Vascular Cell–Derived Heparan Sulfate Shows Coupled Inhibition of Basic Fibroblast Growth Factor Binding and Mitogenesis in Vascular Smooth Muscle Cells

Matthew A. Nugent, Morris J. Karnovsky, Elazer R. Edelman

Basic fibroblast growth factor (bFGF) has been previously shown to be mitogenic for vascular smooth muscle cells (VSMCs) in vivo, but only after vascular injury. We show in the present study that the regulation of bFGF-stimulated VSMC proliferation, by vascular cell–secreted heparin-like compounds, correlates with inhibition of bFGF binding to cell-associated heparan sulfate proteoglycans. The stimulation of cultured VSMC proliferation by bFGF was markedly reduced when these cells were cocultured with confluent endothelial cells or confluent VSMCs (100.8±8.4% and 55.6±2.3% inhibition, respectively) or with conditioned media from these two cell types. Balb/c3T3 fibroblasts had no statistically significant effect on bFGF-stimulated VSMC proliferation. Vascular cell–conditioned media also inhibited bFGF binding to heparan sulfate proteoglycans on VSMCs, and the inhibition of binding correlated linearly with the inhibition of proliferation after a critical amount of binding was inhibited (44%) (r=.952, P<.0001). Heparinase or heparitinase treatment of conditioned media removed the bFGF-inhibitory effects, presumably by degrading heparin-like compounds. Indeed, heparin itself mimicked the inhibitory effects of conditioned media on bFGF-mediated proliferation and binding to heparan sulfate proteoglycans. These results suggest a bFGF regulatory role for vascular cell–produced heparin-like compounds, linking the mitogenic effects with binding to heparan sulfate proteoglycans for this heparin-binding growth factor. (Circ Res. 1993;73:1051-1060.)

KEY WORDS • basic fibroblast growth factor • heparan sulfate • mitogenesis • smooth muscle cells • heparin

Received July 6, 1993; accepted August 11, 1993.

From the Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, Mass (M.A.N., E.R.E.); the Department of Medicine, Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, Mass (E.R.E.); and the Department of Pathology, Harvard Medical School, Boston, Mass (M.J.K.).

This manuscript was sent to Leslie A. Leinwand, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Correspondence to Dr Matthew A. Nugent, Boston University School of Medicine, L912, 80 East Concord Street, Boston, MA 02118.
activation. In addition to mediating receptor binding, bFGF binding to HSPG might directly participate in bFGF signal transduction. Two recent reports have provided evidence for a separate heparan sulfate–mediated pathway for bFGF internalization.27,28

In the present study, we investigated the control of bFGF activity by vascular cells and have identified a potential mechanism through which soluble heparin-like compounds compete with cell-associated heparan sulfate sites to inhibit bFGF binding and activity. This is consistent with the model of vascular growth regulation and repair that is controlled by heparan sulfate and related molecules.10-12,29-31 Indeed, endothelial and smooth muscle cells have previously been demonstrated to produce heparin-like compounds that inhibit the growth of smooth muscle cells in cell culture.11,30,31 and exogenous heparin preparations inhibit neutointimal hyperplasia after vascular injury alone20 and in the presence of bFGF.10 We now report in the present study that the stimulation of cultured bovine aortic VSMC proliferation by bFGF was markedly reduced when these cells were cultured with confluent endothelial cells or confluent VSMCs or with conditioned media from these two cell types. Vascular cell–conditioned media also inhibited bFGF binding to HSPGs on VSMCs, and the inhibition of binding correlated linearly with the inhibition of proliferation after a threshold of binding inhibition was achieved. Heparinase or heparitinase treatment removed this inhibition, and heparin restored it, suggesting a regulatory role for vascular cell–produced heparin-like compounds, linking the mitogenic effects with HSPG binding for this heparin-binding growth factor.

