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Importance of Size and Sulfation of Heparin in Release of Basic Fibroblast Growth Factor from the Vascular Endothelium and Extracellular Matrix†

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ABSTRACT: We have characterized the importance of size, sulfation, and anticoagulant activity of heparin in release of basic fibroblast growth factor (bFGF) from the subendothelial extracellular matrix (ECM) and the luminal surface of the vascular endothelium. For this purpose, ^{125}I-bFGF was first incubated with ECM and confluent endothelial cell cultures, or administered as a bolus into the blood of rats. the immobilized ^{125}I-bFGF was then subjected to release by various chemically modified species of heparin and size-homogeneous oligosaccharides derived from depolymerized heparin. Both totally desulfated and N-desulfated heparin failed to release the ECM-bound bFGF. Likewise, substitution of *N*-sulfate groups of heparin and low molecular weight heparin (fragmin) by acetyl or hexanoyl residues resulted in an almost complete inhibition of bFGF release by these polysaccharides. The presence of *O*-sulfate groups in heparin increased but was not critical for release of ECM-bound bFGF. Similar structural requirements were identified for release of ^{125}I-bFGF bound to low-affinity sites on the surface of vascular endothelial cells. Oligosaccharides derived from depolymerized heparin and containing as little as 8–10 sugar units were, on a weight basis, equivalent to whole heparin in their ability to release bFGF from ECM. Low-sulfate oligosaccharides were less effective releasers of bFGF as compared to medium- and high-sulfate fractions of the same size oligosaccharides. Heparin fractions with high and low affinity to antithrombin III exhibited a similar high bFGF-releasing activity despite a 200-fold difference in their anticoagulant activities. Studies on the fate of intravenously administered ^{125}I-bFGF demonstrated that bFGF sequestered by the luminal surface of the vascular endothelium is accessible to release by heparin species in a manner similar to that observed for release of ECM-bound bFGF. Analysis of the distribution of ^{125}I-bFGF in various organs revealed the presence of intact ^{125}I-bFGF primarily in the rat liver and kidneys. Structural requirements for release of ECM-bound and cell-surface-bound bFGF by heparin and heparin-like molecules were different from those identified for inhibition of heparanase activity and lung colonization of B16 melanoma cells by these heparin molecules. These results indicate that various non-anticoagulant species of heparin having different size, sulfation, and substituted groups can be designed to elicit specific effects such as release of bFGF and inhibition of heparanase and hence for induction of neovascularization and inhibition of tumor metastasis, respectively.

Fibroblast growth factors (FGFs)[1] are a family of structurally related polypeptides characterized by high affinity to heparin (Burgess & Maciag, 1989; Gospodarowicz et al., 1987; Rifkin & Moscatelli, 1989). They are highly mitogenic for vascular endothelial cells (EC) and are among the most potent inducers of neovascularization and mesenchyme formation (Folkman & Klagsbrun, 1987; Kimelman & Kirschner, 1987). Among the FGF gene family are the prototypes acidic FGF (aFGF) and basic FGF (bFGF) which, unlike most other polypeptide growth factors, are primarily cell-associated, consistent with the lack of a conventional signal sequence for secretion (Burgess & Maciag, 1989; Gospodarowicz et al., 1987; Rifkin & Moscatelli, 1989). Despite the lack of a signal peptide, both aFGF and bFGF have been identified in the extracellular matrix (ECM) deposited by cultured myoblasts (Weiner & Swain, 1989) and EC (Baird & Ling, 1987; Vlodavsky et al., 1987). Immunohistochemical staining re-

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[1] Abbreviations: ECM, extracellular matrix; bFGF, basic fibroblast growth factor; HS, heparan sulfate; EC, endothelial cells; DMEM, Dulbecco's modified Eagle's medium; anti-FXa, anti-factor Xa.

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vealed the presence of bFGF in basement membranes of the rat fetus (Gonzalez et al., 1990), bovine cornea (Folkman et al., 1988), and human blood vessels (Cardon-Cardo et al., 1990), suggesting that ECM may serve as a reservoir for bFGF. It appears that bFGF binds specifically to heparan sulfate (HS) and heparin-like molecules in the ECM and cell surface, as indicated by its displacement by heparin, HS, or HS-degrading enzymes (i.e., heparanase, heparitinase), but not by unrelated GAGs or GAG-degrading enzymes (Bashkin et al., 1989). We have demonstrated that heparanase activity expressed by intact platelets, neutrophils, and lymphoma cells releases active bFGF from ECM (Ishai-Michaeli et al., 1990), suggesting its possible involvement in the regulation of blood vessel growth. A similar role can be attributed to heparin, provided that the released bFGF–heparin complex induces EC proliferation.

Heparin is a linear, highly sulfated polysaccharide consisting of alternating uronic acid (either L-iduronic or D-glucuronic) and D-glucosamine residues. It exhibits a high degree of heterogeneity due to variations in the size of the polysaccharide chains and in the degree and distribution of sulfate groups. In the present study, we investigated structural requirements for release of ECM- and cell-surface-bound bFGF by heparin and heparin-like molecules. For this purpose, subendothelial ECM and cultured vascular EC were incubated with ^{125}I-bFGF, washed free of unbound bFGF, and exposed to various chemically modified species of heparin and to size-homogeneous oligosaccharides derived from depolymerized heparin. In other experiments, ^{125}I-bFGF was administered into the blood of rats, and the circulating levels of bFGF were measured before and after injection of native and modified heparins.

Experimental Procedures

Materials. Sodium heparin from porcine intestinal mucosa (PM-heparin) (M_r 14000, anti-FXa 165 IU/mg, sulfur content 12%) was obtained from Hepar Industries (Franklin, OH). A low molecular weight fragment (Kabi 2165, fragmin) of this heparin (M_r 5100, anti-FXa 130 IU/mg, sulfur content 12.4%) was prepared as sodium salt by nitrous acid depolymerization (Thunberg et al., 1980). Partially purified bFGF was isolated from bovine brain, as described (Gospodarowicz et al., 1978). Recombinant human bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM, 1 g of glucose/L), calf serum, fetal calf serum (FCS), penicillin, and streptomycin were obtained from Biological Industries (Beit-Haemek, Israel). 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 were from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Four-well tissue culture plates were from Nunc (Rosklide, Denmark). Na^{125}I was purchased from Amersham (Buckinghamshire, England). Triton X-100, dextran T-40, and all other chemicals were of reagent grade, purchased from Sigma (St. Louis, MO).

