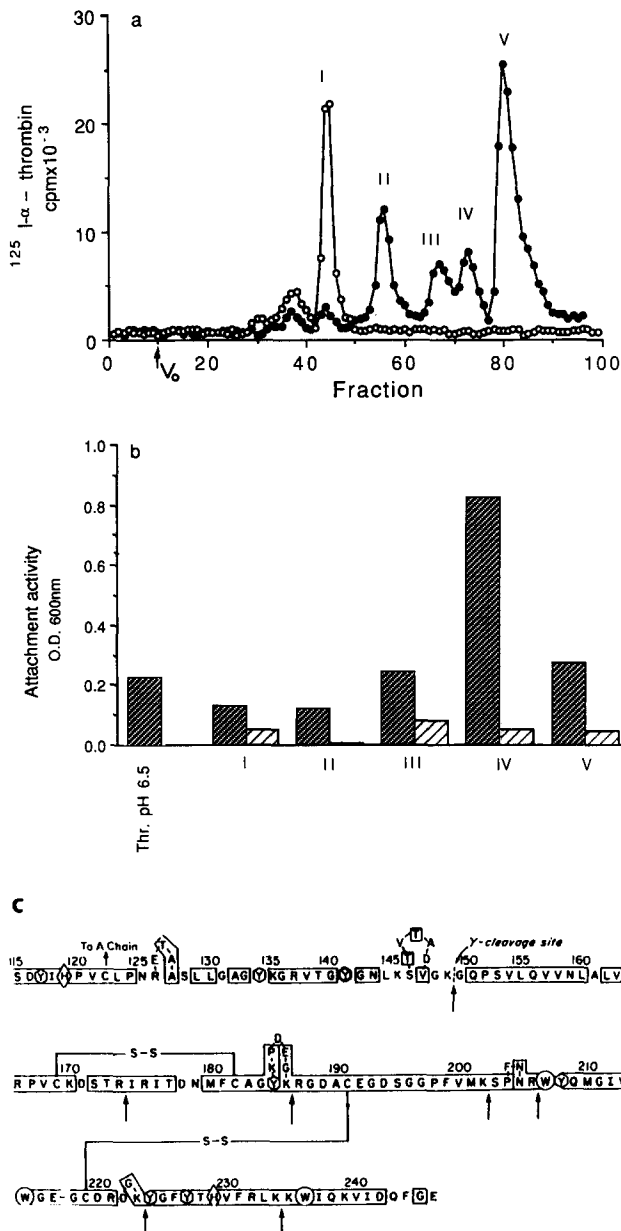


Figure 2. Gel filtration analysis of thrombin-derived fragments cleaved by plasmin. (a) ^{125}I - α -thrombin ($50\ \mu\text{g}$, $5 \times 10^5\ \text{cpm}$) was incubated (4 h, 10 mM Tris-HCl, pH 6.5) in the absence (—○—) or presence (—●—) of $10\ \mu\text{g}/\text{ml}$ plasmin. The reaction mixture was then subjected to gel filtration analysis on FPLC Superdex 75 column (Pharmacia, Uppsala). Fractions (0.4 ml) were collected (1 ml/min) and counted in a γ counter. (b) Attachment activity of thrombin fragments generated by plasmin and separated by gel filtration. Peak fractions were pooled and each peak (I, II, III, IV, V) was used to coat 4-well plates for measurements of EC attachment in the absence (■) and presence (▨) of GRGDSP peptide. The variation between triplicate determinations did not exceed $\pm 12\%$ of the mean. (c) Amino acid sequence of the RGD-containing region of human thrombin B chain. The residues are aligned with residues 115 through 245 of bovine chymotrypsin. Individual residues are identified by the single-letter code. Plasmin cleavage sites are indicated by arrows. The disulfide bonds (S-S) are shown above the sequence line.

