Sulfate Moieties in the Subendothelial Extracellular Matrix Are Involved in Basic Fibroblast Growth Factor Sequestration, Dimerization, and Stimulation of Cell Proliferation*

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The growth promoting activity of the subendothelial extracellular matrix (ECM) is attributed to sequestration of basic fibroblast growth factor (bFGF) by heparan sulfate proteoglycans and its regulated release by heparin-like molecules and heparan sulfate (HS) degrading enzymes. HS is also involved in bFGF receptor binding and activation. The present study focuses on the growth promoting activity and bFGF binding capacity of sulfate-depleted ECM. Corneal endothelial cells (EC) maintained in the presence of chlorate, an inhibitor of phosphoadenosine phosphosulfate synthesis, produced ECM containing 10–15% of the sulfate normally present in ECM. Incorporation of sulfate into HS was reduced by more than 90% Binding of 125I-bFGF to sulfate-depleted ECM was reduced by 50–60% and only about 10% of the ECM-bound bFGF was accessible to release by heparin. Incubation of 125I-bFGF on top of native ECM resulted in dimerization of the ECM-bound bFGF, but there was a markedly reduced binding and dimerization of bFGF on sulfate-depleted ECM. ECM produced in the presence of chlorate contained a nearly 10-fold less endogenous bFGF as compared to native ECM and exerted little or no mitogenic activity toward vascular EC and 3T3 fibroblasts. In other studies, we investigated the interaction between chloride-treated vascular EC and either native or sulfate-depleted ECM. Exogenous heparin stimulated the proliferation of chloride-treated EC seeded on native ECM, suggesting its interaction with ECM-bound bFGF and subsequent presentation to high affinity cell surface receptors. On the other hand, heparin had no effect on chloride-treated cells seeded in contact with sulfate-depleted ECM or regular tissue culture plastic. Altogether, the present experiments indicate that heparan sulfate proteoglycans associated with the cell surface and ECM act in concert to regulate the bioavailability and growth promoting activity of bFGF. While HS in the subendothelial ECM functions primarily in sequestration of bFGF in the vicinity of responsive cells, HS on cell surfaces is playing a more active role in displacing the ECM-bound bFGF and its subsequent presentation to high affinity signal transducing receptors.

Heparan sulfate (HS) is a most ubiquitous glycosaminoglycan present on cell surfaces, in basement membranes and extracellular matrices (Gallagher et al., 1986; Jackson et al., 1991; Kjellen and Lindahl, 1991). Recent interest in heparan sulfate proteoglycans (HSPG) stems from increasing awareness of the functional implications of their interactions with growth factors, matrix molecules, and cytoskeletal elements (Gitay-Goren et al., 1992; Jackson et al., 1991; Ruoslahti and Yamaguchi, 1991; Vladovskv et al., 1993; Yaron et al., 1991). The HS chains have been implicated in a variety of physiological processes including the regulation of glomerular basement membrane permeability to proteins, assembly of basement membranes, regulation of nuclear metabolism, cell attachment and spreading, recruitment of inflammatory cells (chemokines), and the regulation of mammalian cell proliferation and differentiation (Gallagher et al., 1986; Jackson et al., 1991; Ruoslahti and Yamaguchi, 1991; Tanaka et al., 1993). The sulfate residues, which may be present on four different positions of the polysaccharide backbone, are of high interest, since they have been shown to be major factors in the determination of specificity in protein-polysaccharide interactions (Lindahl, 1989). Of particular significance is the interaction between HS and basic fibroblast growth factor (bFGF), involved in bFGF receptor binding and signal transduction (Ornitz et al., 1992, Rapraeger et al., 1991; Yaron et al., 1991). A unique, highly sulfated bFGF-binding fragment of HS was isolated from cell surface HSPG of fibroblasts (Turnbull et al., 1992). Sulfation in critical positions along the polysaccharide chain, particularly 2-O-sulfation, seems necessary to generate a specific bFGF binding motif that can support high affinity bFGF-receptor binding and activation (Aviezer et al., 1994b; Habuchi et al., 1992; Ishihara et al., 1993; Maccarana et al., 1993; Turnbull et al., 1992).

