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Heparinase inhibits neovascularization

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ABSTRACT **Neovascularization is associated with the regulation of tissue development, wound healing, and tumor metastasis. A number of studies have focused on the role of heparin-like molecules in neovascularization; however, little is known about the role of heparin-degrading enzymes in neovascularization. We report here that the heparin-degrading enzymes, heparinases I and III, but not heparinase II, inhibited both neovascularization *in vivo* and proliferation of capillary endothelial cells mediated by basic fibroblast growth factor *in vitro*. We suggest that the role of heparinases in inhibition of neovascularization is through depletion of heparan sulfate receptors that are critical for growth factor-mediated endothelial cell proliferation and hence neovascularization. The differences in the effects of the three heparinases on neovascularization could be due to different substrate specificities for the enzymes, influencing the availability of specific heparin fragments that modulate heparin-binding cytokines involved in angiogenesis.**

Neovascularization, the process of blood vessel formation, is a highly regulated phenomenon characteristic of a number of important physiological events, both normal and pathological (1–3). Heparin-like molecules (such as heparin and heparan sulfate), found on virtually all cell surfaces, determine extracellular matrix physiological properties (4). These molecules bind several cytokines, which are angiogenic, and modulate their function either by stabilizing them or by controlling their bioavailability (5). They also act as low-affinity receptors on cell surfaces and facilitate growth factor activity and receptor binding (6). These observations suggest that enzymes that degrade heparin-like molecules might have a role in modulating neovascularization. Far less is known about the direct role of heparinase on the angiogenic process than is known about that of its substrate, heparin (7–9).

In this study, we investigate the direct effect of heparinases on the process of neovascularization. The source of heparin-degrading enzymes used in this study was *Flavobacterium heparinum*, which produces three heparinases—heparinase I (heparin lyase, EC 4.2.2.7), a 42.5-kDa enzyme that acts at the hexosamine-iduronic acid linkage predominantly found in heparin; heparinase II (no EC number), an 86-kDa enzyme that acts at the hexosamine-uronic acid linkage of heparin-like molecules, not discriminating between the two isoforms of the uronic acid; and heparinase III (heparan-sulfate lyase, EC 4.2.2.8), a 73-kDa enzyme that acts at the hexosamine-glucuronic acid linkage found extensively in heparan sulfate (10, 11).

MATERIALS AND METHODS

Cell Culture. Capillary endothelial cells isolated from bovine adrenal cortex were the generous gift of Katherine Butterfield and J. Folkman (Children's Hospital, Boston). These cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) with 10% calf serum (HyClone) [DMEM/10 supplemented with basic fibroblast growth factor (bFGF) (3 ng/ml)].

Heparinase Purification and Characterization. Heparinases were purified (11, 12) and extensively desalted using a Centricon P-30 (molecular size cutoff, 30 kDa) (Amicon) (Heparinases I, II, and III were collected in a microcentrifuge tube and lyophilized (VirTis Freeze mobil model 12, VirTis). Protein concentration was determined by use of the Micro BCA reagent (Pierce) relative to a bovine serum albumin standard.) The purity of the peak was determined by high-pressure liquid chromatography using a Vydac C_{18} reverse-phase column in a HP 1090 (Hewlett–Packard), with diode array detection, in a gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid for 60 min. Protein was monitored at 210 and 277 nm. Mass spectrometry was performed on the heparinase preparations; ≈2 μg of heparinase was mixed with 1 μl of sinapinic acid (10 mg/ml) (in 80% acetonitrile/0.1% trifluoroacetic acid in water) (1:1, vol/vol) and then analyzed by laser desorption mass spectrometry (Laser MAT, Finnigan, CA).

***In Vivo* Neovascularization Assay.** To determine whether heparinases inhibited neovascularization *in vivo*, the chorioallantoic membrane (CAM) assay was performed as described (13, 24). Briefly, on day 3 of the development, fertilized chicken embryos were removed from their shells and placed in plastic Petri dishes. On day 6, heparinase I (100 pmol) was mixed in methylcellulose disks and applied to the surfaces of the growing CAMs above the dense subectodermal plexus. After a 48-hr exposure of the CAMs to heparinase I, India ink/Liposyn was injected intravascularly as described. For histological analysis, tissue specimens were fixed in formalin, rinsed in 0.1 M cacodylate buffer (pH 7.4), and embedded in JB-4 plastic (Polyscience) at 4°C. Three-micrometer sections were cut with a Reichert 2050 microtome and stained with toluidine blue; micrographs were taken on a Zeiss photomicroscope with Kodak TM (×100) and a green filter.

