# Basic Fibroblast Growth Factor Binds to Subendothelial Extracellular Matrix and Is Released by Heparitinase and Heparin-like Molecules†

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*Received June 10, 1988; Revised Manuscript Received September 21, 1988*

ABSTRACT: Basic fibroblast growth factor (bFGF) exhibits specific binding to the extracellular matrix (ECM) produced by cultured endothelial cells. Binding was saturable as a function both of time and of concentration of $^{125}$I-bFGF. Scatchard analysis of FGF binding revealed the presence of about $1.5 \times 10^{12}$ binding sites/mm$^2$ ECM with an apparent $k_D$ of 610 nM. FGF binds to heparan sulfate (HS) in ECM as evidenced by (i) inhibition of binding in the presence of heparin or HS at 0.1–1 μg/mL, but not by chondroitin sulfate, keratan sulfate, or hyaluronic acid at 10 μg/mL, (ii) lack of binding to ECM pretreated with heparitinase, but not with chondroitinase ABC, and (iii) rapid release of up to 90% of ECM-bound FGF by exposure to heparin, HS, or heparitinase, but not to chondroitin sulfate, keratan sulfate, hyaluronic acid, or chondroitinase ABC. Oligosaccharides derived from depolymerized heparin, and as small as the tetrasaccharide, released the ECM-bound FGF, but there was little or no release of FGF by modified nonanticoagulant heparins such as totally desulfated heparin, N-desulfated heparin, and N-acetylated heparin. FGF released from ECM was biologically active, as indicated by its stimulation of cell proliferation and DNA synthesis in vascular endothelial cells and 3T3 fibroblasts. Similar results were obtained in studies on release of endogenous FGF-like mitogenic activity from Descemet's membranes of bovine corneas. It is suggested that ECM storage and release of bFGF provide a novel mechanism for regulation of capillary blood vessel growth. Whereas ECM-bound FGF may be prevented from acting on endothelial cells, its displacement by heparin-like molecules and/or HS-degrading enzymes may elicit a neovascular response.

Strong affinity for heparin is a characteristic feature of most, if not all, endothelial cell (EC)[1] growth factors (Folkman & Klagsbrun, 1987). Consequently, heparin affinity chromatography has been used to purify, from various normal and malignant tissues, a variety of EC growth factors which are structurally and functionally related to either basic or acidic fibroblast growth factor (FGF) (Gospodarowicz et al., 1984; Shing et al., 1984; Thomas et al., 1984; Maciag et al., 1985; Lobb et al., 1986; Klagsbrun et al., 1986; Schweigerer et al., 1987). Recently, we have demonstrated that cultured bovine vascular and corneal endothelial cells synthesize a basic FGF-like growth factor that remains mostly cell-associated (Vlodavsky et al., 1987a) but up to 30% of which can be extracted from the subendothelial extracellular matrix (ECM) deposited by these cells (Vlodavsky et al., 1987b). Moreover, we have also identified the presence of basic FGF-like molecules in basement membranes of bovine cornea in vivo (Folkman et al., 1988). These results suggest that basement membranes and extracellular matrices may function as storage sites for basic FGF. Displacement of FGF from its storage within basement membranes and ECMs may provide a novel mechanism for induction of neovascularization in normal and pathological situations (Vlodavsky et al., 1987b; Baird & Ling, 1987).

In the present study, we show that (i) bFGF binds to ECM in a specific and saturable manner, (ii) ECM-bound FGF is specifically released by treating the ECM with heparan sulfate (HS) degrading enzymes or by exposure to HS, heparin, or various oligosaccharides derived from depolymerized heparin, and (iii) FGF released from storage in ECM is fully active in induction of endothelial cell proliferation. These results indicate that FGF binds to ECM via HS binding sites and that its release may mediate endothelial cell proliferation and neovascularization.

## Experimental Procedures

*Materials.* Partially purified basic fibroblast growth factor (bFGF) was isolated from bovine brain, as described (Gospodarowicz et al., 1978). Recombinant human basic FGF was kindly provided by Takeda Chemical Industries (Japan). Human hepatoma (Sk-hep-1) derived basic FGF was purified as described (Klagsbrun et al., 1986). Glycosaminoglycans (heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate) and bacterial heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) (*Flavobacterium heparinum*) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). The bacterial heparitinase preparation degraded heparin to an extent which did not exceed 1.2% of its heparan sulfate degrading activity.

† This work was supported by National Cancer Institute Grants CA-30289 to I.V. and CA-37392 to M.K., by the USA–Israel Binational Science Foundation, and by a grant from KabiVitrum AB, Sweden, to I.V. I.V. is a Leukemia Society of America Scholar.

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[1] Abbreviations: ECM, extracellular matrix; bFGF, basic fibroblast growth factor; HS, heparan sulfate; EC growth factors, endothelial cell growth factors; GAGs, glycosaminoglycans.

0006-2960/89/0428-1737$01.50/0 

Contamination with other glycosaminoglycan degrading enzymes did not exceed 0.06% of its heparan sulfate degrading activity. Chondroitinase ABC, chondroitinase AC, collagenase type I, and trypsin were purchased from Sigma Chemicals (St. Louis, MO). Heparin–Sepharose was from Pharmacia (Upssala, Sweden). Dulbecco's modified Eagle's medium (DMEM, H-16), calf serum,, fetal calf serum (FCS), penicillin, and streptomycin were obtained from GIBCO (Grand Island, NY). Saline containing 0.05% trypsin, 0.01 M sodium phosphate, 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 (Oxnard, CA). Four-well tissue culture plates were from Nunc (Roskilde, Denmark). [*methyl*-$^3$H]Thymidine (500 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Triton X-100, dextran T-40, and all other chemicals were of reagent grade, purchased from Sigma.