Preparation and Characterization of Oligosaccharides from Nitrous Acid Depolymerized Heparins. Heparin fragments consisting of saccharide chains of different sizes were obtained by partial deaminative cleavage of heparin from porcine intestinal mucosa using nitrous acid, as described (Thunberg et al., 1980). Sizes ranged from disaccharides to chains with an average length of the starting heparin, i.e., chains of 40–50 saccharides. This mixture of heparin fragments was subjected to ion-exchange chromatography on DEAE-Sepharose using sodium chloride as an eluent. Three fractions were collected: low-sulfate fraction, sulfur/carbon ratio (by elemental analysis) 0.14; medium-sulfate fraction, sulfur/carbon ratio 0.19; and high-sulfate fraction, sulfur/carbon ratio 0.21 (Sudhalter et al., 1989). Each of the low-sulfate, medium-sulfate, and high-sulfate fractions was further separated into size-homogeneous, even-numbered oligosaccharides by gel permeation chromatography on Sephadex G-50 Superfine according to the method of Lane et al. (1984). This procedure resulted in a set of oligosaccharides of different sizes and degrees of sulfation. The oligosaccharides having a higher sulfur/carbon ratio than heparin were found by ^{1}H NMR and ^{13}C NMR spectroscopy to have their additional sulfate groups mainly in the glucosamines as indicated by a larger proportion of *N*-sulfate groups over *N*-acetyl groups and of 6-*O*-sulfate groups over 6-OH groups. The low-sulfate-containing oligosaccharides had a considerably reduced sulfate content in their glucosamines as well as in their iduronic acids (Sudhalter et al., 1989). Oligosaccharides smaller than about 18 monosaccharides did not show any appreciable anticoagulant activity when measured by a clotting time assay (activated partial thromboplastin time) (Holmer et al., 1980). Size-homogeneous oligosaccharides, similar to the starting heparin in their sulfur/carbon ratio, were prepared also by alkaline treatment of heparin methyl ester (β-elimination), as described (Bashkin et., 1989).

Modified Heparins. Chemically modified non-anticoagulant species of heparin were prepared from native heparin and heparin fragment (fragmin, M_r ~5100). Briefly, the pyridinium salt of heparin and heparin fragment underwent complete N-desulfation by incubation with dimethyl sulfoxide and methanol (Inoue & Nagasawa, 1976). Total desulfation of *N*- and *O*-sulfate groups was obtained by exhaustive desulfation with dimethyl sulfoxide containing 10% methanol and 0.4% trifluoroacetic acid. The N-desulfated heparin fragment was N-acetylated with acetic anhydride in water at pH 7–8, as described (Nagasawa & Inoue, 1980), or N-resulfated with sulfur trioxide–trimethylamine complex, as described by Lloyd et al. (1971). The N-acetylated heparin fragment was oversulfated (Ogamo et al., 1985) and yielded an N-acetylated, oversulfated heparin fragment. An O-desulfated, N-acetylated heparin fragment was obtained by O-desulfating an N-acetylated heparin fragment, as described by Nagasawa et al. (1977). Intact heparin was chemically modified by the same procedures. These modified heparins exhibited <5% of the anticoagulant activity of heparin (Bar-Ner et al., 1987). Similar results were obtained with modified species of the heparin fragment.

Fractions with high and low affinity to antithrombin III were separated using an antithrombin–Sepharose column (Andersson et al., 1976; Ekre et al., 1986). The column was equilibrated with 0.05 M Tris-HCl containing 0.15 M NaCl, and the nonbinding saccharide was eluted with the same buffer. The binding fraction was eluted with 2.0 M NaCl in the same buffer. Fractions were pooled, desalted, and lyophilized (Sudhalter et al., 1989).

The chemical modifications made in the heparin fragments were assessed by ^{1}H NMR and ^{13}C NMR spectroscopy using a JEOL GX-400 instrument, 400 MHz for ^{1}H and 100 MHz for ^{13}C, and TSP [2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionate] as an internal standard. N-Desulfation was ascertained by following the change in the chemical shift of the H-2 proton of D-glucosamine from 3.3 to 2.7 ppm, when the spectrum was recorded in in D_2O/NaOD. This change was quantitative. On N-acetylation, the signal at 2.7 ppm disappeared completely, and that at 2.05 ppm (CH_3 of acetyl) became correspondingly larger. On N-resulfation, the signal

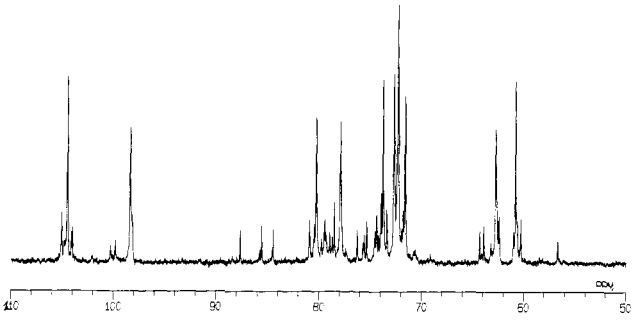

FIGURE 1: ^{13}C NMR spectrum of N/O-desulfated, N-resulfated heparin fragment. ^{13}C NMR spectroscopy was performed as described under Experimental Procedures. The absence of a peak at 102.0 ppm (relative to internal TSP) indicates the absence of sulfate groups at C-2 on L-iduronic acid, and the absence of a peak at 69.2 ppm indicates loss of sulfate groups at C-6 of D-glucosamine. Large peaks at 104.5 and 62.7 ppm indicate the presence of C-1 of nonsulfated L-iduronic acid and of nonsulfated C-6 of D-glucosamine, respectively. Complete N-resulfation was indicated by the absence of a peak at 93.7 ppm (of C-1 of N-desulfated D-glucosamine).

at 2.7 ppm also disappeared completely while that at 3.3 ppm reappeared. O-Desulfation was shown to be complete by ^{13}C NMR (Figure 1).

The degree of sulfation of the N-acetylated, oversulfated heparin fragment was assessed by the molar ratio of sulfate to carboxyl groups obtained by conductimetric titrations according to Casu and Gennaro (1975). This ratio was 1.57 for the N-acetylated heparin fragment and 3.31 for the N-acetylated oversulfated fragment, showing a high increase in O-sulfation. According to ^{13}C NMR, these additional sulfate groups were located both in the glucosamines and in the uronic acids of the heparin fragment.

Cells. Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (Gospodarowicz et al., 1977). Stock cultures were maintained in DMEM supplemented with 10% bovine calf serum, 5% FCS, penicillin (50 units/mL), and streptomycin (50 μg/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 (Bashkin et al., 1989; Vlodavsky et al., 1987). Bovine aortic endothelial cells were cloned and cultured as described (Gospodarowicz et al., 1976).