Endothelial Cell Proliferation Assay. Bovine capillary endothelial cells (BCEs) [10^4 cells per 0.5 ml with 5% calf serum (DMEM/5)] were plated onto gelatin-coated 24-well tissue culture dishes and allowed to attach overnight. On day 2, unattached cells were removed and the attached cells were fed DMEM/5 again. Heparinases and bFGF (12 ng/ml; Takeda, Osaka) were added. Wells containing phosphate-buffered saline (PBS) alone and PBS and bFGF were included as controls. On day 5, medium was aspirated and cells

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Abbreviations: BCE, bovine capillary endothelial cell; CAM, chorioallantoic membrane; bFGF, basic fibroblast growth factor; HSPG, heparan sulfate proteoglycan.

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were washed with 0.5 ml of PBS, removed by treatment with trypsin, and counted (13).

FGF Binding to Endothelial Cells. Binding of ^{125}I-labeled bFGF (^{125}I-bFGF) to triplicate BCE cultures treated with heparinases was compared to control untreated cultures, and the average ± SE is presented. The control binding level (100%) was 1592 ± 69 cpm for heparan sulfate proteoglycan (HSPG) and 821 ± 27 cpm for receptors. Similar results were observed in five separate experiments. Confluent capillary endothelial cell monolayers were prepared by plating 1×10^5 cells per 4-cm^2 well (12-well plates; Costar) in DMEM/10% calf serum (1 ml per well) and incubating (at 37°C) for 3–5 days. To initiate enzyme treatment, the medium was removed, the cells were washed once with DMEM (1 ml per well), and heparinase was added at the indicated concentration in 0.25 ml of DMEM containing bovine serum albumin (5 mg/ml). Enzyme treatment was carried out at 37°C for 30 min. When treatment was complete, the enzyme medium was removed and the monolayers were washed twice (1 ml per well per wash) with cold (4°C) binding buffer (DMEM/25 mM Hepes/0.5% gelatin) and then incubated for 10 min at 4°C in 0.5 ml of binding buffer to precool the cells. ^{125}I-bFGF (14) [5 ng; 0.66 nM ($1.25–5 \times 10^5$ cpm)]** was then added. The plates were incubated at 4°C for 2 hr, at which point the binding buffer was removed and each well was washed three times with cold binding buffer (1 ml per well per wash). The amount of ^{125}I-bFGF bound to HSPG and receptor was determined sequentially in each culture by a modification of the salt/acid washing technique. ^{125}I-bFGF bound to HSPG was released by exposure to high salt buffer (2 M NaCl/20 mM Hepes, pH 7.4; 0.5 ml per well; 5 s), and then the ^{125}I-bFGF bound to receptors was extracted by incubation of the monolayers in low pH buffer (2 M NaCl/20 mM sodium acetate, pH 4.0; 0.5 ml per well; 5 min) 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 γ counter (LKB). The ^{125}I-bFGF bound that was not competed by an excess (5 μg; 555 nM) of unlabeled bFGF was defined as nonspecific and was subtracted from the experimental points. The number of cells attached to the culture plates before and after the salt and acid washes was similar. Heparinase treatment did not result in any change in cell viability, as determined by trypan blue exclusion.

RESULTS

Heparinase Purification and Homogeneity. Heparinases I, II, and III were purified to homogeneity by chromatographic techniques (10, 11), and the purity was confirmed by reverse-phase HPLC, mass spectrometry, and amino acid analysis (11, 12) (Fig. 1).

Heparinase Inhibition of Neovascularization *in Vivo*. To determine whether heparinases I, II, and III inhibited neovascularization *in vivo*, we investigated their effect on chicken CAM neovascularization. Approximately 4 μg of purified heparinases I and III (105 and 54 pmol, respectively) in methylcellulose disks caused 100% inhibition of embryonic neovascularization (n = 12 and 4, respectively), resulting in large avascular zones (Fig. 2*A*). In contrast, neither 4 μg of heparinase II (46 pmol; n = 4) in methylcellulose disks nor empty methylcellulose disks implanted on CAMs develop avascular zones (Fig. 2*B*). Histological analysis of the heparinase-treated CAMs revealed a mesoderm that was thinner than normal, containing dividing fibroblasts and nearly no capillary endothelial cells (Fig. 2 *C* and *D*).