Materials and Methods

**Cell Culture**

Endothelial cells and VSMCs, isolated as described,32,33 were used as a model for bFGF interaction with the cells in the arterial wall. Endothelial cells were used between passages 10 and 18, and VSMCs were used between passages 3 and 7. Balb/c3T3 fibroblasts (American Type Culture Collection, Rockville, Md) were used as a nonvascular-derived control cell type, since this stable well-characterized cell line is responsive to bFGF and produces little of its own bFGF.24,35 Fresh vials of Balb/c3T3 fibroblasts were thawed every 2 months. Chinese hamster ovary (CHO) cells and CHO-745 cells were obtained from Dr Jeffrey Esko, University of Alabama, Birmingham. All cells were maintained in 100-mm culture dishes (Corning, Corning, NY) in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY), supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% calf serum (Hyclone, Logan, Utah). For coculture experiments, cell culture inserts (0.45-μm pore size, CycloRose, Falcon, Lincoln Park, NJ) containing a confluent monolayer of one of these cell types were placed on top of sparse VSMCs cultured in six-well plates (Falcon), and VSMC cell number was measured over time with a Coulter counter. bFGF was continuously administered by controlled release from an alginate microsphere system.36 This stabilized and controlled the bFGF release, avoiding the rapid inactivation of bFGF under standard cell culture conditions.21,36-38 The VSMC proliferation assays with cell-conditioned medium were carried out over 7 days with alginate microspheres as described in the legends to the figures. In all experiments, cell number was determined by counting trypsin-suspended cells with a Coulter counter.

**Materials**

Pure human recombinant bFGF was from Chiron Inc, Emeryville, Calif. bFGF was determined to be fully active at stimulating DNA synthesis in quiescent Balb/c3T3 cells with half-maximal activity observed at 0.2 to 0.5 ng/mL and activity peaking at 1 to 5 ng/mL.12 I-bFGF was prepared by a modification of the Bolton-Hunter procedure.26,39 This technique has been demonstrated to produce active 125I-bFGF as determined by its ability to bind heparin-Sepharose and to stimulate DNA synthesis in Balb/c3T3 and bovine capillary endothelial cells.26,35,39 The specific activity of the 125I-bFGF was assessed by stimulation of quiescent Balb/c3T3 cells and was 25 to 100 μCi/μg.26 Chondroitinase ABC, gelatin, HEPES, and trypsin and chymotrypsin linked to agarose beads were from Sigma Chemical Co, St Louis, Mo. Heparin (porcine intestinal mucosa) was from Hepar Inc, Franklin, Ohio. Heparinase and heparitinase, purified from Flavobacterium heparinum, were provided by Drs Robert Langer, Arthur Lander, and Ramnath Sasisekharan, Massachusetts Institute of Technology, Cambridge.

Calcium cross-linked alginate microcapsules containing heparin-Sepharose beads were prepared, and bFGF was incorporated as described.10 Microcapsules were incubated in 150 mmol/L NaCl, 1 mmol/L CaCl2, and 0.05% gelatin (15 μL per microcapsule) containing bFGF (100 ng per microcapsule) overnight at 4°C with gentle shaking. Under these conditions, 71 ±8% of the bFGF (71 ng per microcapsule) became incorporated within the alginate capsules. To determine the release kinetics, microcapsules containing 125I-bFGF were placed in cell cultures and retrieved, and the radioactivity retained in the microcapsule was detected over time. Near-linear release rates were observed over the course of the experiments, with an average rate from each microcapsule of 0.042 ng/h.