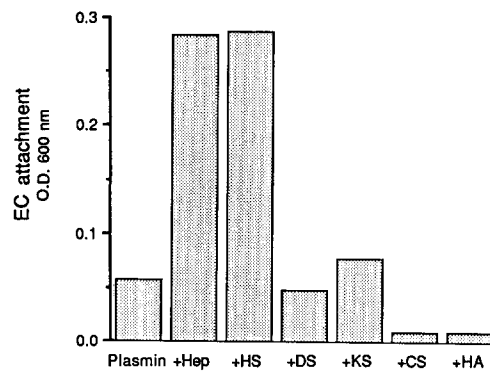


Figure 3. Effect of GAGs on thrombin adhesive properties. Thrombin was treated with a low concentration of plasmin ($0.2\ \mu\text{g}/\text{ml}$) in the absence or presence of $100\ \mu\text{g}/\text{ml}$ GAGs (Hep, heparin; HS, heparan sulfate; DS, dermatan sulfate; KS, keratan sulfate; CS, chondroitin sulfate; HA, Hyaluronic acid) before coating dishes. ECs were allowed to attach for 2 h at 37°C and the extent of attached cells was evaluated by the methylene blue uptake assay. Attachment to dishes coated with the GAGs and native thrombin alone ($<0.02\ \text{OD}$) was subtracted. There was no attachment of cells to dishes coated with the GAGs alone. The variations between triplicate determinations did not exceed $\pm 10\%$.

associated proteoglycans in plasmin cleavage of thrombin and its subsequent ability to promote EC attachment. For this purpose we have used CHO cell mutants (15–17, 34), expressing specific defects in various aspects of GAG biosynthesis. We compared the abilities of CHO wild type and mutant cells to promote plasmin mediated conversion of thrombin to an adhesive molecule, as monitored by the stimulation of EC attachment. Mutant 745 CHO cells, fail to produce all types of proteoglycans due to a deficiency in enzymes required to form the common tetrasaccharide that links GAG chains to core protein (16, 17). The CHO mutant 677, synthesizes all GAGs except heparan sulfate due to a defect in the enzymatic activity required to form the repeating disaccharide unit characteristic of heparan sulfate (15). Exposure of thrombin to plasmin was carried out by incubation on the surface of wild-type and mutant CHO cells. To prevent secretion of adhesive proteins by the CHO cells, monolayers were pretreated with monensin (4), or were fixed with 3% formaldehyde. Under these conditions no secretion of cellular adhesive proteins was observed, as indicated by the lack of EC adhesion when supernatants of the CHO cells were used to coat plastic surfaces. We co-incubated thrombin and plasmin on the surface of fixed CHO wild-type and mutant cells and used the supernatants for coating dishes prior to monitoring the levels of EC attachment. As can be seen in Figs. 4 and 5, a marked enhancement of EC attachment was observed following incubation of thrombin and plasmin on the wild-type CHO cells, as compared to minimal levels of cell attachment obtained following a similar incubation with mutant cells lacking all GAGs or only heparan sulfate. Mutant 677 cells expressing a threefold higher level of chondroitin sulfate than the wild-type cells and mutant 745 cells failed to promote EC attachment to thrombin (Figs. 4 and 5). The residual extent of cell adhesion and spreading observed following incubation of plasmin and thrombin on top of HS-deficient CHO mutants (Fig. 5, b and c) may be