*Modified and Low Molecular Weight Heparins.* Modified nonanticoagulant desulfated heparins (Bar-Ner et al., 1987) were prepared and kindly provided to us by Dr. Lina Wasserman (Rogoff-Wellcome Medical Research Institute, Beilinson Medical Center, Petah-Tikva, Israel). In brief, pyridinium heparin (Nagasawa & Inoue, 1980) underwent exhaustive desulfation with dimethyl sulfoxide (Merck, Rahway, NJ) containing 10% water to yield totally desulfated heparin. N-Desulfated heparin was obtained from the respective pyridinium salt with dimethyl sulfoxide containing 5% water. Totally desulfated heparin and N-desulfated heparin were N-acetylated with acetic anhydride (Nagasawa et al., 1980). Resulfation of the free amino residues of totally desulfated or N-desulfated heparin was performed with sulfur trioxide trimethylamine complex (Aldrich Chemical, Milwaukee, WI) as described (Nagasawa et al., 1980). These modified heparins exhibited <7% of the anticoagulant activity of native heparin (Bar-Ner et al., 1987).

Size-homogeneous oligosaccharides composed of the uronic acid–glucosamine disaccharide building block of heparin were prepared by alkaline treatment of heparin methyl ester ($\beta$-elimination). Similar results were obtained with heparin fragments prepared by controlled nitrous acid depolymerization (Holmer et al., 1986). Heparin methyl ester was obtained by alkylation with methyl iodide of the Hyamine salt of heparin (porcine intestinal mucosa) in a solution of dichloromethane. The heparin methyl ester (1 g) was dissolved in water (5 mL) and heated to 60 °C. Sodium hydroxide (0.4 M, 5 mL) was added, and the solution was stirred for 90 min at 60 °C. The solution (average $M_r$ 3.5K) was neutralized with Dowex 50-W-X8H, and the resin was filtered off with 1 mL of water. The combined solutions were adjusted to pH 7.0, freeze-dried, and applied in 5 mL of 0.25 M NaCl to a gel permeation column (5 × 180 cm, P-6, Bio-Rad). The column was eluted with 0.25 M NaCl at 5.8 cm/h using UV detection at 214 mm. Fractions were desalted on Sephadex G-10, detected by refractive index measurements, and freeze-dried.

Heparin oligosaccharides prepared by nitrous acid depolymerization were gel filtered on two Sephadex G-50 superfine columns (2.6 × 90 cm) coupled in series and eluted with 0.3 M NaCl. Fractions corresponding to the elution positions of 2, 4, 6, 8, 10, 12, and 14 saccharides were pooled, recovered by ethanol precipitation, and rechromatographed separately on the same columns. All fragments had significant anti-factor Xa activity but no anticoagulant activity (Holmer et al., 1986).

*Cells.* Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (Gospodarowicz et al., 1977; Vlodavsky et al., 1987b). Stock cultures were maintained in DMEM (1 g of glucose/L) supplemented with 10% bovine calf serum, 5% FCS, penicillin (50 units/mL), and streptomycin (50 $\mu$g/mL) at 37 °C in 10% $CO_2$ humidified incubators. Brain-derived bFGF (100 ng/mL) was added every other day during the phase of active cell growth. Bovine aortic and capillary endothelial cells were cultured as described (Shing et al., 1984; Vlodavsky et al., 1987b).

*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-well and 96-well plates at an initial density of $5 \times 10^4$ cells/mL. Cells were maintained as described above, except that FGF was not added, and 5% dextran T-40 was included in the growth medium. Six to eight days 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 amd 20 mM $NH_4OH$ in phosphate-buffered saline (PBS), followed by four washes in PBS (Vlodavsky et al., 1987b). The ECM remained intact and firmly attached to the entire area of the tissue culture dish. One square centimeter of this ECM has been shown to contain a total of about 1.8 units (approximately 0.36 ng) of endogenous bFGF activity (Vlodavsky et al., 1987; Folkman et al., 1988).

We have previously demonstrated that the polar secretion of ECM by corneal endothelial cells, and the firm interaction of this ECM with the tissue culture plastic, provides a most appropriate system to obtain a basement membrane like substrate, free of cellular elements (Vlodavsky et al., 1980). The presence of nuclei or cytoskeletal elements could not be detected on the denuded ECM when plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence using antibodies directed against actin, vimentin, or the benzimidazole derivative Hoechst 33258 for nuclear staining (Gospodarowicz et al., 1983). Moreover, ECM prepared following a 24-h exposure of subconfluent corneal endothelial cell cultures to [$^3$H]thymidine was devoid of labeled material. Likewise, no labeled cell-surface components remained associated with the ECM following solubilization (Triton/$NH_4OH$) of lactoperoxidase-iodinated corneal endothelial cells that were plated on ECM for 24 h. No serum proteins could be identified in the ECM (Gospodarowicz & Ill, 1980).

*Growth Factor Activity.* Assay for DNA synthesis in 3T3 cells was performed as described (Vlodavsky et al., 1987a,b). Briefly, Balb/C 3T3 cells were plated at half-confluence on 0.3 cm$^2$ microtiter wells in DMEM supplemented with 10% calf serum. After reaching confluence (2–3 days), the cells were further incubated for a minimum of 5 days. Samples and [$^3$H]thymidine (1 $\mu$Ci/well) were than added to the quiescent cells, and after an incubation period of 32–40 h, DNA synthesis was assayed by measuring the radioactivity incorporated into TCA-insoluble material. A unit of 3T3 cell growth factor activity (approximately 70 000 cpm) was defined as the amount of growth factor in 0.25 mL required to stimulate half-maximal DNA synthesis in 3T3 cells (Vlodavsky et al., 1987a). On the basis of previous studies, it is estimated that pure bFGF has a specific activity of about 5 units/ng (Shing et al., 1984; Klagsbrun & Shing, 1985).