125I-bFGF Binding

bFGF binding to HSPGs and receptors on VSMCs was carried out as described previously for endothelial and Balb/c3T3 cells.20,26,35 VSMCs (5 × 104 per well) were placed in 24-well culture plates in DMEM supplemented with 10% calf serum. When the cells were confluent (4 days), the medium was removed, the monolayers were washed once at 4°C with binding buffer (DMEM, 25 mmol/L HEPES [pH 7.2], and 0.05% gelatin, at 1.0 mL per well), and then 0.25 mL cold binding buffer plus a combination of DMEM containing 2% calf serum and conditioned medium to equal 0.25 mL was added (0.25 mL conditioned medium with no added DMEM containing 2% calf serum is equivalent to 0.5 fraction concentration of conditioned medium). The cells were incubated for 10 minutes at 4°C, and then 0.5 ng per well 125I-bFGF was added, and the incubation continued until equilibrium (2 hours) at 4°C. Nonbound bFGF was removed by washing the monolayers three times with cold binding buffer (1.0 mL per
well per wash). Then the amount of $^{125}$I-bFGF bound to HSPG and receptors was determined sequentially in each culture using a modification of the salt/acid washing technique.19,26 The $^{125}$I-bFGF bound to HSPG was released by exposure to high salt buffer (2 mol/L NaCl and 20 mmol/L HEPES [pH 7.4], at 0.5 mL per well for 5 seconds), and then the receptor-bound $^{125}$I-bFGF was extracted by 5-minute incubation of the monolayers in low pH buffer (2 mol/L NaCl and 20 mmol/L sodium acetate [pH 4.0], at 0.5 mL per well) followed by a wash with the same buffer (0.5 mL per well). $^{125}$I-bFGF was determined in all samples by counting in a 1272 Clinigamma gamma counter (LKB Nuclear, Gaithersburg, Md). The $^{125}$I-bFGF bound that was not competed by an excess (5 μg, 555 nmol/L) of unlabeled bFGF was defined as nonspecific and was subtracted from the experimental points. The salt and acid washing procedure did not alter VSMC attachment.

Conditioned Medium

Conditioned medium was isolated from confluent endothelial cells (3.7×10⁶ cells/mL), VSMCs (5.5×10⁶ cells/mL), and Balb/c3T3 cells (2.4×10⁵ cells/mL) by incubating monolayers in 100-mm culture dishes (Corning) in DMEM containing 2% calf serum (10 mL per dish) for 4 days. Medium was collected and centrifuged (30 minutes at 10 000g) to remove floating cells and debris. Conditioning was carried out in medium containing 2% calf serum because cell viability was significantly reduced at lower calf serum concentrations. To characterize the biochemical composition of the conditioned medium from endothelial cells, the medium was subjected to various enzyme and environmental treatments. Trypsin and chymotrypsin treatment was carried out using enzymes immobilized to agarose beads. Immobilized enzymes were removed by centrifugation at 10 000g. After treatment with heparinase, heparitinase, and chondroitinase ABC, the enzymes were inactivated by heat treatment (65°C for 15 minutes) before addition to the cell cultures. Similar results were observed when the enzymes were inactivated by treatment with trypsin immobilized to agarose (10 U/mL for 1 hour at 37°C). As controls for each treatment, DMEM containing 2% calf serum was treated identically and added to VSMC cultures at the same concentration as the conditioned medium.

Results

Vascular Cells Inhibit bFGF-Stimulated Proliferation of VSMCs

The proliferation of cultured vascular cells in the presence of bFGF was investigated to determine if endothelial cells directly control VSMC responsiveness to bFGF. VSMC growth was stimulated dramatically by the controlled release of bFGF, at 1 ng/d (Fig 1A). The cell number with bFGF was 4.2- and 6.4-fold greater than control after 7 and 9 days, respectively, and the cell doubling time decreased by 2.4-fold. In contrast, when confluent endothelial cells were cultured above VSMCs, bFGF did not stimulate VSMC proliferation (Fig 1B). Inhibition of bFGF-stimulated mitogenesis in VSMCs by endothelial cells was identical whether bFGF was delivered from the top chamber above the endothelial cells or in the bottom chamber beneath the endothelial cells (97.7±5.4% and 95.3±1.6% inhibition of bFGF activity in the presence of endothelial cells after 7 days when delivered from the top and bottom, respectively; P=NS). Even though cultured endothelial cells do not form as a tight barrier as an intact vascular endothelium, these results suggest that the ability of endothelial cells to control VSMC proliferation may extend beyond a mechanical barrier to bFGF delivery in endothelial cell/VSMC cocultures.

