Thrombin-Induced Release of Active Basic Fibroblast Growth Factor-Heparan Sulfate Complexes From Subendothelial Extracellular Matrix

By Miriam Benezra, Israel Vlodavsky, Rivka Ishai-Michaeli, Gera Neufeld, and Rachel Bar-Shavit

The angiogenic factor, basic fibroblast growth factor (bFGF), is sequestered and protected by binding to heparan sulfate proteoglycans (HSPG) in the subendothelial extracellular matrix (ECM). Release of ECM-bound bFGF provides a novel mechanism for regulation of cell proliferation and neovascularization in normal and pathologic situations. Exposure of ECM to thrombin, the final activation product of the clotting cascade, resulted in release of high molecular weight HSPG-bFGF complex, as indicated by its immunoprecipitation with anti-bFGF antibodies, susceptibility to degradation by bacterial heparinase, and inhibition of its mitogenic activity in the presence of neutralizing anti-bFGF antibodies. The ECM-resident bFGF-HSPG complex was not released by thrombin in the presence of hirudin or antithrombin III, or by catalytically blocked thrombin preparations. A threefold to fivelfold higher mitogenic activity was released by thrombin from ECM that was preheated (1 hour, 80°C), as compared with native ECM. This difference is attributed to heat stable bFGF-HSPG complexes that are more readily released after heat treatment of the ECM and to activation and release of ECM-resident transforming growth factor-β (TGF-β) activity. Our results indicate that the large reservoir of proteolytic activity present in plasma in the form of prothrombin may participate in release from the subendothelial ECM of biologically active bFGF and TGF-β, depending on the accessibility of thrombin. Thrombin may gain access to the subendothelium on clot formation after tissue injury and as a result of the conversion of prothrombin to thrombin induced by the ECM itself.

© 1993 by The American Society of Hematology.

CULTURED bovine endothelial cells produce an underlying extracellular matrix (ECM) similar in organization and macromolecular composition to naturally occurring basement membranes.1,3 This ECM contains collagens (mostly types III and IV, with smaller amounts of types I, V, and VI), proteoglycans (mostly heparan sulfate- and dermatan sulfate-proteoglycans, with smaller amounts of chondroitin sulfate proteoglycans), laminin, fibronectin, enactin, and elastin.1,3 Endothelial cells and other cell types plated in contact with the subendothelial ECM no longer require the addition of soluble basic fibroblast growth factor (bFGF) to proliferate and express their differentiated functions.2,3 This result and the identification of ECM-bound growth factors, enzymes, and plasma proteins,4,6 indicate that the ECM provides a storage depot for active molecules and that some of the effects of ECM can be attributed to the combined action of structural components and of ECM-immobilized molecules that are thereby protected and stabilized.6,7 This may allow a more localized, regulated, and persistent mode of action, as compared with the same molecules in a fluid phase. Proteoglycans have been found to function as the principal binders of growth factors and cytokines and more than 20 known heparin-binding proteins contain a consensus structural motif that participates in this binding.5,10 Because proteoglycans are abundant and ubiquitous components of ECM, basement membranes, and cell surfaces, they are likely to be able to capture most of those growth factors and cytokines that have affinity for the glycosaminoglycan (GAG) or protein part of proteoglycans.5,10 Among these growth factors is bFGF, which was identified as a complex with heparan sulfate proteoglycans (HSPG) in the subendothelial ECM produced in vitro4 and in basement membranes of diverse tissues and blood vessels.5,11,12 Members of the FGF family exhibit a high affinity to heparin, are highly mitogenic for mesoderm- and neuroectoderm-derived cells, and are among the most potent inducers of neovascularization and mesenchyme formation.13,14 ECM-bound bFGF, unlike soluble recombinant bFGF, is stable to heat and acid inactivation and to proteolytic degradation.15,16 Release of bFGF from its storage in ECM by heparin-like molecules, heparan sulfate (HS)-degrading enzymes (ie, heparanase),17,19 or proteases (ie, plasmin)20 was suggested to elicit a localized neovascularization in processes such as wound healing, inflammation, and tumor development.