Chlorate, an inhibitor of ATP sulfurylase and hence of the production of phosphoadenosine phosphosulfate, the active sulfate donor for sulfotransferases (Baeuerle and Huttner, 1986), has been shown to abolish sulfation on proteins and carbohydrate residues in intact cells without inhibiting cell growth or protein synthesis (Baeuerle and Huttner, 1986; Keller et al., 1989). Exposure to chlorate markedly reduced binding of bFGF to high affinity cell surface receptors and the ability of 3T3 fibroblasts to proliferate in response to bFGF (Guimond et al., 1993; Rapraeger et al., 1991).

Our studies on the control of cell proliferation by its local environment focus on the interaction of cells with the extracellular matrix (ECM) produced by cultured corneal endothelial cells (EC) (Gospodarowicz et al., 1980; Vladovskv et al., 1980, 1993). This ECM closely resembles the subendothelium in vivo blast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; EC, endothelial cells; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; PBS, phosphate-buffered saline; DSS, disaccharinimidydi suberate; PAGE, polyacrylamide gel electrophoresis.

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‡The abbreviations used are: HS, heparan sulfate; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycans; PBS, phosphate-buffered saline; DSS, disaccharinimidydi suberate; PAGE, polyacrylamide gel electrophoresis.
Properties of Sulfate-depleted Extracellular Matrix

in its morphological appearance and molecular organization. It contains collagens (mostly types III and IV, with smaller amounts of types I and V), proteoglycans (mostly HS- and dermatan sulfate proteoglycans, with smaller amounts of chondroitin sulfate proteoglycans), laminin, fibronectin, entactin, and elastin. EC and other cell types plated in contact with this ECM no longer require the addition of soluble bFGF in order to proliferate and express their differentiated functions (Gospodarowicz et al., 1980; Vlodavsky et al., 1980). In subsequent studies bFGF was identified as a complex with HSPG in the subendothelial ECM produced in vitro (Bashkin et al., 1989; Vlodavsky et al., 1987) and on cell surfaces and basement membranes of diverse tissues and blood vessels (Cardon-Cardo et al., 1990; Gonzalez et al., 1990). HS-bound bFGF is protected against heat inactivation and proteolytic degradation (Saksela et al., 1990; 1991) or by proteases (Benezra 1989; Ishai-Michaeli et al., 1990). HS-bound bFGF is also protected against heat inactivation and proteolytic degradation by sulfate. The specific activity of HS-bound bFGF was determined by gel filtration and detected on a Peptide-Blot. The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995).

**EXPERIMENTAL PROCEDURES**

Materials—Recombinant human bFGF was kindly provided by Takada Chemical Industries (Osaka, Japan). Sepharose 6B was from Pharmacia (Uppsala, Sweden). Sodium heparin from porcine intestinal mucosa (PM-heparin, M, 14,000, anti-Xa 165 IU/mg) was obtained from Hepar Industries (Franklin, OH). Dulbecco's modified Eagle's medium (DMEM, 1 g of glucose/liter (or 4.5 g glucose/liter), Fisher Chemical, PA) was supplemented with 10% newborn calf serum, 5% fetal calf serum, 50 units/ml penicillin, 50 g/ml streptomycin (50 g/ml), 24 h, 37°C) the cell layer with PBS containing 0.5% Triton X-100, followed by washing three times with PBS followed by incubation (30 min, 24°C) with 0.15 mM DSS in PBS. The cross-linking reaction was quenched with 10 mM ethanolamine-HCl (pH 8.0) for 30 min, the incubation medium removed, the remaining ECM scraped and dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (150 mM Tris, pH 8.0, 37°C). The soluble material was analyzed by 15% SDS-PAGE. The cross-linked bFGF was visualized by autoradiography using Kodak XAR film. The cross-linking of bFGF was determined by gel filtration and detected on a Peptide-Blot. The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995). The estimated amount of sulfate labeled HS was determined by a method described elsewhere (Gospodarowicz et al., 1995).