Vascular Cell–Derived Heparin-Like Compounds Inhibit bFGF-Stimulated Proliferation of VSMCs

To determine the cell specificity of the coculture effect, bFGF-stimulated VSMC proliferation in the presence of confluent endothelial cells, VSMCs, and Balb/c3T3 fibroblasts was compared (Fig 2). Endothelial cells completely inhibited bFGF mitogenesis, and confluent VSMCs had a significant partial effect (55.6±2.3% inhibition); however, Balb/c3T3 fibroblasts had no statistically significant effect. None of these cell types inhibited cell proliferation in the absence of bFGF. Thus, the extent of the effect observed was not a general phenomenon of the coculture system but, instead, appears to reflect specific responses to the three cell types. The additional possibility that bFGF was catabolized or depleted by the presence of additional cells in the coculture system was further eliminated by the observation that conditioned media from these three cell types mimicked the effects of the cocultures at inhibiting bFGF mitogenesis (Fig 3). The same cell specificity was observed with conditioned media. Endo-
thelial cell–conditioned medium had the most dramatic effect on bFGF-stimulated VSMC proliferation over the concentration range tested. Conditioned media from VSMCs and Balb/c3T3 fibroblasts partially inhibited bFGF-induced mitogenesis. As was observed with the cocultures, conditioned medium from VSMCs was less effective than that from endothelial cells and more effective than that from Balb/c3T3 fibroblasts. When endothelial cell–conditioned medium was added to a final concentration of 10%, bFGF-induced mitogenesis was reduced by 84%, a greater inhibition than when VSMC-conditioned medium was added at 50% concentration (66% inhibition of bFGF activity) (Fig. 3). The effects of conditioned media on bFGF-stimulated mitogenesis were reversible. When VSMCs were pretreated with endothelial cell–conditioned medium at 50% concentration for 7 days, then washed, trypsinized, and replated, bFGFs stimulated cell proliferation to an extent similar (2.8-fold after 7 days) to that found in control cells pretreated with DMEM containing 2% calf serum. This implies that the effects of the conditioned media are not the result of a permanent change in the VSMCs but instead might involve an interaction of soluble factors with bFGF or VSMCs that depends on the continuous presence of these factors.

Direct evidence that the bFGF inhibitory activity is related to heparin-like compounds derives from the results in which conditioned medium was treated with glycosaminoglycan-specific degradative enzymes (Fig. 4). When endothelial cell–conditioned medium was treated with purified heparinase and heparitinase,40 enzymes that degrade heparin and heparin-like compounds, the inhibitory activity was significantly reduced. Treatment with a crude preparation of the chondroitin sulfate– and dermatan sulfate–specific enzyme chondroitinase ABC reduced the bFGF inhibitory activity of the conditioned medium by 25%. The crude chondroitinase ABC contains small amounts of contaminating lyase activity (Sigma). Therefore, the partial effect of chondroitinase ABC might result from contaminating heparinase-like activity. Alternatively, a contributing effect of chondroitin/dermatan sulfate–like compounds might also exist. Exposure of endothelial cell–conditioned medium to elevated temperatures or protease, conditions that should inactivate most proteins, also had little to no effect on the bFGF inhibitory activity (<10% loss in activity). Taken together, these results strongly
Fig. 4. Bar graph showing that inhibition of basic fibroblast growth factor (bFGF) mitogenesis by conditioned media depends on heparin-like compounds. Conditioned medium from endothelial cells was subjected to the indicated treatments and then added (0.5 mL of 1.0 mL total) to growing vascular smooth muscle cells in the absence and presence of controlled continuous bFGF treatment. The treatments were as follows: 65°C for 15 minutes; trypsin, 10 U/mL for 1 hour at 37°C; chymotrypsin (CHYMO), 10 U/mL for 1 hour at 37°C; chondroitinase ABC (CTase), 10 μg/mL for 30 minutes at 37°C; heparinase (HPase), 10 μg/mL for 30 minutes at 37°C; and heparitinase (HTase), 1 μg/mL for 30 minutes at 37°C. Cell number was determined after 7 days, and bFGF stimulation (the ratio of the cell number with bFGF to that without bFGF) with conditioned medium was compared with the control preparation with DMEM containing 2% calf serum subjected to the above treatments. The data are presented as percent inhibition of bFGF mitogenesis by the conditioned medium, which was equal to the stimulation by bFGF in control cultures minus that in the presence of conditioned medium divided by control stimulation minus one, times 100. Thus, complete inhibition of bFGF activity (100%) occurs when there is no difference between the cell numbers (±bFGF) in the presence of conditioned medium. The data are a composite from several experiments. The percent inhibition of bFGF with untreated conditioned medium represents the average from six separate experiments.