*Iodination of FGF.* Recombinant basic FGF (0.4 $\mu$mol, 7 $\mu$g) was incubated with 1 mCi (0.6 $\mu$mol) of $^{125}$I-labeled Bolton–Hunter reagent in 30 $\mu$L of 100 mM sodium phosphate buffer, pH 8.5, for 2.5 h on ice. Excess Bolton–Hunter reagent was quenched by adding 200 $\mu$L of 0.2 M glycine and incubating for 45 min on ice; 20 $\mu$L of 0.5% gelatin was then added,

and the reaction vial was washed with an additional 250 μL of gel filtration buffer (50 mM Tris-HCl, 0.05% gelatin, 1 mM dithiothreitol, and 0.3 M NaCl, pH 7.5). The combined sample was subjected to gel filtration on a Sephadex G-25 column equilibrated with the same buffer. The specific activity of $^{125}$I-bFGF, assessed by stimulation of DNA synthesis in 3T3 cells and bovine capillary endothelial cells, was approximately 1960 Ci/mmol ($1.7 \times 10^5$ cpm/ng) at first assay date. This value corresponds to approximately 0.9 mol of Bolton–Hunter reagent per mole of bFGF. The $^{125}$I-FGF fully retained its mitogenic activity and its high affinity for heparin–Sepharose, and its purity was >99% as assessed by SDS–PAGE and autoradiography. In some experiments, FGF was iodinated with $^{125}$I and IodoGen, as described (Neufeld & Gospodarowicz, 1985). Results were essentially the same.

*Binding Studies.* To analyze the concentration dependency of bFGF binding to ECM, each set of triplicate ECM-coated wells (96-well plates) was incubated with a different amount of bFGF ($10^4$ cpm of $^{125}$I-FGF tracer plus unlabeled bFGF) at concentrations ranging from 0.3 to 20 μg/mL. The incubation volume was 0.1 mL per 30 mm$^2$ well, and the binding medium was PBS, pH 7.4, containing 0.05% gelatin. Following incubation at 22 °C for 90 min, the medium was removed and the plate washed by rapid dipping in 3 × 1 L PBS at 4 °C. The bound bFGF was solubilized in 1 M NaOH and counted for $^{125}$I in Beckman Model 5500 γ counter. The picomoles of bFGF bound in each well was calculated by using the specific activity of bFGF in each concentration (5.6–358 cpm/pmol). The binding of tracer $^{125}$I-bFGF alone to ECM was specific, in that >90% of the counts were displaced by 20 μg/mL unlabeled bFGF (the amount bound in the absence and presence of excess bFGF was 3151 ± 84 and 296 ± 6 cpm, respectively). For estimation of $k_D$ and $B_{max}$, the data were analyzed by the method of Scatchard. For competition with various glycosaminoglycans (GAGs), ECM-coated wells were incubated with $^{125}$I-FGF ($2 \times 10^4$ cpm/well) in the presence of increasing concentrations of each of the native GAGs, modified heparins, or low molecular weight heparin fragments. In some experiments, ECM was treated with various enzymes and washed prior to incubation with labeled FGF.

*Release of ECM-Bound FGF.* ECM-coated wells (four-well plates) were incubated either with unlabeled bFGF (50 3T3 units in 0.2 mL of DMEM per well, overnight, 4 °C) or with iodinated FGF [(1–2) × $10^4$ cpm/well, 1 h, room temperature], and the unbound factor was washed 4 times with PBS containing 0.05% gelatin. ECM was then incubated with various GAGs or enzymes, and, after various time periods, aliquots (20 and 30 μL) of the 0.4-mL incubation medium were tested for mitogenic activity (stimulation of DNA synthesis in 3T3 cells) or amount of released iodinated material. Under the experimental conditions of the present study, the various enzymes and GAGs had no effect on the basal and bFGF-stimulated DNA synthesis. We have previously reported that the subendothelial ECM contains endogenous bFGF [about 0.1 and 0.7 ng per ECM-coated well of 96-well and 4-well plates, respectively (Vlodavsky et al., 1987)]. Nevertheless, incubation of control ECM-coated wells, with or without the various GAGs and ECM-degrading enzymes, yielded no detectable stimulation of DNA synthesis when aliquots of the incubation medium were added to growth-arrested 3T3 cells.