For preparation of sulfate-labeled ECM, corneal EC were plated into four-well plates and cultured as described above. Na235SO4 (540–590 mCi/mmol) was added (20 μCi/ml) 1 and 5 days after seeding, and the cultures were incubated with the label without medium change. Ten to 12 days after seeding, the cell monolayer was dissolved and the ECM from the entire cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH4OH, followed by washing three times with PBS. The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish (Gospodarowicz et al., 1980, 1983; Vlodavsky et al., 1980, 1987).

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Fate content was obtained in ECM produced in the presence of 30 mM chlorate (Fig. 1, inset). Similar results were obtained when the stock of corneal EC was treated (48 h) with 30 mM chlorate before seeding into the four-well plates and thereafter. At this concentration, chlorate had little or no effect on the total amount of ECM protein deposited by the cells, nor on the morphology and organization of the ECM as revealed by phase and scanning electron microscopy. Exposure to 60 mM chlorate resulted in an almost complete inhibition of sulfate incorporation, but this was associated with a slight decrease (20%) in ECM deposition. In subsequent experiments, sulfate-depleted ECM was produced by cells maintained in the presence of 30 mM chlorate, as described above. Specific incorporation of sulfate into HS was analyzed by measurements of sulfate-labeled material released from ECM during incubation (24 h, 37 °C) with 2 μg/ml of a purified preparation of human placental heparanase. Sulfate-labeled HS degradation products released into the incubation medium were analyzed by gel filtration on Sepharose 6B. Inset, total amount of labeled sulfate determined following trypsin digestion of the ECM. Aliquots of the trypsinized material were counted in a β-counter. Each data point (cpm/well) is the mean ± S.D. of four wells.

**Fig. 1. Effect of chlorate on sulfate incorporation into HS in ECM.** Corneal EC were seeded at a confluent density (2 × 10^5 cells/16-mm well) in the absence (□) or presence of increasing concentrations (▲, 1 mM; ○, 10 mM; ■, 20 mM; ●, 30 mM; ▲, 60 mM) of chlorate. The cells were maintained in Fisher’s medium in the presence of Na_2^{35}SO_4 (20 μCi/well) added on day 1 and 5. ECM was prepared on day 10, followed by incubation (24 h, 37 °C, pH 6.2) with 2 μg/ml of a purified preparation of human placental heparanase. Sulfate-labeled HS degradation products released into the incubation medium were analyzed by gel filtration on Sepharose 6B. Inset, total amount of labeled sulfate determined following trypsin digestion of the ECM. Aliquots of the trypsinized material were counted in a β-counter. Each data point (cpm/well) is the mean ± S.D. of four wells.

Effect of Chlorate on bFGF Binding Capacity and Growth Promoting Activity of ECM—Sulfate groups are involved in bFGF binding to isolated heparin and HS (Aviezer et al., 1994b; Habuchi et al., 1992; Ishai-Michaeli et al., 1992, 1993; Maccarana et al., 1993; Turnbull et al., 1992). The availability of sulfate-deficient ECM provided an appropriate means to investigate to what extent sulfate groups are involved in bFGF binding to a multimolecular structure such as intact subendothelial ECM. Binding of 125I-bFGF to ECM produced in the presence of 30 mM chlorate was inhibited by 50–60%. Sulfate depletion had a more pronounced effect on bFGF binding to HS in the ECM, as revealed by 70–80% reduction in the amount of ECM-bound bFGF displaced by heparin (Fig. 2). A similar value was obtained when the ECM-HS was degraded by human placental heparanase (data not shown). While heparin or heparanase treatment released 50–60% of the 125I-bFGF bound to native ECM, only 10–15% of the bFGF bound to sulfate-depleted ECM was released under the same conditions. It is therefore conceivable that binding of bFGF to sulfate-depleted ECM produced in the presence of 30 mM chlorate was due primarily to binding to other components of the ECM (i.e. fibronectin) or to glycosaminoglycan side chains deprived of their sulfate moieties (Ornitz et al., 1995).