Table 1. Removal of Heparan Sulfate Proteoglycans From Vascular Smooth Muscle Cells by Heparinase Treatment Reduces Basic Fibroblast Growth Factor Binding to its Receptors

<table>
<thead>
<tr>
<th>Heparinase Treated</th>
<th>125I-bFGF Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>HSPG</td>
<td>3117±160</td>
</tr>
<tr>
<td>Receptor</td>
<td>3084±133</td>
</tr>
</tbody>
</table>

bFGF indicates basic fibroblast growth factor; HSPG, heparan sulfate proteoglycan. Values are mean ± SEM of triplicate determinations.

125I-bFGF binding to HSPGs and receptors on untreated (control) and heparinase-treated (10 μg/mL for 30 minutes at 37°C) vascular smooth muscle cells was carried out at 4°C for 2 hours in 0.5 mL binding buffer as described in “Materials and Methods.” Similar results were observed in an additional identical experiment (HSPG binding was reduced to 7.1 ± 2.0% and receptor binding was reduced to 15.1 ± 0.1% of the untreated control value). Heparinase treatment had no effect on cell viability or cell attachment.

The inhibitory activity of conditioned media may involve regulation of bFGF at several levels: binding, signal transduction, transcriptional activation, etc. We investigated the effects of conditioned media on bFGF binding to VSMCs. As noted in Fig 5A, bFGF binding to cell-associated HSPGs in VSMC cultures was significantly reduced when endothelial cell–conditioned medium, at concentrations that were effective at inhibiting mitogenic activity, was included in the binding medium. Conditioned media from VSMCs or Balb/c3T3 cells were much less effective than endothelial cell–conditioned medium at reducing bFGF binding to HSPGs (Fig 5B and 5C). The dose required to inhibit bFGF binding to HSPGs by 50% was 0.03 fractional concentration for endothelial cell–conditioned medium compared with 0.3 for VSMC-conditioned medium and a predicted value of 0.6 for Balb/c3T3 cells. When these values were normalized to cell number, it was calculated that endothelial cells secrete 15 times more HSPG competing activity per cell than do VSMCs and Balb/c3T3 cells. Once again, the relative effects of the three conditioned media preparations on bFGF binding were similar to those observed on bFGF–induced mitogenesis with cocultures and conditioned media (Table 2). Consistent with a role for HSPG binding in controlling bFGF receptor binding, bFGF receptor binding was also inhibited by conditioned media. When the binding of bFGF to its receptor on VSMCs was measured, endothelial cell–conditioned medium caused a significant decrease in bFGF binding to its receptor (Table 1). We recently investigated this cooperative binding interaction in Balb/c3T3 cells and determined that it results from an increase in the stability of bFGF-receptor complexes when cell-associated HSPGs are present and not from heparan sulfate facilitating the association of bFGF with its receptor. This mechanism suggests that a soluble heparin-like compound(s) might act in a competitive manner to inhibit, rather than enhance, the binding of bFGF to cell-associated HSPG and thus prevent the formation of stable bFGF-receptor complexes.