Preparation of Dishes Coated with ECM. Bovine corneal endothelial cells were dissociated from stock cultures (2nd–5th passage) with STV and plated into four-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. Six to eight days after the cells reached confluency, we exposed the subendothelial ECM by dissolving (3 min, 22 °C) the cell layer with a solution containing 0.5% Triton X-100 and 20 mM NH_4OH in PBS followed by four washes in PBS (Bashkin et al., 1989; Ishai-Michaeli et al., 1990; Vlodavsky et al., 1987). The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish. The presence of nuclei or cytoskeletal elements could not be detected in the denuded ECM when plates were examined by phase-contrast microscopy, scanning electron microscopy, or indirect immunofluorescence using anti-actin and anti-vimentin antibodies or the benzimidazole derivative Hoechst 33258 for nuclear staining. No serum proteins could be identified in the ECM (Gospodarowicz et al., 1983). Main constituents of the corneal endothelial ECM were fibronectin, aminin, collagen types I, III, and IV, elastin, and sulfated proteoglycans (mostly heparan sulfate and dermatan sulfate proteoglycans and to a lesser extent chondroitin sulfate proteoglycans) (Vlodavsky et al., 1980, 1983).

Iodination of bFGF. Recombinant bFGF was iodinated with ^{125}I and IodoGen (Pierce Chemicals Co., Rockford, IL) as described (Bashkin et al., 1989; Neufeld & Gospodarowicz, 1985). Briefly, bFGF (3.3 μg in 50 μL of 10 mM Tris-HCl, pH 7.1, and 2 M NaCl), together with 60 μL of 0.2 M sodium phosphate, pH 7.2, was added to a glass tube containing 1.6 μg of Iodogen. The reaction was started by the addition of a 2-fold molar excess of $Na^{125}I$ and stopped after 15 min at room temperature by the addition of 60 μL of 0.1% sodium metabisulfite and 30 μL of 0.1 mM KI. The reaction mixture was applied onto a small (0.3 mL) heparin–Sepharose column and the ^{125}I-bFGF eluted with 1.5 mL of buffer containing 20 mM sodium phosphate, pH 7.2, 2 M NaCl, and 0.2% gelatin. The specific activity was usually 1.2×10^5 cpm/ng of bFGF, and the labeled preparation was kept for up to 3 weeks at 4 °C. The iodinated material retained full biological activity and yielded a single band in the position of unlabeled bFGF when subjected to $NaDodSO_4$–PAGE and autoradiography. In some experiments, bFGF was iodinated with ^{125}I and chloramine T. Results were essentially the same.

Release of ECM-Bound bFGF. ECM-coated wells (four-well plates) were incubated with iodinated bFGF [$(1.5–2.5) \times 10^4$ cpm $(0.25\ mL)^{-1}$ $well^{-1}$, 3 h, room temperature], and the unbound factor was removed by four washes with PBS containing 0.02% gelatin. ECM was then incubated (3 h, 24 °C) with the various types of modified and low molecular weight species of heparin, 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 and solubilized with 1 M NaOH, and the radioactivity was counted in a γ-counter. The percentage of released ^{125}I-bFGF was calculated from the total ECM-associated radioactivity. "Spontaneous" release of ^{125}I-bFGF in the presence of incubation medium alone was 7–12% of the total ECM-bound bFGF. This value was subtracted from the experimental values. Each experiment was performed 3–5 times, yielding similar results. Each data point is the average of triplicate wells, and the standard deviation did not exceed 10%.

Release of Cell-Bound bFGF. Confluent endothelial cell monolayers (10^6 cells/35-mm dish) were incubated (2 h, 37 °C) with ^{125}I-bFGF [$(4–6) \times 10^5$ cpm mL^{-1} $dish^{-1}$] in PBS, pH 7.4, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% bovine serum albumin (PBS/BSA). The cell monolayers were washed twice with ice-cold PBS and twice with PBS/BSA, followed by incubation (2 h, 37 °C) with native and modified species of heparin fragment in PBS/BSA. Released radioactivity was counted in a γ-counter. ^{125}I-bFGF released during incubation with PBS/BSA alone amounted to about 25% of the total cell-bound bFGF. To determine the remaining low-affinity-bound bFGF, the cells were incubated for 5 min with a cold solution containing 1.6 M NaCl/20 mM HEPES, pH 7.4, and the incubation medium was counted in a γ-counter. High-affinity-bound bFGF was determined by 2 M NaCl (20 mM acetate buffer, pH 4.0) extraction (Moscatelli, 1987; Yayon et al., 1991). Experiments were performed 3 times, each in triplicate dishes.

Fate of Intravenously Administered bFGF. ^{125}I-bFGF diluted in saline was administered into rats (Sabra, mean weight 291 g) intravenously as a bolus. Samples of blood (0.3 mL) were drawn from the arterial line at various time intervals and counted in a γ-counter (Chajek-Shaul et al., 1988; Walen et al., 1989). Native and modified species of heparin were injected either together with the ^{125}I-bFGF or 5 min afterward.

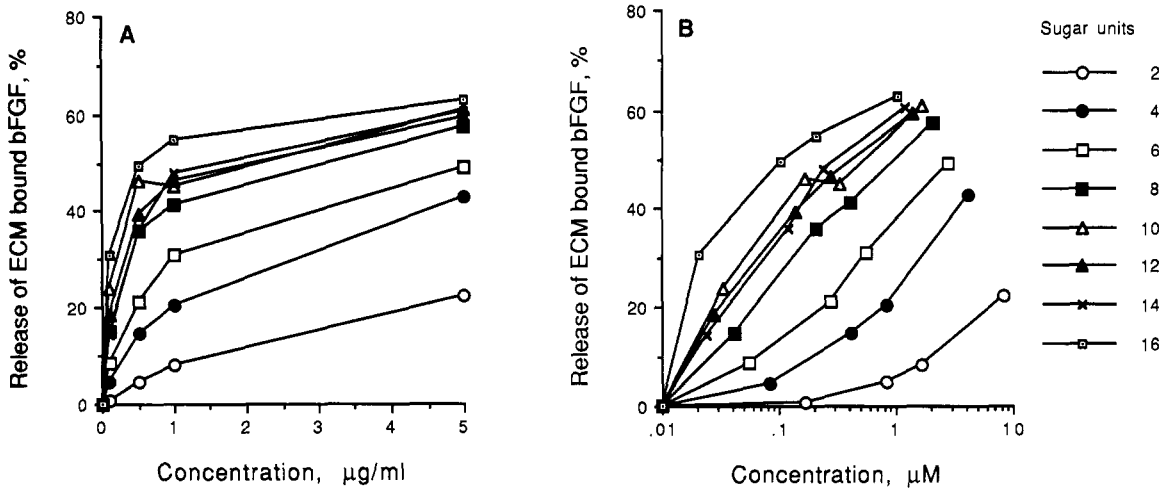

FIGURE 2: Release of ECM-bound bFGF by heparin-derived oligosaccharides of varying sizes. ECM-coated wells of four-well plates were incubated (24 °C, 3 h) with ^{125}I-bFGF (2×10^4 cpm/well). The ECM was washed 4 times and incubated (24 °C, 3 h) with various concentrations of size-homogeneous oligosaccharides prepared by nitrous acid depolymerization. Released radioactivity is expressed as the percent of total ECM-bound ^{125}I-FGF (1×10^4 cpm/well; 34 pg of bFGF/well). Release of ^{125}I-bFGF in the absence of oligosaccharides was 14% of the total ECM-bound bFGF. This value was subtracted. Concentrations of the oligosaccharides are expressed in micrograms per milliliter (A) or micromolar (B) (average molecular weight of a monosaccharide = 320). The number of sugar units is presented next to each symbol. Each data point is the mean of triplicate wells, and the standard deviation did not exceed ±6%.