We have previously demonstrated that EC and other cell types plated in contact with the subendothelial ECM no longer require the addition of soluble bFGF in order to proliferate (Gospodarowicz et al., 1980; Vlodavsky et al., 1987). This mitogenic effect was attributed primarily to the presence of bFGF in ECM, although the mode of bFGF deposition was not elucidated (Vlodavsky et al., 1987). ECM produced in the absence and presence of chlorate was tested for mitogenic activity toward vascular EC and 3T3 fibroblasts. For this purpose, vascular EC were seeded at a low cell density (1,000 cells/16-mm well) on top of ECM produced in the absence and presence of 30 mM chlorate. Six days after seeding, the cultures were exposed to [3H]thymidine and the amount of trichloroacetic acid-precipitable radioactivity was determined 3 h afterwards (Fig. 3A).
EC were also seeded at a clonal cell density (300 cells/35-mm dish) on top of native and sulfate-depleted ECM and cell colonies were stained 10 days after seeding (Fig. 3, inset). As demonstrated in Fig. 3A, ECM produced in the presence of chlorate exerted a greatly reduced mitogenic activity toward vascular EC seeded at a low or clonal cell density. In other experiments (Fig. 3B), ECM produced in the presence of increasing concentrations of chlorate was subjected to trypsin digestion and aliquots of the solubilized material were added to confluent, growth-arrested 3T3 fibroblasts (Fig. 3B) or to sparsely seeded EC (1,000 cells/16-mm well) maintained in the presence of 10% heat-inactivated calf serum (data not shown).

A trypsin digest of native ECM was highly mitogenic to growth arrested 3T3 fibroblasts (Fig. 3B), and this activity was inhibited by neutralizing anti-bFGF antibodies (data not shown). In contrast, ECM produced in the presence of 30 mM chlorate was devoid of mitogenic activity toward 3T3 fibroblasts (Fig. 3B). Likewise, chlorate markedly reduced (~60%) the growth promoting activity of ECM extracts toward sparsely seeded EC (data not shown). As demonstrated in Fig. 3A, EC plated on chlorate-treated ECM responded to exogenously added bFGF in a manner similar to cells plated on regular tissue culture plastic. These results indicate that sulfation is critical for the growth promoting activity of the ECM.

In other experiments, ECM produced in the absence and presence of 30 mM chlorate was digested (3 h, 37°C) with 0.1 μg/ml trypsin and the amount of bFGF in the solubilized material was determined by an immunassay (Quantikine human bFGF, R&D Systems, Minneapolis, MN). The amount of bFGF in sulfate-depleted ECM was about 10-fold lower than that determined in native ECM (i.e. 11 and 121 pg of bFGF/ECM-coated 16-mm culture well, respectively). Similar results were obtained when the ECM was digested with bacterial (Flavobacterium heparinum) heparinase I (IBEX Technologies, Montreal, Canada) rather than trypsin. The heparinase-treated ECM exerted little or no mitogenic activity on vascular EC.

Effect of Heparin on bFGF Sequestration and Growth Promoting Activity of Sulfate-depleted ECM—We investigated the effect of heparin on bFGF sequestration and the growth promoting activity of ECM produced by corneal EC maintained in the absence and presence of chlorate. Heparin was included in the cell lysis solution to prevent binding of intracellular bFGF to ECM-HS when the ECM-producing cells are lysed. When 125I-bFGF was added to the lysis solution, it was found that only about 2 and 0.2% of the added bFGF was sequestered by...
Heparin (10–20 μg/ml) inhibited by about 80% the deposition of bFGF on top of ECM produced in the absence of chlorate, and there was little or no effect to heparin on the residual binding of 125I-bFGF to ECM produced in presence of chlorate (Fig. 4).