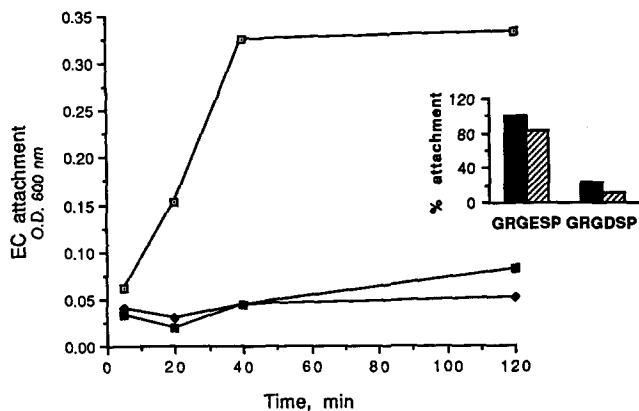


Figure 4. Adhesive properties of thrombin after incubation on wild-type and HS-deficient CHO mutant cells. Thrombin (10 $\mu\text{g/ml}$) and plasmin (0.2 $\mu\text{g/ml}$) were preincubated (1 h, 37°C) on the surface of formaldehyde fixed (3%, 15 min) monolayers (0.5×10^6 cells/well) of either CHO-KI wild-type cells (\square) or the cell mutants 677 (\blacklozenge) and 745 (\square). The preincubation was carried out for various periods of time, as indicated. Supernatants were collected and used for coating the dishes. The values were corrected for the attachment obtained in the presence of CHO cells incubated with thrombin alone (<0.025 OD). ECs were then allowed to adhere and the extent of attached cells was evaluated. (Inset) RGD dependence of thrombin adhesive properties acquired upon incubation with CHO-KI cells. Thrombin (10 $\mu\text{g/ml}$) and plasmin (0.2 $\mu\text{g/ml}$) were preincubated (60 min, 24°C) on fixed CHO-KI cells, and then collected and used to coat dishes. EC attachment was performed in the presence of 0.3 $\mu\text{g/ml}$ (\blacksquare) and 1 $\mu\text{g/ml}$ (\square) of the synthetic peptides GRGSDP and GRGESp. The level of EC attachment was evaluated. The variation between different determinations did not exceed $\pm 12\%$ of the mean.

due to a very limited exposure of the RGD sequence in the presence of a suboptimal amount of plasmin (0.2 $\mu\text{g/ml}$; see Fig. 1) alone and residual HS ($<5\%$) expressed by the mutant cells. These results indicate that cell surface-associated HS is involved in plasmin-mediated conversion of thrombin to an adhesive molecule. In control experiments, [^{125}I]plasmin (0.2 $\mu\text{g/ml}$, 184,500 cpm) was incubated (2 h, 25°C) with CHO wild type and mutant cells to investigate whether differences in cell attachment can be attributed to a difference in plasmin binding to the cells, resulting in a different conversion of thrombin to an adhesive protein. 8–10% of the added plasmin was bound to the various CHO cell types and to ECM, indicating that the observed differences in cell adhesion are not due to differences in the amount of available plasmin. Likewise, the various CHO cell types did not differ in their ability to bind [^{125}I]thrombin (0.7–1% of the added thrombin). Gel filtration analysis and cell adhesion promoting activity of thrombin fragments generated during incubation on top of wild-type CHO cells in the presence of a suboptimal concentration (0.2 $\mu\text{g/ml}$) of plasmin was similar to the pattern obtained upon incubation of thrombin and plasmin (2 $\mu\text{g/ml}$) in solution (not shown).

The enhanced attachment of EC after incubation of thrombin and plasmin on top of fixed wild-type CHO cells was mediated through the RGD domain of thrombin. This was shown by the complete inhibition of EC attachment obtained when the synthetic peptide GRGDSP (Fig. 4, inset), or anti-

prothrombin antibodies (not shown) were present. There was no effect in the presence of GRGESp peptide (Fig. 4, inset), or nonimmune serum. The extent of EC attachment to thrombin following its incubation with plasmin in contact with CHO wild-type cells was linearly dependent on the number of CHO cells (Fig. 6). It is therefore conceivable that cell membrane-associated HS is directly involved in the conversion of thrombin by plasmin to an adhesive protein. We, therefore, postulated that cell-associated HS modulates thrombin in a manner that renders the molecule more accessible to cleavage by plasmin. This possibility gains support from data (Fig. 7) showing that when thrombin alone was preincubated on the surface of fixed CHO cells and then collected and subjected to cleavage by a suboptimal concentration of plasmin, a marked stimulation of EC attachment was obtained (Fig. 7 C), similar to the effect observed when both plasmin and thrombin were incubated on the cell monolayers (Fig. 7 B). When plasmin, on the other hand, was first preincubated with CHO cells, no induction of EC attachment to immobilized thrombin was obtained (Fig. 7 D). These results indicate that thrombin has to interact with the cell surface for its efficient conversion to an adhesive molecule. Anti-prothrombin antibodies efficiently inhibited the adhesive properties of thrombin induced by plasmin on the surface of fixed CHO-KI cells (Fig. 7 E).