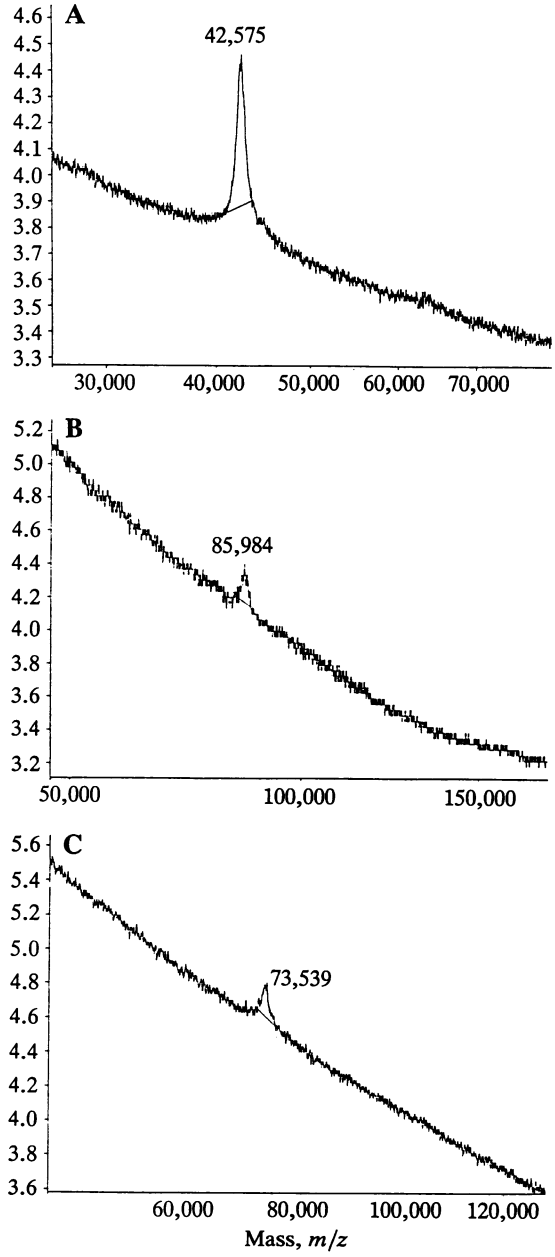

FIG. 1. Mass spectrometry profiles of heparinases I, II, and III. Molecular mass is represented in Da on the x axis. Laser desorption mass spectrometry was performed using Laser MAT (Finnigan-MAT, San Jose, CA) on heparinase I (*A*), heparinase II (*B*), and heparinase III (*C*).

Heparinase Inhibition of Endothelial Cell Proliferation. bFGF is a potent mitogen for BCEs and is considered an important mediator of neovascularization (16). We tested the direct effect of the three heparinases on BCE proliferation *in vitro* in the presence and absence of bFGF. Heparinases III and I significantly inhibited FGF-mediated BCE proliferation, with IC_{50} values of 21 and 6 nM, respectively (Fig. 3). Importantly, in the absence of exogenous bFGF, heparinase I did not inhibit BCE proliferation. Interestingly, at very low concentration ranges (0.1–1 nM), in the presence of bFGF, heparinase III potentiated BCE proliferation by ≈40%; on the other hand, at the same concentration range, heparinase I had no significant effect on BCE proliferation. Heparinase

**^{125}I-bFGF was prepared by a modification of the Bolton–Hunter procedure (15). This technique has been demonstrated to produce active ^{125}I-bFGF as determined by its ability to bind heparin Sepharose and to stimulate DNA synthesis in BALB/c 3T3 and BCEs. The specific activity of ^{125}I-bFGF was assessed by stimulation of quiescent BALB/c 3T3 cells and was 25–100 μCi/μg (1 Ci = 37 GBq).