We next analyzed the effect of heparin on the growth promoting activity of ECM produced in the absence and presence of chlorate. Heparin, present during the 5-min cell lysis period, had little or no effect on the growth promoting activity of native ECM toward vascular EC seeded on ECM at a low (Fig. 5) or clonal (data not shown) cell densities. Surprisingly, the mitogenic activity toward EC of ECM produced in the presence of chlorate was stimulated (1.5–4-fold, in different experiments) when heparin was included in the cell lysis solution. This stimulation was observed both when the endothelial cells were seeded directly on the ECM (Fig. 5) and when the ECM was first digested with trypsin and aliquots of the solubilized material were tested for mitogenic activity on vascular EC (data not shown). Measurements of 125I-heparin binding revealed that under the experimental conditions applied in Fig. 5, <0.5% of the heparin was bound to the ECM and there was no difference in heparin binding to ECM produced in the absence or presence of chlorate (data not shown).

Mitogenic Response of Chlorate-treated Endothelial Cells Plated on ECM—It has been previously demonstrated that heparin restores the ability of chlorate-treated 3T3 fibroblasts to proliferate in response to bFGF (Rapraeger et al., 1991). We investigated whether proliferation of chlorate-treated EC can be similarly restored by native ECM, in the absence and presence of added heparin. For this purpose, vascular EC were pretreated for 24 h with 30 mM chlorate, dissociated with STV, and seeded (2,000 cells/16-mm well) into regular tissue culture wells (open box), and wells coated with ECM produced in the presence of chlorate (data not shown). Heparin (1 μg/ml) was added to some of the wells on day 1 and 3 and the cells counted in a Coulter counter on day 5 after seeding. Each data point (cells/well) is the mean ± S.D. of four wells.

Mitogenic response was obtained when the chlorate-treated EC were seeded in contact with native ECM, and best results were obtained when heparin (1 μg/ml) was added to the culture medium (Fig. 6). Under these conditions (chlorate-treated EC maintained on native ECM in the presence of heparin), there was little or no further stimulation of cell proliferation in response to exogenously added bFGF (data not shown). These results suggest that sulfate moieties on cell surfaces play an active role in the presentation of ECM-bound bFGF to its high affinity cell surface receptors and that soluble heparin may exert a similar effect in chlorate-treated, undersulfated endothelial cells.

Dimerization of bFGF on Native and Sulfate-depleted ECM—
Heparan sulfates are heterogeneous molecules that vary both in their basic disaccharide subunits and in their degree and position of sulfation (Gallagher et al., 1986; Jackson et al., 1991; Kjellen and Lindahl, 1991). Both the level of sulfation and position of sulfate groups are major determinants in the interaction between bFGF and HS and the ability of heparin and HS to promote bFGF receptor binding and mitogenic activity (Aviezer et al., 1994b; Habuchi et al., 1992; Ishihara et al., 1993; Maccarana et al., 1993; Ornitz et al., 1992; Turnbull et al., 1992). Using chlorate, an inhibitor of phosphoadenosine phosphosulfate synthase, we investigated the involvement of sulfate groups in the growth promoting activity of the subendothelial ECM. Sulfate-depleted ECM exhibited a greatly reduced mitogenic activity toward vascular EC and 3T3 fibroblasts, as compared to native ECM. Similar results were obtained, regardless of whether the vascular EC were seeded on top of chlorate-treated ECM or whether the sulfate-depleted ECM was first digested with trypsin and aliquots of the solubilized ECM added to EC seeded on regular tissue culture plastic. The lack or low mitogenic activity may be due to (i) reduced amounts of bFGF in ECM produced in the presence of chlorate and (ii) inability of this ECM to present the ECM-bound bFGF to its high affinity cell surface receptors. Measurements of bFGF binding revealed a 50–60% reduction in bFGF binding to ECM produced by chlorate-treated corneal EC, as compared to untreated cells. The amount of HS-bound, heparin/heparanase releasable, 125I-bFGF was reduced by 70–80% in chlorate-treated ECM, suggesting that 125I-bFGF may bind also to sulfate-depleted glycosaminoglycan side chains (Ornitz et al., 1995), ECM components other than HS (i.e. fibronectin), and possibly ECM-bound bFGF receptors (Hanneken et al., 1995).