## Results

*Binding of $^{125}$I-FGF to ECM.* Binding of $^{125}$I-bFGF to ECM-coated wells was analyzed as a function of FGF concentration, as described under Experimental Procedures. Apparent saturation of binding was achieved at 5–10 μg/mL (Figure 1A). Scatchard analysis of the data indicated that a single class of binding sites is likely over the concentration range of FGF studied (Figure 1B). The apparent dissociation constant for the interaction of bFGF and ECM was $6.1 \times 10^{-7}$ M, much higher than that reported for binding of bFGF to high-affinity ($k_D$ approximately $2.7 \times 10^{-10}$ M) (Neufeld & Gospodarowicz, 1985; Moscatelli, 1987) and low-affinity ($k_D$ approximately $2 \times 10^{-9}$ M) (Moscatelli, 1987) cell-surface receptor sites. Approximately $1.5 \times 10^{12}$ FGF molecules were bound per millimeter squared of ECM, corresponding to the area occupied by about $10^3$ confluent bovine aortic endothelial cells. The time course of association of FGF to ECM was investigated at 4 °C. Apparent equilibrium was reached after 2 h (not shown), and the amount of bound $^{125}$I-FGF decreased by up to 10% when ECM was rinsed with PBS and further incubated with fresh binding medium for 3 h at 4 °C. Similar results were obtained in studies with the ECM produced by a mouse endodermal cell line, PH-HR9 (Kramer & Vogel, 1984). These cells produce no detectable amounts of bFGF, and their ECM is devoid of endogenous FGF and is not mitogenic for vascular EC (Vlodavsky et al., 1988). $^{125}$I-FGF binding to ECM was specific, since neither insulin (5 μg/mL), epidermal growth factor (2 μg/mL), platelet-derived growth factor (1 μg/mL), fibronectin (50 μg/mL), nor transferrin (10 μg/mL) competed with $^{125}$I-FGF on binding to ECM. In

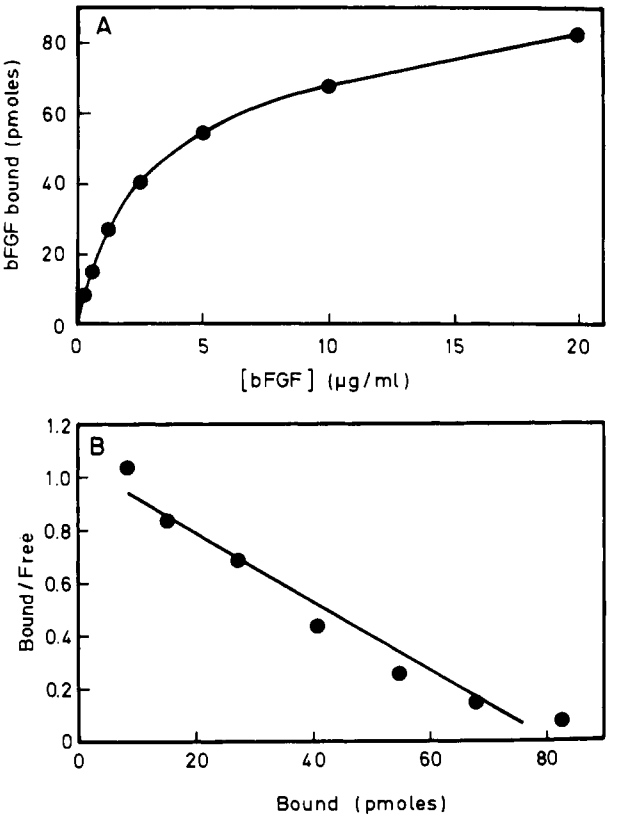


FIGURE 1: Binding of $^{125}$I-bFGF to ECM. ECM-coated wells of a 96-well plate were incubated (22 °C, 90 min) with $^{125}$I-bFGF ($1 \times 10^4$ cpm/well) in the presence of unlabeled bFGF at concentrations ranging from 0.3 to 20 μg/mL. Binding was performed as described under Experimental Procedures, and picomoles of bFGF bound in each well were calculated from the specific activity of bFGF for each concentration point. Data are plotted as (A) a function of FGF concentration and (B) according to Scatchard. A molecular weight of 18000 for bFGF was used to calculate $k_D$ and the number of binding sites. Each point is the average of triplicate wells, and the standard deviation did not exceed ±5%.

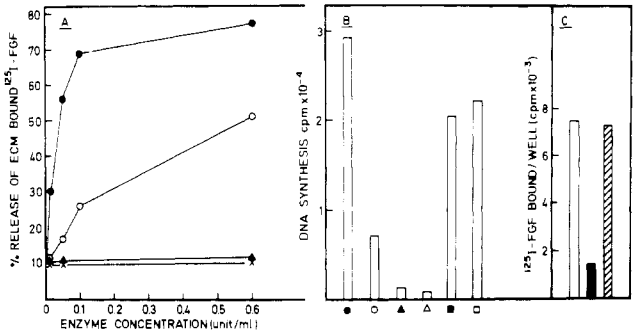


FIGURE 2: Effect of enzymes on ECM binding and release of FGF. (A) ECM-coated wells of a 96-well plate were incubated (22 °C, 90 min) with $^{125}$I-bFGF ($1 \times 10^4$ cpm/well), washed, and treated (37 °C, 60 min) with heparitinase (●), heparinase (○), chondroitinase ABC (▲), or chondroitinase AC (×). Radioactivity released into the incubation medium is expressed as percent of total ECM-bound $^{125}$I-FGF (100% = 26 pg). "Spontaneous" release in the presence of buffer alone was 7–10% of the total ECM-bound FGF. (B) ECM-coated 4-well plates were incubated (22 °C, 90 min) with brain-derived bFGF (50 units/well), washed, and treated (0.1 unit/mL, 37 °C, 60 min) with heparitinase (●), heparinase (○), chondroitinase ABC (▲), hyaluronidase (△), collagenase (■), or 5 μg/mL trypsin (□). Aliquots (20 μL) of the incubation medium were tested for stimulation of DNA synthesis in growth-arrested 3T3 cells, as described under Experimental Procedures. About 5 ng of bFGF was bound to the ECM. Spontaneous release of mitogenic activity in the presence of incubation medium alone was subtracted and did not exceed 10% of that released by heparitinase. (C) ECM-coated four-well plates were untreated (open bars) or pretreated with bacterial heparitinase (0.1 unit/mL, 37 °C, 60 min) (solid bars) or chondroitinase ABC (0.5 unit/mL, 37 °C, 60 min) (hatched bars). ECM was washed and incubated (22 °C, 90 min) with $^{125}$I-FGF ($1.5 \times 10^4$ cpm/well), and the amount of ECM-bound FGF was determined. About 65 pg of $^{125}$I-bFGF was bound to untreated ECM.

contrast, bFGF purified from bovine brain or human hepatoma cells (Sk-hep-1) was as effective as recombinant bFGF in displacing $^{125}$I-FGF from ECM.