Thrombin (E.C. 3.4.21.5), the final activation product of the clotting cascade, is responsible for converting fibrinogen to fibrin monomers that polymerize spontaneously to form a typical clot mesh. In addition to its major role in hemostasis, thrombin, a multifunctional serine protease, degrades various constituents of the ECM13 and induces cellular responses such as proliferation, chemotaxis, and endothelial cell adhesion.22-25 We have shown that thrombin binds to the subendothelial ECM through a short anchorage binding site, leaving the majority of the molecule functional and available for cellular interactions.26 ECM-immobilized thrombin was found to be protected from inactivation by its circulating inhibitor antithrombin III (ATIII).26 Thus,
thrombin when sequestered by the ECM may exhibit a localized long-acting stimulation of surrounding tissues.

Under normal conditions, 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. Thus, thrombin may pass through the endothelial cell layer and reach subendothelial structures. Moreover, thrombin may also be accessible to the vascular subendothelium even at postoccluding events because on fibrinolysis, it can be released from a fibrin clot, intact and functionally active.

In view of thrombin accessibility to the subendothelial basement membrane, we have addressed the possibility that thrombin may also function in release of active bFGF and possibly other growth-promoting factors from ECM. Our findings indicate that bFGF within the subendothelial ECM is accessible to release by thrombin in an active and stable form as a high molecular weight complex with HSPG.

MATERIALS AND METHODS

Materials. Purified preparations of human thrombin and catalytically modified thrombin preparations (diisopropylfluorophosphate-α-thrombin [DIP-α-thrombin]; D-Phenylalanyl-L-Prolyl-L-Arginine chloromethyl-ketone-α-thrombin [PPACK-α-thrombin]) were kindly provided by Dr. W. F. Fenton II (Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany). ATIII was a generous gift from Behring Institute (Marburg, Germany) and hirudin was purchased from American Diagnostica (New York, NY). Dulbecco’s Modified Eagle’s Medium (DMEM, 1 g glucose/L or 4.5 g glucose/L), Fisher Medium, calf serum, fetal calf serum [FCS], penicillin, streptomycin, and saline containing 0.05% trypsin, 0.01 mol/L sodium phosphate, and 0.02% EDTA (sodium-trypsin-versen [STV]) were obtained from Biological Industries (Beit Haemek, Israel). Recombinant human bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Triton X-100, dextran T-40, and bovine serum albumin (BSA) were from Sigma Chemical Co (St Louis, MO). Bacterial (flavobacterium heparinum) heparinase I (EC 4.2.2.7, IBEX 101) was kindly provided by Dr. Affinity-purified rabbit anti-bFGF IgG was obtained from R&D Systems (Minneapolis, MN). Bovine corneal endothelial cell serum, 5% FCS, penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37°C in 10% CO2-humidified incubators. Partially purified brain-derived 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 (2nd to 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, NaCl (150 mM) was added (40 μCi/mL) 3 and 7 days after seeding the cells and the cultures were incubated with the label with no medium change. Six to 8 days after the cells reached confluence, the subendothelial ECM was exposed by dissolving (3 minutes, 22°C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mmol/L NH4OH in phosphate-buffered saline (PBS), followed by four washes in PBS. 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.

Iodination of bFGF. Recombinant bFGF was iodinated with chloramine T as described. Brieﬂy, bFGF (5 μg) was added to 60 μL of 0.2 mol/L sodium phosphate, pH 7.2, containing 1 μCi Na12'I. Chloramine T (10 μL of 1 mg/mL) was added for 45 seconds at room temperature and the reaction was stopped by the addition of 50 μL 0.05% sodium metabisulfite and 50 μL of 10 mmol/L KI. The reaction mixture was then applied onto a small (0.3 mL) heparin-Sepharose column equilibrated with 0.6 mol/L NaCl in 20 mmol/L phosphate buffer, pH 7.2. The column was washed with the same buffer and the eluted bFGF eluted with 1.5 mL phosphate buffer containing 2 mol/L NaCl. 0.3% 3-(3-cholamidopropyl)-di-methylammoniomethyl)propansulfonate (CHAPS), and 0.2% gelatin. The specific activity was 1.2 to 1.7 × 10^7 cpm/ng bFGF and the labeled preparation was kept for up to 3 weeks at -70°C.