Data on the disappearance of bFGF from the blood are presented as a percentage of the expected blood concentration at zero time. At the end of the experiment, rats were infused with saline, various organs were removed, and the radioactivity was counted in a γ-counter (Chajek-Shaul et al., 1988; Whalen et al., 1989). In some experiments, tissue extracts were subjected to immunoprecipitation with anti-bFGF antibodies and Sepharose A beads, followed by SDS–polyacrylamide gel electrophoresis and autoradiography, as described (Rogelj et al., 1989).

Results

Effect of Oligosaccharides Derived from Depolymerized Heparin. ECM was incubated (3 h, 24 °C) with ^{125}I-bFGF, washed free of unbound bFGF, and exposed (3 h, 24 °C) to various size homogeneous oligosaccharides prepared from heparin by either nitrous acid depolymerization (Figure 2) or alkaline treatment (not shown). As demonstrated in Figure 2A, maximal release of ^{125}I-bFGF (i.e., 50–60% of the ECM-bound bFGF) was achieved by 5 μg/mL octasaccharide. Release of ECM-bound bFGF by the hexasaccharide was somewhat lower. Exposure of ECM to higher molecular weight oligosaccharides containing up to 16 sugar units (Figure 2A), or to either heparin fragment or intact heparin (not shown), yielded results which were, on a weight basis, similar to those obtained with the octasaccharide. About 30% of the ECM-bound ^{125}I-bFGF was not accessible to release even by 10 μg/mL heparin. Exposure of ECM to 5 μg/mL tetrasaccharide resulted in release of about 40% of the ECM-bound bFGF, and there was little release by the disaccharide. Oligosaccharides prepared by nitrous acid depolymerization or alkaline treatment (β-elimination) yielded similar results. Depending on the batch of ECM and ^{125}I-bFGF, 7–15% of the ECM-bound ^{125}I-bFGF was released during incubation with PBS alone. This "spontaneous" release was subtracted and could be markedly reduced (70–80%) by first heating (80 °C, 60 min) the ECM to inactivate its intrinsic proteolytic, most likely plasminogen activator, activity (Bar-Ner et al., 1986; Vlodavsky et al., 1990). Size-dependent differences in bFGF-releasing activity were more pronounced when the various oligosaccharides were compared on an estimated molar basis. As demonstrated in Figure 2B, half-maximal release of ECM-bound ^{125}I-bFGF was obtained at 0.1 μM octasaccharide, as compared to 2 μM tetrasaccharide. Oligosaccharides containing 10, 12, and 14 sugar units yielded a half-maximal release at about 0.06 μM (Figure 2B).

We compared the effect of oligosaccharides derived from nitrous acid degraded heparin and having varying degrees of sulfation. While medium- and high-sulfate fractions of a given oligosaccharide (ratio of sulfur to carbon ~0.18 and ~0.21, respectively) showed virtually the same bFGF-releasing activity, low-sulfate oligosaccharides (sulfur to carbon ratio = 0.11) were less efficient releasers of bFGF, especially when compared at low concentrations (0.1–1 μg/mL) (Figure 3). This effect was more evident with oligosaccharides containing 4–8 sugar units (Figure 3A) as compared to 12 sugar units or more (Figure 3B).

Effect of Chemically Modified Species of Heparin. In order to analyze the involvement of *O*-sulfate and *N*-sulfate residues of heparin in release of ECM-bound bFGF, both heparin and heparin fragment were either N/O-desulfated or selectively N-desulfated, as described under Experimental Procedures. *N*-Sulfate groups of these heparins were also partially or fully substituted with either acetyl or hexanoyl groups. As demonstrated in Figure 4, both N/O-desulfated and N-desulfated heparin fragments failed to release the ECM-bound bFGF. In contrast, N-resulfation of N/O-desulfated heparin fragment restored its bFGF-releasing activity to a large extent. Similar results were obtained with the respective modified species of intact heparin. Total N-acetylation of heparin or heparin fragment resulted in an almost complete inhibition of their bFGF-releasing activity (Figure 4). Oversulfation of these N-acetylated molecules resulted in partial restoration of their ability to release bFGF from ECM (Figure 4). Likewise, total substitution of *N*-sulfate groups of heparin fragment with hexanoyl groups resulted in an almost complete inhibition of its FGF-releasing activity (Figure 4), but there was only a small inhibitory effect to a partial hexanoyl substitution of *N*-sulfate groups (not shown). These results indicate that *N*-sulfate groups of heparin are critical for efficient release of ECM-bound bFGF. N-Desulfation or substitution of the N positions with acetyl or hexanoyl residues abolished the FGF-releasing activity of heparin despite a normal content of *O*-sulfate groups.

Table I: Release of ECM-Bound bFGF and Inhibition of Heparanase Activity by Chemically Modified and Low Molecular Weight Species of Heparin

	N-sulfated	O-sulfated	% sulfur[a]	anti-FXa[b] act. (IU/mg)	inhibn of heparanase[c] (%)	release of ECM-bound bFGF[d] (%)
heparin fragment	+	+	12.4	130	100	61
N/O-desulfated	−	−	<1	<1	0	<1
N-desulfated	−	+	9.7	<1	0	<1
N-acetylated	−	+	8.7	<1	100	5
N-hexanoyl	−	+	7.5	1	100	4
N/O-desulfated, N-resulfated	+	−	5.3	<1	3	41
hexasaccharide	+	+	10.7	<1	0	50
tetradecasaccharide	+	+	9.3	44	50	63
low affinity[e]	+	+	12.3	2	95	54
high affinity[e]	+	+	11.4	360	65	45

[a] Determined by elemental analysis. [b] Anti-FXa activity was determined using the chromogenic substrate S-2222 according to Tein et al. (1976). [c] Determined by release of low molecular weight sulfate-labeled degradation products ($0.5 < K_{av} < 0.8$, Sepharose 6B) from metabolically labeled ECM, measured in the absence and presence of 2.5 μg/mL each of the indicated molecules (Bar-Ner et al., 1987). [d] Determined in the presence of 5 μg/mL various compounds, as described under Experimental Procedures. [e] Fractions of heparin tetradecasaccharide with low and high affinity to antithrombin III, prepared as described under Experimental Procedures.