Direct immunoquantitation of bFGF in solubilized ECM revealed about a 10-fold reduction in the amount of endogenous bFGF in ECM produced in the presence of chlorate as compared to native ECM. In this assay the ECM was first digested with trypsin to solubilize the matrix and hence the reduced amounts of bFGF determined in sulfate-depleted ECM may be attributed, in part, to tryptic degradation of bFGF that is no longer protected by properly sulfated HS (Saksela et al., 1988). It should be noted, however, that a similar decrease in bFGF content was obtained when the ECM was digested with bacterial heparinase, rather than with trypsin, resulting in solubilization of >90% of the ECM-resident bFGF. These results, together with the lack of or greatly reduced mitogenic activity exerted by intact, undegraded sulfate-depleted ECM, suggest that this ECM exhibit little or no growth promoting activity simply because its HS chains fail to sequester bFGF and hence can not function as a secured depot of this growth factor in the vicinity of cells. Measurements of the cellular content of bFGF revealed no difference between chlorate-treated and untreated EC, suggesting that chlorate did not affect the synthesis of bFGF. An inhibitory effect on bFGF deposition, possibly as a complex with cell-associated HS, cannot be excluded.

A major concern in the study of the growth promoting activity of the subendothelial ECM is whether the ECM-bound bFGF is deposited into the ECM by intact EC, prior to denudation of the ECM, or sequestered by HS and other components of the ECM when the bFGF-containing ECM are lysed and the ECM exposed. In the present study, heparin was included in the cell lysis solution in order to eliminate the latter possibility. Measurements of 125I-bFGF binding revealed that heparin (10 μg/ml), present in the cell lysis solution, inhibited by about 90% the binding of 125I-bFGF to the newly exposed native ECM. However, there was no effect to this heparin on the growth
promoting activity of the ECM, indicating that the mitogenic activity of native ECM is not due to sequestration of bFGF occurring when the ECM-producing cells are lysed. An unexpected result was obtained when the effect of heparin on the mitogenic activity of sulfate-depleted ECM, was investigated. Unlike the results with native ECM, heparin, present during the 5-min cell lysis period, stimulated the growth promoting activity of sulfate-depleted ECM. A possible explanation for this stimulation is the ability of heparin to bind to bFGF in the ECM and function in the displacement and presentation of ECM-bound bFGF to high affinity receptor sites on the cell surface. Alternatively, heparin may bind to intracellular bFGF and then to the ECM, increasing the concentration of bFGF in the undersulfated matrix. ECM binding of $^{125}$I-heparin was low and there was no difference between native and sulfate-depleted ECM, but this may result from the iodination procedure, which could significantly alter the ability of heparin to bind to heparin-binding proteins (i.e. fibronectin, vitronectin) in the ECM. Heparin was previously shown to restore the mitogenic response of chlorate-treated 3T3 fibroblasts and endothelial cells to bFGF (Guimond et al., 1993; Rapraeger et al., 1991).