Conversion of thrombin to an adhesive molecule was also accelerated upon incubation of thrombin and plasmin on top of a fixed subendothelial ECM (Fig. 8). Similar to the results observed with CHO cells, exposure of thrombin to a low concentration plasmin (0.2 $\mu\text{g/ml}$) resulted in its conversion to an adhesive molecule only in the presence of intact ECM, or when thrombin was first incubated with ECM (Fig. 8, B–D). This accelerated conversion was mediated by the ECM HS since it was markedly inhibited when the ECM HS was first extensively degraded by bacterial heparinase (0.5 U/ml, 2 h, 37°C) (Fig. 8 F). Use of metabolically sulfate ($\text{Na}_2^{35}\text{SO}_4$) labeled ECM revealed that $\sim 90\%$ of the total incorporated radioactivity was released by treatment with the heparinase enzyme. EC attachment to thrombin following exposure to plasmin on fixed ECM was inhibited in the presence of GRGDSP peptide (Fig. 8 G) or anti-thrombin/prothrombin antibodies (not shown).

Discussion

Adhesive interactions of cells with their extracellular environment is a complex process associated with morphological alterations, cell migration, and formation of focal contact structures. These adhesive interactions play a leading role in the progression of vascular thrombosis and subsequent wound healing. After vascular injury, the fibrin-dependent aggregation of platelets adherent to the exposed subendothelium (6, 36) contributes to the formation of thrombus that initially seals the vessel to prevent excessive blood loss. Subsequently, a local repair mechanism is initiated, involving EC attachment, migration, and proliferation to renew the damaged vessel. Therefore, to understand the molecular events involved in thrombus formation and wound healing, it is necessary to delineate structural interactions between cells and molecules participating in this process.

The vascular endothelium forms an active boundary be-

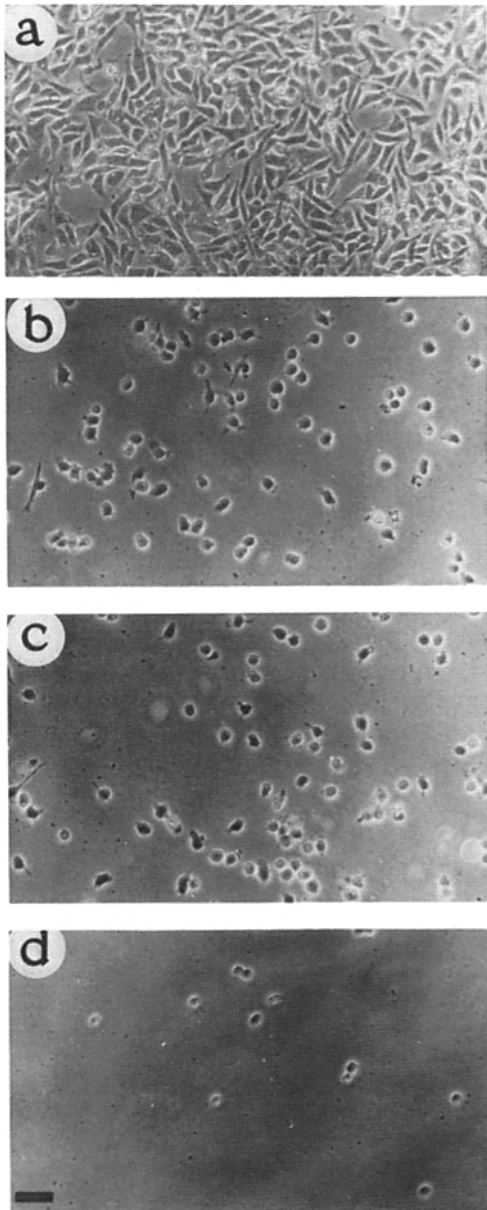


Figure 5. Attachment of endothelial cells to plasmin-treated thrombin, preincubated on CHO-KI wild-type and HS-deficient CHO cells. Thrombin ($10 \mu\text{g/ml}$) and plasmin ($0.2 \mu\text{g/ml}$) were preincubated (60 min, 24°C) on formaldehyde fixed cells: (a) CHO-KI wild type; (b) CHO mutant 745; (c) CHO mutant 677. Supernatants were then used to coat dishes and the extent of EC attachment was evaluated as compared to; (d) dishes coated with thrombin that was preincubated on fixed CHO-KI cells in the absence of plasmin. Phase contrast micrographs were taken after a 1-h incubation at 37°C with ECs. Bar, $75 \mu\text{m}$.