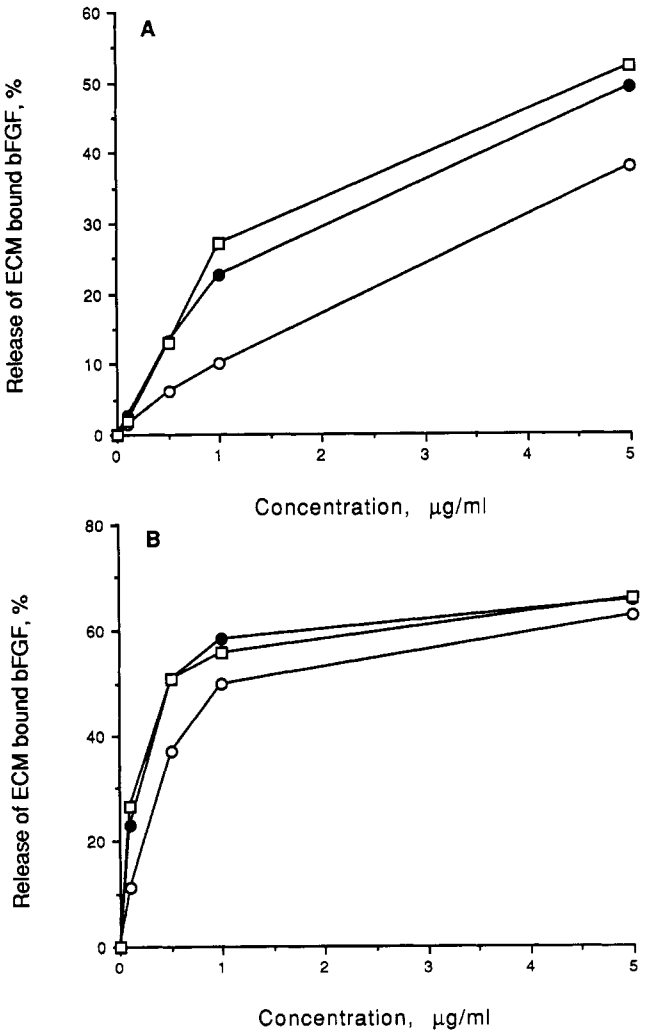

FIGURE 3: Release of ECM-bound bFGF by heparin-derived oligosaccharides of varying sizes and degrees of sulfation. ECM-coated wells of four-well plates were incubated (24 °C, 3 h) with ^{125}I-bFGF (2×10^4 cpm/well). The ECM was washed 4 times and incubated (24 °C, 3 H) with increasing concentrations of low-sulfate (○), medium-sulfate (●), and high-sulfate (□) fractions of (A) hexasaccharide and (B) hexadecasaccharide obtained from heparin by nitrous acid depolymerization and ion-exchange chromatography on DEAE-Sepharose. Released radioactivity is expressed as the percent of total ECM-bound ^{125}I-bFGF (i.e., 1×10^4 cpm/well). Release of ^{125}I-bFGF in the absence of oligosaccharides (11% 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%.

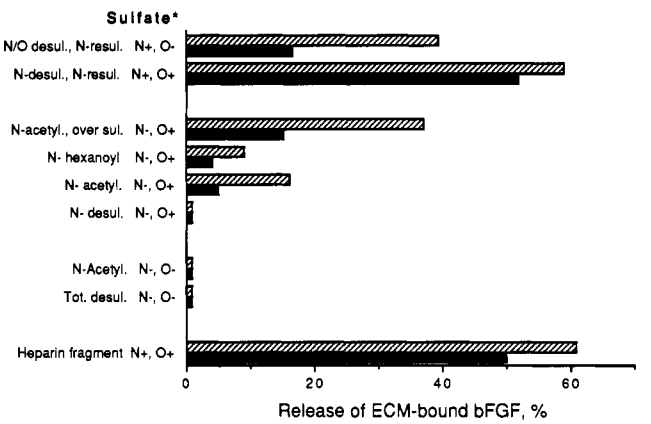

FIGURE 4: Release of ECM-bound bFGF by chemically modified species of heparin fragment. ECM-coated wells of four-well plates were incubated (24 °C, 3 h) with ^{125}I-bFGF (2×10^4 cpm/well). Unbound bFGF was removed and the ECM incubated (24 °C, 3 h) with 1 (black bars) and 5 μg/mL (hatched bars) heparin fragment and species of heparin fragment chemically modified to contain sulfate groups at either the N position, the O position, or both, as indicated under *sulfate* by (−) or (+). Similar results were obtained by the respective chemically modified species of intact heparin. Released radioactivity is expressed as the percent of total ECM-bound ^{125}I-bFGF (1×10^4 cpm/well). The amount of ^{125}I-bFGF released in the presence of PBS alone (7%) was subtracted. The variation between triplicate wells did not exceed ±10% of the mean.

Figure 4 and Table I indicate that the ability of a given compound to release bFGF from ECM is dependent primarily on the position of the sulfate group rather than on the total level of sulfation (i.e., percent sulfur). Thus, N/O-desulfated, re-N-sulfated heparin fragment (percent sulfur = 5.3) exhibited a much higher bFGF-releasing activity than either N-desulfated, *N*-acetyl, or *N*-hexanoyl species of heparin fragment containing 9.7%, 8.7%, and 7.5% sulfur, respectively. As indicated in Figure 4, *O*-sulfate groups of heparin fragment contributed to its bFGF-releasing activity, since oversulfation of N-acetylated heparin fragment (percent sulfur = 13.9) resulted in partial restoration of its bFGF-releasing activity. The involvement of *O*-sulfate groups in bFGF release was also indicated by the finding that N-resulfation of N/O-desulfated heparin fragment resulted in a partial restoration of activity, as compared to a full restoration induced by N-resulfation of N-desulfated heparin fragment (Figure 4).