*Release of ECM-Bound FGF by ECM-Degrading Enzymes.* ECM was incubated with $^{125}$I-bFGF, or unlabeled bFGF, washed free of unbound FGF, and exposed to various ECM-degrading enzymes. The incubation medium was then assayed for amount of released $^{125}$I radioactivity and stimulation of DNA synthesis in 3T3 fibroblasts.

Most efficient release of ECM-bound $^{125}$I-FGF was obtained by treating ECM with either bacterial heparitinase (eliminase) or heparanase (endo-β-D-glucuronidase) that we have purified from a human hepatoma cell line (Sk-hep-1). These enzymes released about 70% of the ECM-bound FGF within 1 h of exposure to 0.1 unit of enzyme activity. Similar results were obtained in the presence of protease inhibitors such as PMSF and aprotinin. Studies with metabolically sulfate-labeled ECM revealed that, although about 70% of the ECM heparan sulfate was degraded under these conditions (Bar-Ner et al., 1985), the ECM remained firmly bound to the entire area of the tissue culture dish and retained the morphological appearance of untreated ECM. Heparinase from *Flavobacterium heparinum* was 5–7-fold less effective than bacterial heparitinase (Figure 2A). In contrast, there was no release of ECM-bound FGF by treating the ECM with chondroitinase ABC, chondroitinase AC, or hyaluronidase (Figure 2A). Similar results were obtained with the ECM produced by the mouse endodermal cell line PF-HR9. Proteolytic digestion of ECM with trypsin or collagenase released up to 80% of the ECM-bound FGF, but in contrast to treatment with the GAG-degrading enzymes, this was associated with destruction and solubilization of the entire ECM. FGF released by each of these enzymes was biologically active, as measured by its stimulation of [$^3$H]-

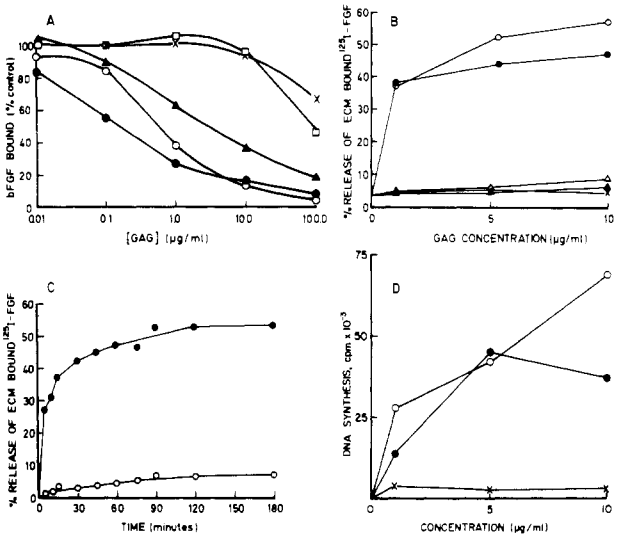


FIGURE 3: Effect of various GAGs on ECM binding and release of bFGF. (A) ECM-coated wells of a 96-well plate were incubated (22 °C, 90 min) with $^{125}$I-bFGF ($1 \times 10^4$ cpm/well) in the presence of increasing concentrations of chondroitin sulfate (□) (similar results were obtained with hyaluronic acid), keratan sulfate (×), dermatan sulfate (▲), heparan sulfate (○), and heparin (●). FGF binding was performed as described under Experimental Procedures. 100% binding of $^{125}$I-FGF in the absence of GAGs was 37 pg per well. (B and C) ECM-coated wells of four-well plates were incubated (22 °C, 90 min) with $^{125}$I-FGF ($1 \times 10^4$ cpm/well). ECM was washed 4 times with PBS containing 0.05% gelatin and incubated (22 °C) (B) for 90 min with increasing concentrations of heparin (●), heparan sulfate (○), chondroitin sulfate (×), hyaluronic acid (△), or keratan sulfate (▲), and (C) for various time periods in the absence (○) or presence of 5 μg/mL heparin (●). Amount of radioactive material released into the incubation medium is expressed as percent of total ECM-bound $^{125}$I-FGF (100% = approximately 46 pg of bFGF per well). (D) Inner layers of bovine corneas, dissected as described (Folkman et al., 1988), were washed 3 times in PBS and incubated (24 °C, 1 h) in the absence or presence of heparin (●), heparan sulfate (○), or chondroitin sulfate (×) in PBS. Aliquots (30 μL) of the incubation medium were tested for stimulation of [$^3$H]thymidine incorporation into DNA of quiescent 3T3 cells. Mitogenic activity released from corneas in the presence of PBS alone was subtracted and did not exceed 20% of that released in the presence of 10 μg/mL HS.

thymidine incorporation in quiescent 3T3 cells (Figure 2B) and capillary endothelial cells (not shown). Measurements of released mitogenic activity and ECM-bound $^{125}$I-FGF yielded similar results (percent release). These results suggest that bFGF binds to HS and heparin-like molecules in ECM. This conclusion was supported by the finding that pretreatment of ECM with bacterial heparitinase (0.1 unit/mL, 1 h, 37 °C) resulted in a 75% decrease in the ECM FGF binding capacity. In contrast, there was no effect of pretreating the ECM with chondroitinase ABC (Figure 2C).