tween the circulating blood and underlying tissues. The integrity of the system is an essential requirement for the controlled vascular tone and permeability and for maintaining the non-thrombogenic properties of the EC apical surface. Thrombin, the central bioregulatory enzyme in hemostasis, may be present within the vascular system, immobilized to the subendothelial basement membrane, active and protected from inactivation (3). ECM-bound thrombin may thus exhibit prolonged and localized functions within the vascular system. An internal source for thrombin in the vessel wall

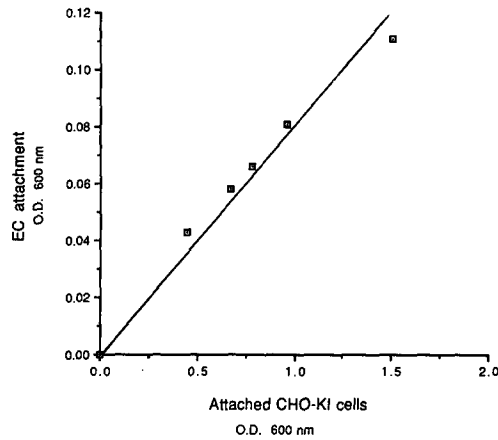


Figure 6. Effect of CHO-KI cell number on the adhesive property of thrombin. EC attachment to dishes coated with thrombin ($10 \mu\text{g/ml}$) that was first incubated (60 min) with plasmin ($0.2 \mu\text{g/ml}$) on the surface of increasing numbers of fixed CHO-KI cells (i.e., 5×10^3 , 5×10^4 , 5×10^5 , 10^6 , 2×10^6 , 3×10^6 cells/well). Correlation plot between the extent of EC attachment and the number of CHO-KI cells was obtained. The level of attachment obtained to native thrombin alone was subtracted. Each data point is a mean of five determinations and the variation between different determinations did not exceed $\pm 15\%$ of the mean.

may be provided by the thrombus, from which it can be released intact and active during fibrinolysis or by transendothelial passage through gaps formed between adjacent EC (20, 33, 53).

In this report we present data showing that plasmin, the major enzyme generated during fibrinolysis, acts on thrombin in a manner that converts it to a potent adhesive molecule. This conversion was greatly enhanced in the presence of heparin or heparan sulfate, but not by other species of

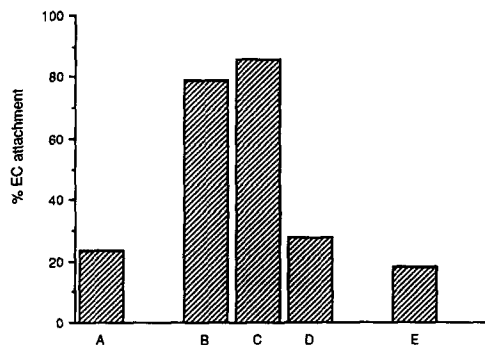


Figure 7. Effect of incubation with CHO cells on the induction of thrombin adhesive properties by plasmin. The following modes of sequential incubations were performed: (A) Thrombin ($10 \mu\text{g/ml}$) incubated (60 min, 24°C) with plasmin ($0.2 \mu\text{g/ml}$) prior to coating; (B) thrombin incubated with plasmin on formaldehyde fixed CHO-KI cell monolayers; (C) thrombin preincubated on fixed CHO-KI cell monolayers and then collected and incubated (60 min, 24°C) with plasmin; (D) plasmin preincubated on CHO-KI cell monolayers and then collected and incubated with thrombin; and (E) preincubation similar to B with anti-thrombin/prothrombin antibodies (1:100) present during the attachment assay. The number of EC attachment to $\text{NO}_2\text{-}\alpha$ -thrombin (0.32 OD) was evaluated as 100%. Each data point is a mean of six determinations and the variation between different determinations did not exceed $\pm 12\%$ of the mean.