In order to correlate the anticoagulant activity of heparin to its bFGF-releasing activity, heparin was separated on an antithrombin–Sepharose column into a nonbinding fraction

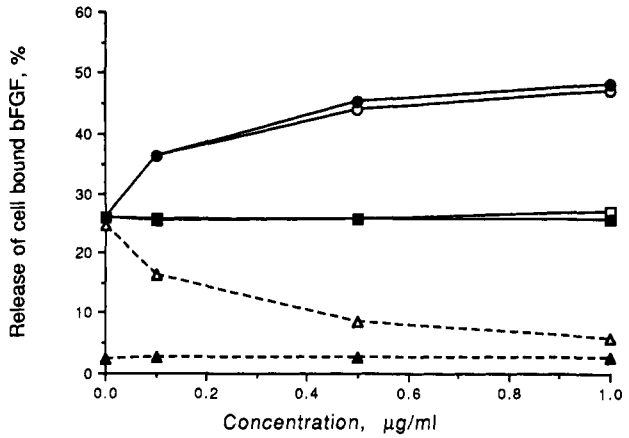

FIGURE 5: Release of cell-bound bFGF. Confluent endothelial cell monolayers were incubated (37 °C, 2 h) with ^{125}I-bFGF [(4–6) × 10^5 cpm/35-mm dish]. Unbound bFGF was removed, and the cells were incubated (2 h, 37 °C) with various concentrations of heparin (○), heparin fragment (●), N-acetylated heparin fragment (□), or totally desulfated heparin fragment (■). The amount of ^{125}I-bFGF released into the incubation medium (○, ●, □, ■) and the amount of ^{125}I-bFGF remaining (after release by heparin) bound to low-affinity (△) and high-affinity (▲) cell-surface receptor sites were determined as described under Experimental Procedures. Released radioactivity is expressed as the percent of total cell-bound ^{125}I-bFGF. The variation between triplicate dishes did not exceed 8% of the mean.

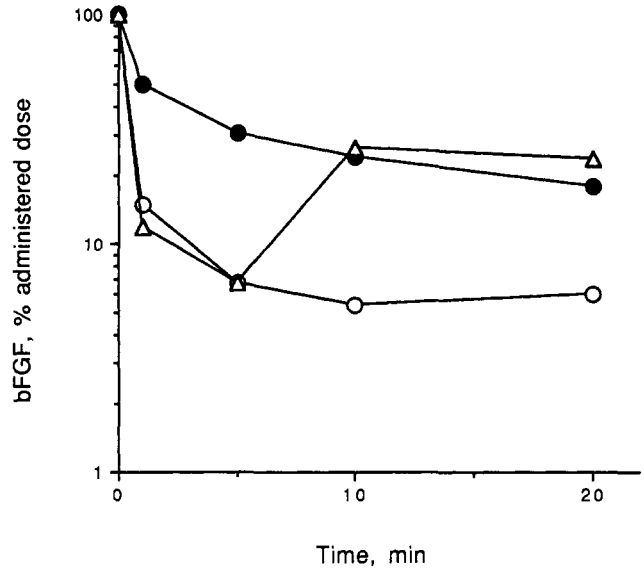

FIGURE 6: Effect of heparin on the rate of bFGF disappearance from the bloodstream. ^{125}I-bFGF (1 × 10^6 cpm, 1.5 × 10^5 cpm/ng) was administered as a bolus (0.3 mL) into the bloodstream of rats (n = 6) in the absence (○) or presence (●) of 5 μg of heparin. Two rats received heparin (5 μg/rat) 5 min after the administration of ^{125}I-bFGF (△). Samples (0.3 mL) of blood were drawn from the arterial line at the indicated time intervals and counted in a γ-counter. Data are presented as the percent of the expected blood concentration of ^{125}I-bFGF at zero time (30900 cpm/0.3 mL).

of virtually no anti-FXa activity and a binding fraction of high anti-FXa activity (Andersson et al., 1976; Ekre et al., 1986; Sudhalter et al., 1989). As demonstrated in Table I, fractions of tetradecasaccharides with high and low affinity to antithrombin III exhibited a similar high bFGF-releasing activity, despite a nearly 200-fold difference in their anti-FXa activity (Table I). This result together with the other data presented in Table I indicates that release of ECM-bound bFGF and the anti-coagulant activity of heparin are unrelated properties.

Structural Requirements for Release of ECM-Bound bFGF and for Inhibition of Heparanase Activity by Heparin. Table I demonstrates that different structural properties of heparin are required for release of ECM-bound bFGF and for inhibition of heparanase activity. This endo-β-D-glucuronidase is expressed by highly metastatic tumor cells (Nakajima et al., 1983, 1988; Vlodavsky et al., 1983) and by activated cells of the immune system (Laskov et al., 1991; Naparstek et al., 1984; Savion et al., 1987). Native and modified species of heparin which inhibit the enzyme activity in vitro also inhibit the incidence of tumor metastasis (Nakajima et al., 1988; Parish et al., 1987; Vlodavsky et al., 1990) and autoimmune diseases in experimental animals (Lider et al., 1989; Willenborg & Parish, 1988). As shown in Table I, substitution of the *N*-sulfates of heparin fragment with acetyl or hexanoyl groups had little or no effect on its ability to inhibit heparanase, as opposed to a greatly reduced ability of the substituted heparin molecules to release ECM-bound bFGF. On the other hand, heparin-derived oligosaccharides containing 6–10 sugar units exhibited a high bFGF-releasing activity but failed to inhibit the heparanase enzyme. Likewise, release of bFGF from ECM, but little or no inhibition of heparanase activity, was brought about by N/O-desulfated, N-resulfated heparin fragment (Table I).

Effect of Heparin on ^{125}I-bFGF Release from Endothelial Cell Monolayers and from the Luminal Surface of Blood Vessels. (A) Cultured Vascular Endothelial Cells. Confluent endothelial cell monolayers were incubated (2 h, 37 °C) with ^{125}I-bFGF, washed free of unbound bFGF, and exposed (2 h, 37 °C) to various species of heparin. As demonstrated in Figure 5, exposure to 1 μg/mL heparin or heparin fragment resulted in release of nearly 50% of cell-associated ^{125}I-bFGF, but there was little or no release of bFGF by 1 μg/mL N-acetylated heparin fragment. N/O-Desulfated heparin failed to release the cell-bound bFGF even at 10 μg/mL. About 25% of the cell-bound ^{125}I-bFGF was released, under the same conditions, during incubation with PBS/BSA alone. Similar studies with other types of modified and low molecular weight species of heparin revealed that structural requirements for release of cell-bound bFGF were similar to those observed with ECM-bound ^{125}I-bFGF. Measurements of the remaining cell-bound ^{125}I-bFGF revealed that the bFGF was released specifically from low-affinity, presumably HS and heparin-like cell-surface receptor sites but there was no release of bFGF bound to high-affinity receptors on the cell surface (Figure 5).