Our experiments with chlorate-treated vascular EC plated on intact native ECM, demonstrated that heparin can restore the ability of the cells to proliferate in response to bFGF residing in the ECM. Previous studies revealed that the $K_{d}$ value for interaction of bFGF with the cell surface HS ($2 \times 10^{-9}$ M) is lower than for interaction with HS in the ECM ($1 \times 10^{-7}$ M) (Bashkin et al., 1989; Moscatelli, 1987; Roghani et al., 1994), suggesting that ECM-bound bFGF interacts first with HS on the cell surface and is then presented to high affinity cell surface receptors. Because the cell surface HSPG, unlike that of the ECM, is mobile in the plane of the membrane and can turn over more rapidly by shedding and internalization, it may readily replenish its bFGF from the ECM reservoir, which serves more as an efficient large capacity bFGF storage depot in the vicinity of cells (Bernfield and Hooper, 1991). Both functions (i.e. sequestration and presentation of bFGF to high affinity receptor sites) may not be fulfilled by non-sulfated HS side chains present in the ECM and surface of chlorate-treated EC. A difference between cell surface- and ECM-derived species of HS in their ability to promote bFGF mitogenicity was also demonstrated in our recent studies on the growth promoting activity of HS degradation fragments released by bacterial heparinase III from ECM and cell surfaces. Using HS-deficient lymphoid cells, we have demonstrated a stimulated cell proliferation induced by bFGF in the presence of HS degradation fragments released from cell surfaces, but not from ECM.2 Altogether, it appears that HS in ECM, unlike on cell surfaces, may not function efficiently as an accessory low affinity receptor capable of directly accelerating the arrival of bFGF at its high affinity signaling receptor.

The essential involvement of sulfate groups in bFGF receptor binding and activation was previously demonstrated by applying undersulfated and oversulfated species of heparin. These studies utilized chlorate-treated cells and HS-deficient cell mutants (Guimond et al., 1993; Rapraeger et al., 1991; Yawon et al., 1991). Best results were achieved in the presence of over- sulfated heparin fragments, regardless of whether the N- position was sulfated or acetylated (Aviezer et al., 1994b). In a recent study (Ornitz et al., 1995), a stimulatory effect was also induced by synthetic, nonsulfated heparan-derived di- and trisaccharides. Our studies with native and sulfate-depleted endothelial cells and ECM demonstrate that properly sulfated HSPG associated with the cell surface and ECM act in concert to regulate the bioavailability of active bFGF and possibly other effector molecules to their signal transducing receptors.

Perlecan, the large basement membrane proteoglycan, was recently identified as a major candidate for a bFGF low affinity accessory receptor and an angiogenic modulator. Other HSPG (e.g. syndecan, fibroglycan, and glypicanc) exhibited only a small activity (Aviezer et al., 1994a). Undersulfated perlecan synthesized in the presence of chlorate is likely to exhibit a much lower capacity to sequester bFGF and subsequently activate the high affinity bFGF cell surface receptor site. This may result in a marked inhibition of the EMC-induced EC proliferation and indirect involvement in neovascularization. It was also demonstrated that acidic FGF binding to its low affinity accessory receptor caused oligomerization of the FGF molecules, thereby indirectly cross-linking and activating the high affinity receptors, resulting in transmembrane signaling and cell proliferation (Spivak-Kroizman et al., 1994). A similar ligand oligomerization was observed during incubation of acidic FGF with bovine lens epithelial cells (Mascarelli et al., 1993).

In the present study, intact ECM was found to induce dimerization of $^{125}$I-bFGF to a much higher extent as compared to sulfate-depleted ECM. The markedly reduced dimerization was attributed primarily to the decrease in bFGF binding and sequestration by HSPG in the sulfate-deficient ECM. Dimerization of bFGF observed on sulfate-depleted ECM may be mediated by both sulfated and nonsulfated HS derived saccharides remaining in this ECM. The latter possibility was recently reported (Ornitz et al., 1995). The highly reduced ability of sulfate-depleted ECM to sequester and dimerize bFGF is in all likelihood responsible for the impaired mitogenic activity of this ECM. Oligomerization of ECM-bound bFGF and possibly other heparin-binding growth factors may contribute to the potent growth- and differentiation-promoting activities of the ECM. This oligomerization is induced by properly sulfated HSPG found in native, but not sulfate-depleted ECM. Specific alterations in the level and pattern of sulfation alone on the HS side chains may thus provide a means to modulate the involvement of HS and ECM in the control of cell proliferation and differentiation and in processes such as neovascularization and tissue remodeling.

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