Release of ECM-bound bFGF. ECM-coated wells (four-well plates) were preheated (1 hour, 80°C) to inactivate endogenous proteolytic activity residing in the ECM. The ECM was then incubated with iodinated bFGF (1.5 to 2.5 × 10^4 cpm/well, overnight, room temperature) and the unbound factor was removed by four washes with PBS containing 0.02% gelatin. The ECM was then incubated (7 hours, 37°C, PBS containing 0.02% gelatin) with thrombin and aliquots of the 0.25 mL incubation medium were counted in a γ-counter to determine the amount of released iodinated material. The remaining ECM was washed twice with PBS, solubilized with 1 mol/L NaOH, and the radioactivity counted in a γ-counter. The percentage of released 125I-bFGF was calculated from the total ECM-associated radioactivity. "Spontaneous" release of 125I-bFGF in the presence of incubation medium alone was 7% to 12% of the total ECM-bound bFGF. Native and heat-treated ECM-coated wells that were not exposed to 125I-bFGF were incubated with thrombin under similar conditions and mitogenic activity released into the incubation medium was determined using growth-arrested 3T3 fibroblasts.

Growth factor activity. Assay for DNA synthesis in 3T3 cells was performed as described. Brieﬂy, Balb/c 3T3 cells were plated at half confluence onto 0.3-cm2 microtiter wells in DMEM (4.5 g glucose/L) supplemented with 10% FCS. After reaching confluence (3 to 5 days), the cells were incubated with DMEM containing 0.2% FCS for 48 hours. Samples and 3H-thymidine (1 μCi/well) were then added to the quiescent cells and after an incubation period of 48 hours, DNA synthesis was assayed by measuring the incorporated radioactivity, as described.

Immunoprecipitation of heparan sulfate-bound bFGF. 35SO42-labeled, preheated ECM-coated 35-mm plates were preincubated (5
hours, 25°C) with or without 2 μg/mL bFGF, followed by three washes with PBS containing 0.02% gelatin to remove the unbound bFGF. The ECM was then incubated (48 hours, 37°C) in 0.5 mL PBS containing 0.02% gelatin in the absence or presence of 1 μmol/L thrombin. The incubation medium was collected and incubated (16 hours, 4°C, on an end over rotator) with rabbit anti-bFGF (1 to 24 N-terminal amino acids) antibodies immobilized onto protein A-Sepharose beads (200 μL beads containing 10 μL anti-bFGF antibodies). The beads were then washed three times with PBS and the bound molecules were eluted by boiling in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by electrophoresis in a 5% to 15% gradient polyacrylamide gel. The radiolabeled molecules were visualized by autoradiography.

**RESULTS**

*Release of ECM-bound 125I-bFGF.* In previous studies we have shown that 125I-bFGF binds to HSPG in the ECM and is efficiently released by HS-degrading enzymes. Because thrombin may, under certain conditions, gain access to the subendothelial ECM, we investigated whether ECM-bound bFGF is accessible to release by thrombin as well. For this purpose, ECM was preheated (1 hour, 80°C) to inactivate any intrinsic proteolytic activity residing in the ECM. The ECM was then incubated (overnight, 25°C) with 125I-bFGF, washed free of unbound bFGF, and exposed (9 hours, 37°C) to increasing concentrations of α-thrombin (0.01 to 5 μmol/L). As shown in Fig 1A, 0.01 μmol/L α-thrombin induced a minimal release (5.6%) of 125I-bFGF, while 5 μmol/L α-thrombin released up to 45% of the ECM-bound 125I-bFGF. Depending on the batch of ECM and 125I-bFGF, 5% to 12% of the ECM-bound 125I-bFGF were released during a similar incubation with PBS alone. This spontaneous release was subtracted from the total amount of bFGF released in the presence of α-thrombin. When the ECM was incubated with 0.1 μmol/L α-thrombin, an increased release of 125I-bFGF was observed as a function of time during the first 4 hours, remaining at a similar level thereafter (Fig 1B).

**Effect of catalytically blocked thrombin and thrombin inhibitors on release of ECM-bound bFGF.** DIP-α-thrombin was applied to determine whether alterations in thrombin catalytic site affect its ability to release ECM-bound bFGF. Addition of 0.1 μmol/L catalytically blocked thrombin elicited a basal level of bFGF release, similar to that released from ECM that was incubated with PBS alone (Fig 2). A similar result was obtained with PPACK-α-thrombin, indicating that release of ECM-bound bFGF is dependent on the catalytic activity of thrombin (Fig 2). Likewise, preincubation (90 minutes, 37°C) of α-thrombin (0.1 μmol/L) with hirudin (80 U/mL), a potent nonphysiologic inhibitor of thrombin, resulted in an almost complete inhibition of thrombin-induced release of ECM-bound bFGF. A similar inhibition was also obtained in the presence of the physiologic inhibitor of thrombin, ATIII. As shown in Fig 2, preincubation (90 minutes, 37°C) of α-thrombin (0.1 μmol/L) with 1 μmol/L ATIII, resulted in an almost complete inhibition of bFGF release, further indicating that release of ECM-bound bFGF depends on the proteolytic activity of thrombin and is not caused by a residual proteolytic activity remaining in the heated ECM (Fig 2).