(B) Experimental Animals. We next investigated the effect of heparin and various species of heparin on the fate of ^{125}I-bFGF administered into the blood of rats. For this purpose, heparin was injected intravenously (iv) either 2–3 min prior to a bolus iv administration of ^{125}I-bFGF or 5 min afterward. At various time intervals, samples of blood were drawn from the artery line and counted in a γ-counter. Figure 6 demonstrates that as early as 1 min after the administration of bFGF, only 13–15% of the initial plasma concentration of bFGF remained in the circulation. In contrast, about 40% of the expected initial concentration of ^{125}I-bFGF remained in the circulation when heparin was injected 2–3 min prior to the ^{125}I-bFGF. Administration of heparin 5 min after the ^{125}I-bFGF resulted in a rapid restoration of the plasma concentration of bFGF to a level which was equal to or higher than that measured in animals that received heparin prior to bFGF. At 10 min, the plasma concentration of bFGF was 4–5-fold higher in the presence than in the absence of heparin, regardless of whether the heparin was injected prior or after the ^{125}I-bFGF. Heparin fragment was somewhat less effective than intact heparin, and there was little or no effect to N-acetylated, or N/O-desulfated heparin (not shown). These results suggest that a large proportion of the bFGF in the

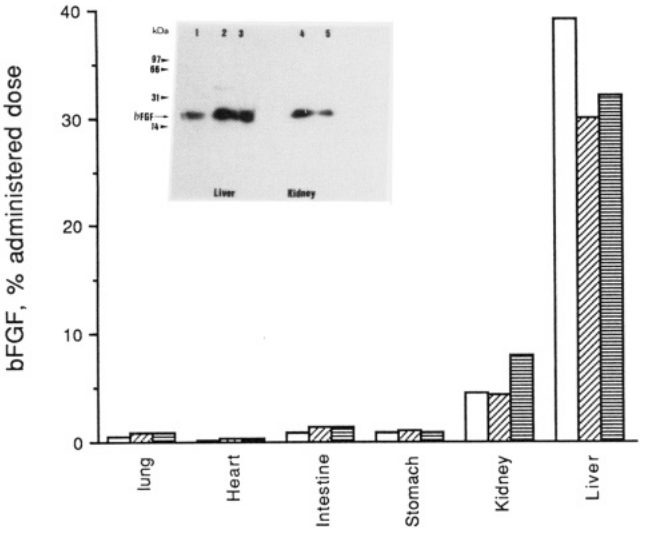

FIGURE 7: Distribution of intravenously administered bFGF in various organs. ^{125}I-bFGF was administered into the bloodstream of rats in the absence (white bars) or presence (hatched bars) of 5 μg of heparin as described in the legend to Figure 6. Two rats received heparin (5 μg/rat) 5 min after the administration of ^{125}I-bFGF (horizontally striped bars). After 20 min, the rats were infused with saline, various organs were removed, and the radioactivity was counted in a γ-counter. Insert: Extracts of the liver and kidneys were subjected to immunoprecipitation with anti-bFGF antibodies and protein A–Sepharose followed by SDS–PAGE and autoradiography, as described under Experimental Procedures. Lane 1, ^{125}I-rbFGF; lanes 2 and 3, liver extracts; lanes 4 and 5, kidney extracts.

bloodstream is rapidly sequestered by heparin-like molecules in the luminal surface of the endothelium and can be released by native heparin and low molecular weight species of heparin, in a manner similar to that observed with ^{125}I-bFGF bound to cultured vascular endothelial cells and to the subendothelial ECM. That bFGF is in fact sequestered by heparin-like molecules in the vessel wall was also indicated by a nearly 2-fold higher circulating level of ^{125}I-bFGF measured in the blood of animals that received iv injections of bacterial heparinase (0.2 unit/rat) 15 min prior to and 1 min after the ^{125}I-bFGF (not shown). Analysis of the distribution of ^{125}I-bFGF in various organs revealed that bFGF was predominantly cleared by the liver and kidneys (Figure 7). Similar results were reported by Whalen et al. (1989). Immunoprecipitation of tissue extracts followed by SDS gel electrophoresis and autoradiography revealed the presence of intact ^{125}I-bFGF in both organs (Figure 7, insert).

Discussion

Despite the ubiquitous presence of bFGF in normal tissues, EC proliferation in these tissues is usually very low, with turnover time measured in years (Denekamp, 1984). This raises the question of how bFGF is prevented from acting on the vascular endothelium continuously and in response to what signals it becomes available for stimulation of capillary EC proliferation. One possibility is that bFGF is sequestered from its site of action by means of binding to HS and heparin-like molecules in the cell surface and ECM and saved for emergencies such as wound repair and neovascularization. We have previously demonstrated that ECM-bound bFGF can be released in an active form by either heparin-like molecules or heparin- and HS-degrading enzymes, both in vitro and in vivo (Folkman et al., 1988; Vlodavsky et al., 1991). Although heparin is best known for its anticoagulant and antithrombotic properties, it affects various physiological processes such as vascular endothelial (Imamura & Mitsui, 1987; Sudhalter et al., 1989) and smooth muscle cell proliferation (Castellot et al., 1986), angiogenesis (Folkman et al., 1983), inflammation (Ekre et al., 1986; Sy et al., 1983), and autoimmunity (Willenborg & Parish; Lider et al., 1989). It is, however, virtually impossible to assign structure–function relationships based on studies with native heparin due to variations in the size of the polysaccharide chains and in the degree and distribution of sulfate groups (Sudhalter et al., 1989). In order to elucidate the structural requirements for release of ECM-bound bFGF by heparin, we have used a number of chemically modified species of heparin fragment and homogeneously sized oligosaccharides obtained from depolymerized heparin. The presence of *N*-sulfates was found to be a critical requirement for release of bFGF, since total substitution of *N*-sulfates with acetyl or hexanoyl groups resulted in an almost complete inhibition of the bFGF-releasing activity of heparin, despite a normal content of *O*-sulfate groups. O-Sulfation facilitated bFGF release, but was not an absolute requirement, as indicated by the high bFGF-releasing activity of totally N/O-desulfated, N-resulfated heparin fragment. The latter compound contained less sulfate than N-acetylated heparin fragment, indicating that bFGF release is determined primarily by the sulfate position and to a lesser extent by its total content. The contributory role of *O*-sulfates was indicated by the finding that oversulfation of N-acetylated heparin fragment resulted in partial restoration of its ability to release ECM-bound bFGF. The effect of sulfation was also demonstrated in experiments with homogeneously sized saccharides obtained from depolymerized heparin and separated into fractions of low, medium, and high degrees of sulfation (Sudhalter et al., 1989). While medium- and high-sulfated fractions of a given oligosaccharide showed virtually the same bFGF-releasing activity, the respective low-sulfate oligosaccharide was a less effective releaser of bFGF. This behavior was more prominent with low-size oligosaccharides (i.e., hexasaccharides) and at low concentrations of the saccharide. A nearly maximal release of ECM-bound bFGF was obtained already by the octasaccharides. The tetrasaccharide exhibited about 10% and 40% of the activity of native heparin when calculated on a molar basis and weight basis, respectively. No correlation was found between the antithrombotic activity (anti-factor Xa activity) of heparin and its bFGF-releasing activity, indicating that the specific pentasaccharide sequence responsible for the binding of anticoagulant heparin to antithrombin III (Lindahl et al., 1984) is not required for release of ECM-bound bFGF. Structural requirements similar to those described above were found for release of ^{125}I-bFGF from low-affinity sites on the surface of cultured vascular endothelial cells. In contrast, bFGF bound to high-affinity cell-surface receptor sites was not accessible to release by heparin and heparin-like molecules. Studies on the fate of intravenously administered ^{125}I-bFGF suggested that bFGF is sequestered by the luminal surface of the vascular endothelium. Structural requirements for release of the bound bFGF into the circulation were similar to those found for release of ECM- and cell-bound bFGF. Analysis of the distribution of ^{125}I-bFGF in various organs revealed the presence of intact bFGF primarily in the liver and kidneys.