Under the present experimental conditions, there was no stimulation or inhibition of [$^3$H]thymidine incorporation by any of the above enzymes, either in the absence or in the presence of FGF. In previous studies, we could not identify growth factors other than bFGF in the subendothelial ECM (Vlodavsky et al., 1987). In support of this observation is the almost complete inhibition of the ECM growth-promoting activity in the presence of anti-bFGF antibodies (Vlodavsky et al., 1989). These antibodies also inhibited the mitogenic activity of material released by the ECM-degrading enzymes.

*Effect of GAGs on ECM Binding and Release of FGF.* Experiments on the effect of various GAGs on FGF binding to ECM indicated that heparin, HS, and, to a lesser extent, dermatan sulfate efficiently inhibited binding of $^{125}$I-bFGF to ECM. A 50% inhibition of FGF binding was obtained in the presence of 0.1–1 μg/mL heparin or HS, but there was

no inhibition of binding by chondroitin sulfate, keratan sulfate, or hyaluronic acid at 10 μg/mL (Figure 3A). These competition experiments corroborate the above-described enzyme digestion studies suggesting preferential and specific binding of FGF to HS in ECM. ECM-bound FGF may serve as a reservoir of bFGF around cells, provided that the bound FGF can be readily released. As demonstrated in Figure 3C, about 40% of ECM-bound $^{125}$I-bFGF was released by either heparin or HS (5 μg/mL) within 15 min of incubation at 22 °C, as compared to less than 3% release of FGF from ECM incubated in medium alone. Heparin and HS at 1 μg/mL released 35–40% of the ECM-bound FGF, as compared to less than 10% release observed in the presence of chondroitin sulfate, keratan sulfate, or hyaluronic acid at 10 μg/mL (Figure 3B). Release of ECM-bound FGF by the latter GAGs was similar to the "spontaneous" release observed during incubation in medium alone (Figure 3B). FGF released by heparin or HS stimulated DNA synthesis in 3T3 fibroblasts and proliferation of capillary endothelial cells. In these experiments, ECM was first incubated with unlabeled brain bFGF or hepatoma-derived bFGF, washed, and incubated for a second period of time with various GAGs. In terms of specificity and extent (percent of total ECM-bound FGF) of FGF release, similar results were obtained regardless of whether release of ECM-bound FGF was evaluated by measurements of $^{125}$I-bFGF or actual mitogenic activity. However, measurements of mitogenic activity released as a function of concentration and incubation time with GAGs revealed quite complex and often biphasic dependencies, as compared to measurements of released $^{125}$I-bFGF. This behavior may be due to direct effects of heparin and HS on the mitogenicity (Thornton et al., 1983) and stability (Gospodarowicz & Cheng, 1986; Saksela et al., 1988) of FGF, and to possible effects on the 3T3 and endothelial cells themselves.

We have previously demonstrated that bFGF-like growth-promoting activity is stored within Descemet's membranes of bovine corneas in vivo (Folkman et al., 1988). Incubation of inner layers of bovine corneas, containing mostly Descemet's membranes with heparin or HS, but not with chondroitin sulfate, resulted in release of mitogenic activity (Figure 3D) which was blocked in the presence of polyclonal anti-bFGF antibodies (not shown). Thus, the corneas in vivo yielded the same results as those observed for release of FGF that was exogenously bound to the Descemet's membranelike ECM produced by cultured corneal endothelial cells.

*Release of ECM-Bound FGF by Heparin Oligosaccharides and Chemically Modified Heparins.* In order to investigate the structural requirements for release of ECM-bound FGF, we analyzed the effect of various heparin oligosaccharides and chemically modified heparin species of low anticoagulant activity. Octasaccharide and higher oligosaccharides isolated from depolymerized heparin, as described under Experimental Procedures, were, on a weight basis, as effective as native heparin in releasing ECM-bound FGF (Figure 4). Exposure to oligosaccharides of lower molecular weight, and as small as the tetrasaccharide, induced release of ECM-bound FGF, albeit to a lower extent than native heparin. Incubation with trisulfated disaccharides isolated from depolymerized heparin resulted in release of ECM-bound FGF to an extent which was only slightly above the amount of $^{125}$I-FGF released during incubation in medium alone. A synthetic pentasaccharide, representing the heparin binding site for antithrombin III (Sinay et al., 1984), released 25–30% of the ECM-bound FGF at 10 μg/mL. FGF released by low molecular weight heparins induced DNA synthesis in growth-arrested 3T3 fibroblasts