**Release of endogenous mitogenic activity from ECM.** The growth-promoting effect of the ECM was attributed, in part, to the presence of bFGF in ECM. The following experiments were performed to test whether this endogenous mitogenic activity is susceptible to release by thrombin. ECM-coated dishes were preheated (1 hour, 80°C) and

---

**Fig 1.** Release of ECM-bound 125I-bFGF by thrombin. (A) Preheated (1 hour, 80°C) ECM-coated wells (4-well plates) were incubated (16 hours, 25°C) with 125I-bFGF (∼4 × 10⁴ cpm/well). The ECM was washed three times to remove the unbound bFGF, and incubated (9 hours, 37°C) with increasing concentrations of α-thrombin (0.01 to 5 μmol/L). (B) Preheated ECM (4-well plates) was incubated (16 hours, 25°C) with 125I-bFGF (∼4 × 10⁴ cpm/well). Unbound bFGF was removed and the ECM was incubated with 0.1 μmol/L α-thrombin for various time periods. Released 125I-bFGF was counted in a γ-counter. Released radioactivity is expressed as percent of total ECM-bound 125I-bFGF (1 × 10⁴ cpm/well). Release of 125I-bFGF in the absence of thrombin (7% percent of the total ECM-bound bFGF) was subtracted. Each data point is the mean of triplicate wells, and the variation between wells did not exceed ±8%.
RELEASE OF bFGF BY THROMBIN

Fig 2. Effect of catalytically blocked thrombin and thrombin inhibitors on release of ECM-bound bFGF. Preheated ECM that was preincubated with labeled bFGF and washed free of unbound bFGF (legend to Fig 1), was incubated (9 hours, 37°C) with (A) PBS containing 0.02% gelatin; (B) 0.1 μmol/L α-thrombin; (C) 0.1 μmol/L DIP-α-thrombin; (D) 0.1 μmol/L PPACK-α-thrombin; (E) 0.1 μmol/L α-thrombin and 20 U/well hirudin; and (F) 0.1 μmol/L α-thrombin and 1 μmol/L antithrombin III. Released labeled bFGF was counted in a γ-counter. Radioactivity released into the incubation medium is expressed as percent of total ECM-bound labeled bFGF. The amount of radioactivity released in the absence of thrombin (7% to 10%) was subtracted. The variation between triplicate wells did not exceed ±8% of the mean.

Fig 3. Release of endogenous mitogenic activity from ECM. (A) Preheated ECM-coated wells (4-well plates) (closed symbols) and regular tissue culture plastic wells (open symbols) were incubated (4 hours, 37°C) with PBS containing 0.02% gelatin (A, □); 1 μmol/L α-thrombin (●, ■); or 1 μmol/L PPACK-α-thrombin (▲, △). (B) Effect of thrombin inhibitors. Preheated ECM-coated wells (4-well plates) were incubated (4 hours, 37°C) with PBS containing 0.02% gelatin (A); 0.1 μmol/L α-thrombin (●); or 0.1 μmol/L α-thrombin and 1 μmol/L antithrombin III (▲). Aliquots (5 to 40 μL) of the incubation medium were tested for stimulation of 3H-thymidine incorporation in growth-arrested 3T3 fibroblasts, as described in Materials and Methods. Each data point is the average of triplicate wells and the standard deviation did not exceed ±10%.