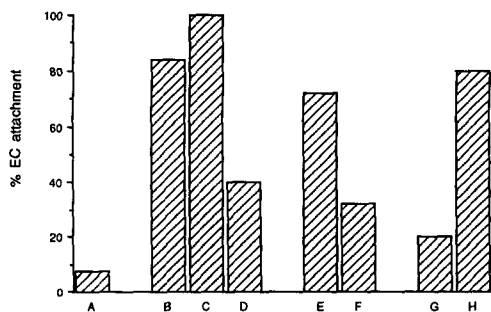


Figure 8. Effect of fixed ECM on the induction of thrombin adhesive properties by plasmin. The following modes of sequential incubations were performed: (A) Thrombin (10 $\mu\text{g/ml}$) incubated (25°C, 1 h) with plasmin (0.2 $\mu\text{g/ml}$) before coating; (B) thrombin and plasmin incubated as in A, but on formaldehyde fixed ECM; (C) thrombin (10 $\mu\text{g/ml}$) incubated (25°C, 1 h) on fixed ECM. The incubation medium was then treated (25°C, 1 h) with plasmin before coating the tissue culture plastic; (D) plasmin (0.2 $\mu\text{g/ml}$) was incubated on fixed ECM followed by treating the incubation medium with thrombin and coating the plastic surfaces. (E and F) ECM was treated (2 h, 37°C) with bacterial heparinase (E, 0.05 U/ml; F, 0.5 U/ml), fixed, and incubated (1 h, 25°C) with thrombin and plasmin. The incubation medium was then used to coat the dishes. (G and H) Plasmin and thrombin incubated on fixed ECM before coating. EC attachment was performed in the presence of (G) GRGDSP, or (H) GRGESP peptides (1 $\mu\text{g/ml}$). The number of EC attached to $\text{NO}_2\text{-}\alpha$ -thrombin (0.35 OD) was evaluated as 100%. Each data point is a mean of four determinations and the variation between different determinations did not exceed $\pm 15\%$ of the mean.

GAGs. Although heparin interacts with both thrombin and its inhibitors, anti-thrombin III (ATIII), and protease nexin-1 (PN-1), the acceleration of its inactivation is believed to result from interaction of thrombin with the inhibitors alone (18, 30). It was therefore suggested that thrombin-heparin interactions are not significant in terms of affecting thrombin functional activities (39). Our data demonstrate, for the first time, the importance of thrombin-heparin interactions, resulting in modulation of thrombin in a manner that markedly enhances its conversion by plasmin to a potent adhesive protein. The role of cell surface heparan sulfate in this conversion was investigated using a series of genetically mutated CHO cells, expressing various defects in GAG synthesis. By analyzing the level of EC attachment to thrombin, following incubation of plasmin and thrombin on the surface of these mutant cells, we demonstrated that cell-associated HS is directly involved in plasmin-induced conversion of thrombin to an adhesive molecule. The possibility, however, that cell surface modulation of thrombin may require also interaction with other cell surface molecules, cannot be excluded. Our results show that thrombin acquires adhesive properties only upon incubation with wild-type CHO-KI cells, known to possess both HS and chondroitin-4-sulfate on the cell surface. No attachment promoting activity was induced when thrombin and plasmin were preincubated on the surface of CHO cell mutants lacking both GAGs or only heparan sulfate, regardless of the content of chondroitin sulfate. Incubation of thrombin with plasmin and soluble heparin or HS was less effective than incubation with wild-type CHO cells.