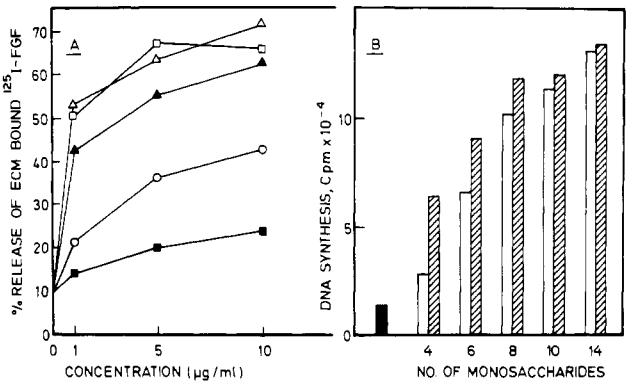


FIGURE 4: Release of ECM-bound FGF by low molecular weight heparins. ECM-coated wells of 96-well plates were incubated (22 °C, 90 min) with (A) $^{125}$I-FGF ($1 \times 10^4$ cpm/well) or (B) brain-derived bFGF (50 3T3 units/well). ECM was washed 4 times and incubated with oligosaccharides derived from depolymerized heparin, as described under Experimental Procedures. (A) $^{125}$I-FGF released from ECM by incubation (22 °C, 90 min) with various concentrations of trisulfated disaccharides (■), tetrasaccharides (○), hexasaccharides (▲), octasaccharides (□), or decasaccharides (△), isolated from depolymerized heparin. Released radioactivity is expressed as percent of total ECM-bound $^{125}$I-FGF (i.e., 34 pg of bFGF per well). Release of $^{125}$I-FGF in the absence of oligosaccharides was 10% of the total ECM-bound FGF. (B) Mitogenic activity released from ECM by incubation (37 °C, 1 h) with 1 μg/mL (open bars) and 5 μg/mL (hatched bars) various oligosaccharides was tested by adding 20-μL aliquots of the incubation medium to growth-arrested 3T3 fibroblasts. DNA synthesis was measured as described under Experimental Procedures. Release of mitogenic activity in the presence of incubation medium alone (solid bar) did not exceed >11% of that released by the 14-mer.

(Figure 4B). For studies on the effect of degree of sulfation of heparin on FGF release, native heparin was first either totally desulfated or N-desulfated, as described under Experimental Procedures. These heparins were left with their N position exposed or were either N-acetylated or N-resulfated. The modified heparins exhibited <7% of the anticoagulant activity of native heparin (Bar-Ner et al., 1987). In contrast to native heparin, the modified heparins (N-acetylated; N-acetylated, O-desulfated; totally desulfated) failed to inhibit binding of $^{125}$I-FGF to ECM (Figure 5A). Totally desulfated heparin and N-desulfated heparin also failed to release ECM-bound FGF, as compared to native heparin or N-resulfated heparin (Figure 5B). N-Acetylated heparin failed to release ECM-bound FGF at 1 μg/mL and was 3–4-fold less effective than native heparin at 5–10 μg/mL (Figure 5B). These results indicate that *N*-sulfate groups of heparin are necessary for its FGF-releasing activity, although a low activity is retained when the N position is acetylated, provided that the *O*-sulfate groups are retained.

## Discussion

Heparin binding FGF-like growth factors are highly mitogenic for capillary endothelial cells in vitro, and picomolar quantities can induce angiogenesis in vivo (Shing et al., 1985; Folkman & Klagsbrun, 1987). Despite the ubiquitous presence of these potent EC growth factors in normal tissues, endothelial cell proliferation in these tissues is usually very low, with turnover time measured in years (Denekamp, 1984). This raises the question of how these growth factors are prevented from acting on the vascular endothelium continuously and what signals make them available for stimulation of capillary endothelial cell proliferation.

We have previously reported the extraction of FGF-like growth factors from the ECM produced by cultured endothelial cells (Vlodavsky et al., 1987b) and from basement

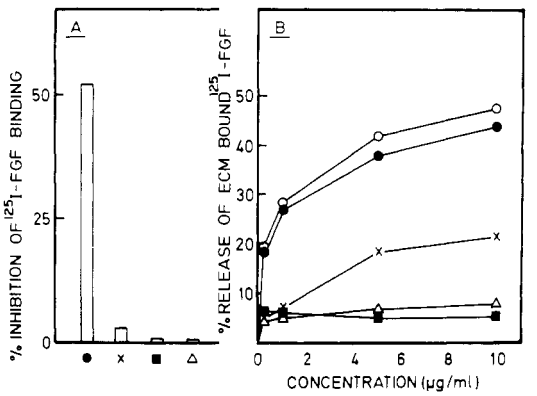


FIGURE 5: Effect of native heparin and modified nonanticoagulant heparins on FGF binding to ECM and release of ECM-bound FGF. (A) ECM-coated wells of 96-well plates were incubated (22 °C, 90 min) with $^{125}$I-FGF in the presence of 5 μg/mL native heparin (●), 5 μg/mL N-acetylated heparin (×), 5 μg/mL totally desulfated heparin (■), or 5 μg/mL N-acetylated, O-desulfated heparin (△). Inhibition of ECM $^{125}$I-FGF binding by each of these heparins is expressed as percent of the amount of FGF bound in the absence of heparin (100% bFGF binding was 29 pg per well). (B) ECM-coated wells of 96-well plates were incubated (22 °C, 90 min) with $^{125}$I-FGF ($1 \times 10^4$ cpm/well). ECM was washed and tested for release of $^{125}$I-FGF, following incubation (22 °C, 90 min), with various concentrations of native heparin (●), totally desulfated heparin (■), N-desulfated heparin (△) (similar results were obtained with N-acetylated, O-desulfated heparin), N-acetylated heparin (×), or totally desulfated, N-resulfated heparin (○). Spontaneous release in the presence of PBS and 0.05% gelatin alone was 4–6% of the total ECM-bound $^{125}$I-FGF.

membranes of the cornea in vivo (Folkman et al., 1988). In the present study, we have analyzed the binding of bFGF to ECM and the requirements for release of ECM-bound FGF. The results provide evidence that (a) ECM may function as a storage depot for EC growth factors through their specific binding to HS proteoglycans and (b) ECM-bound FGF-like growth factors are readily released upon exposure to heparin, HS, or HS-degrading enzymes. These results suggest that regulation of capillary growth and neovascular response may result from displacement of EC growth factors from their storage sites within basement membranes and extracellular matrices.