then incubated (4 hours, 37°C) with various concentrations of thrombin. The incubation medium was collected and 40-μL aliquots added to growth-arrested 3T3 cells. It was found that mitogenic activity, measured by stimulation of 3H-thymidine incorporation, was released by incubation of ECM with 10 nmol/L thrombin reaching a nearly maximal value at 0.1 μmol/L thrombin (not shown). In other experiments, preheated ECM was treated (4 hours, 37°C) with a relatively high concentration (1 μmol/L) of thrombin and increasing amounts of the incubation medium tested for mitogenic activity on 3T3 fibroblasts. Ten microliters of the incubation medium yielded a nearly maximal effect (Fig 3A). No mitogenic activity was observed when thrombin was incubated under the same conditions in regular tissue culture dishes in the absence of ECM (Fig 3A). Moreover, direct incubation of 0.1 μmol/L thrombin with growth-arrested 3T3 fibroblasts failed to stimulate any thymidine incorporation above basal level. Likewise, little or no mitogenic activity was released on incubation of ECM with 1 μmol/L PPACK-α-thrombin (Fig 3A), or with 0.1 μmol/L thrombin that was first incubated (1 hour, 37°C) with 1 μmol/L ATIII (Fig 3B). These results indicate that the observed mitogenic activity is not caused by a direct effect of thrombin on the 3T3 fibroblasts, but rather to release from ECM of an intrinsic growth-promoting factor by active thrombin. In the above described studies we have used ECM that was preheated (1 hour, 80°C) to inactivate any

endogenous proteolytic activity residing in the ECM. Surprisingly, the amount of mitogenic activity released by thrombin from native ECM was threefold to fivefold lower than that released from preheated ECM (Fig 4).

Neutralizing anti-bFGF antibodies were applied to identify the growth-promoting factor released from ECM by thrombin. For this purpose, native and preheated ECM were incubated (24 hours, 37°C) with 0.1 μmol/L thrombin. Aliquots (25 μL) of the incubation medium were then incubated (1 hour, 25°C) with neutralizing anti-bFGF antibodies (8 pg), neutralizing anti-TGF-β antibodies (12 μg), or rabbit anti-IgG (7.5 μg) and tested for stimulation of 3H-thymidine incorporation. The results showed that the release of mitogenic activity was not blocked by neutralizing anti-bFGF antibodies (Fig 4A). However, the release of mitogenic activity was significantly reduced by neutralizing anti-TGF-β antibodies (Fig 4B). These results indicate that the growth-promoting factor released from ECM by thrombin is not bFGF, but rather TGF-β. In conclusion, our results suggest that thrombin can release an intrinsic growth-promoting factor from ECM that stimulates growth of 3T3 fibroblasts. Further studies are needed to identify the nature of this factor and its role in the regulation of cell proliferation.
thymidine incorporation in 3T3 fibroblasts. As shown in Fig 5, the anti-bFGF antibodies inhibited by about 50% the mitogenic activity released from both native and preheated ECM. Antibodies to TGF-β inhibited the mitogenic activity released from native and heated ECM by 20% and 60%, respectively (Fig 5). Release of mitogenic activity from ECM was not inhibited by antirabbit IgG (Fig 5). These results suggest that the increased mitogenic activity released by thrombin from preheated ECM can be attributed to heat stable bFGF-HSPG complexes that are more readily released after heat treatment of the ECM and to activation and release of ECM-resident TGF-β activity.

We next determined whether the bFGF was released as a high molecular weight (Mr) complex with HSPG. For this purpose, recombinant bFGF (2 μg/mL) was incubated (5 hours, 25°C) with metabolically sulfate-labeled ECM that was preheated for 1 hour at 80°C. The ECM was then washed free of unbound bFGF, incubated (48 hours, 37°C) with or without 1 μmol/L thrombin and the incubation media were treated (5 hours, 37°C) or untreated with 0.25 U bacterial hepannase. Media were then subjected to immunoprecipitation with anti-bFGF-conjugated protein A-Sepharose, followed by SDS-PAGE and autoradiography. As shown in Fig 6A (lane 3), incubation of ECM with thrombin resulted in release of high Mr (-300 Kd and 235 Kd) sulfate-labeled material that was precipitated with anti-bFGF antibodies, but not with nonimmune rabbit serum (Fig 6B, lane 4). Pretreatment with hepannase completely abolished the appearance of this high Mr-labeled component (Fig 6A, lane 4), indicating that the immunoprecipitated material was a complex of bFGF with HSPG. In contrast, there was no detectable release of immunoprecipitable 35S04−-labeled material from preheated ECM that was not exposed to thrombin (Fig 6, lane 1). A similar release of endogenous