bFGF Binding to VSMCs Is Inhibited by Conditioned Media

Cell-associated heparan sulfate has been shown to be required for bFGF receptor binding and activity in several cell types.12,13,24-26 and might be directly involved in bFGF signal transduction.27,28 The binding of bFGF to its receptors on VSMCs was also found to depend on heparan sulfate, because heparinase digestion of VSMCs resulted in a significant decrease in bFGF binding to its receptor (Table 1). We recently investigated this cooperative binding interaction in Balb/c3T3 cells and determined that it results from an increase in the stability of bFGF-receptor complexes when cell-associated HSPGs are present and not from heparan sulfate facilitating the association of bFGF with its receptor. This mechanism suggests that a soluble heparin-like compound(s) might act in a competitive manner to inhibit, rather than enhance, the binding of bFGF to cell-associated HSPG and thus prevent the formation of stable bFGF-receptor complexes.
significant decrease. Endothelial cell-conditioned medium inhibited bFGF receptor binding in a dose-dependent manner, saturating at 55% inhibition at the highest concentration tested. However, this same concentration range of VSMC- and Balb/c3T3–conditioned medium resulted in no statistically significant effects on bFGF binding to its receptors.

The inhibition of bFGF binding by the conditioned media, like the inhibition of mitogenesis, also appears to be caused by heparin-like compounds in the conditioned media. When endothelial cell-conditioned medium was treated with heparinase or heparitinase, the bFGF-binding inhibitory activity was lost (bFGF binding in the presence of heparinase- or heparitinase-treated conditioned medium was 95±3% and 91±2% of control, respectively, at 0.5 fractional concentration). The ability of endothelial cell-conditioned medium to inhibit bFGF receptor binding was not affected by treatment with trypsin, chondroitinase ABC, or exposure to heat (65°C).

To further demonstrate that the inhibitory effects of the conditioned media preparations on bFGF binding result from heparin-like compounds in the media, conditioned media from CHO cells and mutant CHO cells (CHO-745) deficient in heparan sulfate synthesis were assayed for bFGF-binding inhibitory activity (Fig 6). Wild-type CHO cells inhibited bFGF binding to HSPG sites on VSMCs to approximately the same extent as did Balb/c3T3 fibroblasts. However, the mutant CHO-745 cells had no effect on bFGF binding to VSMCs, suggesting that inhibition of bFGF binding by conditioned media is a reflection of the ability of the cells to produce heparin-like compounds.

The inhibition of bFGF-mediated VSMC proliferation by the conditioned media preparations correlated directly with the inhibition of bFGF binding to HSPG once a critical amount of binding was inhibited (44%) (r=.952, P<.0001) (Fig 7). When bFGF binding to HSPG was inhibited by less than this threshold value, there was no effect on proliferation (r=.083), and once binding was inhibited by more than 80%, bFGF mitogenesis was inhibited completely. Thus, once at the critical level of bFGF-HSPG binding, there is a one to one relation between bFGF binding to HSPG and bFGF stimulation of mitogenesis in VSMCs. Unlike binding to HSPG, there was no correlation between inhibition of bFGF-induced mitogenesis and inhibition of bFGF binding to its specific tyrosine kinase cell surface receptors.
Heparin Inhibits bFGF Binding to VSMCs

Since the effect of endothelial cell−conditioned medium on bFGF binding seems to be mediated by heparin-like molecules, we investigated the direct effects of heparin on bFGF binding to VSMCs. Heparin inhibited bFGF binding to HSPG and receptor sites on VSMCs with an ID₅₀ of 0.5 μg/mL and 7 μg/mL, respectively (Fig 8). The inhibitory effect of heparin was saturated at 20 to 100 μg/mL. In comparison, half-maximal inhibition of bFGF binding to HSPG on VSMCs was observed with 15 μL of endothelial cell−conditioned medium (0.03 fraction of total), which represents the medium from only approximately 5000 to 10 000 cells. Consistent with these effects of isolated heparin on bFGF binding, heparin inhibited bFGF-stimulated VSMC proliferation (ID₅₀, 1 to 10 μg/mL). Thus, the actions of the endothelial cell−secreted heparin-like compound(s) are, to a certain extent, reflective of those of heparin. However, direct measurement of the concentration of heparin-like material in the crude endothelial cell−conditioned medium (conditioned for 1 day in the absence of serum), using the dimethylmethylen blue dye binding assay, revealed that it was 0.5 to 1.0 μg/mL. Therefore, the concentration of endothelium-produced heparin-like material required to inhibit bFGF binding to HSPG by 50% (0.03 fraction concentration) was equivalent to 0.015 to 0.030 μg/mL heparan sulfate. Thus, the heparin-like compounds secreted by endothelial cells appear to be more active at modulating bFGF than are standard preparations of heparin (on the order of 15− to 30-fold more active on a mass to mass comparison).