Scatchard analysis revealed that bFGF binds to ECM with an affinity ($k_D$ = 610 nM) lower than that reported for binding of FGF to low affinity, presumably heparin-like sites on cell surfaces (Moscatelli, 1987). It appears that FGF binds specifically to HS, since up to 90% of the bound growth factor was displaced by heparin, HS, or HS-degrading enzymes, but not by unrelated GAGs (i.e., chondroitin sulfate, keratan sulfate, hyaluronic acid) or enzymes (chondroitinases AC and ABC). There is very little spontaneous release of ECM-bound FGF. Low affinity but yet specific binding are expected features of a physiologically active reservoir of bFGF around cells. These properties enable ECM-bound FGF to be readily available for cells in response to an appropriate signal. By using both in vitro (i.e., ECM produced by cultured cells) and in vivo (bovine cornea Descemet's membrane) experimental systems, we have demonstrated that release of ECM-bound FGF exhibits the same requirements and specificity regardless of whether FGF is exogenously added and bound to ECM or is an endogenous natural constituent of Descemet's membrane. Apart from the angiogenic factor FGF discussed here, the ECM heparan sulfate was recently shown to bind both GM-CSF and Interleukin-3 (Roberts et al., 1987). These growth factors, once bound, can be presented in an active form to hemopoietic cells, thus providing a mechanistic explanation for the observed total dependence of hemopoietic cells on intimate stromal cell contact (Roberts et al., 1988).

Studies on effects of modified and low molecular weight heparins revealed that FGF was released by heparin fragments as small as the tetrasaccharide, as compared to little release by N-acetylated heparin. Different structural requirements were found in our studies on inhibition of heparanase activity by these heparins (Bar-Ner et al., 1987). Whereas N-acetylated heparin was almost as efficient an inhibitor as native heparin, heparin-derived oligosaccharides—smaller than the hexamer—were practically ineffective (Bar-Ner et al., 1987). These results indicate that different structural features are responsible for various heparin-mediated activities, such as anticoagulation, inhibition of heparanase, and release of ECM-bound FGF. Elucidation of these structural requirements may clarify the complexity of heparin-mediated effects in experimental animals.

Heparitinase and heparanase were found to be the most efficient specific releasers of active FGF from ECM. Moreover, ECM that was pretreated with heparitinase could no longer bind FGF. Heparanase has previously been shown to play a role in extravasation of blood-borne cells and tumor cell metastasis (Nakajima et al., 1983; Vlodavsky et al., 1983). The present study suggests that it may also participate in tumor angiogenesis, through mobilization of ECM-bound EC growth factors. Thus, alterations in basement membrane structure and turnover that are associated with normal (epithelial morphogenesis) (Bernfield & Banarjee, 1978) and pathological (tumor progression) (Ingber et al., 1981) processes may at the same time be responsible for release of ECM-bound growth- and differentiation-promoting activities. In fact, the onset of angiogenesis in carcinoma in situ often coincides with degradation of the basement membrane (Folkman & Cotran, 1986). An indirect involvement in tumor angiogenesis can similarly be ascribed to heparin, which has been shown to elicit an angiogenic response (Taylor & Folkman, 1982), and, as presently observed, to release ECM-bound FGF. It is conceivable, for example, that mast cell heparin and/or heparanase could be involved in angiogenesis (Kessler et al., 1976) by liberating angiogenic factors that are bound to ECM. It has long been postulated that the microenvironment within some organs may be more conducive to neoplastic growth than in other tissues (Naito et al., 1987). Such preferential tumor development in certain organs may be attributed to ECM storage and release of growth-promoting factors that stimulate proliferation and/or vascularization of arrested tumor cells.

A striking feature of FGF is that it is mostly an intracellular protein (Folkman & Klagsbrun, 1987; Vlodavsky et al., 1987a), consistent with the lack of a consensus signal peptide in its gene (Abraham et al., 1986; Jaye et al., 1986). These observations suggest that FGF is released from cells via a nontraditional secretory mechanism, perhaps in response to sublethal cell damage and leakage associated with inflammation, tissue damage, and tumor necrosis. Cells, such as platelets and macrophages, which may contain FGF (Baird et al., 1985), may release their FGF content and induce a neovascular response upon activation and degranulation. Endothelial cells may release bFGF upon blood vessel damage. A possible, but yet unproved, pathway for deposition of FGF into ECM is by forming an intracellular complex with HS, found in the cell cytoplasm and nucleus (Fedarko & Conrad, 1985), which is then inserted into the cell surface and/or liberated into the ECM. Regardless of its mode of secretion, FGF binding to HS (Saksela et al., 1988) and storage in ECM are likely to protect the molecule from inactivation and, at the

same time, to inhibit its mitogenic activity (Gospodarowicz & Cheng, 1986). It remains to be elucidated whether ECM-bound FGF has to be released in order to induce a proliferative response in cells that are placed in contact with ECM. We propose that storage of EC growth factors in ECM provides a novel mechanism for regulation of capillary blood vessel growth. Under normal conditions, it may prevent them from acting on the vascular endothelium, thus maintaining a very low rate of endothelial cell turnover and vessel growth. On the other hand, release from storage in ECM may elicit endothelial cell proliferation and neovascularization.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Choay (Institute Choay, Paris) for providing the synthetic pentasaccharide.

**Registry No.** bFGF, 106096-93-9; heparin, 9005-49-6; heparitinase, 52227-76-6; heparan sulfate, 9050-30-0.

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