---

**Fig 4.** Thrombin-mediated release of mitogenic activity from native versus heat-inactivated ECM. (A) Dose response. Preheated (1 hour, 80°C) ECM-coated wells (4-well plates) (e), native ECM (C), and regular tissue culture plastic (a) were incubated (24 hours, 37°C) with 0.1 μmol/L α-thrombin. Aliquots (5 to 60 μL) of the incubation medium were tested for stimulation of 3H-thymidine incorporation by growth-arrested 3T3 fibroblasts. Aliquots (25 μL) of the incubation medium were preincubated (1 hour, 25°C) in the absence (A, a) and presence of either neutralizing anti-bFGF antibodies (8 pg/well) (B, b); neutralizing TGF-β antibodies (12 μg/well) (C, c); or antirabbit IgG (7.5 μg/well) (D). These aliquots were then tested for stimulation of 3H-thymidine incorporation in 3T3 fibroblasts. Data are expressed as percentage of mitogenic activity released by thrombin from native and preheated ECM (100% = 42,000 cpm and 60,737 cpm, respectively). Mitogenic activity released in the absence of thrombin was 4,300 cpm/well. Each data point is the average of triplicate wells and the variation did not exceed ±10%.

**Fig 5.** Effect of antibodies to bFGF and TGF-β on mitogenic activity released by thrombin from native and preheated ECM. Native (A through D) and preheated (a through c) ECM-coated wells (4-well plates) were incubated (24 hours, 37°C) with 0.1 μmol/L α-thrombin. Aliquots (25 μL) of the incubation medium were preincubated (1 hour, 25°C) in the absence (A, a) and presence of either neutralizing anti-bFGF antibodies (8 pg/well) (B, b), neutralizing anti-TGF-β antibodies (12 μg/well) (C, c), or antirabbit IgG (7.5 μg/well) (D). These aliquots were then tested for stimulation of 3H-thymidine incorporation in 3T3 fibroblasts. Data are expressed as percentage of mitogenic activity released by thrombin from native and preheated ECM (100% = 42,000 cpm and 60,737 cpm, respectively). Mitogenic activity released in the absence of thrombin was 4,300 cpm/well. Each data point is the average of triplicate wells and the variation did not exceed ±10%.
Fig 6. Release of bFGF-HSPG complexes from ECM by thrombin. Preheated sulfate-labeled ECM (35-mm plates) was incubated (6 hours, 25°C) with or without 2 μg/mL bFGF. Unbound bFGF was washed (three times) and the ECM was then incubated (48 hours, 37°C) with and without 1 μmol/L α-thrombin in a total volume of 0.5 mL/dish. The incubation medium was subjected to immunoprecipitation by anti-bFGF antibodies directed against the N-terminal (1 to 24) portion of bFGF, as described in Materials and Methods. The samples were then analyzed by a 5% to 15% gradient SDS-PAGE, and the sulfate-labeled molecules were visualized by autoradiography. (A) Lane 1, untreated ECM; lane 2, ECM incubated with 1 μmol/L α-thrombin; lane 3, ECM that was first incubated with bFGF followed by incubation with 1 μmol/L α-thrombin; lane 4, same incubation as lane 3 followed by treatment (5 hours, 37°C) with 0.25 U bacterial heparinase. (B) Lane 1, untreated ECM; lane 2, ECM that was first incubated with bFGF followed by incubation with 1 μmol/L α-thrombin; lane 3, same as in lane 2 except that the immobilized bFGF antibodies, rather than the ECM, were first incubated with bFGF; lane 4, as described in lane 2 except that nonimmune rabbit serum was used instead of the anti-bFGF antiserum.

DISCUSSION

Previous studies on release of ECM-bound bFGF focused on the liberation of exogenously added 125I-bFGF by plasmin and HS degrading enzymes. The growth factor was released in an active form as a complex with HSPG or HS degradation products. We now report that the endogenous thrombin-releasable ECM-associated bFGF, as also indicated by a lower intensity of the immunoprecipitated complex, as compared with that released under the same conditions, but in the presence of exogenously added bFGF.

High Mr bFGF-HSPG complex was detected when the ECM was incubated with thrombin in the absence of exogenously added bFGF. Thus, anti-bFGF antibodies immunoprecipitated an ~235-kD sulfate-labeled material from the incubation medium of thrombin-treated ECM (Fig 6A, lane 2). This material represents the endogenous thrombin-releasable ECM-associated bFGF, as also indicated by a lower intensity of the immunoprecipitated complex, as compared with that released under the same conditions, but in the presence of exogenously added bFGF.