Discussion

It appears that the anatomic continuity of the endothelial monolayer and the underlying VSMCs provides biochemical control of vascular physiology as well as structural integrity. We now demonstrate that one manner in which smooth muscle cell proliferation is regulated involves a factor(s) that inhibits the binding and activity of growth factors. We found that the differential ability of vascular cells and their conditioned media to inhibit the mitogenic effects of bFGF on VSMCs was directly related to their ability to inhibit the binding of bFGF to HSPGs on VSMCs. Thus, endothelial cells and endothelial cell−conditioned medium completely inhibit bFGF-stimulated VSMC proliferation, whereas VSMCs had a partial effect. These results suggest that the mitogenic activity of bFGF within the blood vessel wall might be tightly controlled by the local concentration of heparin-like compounds in the extracellular space. It is known that heparin and endothelium-derived heparan sulfate are potent antiproliferative agents for VSMCs in vitro and in vivo. Our results suggest that heparin-like compounds might inhibit proliferation, in part, by interacting with heparin-binding growth factors in the extracellular environment, preventing binding to cell-surface HSPG. It is interesting to note that similar heparin and heparan sulfate structural requirements, polymer chain length and degree of sulfation, have been observed for VSMC growth inhibition and bFGF binding.
The correlation between inhibition of bFGF binding to HSPGs and bFGF mitogenesis and the lack of a direct correlation between mitogenesis and receptor binding suggest that the bFGF-HSPG interaction might be directly involved in bFGF signal transduction. A direct role for HSPGs in bFGF action has been suggested by two recent studies reporting the existence of HSPG-mediated receptor-independent bFGF internalization pathways. The results indicate that interfering with the bFGF-HSPG binding interaction alone, with soluble heparin-like compounds, might be sufficient to inhibit the mitogenic activity of bFGF on VSMCs. These results reinforce those previously reported in which the activity of bFGF was inhibited if cell-associated HSPG binding sites were degraded or metabolically undersulfated. Consistent with this are our previous results, in which we found that the bFGF-induced stimulation of intimal hyperplasia in injured rat arteries was inhibited by the perivascular administration of heparin. Comparison of the effects of heparin and conditioned media on bFGF binding to VSMCs (Figs 5 and 6) shows similar effects on HSPG binding. However, the endothelial cell–derived heparin-like compound(s) appears, on a relative scale, to be more potent than heparin. This difference might reflect specificity in the binding of bFGF to intact proteoglycans as compared with heparin. The extent to which the effects of the conditioned media on bFGF action represent those of heparin and related compounds in general is not known at this time.

The inhibitory effects of conditioned media heparin-like compounds are also consistent with a mechanism in which the sole function of cell-associated HSPG is to facilitate the formation of “functional” bFGF receptor complexes. Receptor binding was also significantly reduced when HSPG binding was inhibited (Figs 5 and 8). Although a considerable amount of receptor binding exists under conditions in which bFGF mitogenesis is completely inhibited, the bFGF receptor binding might not represent functional high-affinity complexes. We have previously shown that bFGF is able to associate with its receptors in the absence of HSPG; however, the complexes that are generated dissociate at a 15- to 20-fold faster rate than do those on cells containing HSPG.