There was no correlation between the ability of the various modified and low molecular weight species of heparin to release bFGF and their ability to inhibit the enzyme heparanase (Table I) and tumor metastasis in experimental animals (not shown) (Vlodavsky et al., 1990). Unlike release of bFGF, inhibition of heparanase activity required larger size oligosaccharides (tetradecasaccharide and higher) and was only slightly affected by substitution of *N*-sulfates with acetyl

(Bar-Ner et al., 1987) or hexanoyl groups. These results indicate that different effects of heparin are mediated by different sugar sequences and that specific heparin-like molecules can be designed to elicit or inhibit a specific effect. for example, N-substituted species of heparin, rather than native heparin, could be applied to inhibit tumor metastasis, since their efficient inhibition of heparanase activity was not associated with a significant release of active bFGF from cells and ECM. These compounds are therefore expected to inhibit metastases formation by certain tumor cells, correlated with their inhibition of heparanase activity (Nakajima et al., 1988; Parish et al., 1988; Vlodavsky et al., 1983, 1990), with little or no potential induction of tumor angiogenesis in response to bFGF release. A recent study performed with HS-deficient CHO mutant cells demonstrated that binding of bFGF to heparin-like molecules on the cell surface is a prerequisite for binding of bFGF to its high-affinity cell-surface receptors (Yayon et al., 1991). Likewise, fibroblasts and muscle cells treated with heparin-degrading enzymes or with inhibitors of sulfation were found to bind bFGF and respond to the bound factor only when heparin was added to the medium (Rapraeger et al., 1991). The exact structural requirements for restoration of bFGF high-affinity binding and response are currently being investigated in our laboratory, using the chemically modified and low molecular weight species of heparin applied in the present study. Previous studies demonstrated a correlation between the degree of sulfation of heparin-like molecules and their relative potencies in stabilizing (Gospodarowicz & Chen, 1986) and potentiating basic and acidic FGF (Sudhalter et al., 1989). Low-affinity receptor binding of bFGF is significantly reduced in cells expressing low-sulfated HSPGs (Yayon et al., 1991). Altogether, these results suggest that the charge density and the appropriate distribution of this charge (e.g., sulfate groups) on the polysaccharide may lead to the formation of a stable, receptor-compatible, and biologically active conformation of bFGF.

Our present and previous studies on the sequestration and release of bFGF (Vlodavsky et al., 1991) suggest that bFGF may acquire an immobilized storage form that is stable but relatively inactive and a soluble form that is labile but highly active. Release of HS-bound bFGF by heparin-derived oligosaccharides containing 8–10 sugar units, as well as by heparin- and HS-degrading enzymes, may yield an intermediate type of molecule that is relatively stable (Gospodarowicz & Chen, 1986) and may readily diffuse through the stroma (Flaumenhaft et al., 1990), as compared to free bFGF. This released form is also capable of binding to high-affinity plasma membrane receptors (Saksela & Rifkin, 1990), resulting in proliferative and differentiation responses in endothelial cells and other mesoderm-derived cells. Sequestration and release of FGF-like factors may thus provide a novel mechanism for regulation of capillary blood vessel growth. Under normal conditions, it may prevent them from acting on the vascular endothelium, while perturbation of the ECM and/or exposure to heparin-like molecules may elicit localized EC proliferation and neovascularization.

Registry No. bFGF, 106096-93-9; heparin, 9005-49-6; heparanase, 89800-66-8; antithrombin III, 9000-94-6.

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Does Pyrophosphate Bind to the Catalytic Sites of Mitochondrial F_1-ATPase?†

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ABSTRACT: The interactions between the pyrophosphate (PP_i) binding sites and the nucleotide binding sites on mitochondrial F_1-ATPase have been investigated, using F_1 preparations containing different numbers of catalytic and noncatalytic nucleotide-binding sites occupied by ligands. In all cases, the total number of moles of bound nucleotides and PP_i per mole of F_1 was less than or equal to six. F_1 preparations containing either three or two filled noncatalytic sites and no filled catalytic sites (referred as $F_1[3,0]$ and $F_1[2,0]$) were found to bind 3 mol of PP_i/mol of F_1. Tight binding of ADP–fluoroberyllate complexes to two of the catalytic sites of F_1 converted the three heterogeneous PP_i-binding sites into three homogeneous binding sites, each exhibiting the same affinity for PP_i. The addition of PP_i at saturating concentrations to F_1 containing GDP bound to two catalytic sites ($F_1[2,2]$) resulted in the release of 1 mol of GDP. Furthermore, the addition of PP_i to F_1 filled with ADP–fluoroberyllate at the catalytic sites resulted in the release of 1 mol of tightly bound ADP/mol of F_1. Taken together, these results indicate that PP_i binds to specific sites that interact with both the catalytic and the noncatalytic nucleotide-binding sites of F_1.

Despite recent advances in the understanding of the mechanism by which the catalytic sector F_1 of H^+-ATPase[1] undergoes ATP hydrolysis, there remains a number of unsolved problems, some of which concern the status of the nucleotide-binding sites. The presence of six nucleotide-binding sites located in the three α and the three β subunits of mitochondrial, bacterial, and chloroplastic F_1 has been demonstrated (Dunn & Futai, 1980; Ohta et al., 1980; Wagenwoord et al., 1980; Cross & Nalin, 1982; Lunardi & Vignais, 1982; Issartel & Vignais, 1984; Gromet-Elhanan & Khananshvili, 1984; Perlin et al., 1984; Issartel et al., 1986; Hisabori et al., 1986). Three of these sites rapidly exchange their bound ADP and ATP with added nucleotides (Cross et al., 1982; Gresser et al., 1982; Melese & Boyer, 1985), and they are competent for catalysis. When prepared by the method of Knowles and Penefsky (1972), beef heart mitochondrial F_1 contains three

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[1] Abbreviations: F_1-ATPase, catalytic sector (soluble) of the beef heart mitochondrial H^+-ATPase complex; $F_1[x,y]$, F_1 with x noncatalytic and y catalytic sites occupied by nucleotides; AdN, adenine nucleotide; PP_i, pyrophosphate.

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