Cell attachment and proliferation are actively involved in the initiation of tissue repair and wound healing. Apart from its ability to release bFGF and possibly other ECM-bound active molecules, thrombin may contribute to local repair mechanisms and maintenance of the integrity of the vessel wall by means of its own stimulation of cell attachment and proliferation. The RGD domain (residues 187 to 189) of thrombin B-chain renders on activation by plasmin.
and heparan sulfate, potent adhesive properties to the molecule, inducing endothelial cell attachment, spread, and cytoskeletal reorganization via the \(\alpha_\beta_1\) integrin.\(^\text{25}\) Thrombin also induces the chemotaxis of macrophages,\(^\text{25}\) and exerts a direct stimulation of the growth of fibroblasts\(^\text{22}\) and smooth muscle cells.\(^\text{24,40}\) As shown in the present study, it may also stimulate the proliferation of vascular endothelial cells by means of releasing the ECM-resident bFGF. The mitogenic activity of the released bFGF may be further enhanced by thrombin that acts in combination with bFGF to elicit a higher proliferative response.\(^\text{41}\)

Basic FGF is released by both plasmin\(^\text{20}\) and thrombin as a complex with HSPG. This complex is resistant to inactivation in the proteinase-rich milieu of the wound as was demonstrated by showing that plasmin degrades free bFGF, but not bFGF that was first incubated with heparin or heparan sulfate.\(^\text{20}\) Moreover, heparan sulfate-bound bFGF could more readily diffuse to distant sites, as compared with uncomplexed bFGF.\(^\text{46}\) Most of the present experiments were performed with heat-treated ECM to inactivate any ECM-resident proteolytic activity\(^\text{27}\) and to ascribe the released mitogenic activity solely to thrombin. Surprisingly, a threefold to fivefold higher growth-promoting activity was released from heat-inactivated ECM compared with native ECM. This may be caused by weakening of the supramolecular structure of the ECM, resulting in a facilitated release of active molecules residing in the ECM. This result further shows that interaction of bFGF with HSPG stabilizes the molecule and protects it from heat inactivation. Heat treatment may also inactivate a putative growth factor inhibitor, or activate a latent growth-promoting factor, resulting in an increased release of mitogenic activity on exposure of the ECM to thrombin. In fact, our results indicate that native ECM contains a latent TGF-\(\beta\) that is released by thrombin in an active form provided that the ECM was first exposed to heat treatment. Both plasmin and thrombin were recently found to release latent TGF-\(\beta_1\) from the pericellular matrix of cultured fibroblast and fibrosarcoma cells.\(^\text{43}\)

In a previous study we have shown that degradation of heparan sulfate in the ECM by cellular heparanase is stimulated threefold to fivefold in the presence of thrombin.\(^\text{46}\) This stimulation was attributed to a better accessibility of heparan sulfate side chains to the heparanase enzyme. Thus, thrombin may enhance cell invasion and angiogenesis through stimulation of both heparanase activity and bFGF release in normal and pathologic processes such as tumor metastasis, inflammation, and autoimmunity.\(^\text{46}\) Thrombin also stimulates tumor metastasis in vivo through an enhanced adhesion of tumor cells to platelets and endothelial cells.\(^\text{46}\)

In a recent study we have shown that prothrombin, the circulating zymogen form of thrombin, is converted to thrombin on incubation with the subendothelial ECM.\(^\text{44,47}\) This conversion was attributed to the activity of ECM-resident proteolytic enzymes, most likely tissue plasminogen activator.\(^\text{37,47}\) Thus, the large reservoir of proteolytic activity present in plasma in the form of prothrombin may participate in ECM degradation, bFGF release, and the related cellular effects (ie, wound healing) in a continuous manner, both before and after clot formation, provided that prothrombin gains access to the subendothelial ECM.

ACKNOWLEDGMENT

We thank Dr John W. Fenton II for the generous gift of thrombin preparations.

REFERENCES

RELEASE OF BFGF BY THROMBIN


47. Beneza M, Vlodavsky I, Bar-Shavit R: Prothrombin is converted to thrombin by plasminogen activator residing in the subendothelial extracellular matrix. Semin Thromb Hemost (in press)
Thrombin-induced release of active basic fibroblast growth factor-heparan sulfate complexes from subendothelial extracellular matrix

M Benezra, I Vlodavsky, R Ishai-Michaeli, G Neufeld and R Bar-Shavit