Heparin-like compounds produced by endothelial cells inhibit VSMC growth, and VSMCs themselves have also been reported to produce heparin-like growth inhibitory compounds, suggesting that an autocrine mechanism for growth control might also be important. The results in Figs 2, 3, 5, and 6 suggest that VSMCs, in addition to endothelial cells, also produce bFGF inhibitory compounds. Although there is a clear distinction in the relative production by endothelial cells and VSMCs, on a per cell basis, there are far more smooth muscle cells than endothelial cells within the wall of large blood vessels in vivo. Therefore, smooth muscle cell–derived heparan sulfate might be an important, or even the predominant, heparin-like compound in certain in vivo settings. Our results demonstrate that the response of VSMCs to bFGF can be controlled by heparin-like compounds produced by vascular cells and suggest that the smooth muscle cell proliferative state in vivo might similarly be regulated by the production of heparin-like compounds within the blood vessel wall. Several smooth muscle cell mitogens in addition to bFGF, including platelet-derived growth factor and heparin-binding epidermal growth factor–like growth factor, bind avidly to heparin and heparan sulfate.

It is possible that the cellular response to these compounds, as well as bFGF, is controlled by their interaction with heparin-like compounds. Thus, heparin-like compounds produced by vascular cells might act as general regulators of the bioavailability of a wide spectrum of heparin-binding growth factors within the blood vessel wall. Damage to the endothelial and media cells might be expected to lead to a loss of these compounds, creating a situation in which growth factor–stimulated mitogenesis is permitted. This type of mechanism is consistent with previous findings that bFGF only stimulates intimal hyperplasia in the presence of vascular injury.

It is widely accepted that endogenous cell–associated heparin-like compounds facilitate bFGF binding and activity; however, apparently conflicting results have been reported for the effects of exogenous preparations of soluble heparin-like compounds. Our results and those of others suggest that soluble heparin-like compounds might act with cell-associated bFGF binding sites to inhibit bFGF action. However, studies have also found that the binding of bFGF to its receptors in heparan sulfate–deficient cells is recovered by the addition of soluble heparin. Thus, the effects of soluble heparin on bFGF binding might depend on the level of endogenous heparan sulfate relative to bFGF receptors within the cells and might be complicated by the binding of soluble heparin to bFGF receptors and to cells. The ability of exogenous heparin to regulate bFGF receptor binding appears to be concentration dependent. At low concentrations (5 to 100 ng/mL), heparin facilitates bFGF receptor binding to heparanase-treated Balb/c3T3 cells, but not to untreated cells. When the concentration of heparin is raised, bFGF receptor binding on both
untreated and heparinase-treated cells is inhibited (authors' unpublished data). Thus, it is possible that, when the cellular heparan sulfate levels are below those required for bFGF binding and activity, a considerable amount of the soluble heparin binds to the cell surface and inserts to facilitate bFGF binding. At higher concentrations, increasing amounts of heparin remain soluble and might then inhibit bFGF binding.

Thus, previous results combined with those presented here suggest that bFGF activity might be mediated by the balance of cell-associated and soluble heparin-like compounds. Conditions that result in a shift toward soluble heparin-like compounds might lead to inhibition of bFGF activity by effectively sequestering bFGF away from the cell surface, whereas a shift toward cell-associated HSPG might result in amplification of the bFGF response. Indeed, agents such as transforming growth factor β, which upregulate soluble and cell-associated bFGF-binding HSPGs, have been reported to both potentiate and inhibit the mitogenic activity of bFGF, depending on the conditions. The particular array of compounds present in the local extracellular environment likely dictates the cellular response to growth factors, in part, by controlling their bioavailability.

Acknowledgments

This study was supported by National Institutes of Health Grant HL-17747, Postdoctoral Fellowship F32 GM14003 (Dr Nugent), and Physician-Scientist Award K12 AG00294 (Dr Edelman). We thank Anna Browne and Oliver Chen for technical assistance. We are grateful to Dr Patricia D'Amore for providing bovine aortic endothelial and VSMCs. We thank Drs Patricia D'Amore, Michael Klagesmarn, Laurie Pukac, and James San Antonio for their critical review of the manuscript.

References


Vascular cell-derived heparan sulfate shows coupled inhibition of basic fibroblast growth factor binding and mitogenesis in vascular smooth muscle cells.
M A Nugent, M J Karnovsky and E R Edelman

_Circ Res._ 1993;73:1051-1060
doi: 10.1161/01.RES.73.6.1051

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/73/6/1051