HS proteoglycans are a diverse group of macromolecules containing at least one covalently bound HS chain, with

N- and O-linked sulfate groups. They are widely distributed throughout animal tissues associated with cell surfaces and basement membranes and are becoming increasingly recognized as mediators of the binding and function of heparin-binding growth factors (e.g., bFGF, VEGF, HB-EGF) (21, 28, 32, 51, 52), adhesive proteins (e.g., N-CAM, fibronectin) (12), plasma proteins (e.g., vitronectin) (43), enzymes (e.g., LPL) (14, 32, 52), enzyme inhibitors (ATIII, PN-1) (18, 52) and viruses (47). The structural diversity of HS is particularly suited for generating specific domain structures that can be used for biological recognition of specific proteins. A specific pentasaccharide domain is critical for the interaction between heparin and ATIII (32) and has also been identified in HS proteoglycans on the surface of capillary EC (29, 35). Fibroblasts produce a different, not yet identified, species of cell surface and ECM-associated HS that accelerate the inhibition of thrombin by PN-1 (18). A bFGF-binding sequence has recently been identified in fibroblast HS (49). Studies are underway to determine whether a specific type of cell surface and ECM heparan sulfate is involved in the observed plasmin-mediated conversion of thrombin to an adhesive molecule. This conversion provides another example of a localized reaction occurring in contact with the cell surface and ECM and which is greatly accelerated by HS and heparin-like molecules. Recently, it has been shown that cell surface HS, but not other GAGs, is the principal site for binding of herpes simplex to cells and for subsequent infection of the cells (47). Transfection of CHO cell mutants lacking HS with FGF receptor revealed that binding of bFGF to the receptor requires the presence of either cell surface heparan sulfate or soluble heparin (44, 56). In other cases, the protein portion in the extracellular domain of HS proteoglycans appears to be involved in direct interactions with other molecules, such as TGF- β (2, 54), acidic FGF receptor (45), and hyaluronate (10).

Plasmin is generated enzymatically from plasminogen by a highly regulated mechanism of a widely distributed class of serine proteases referred to as plasminogen activators. The enhanced kinetics of plasminogen activation by tissue type plasminogen activator obtained on fibrin as compared to solution, provides an extremely efficient mechanism for intravascular thrombolysis. Once formed on the fibrin surface, plasmin initiates the process of substrate degradation. As a consequence of the initial proteolysis, carboxy-terminal lysyl residues are generated which provide additional binding sites for plasminogen and plasmin (27, 54). Moreover, when complexed to fibrin, plasmin is protected from inhibition by α_2 -plasmin inhibitor (26, 42) that effectively inhibits circulating plasmin. Fibrin- or cell surface-immobilized plasmin may thus actively release thrombin trapped within a clot and at the same time may expose the cell adhesion domain of thrombin.

Gel filtration analysis of cleavage products obtained during incubation of thrombin with plasmin suggests that the cell adhesion promoting activity of thrombin may reside in a specific fragment, about 31-amino acids in size, which contains the RGD sequence. It should be noted that the RGD site in thrombin is located next to a unique domain which is not present in other serine proteases (9). Exposure of this specific domain together with the RGD sequence may elicit the entire cell adhesion activity induced by thrombin.

Thrombin-mediated acceleration of cell adhesion may function in maintaining the integrity of the vessel wall. Ex-

periments using antibodies directed against the RGD epitope of thrombin will hopefully clarify whether such a role is indeed fulfilled by thrombin. In conclusion, we present evidence that under certain conditions thrombin may acquire adhesive properties and hence be actively engaged in supporting EC adhesion. Adoption of this unique function of thrombin is accelerated by heparin and HS, particularly when present on the cell surface and ECM. Chemical modifications of thrombin revealed that Lys₂₄₀ and Lys₁₆₉, positioned near Arg₁₈₇, render thrombin accessible to heparin (9), suggesting that these positively charged groups are crucial for the conversion of thrombin to an adhesive protein. Moreover, it is conceivable that heparin may further protect Arg₁₈₇ from cleavage by plasmin. Altogether these results ascribe a new physiological significance to the heparin binding property of thrombin.

This work was supported by grants from the German-Israel Foundation for Scientific Research, and the GSF (Forschungszentrum fuer umwelt und gesundheit) awarded to R. Bar-Shavit, National Institutes of Health grant GM 33063 awarded to J. D. Esko, and by the Israeli Ministry of Health to R. Bar-Shavit.

Received for publication 8 December 1992 and in revised form 18 July 1993.

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