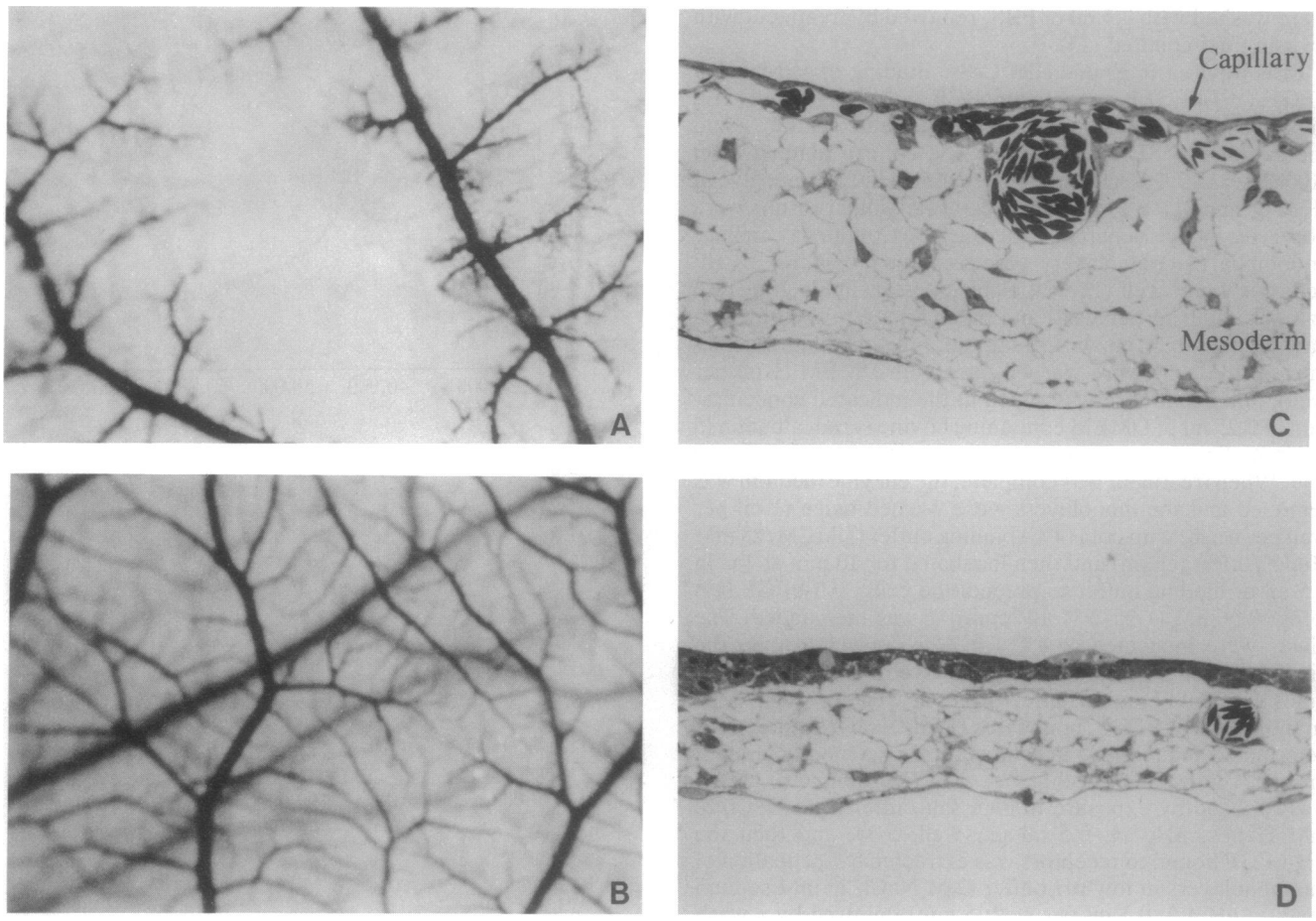

FIG. 2. Inhibition of neovascularization by heparinase I. (*A*) CAM with heparinase I (4 μg)-containing disk: 100% of the eggs tested (n = 12) on several different batches of heparinase I had avascular zones. (*B*) Normal CAM containing an empty methylcellulose disk. (*C*) Histological sections of day 8 normal CAMs. (×616.) (*D*) Histological sections of day 8 CAM treated with heparinase I. CAM treated with heparinase III (not shown) appears nearly identical to heparinase I, and CAM treated with heparinase II (also not shown) appears similar to normal CAM containing an empty methylcellulose disk.

II had little effect on BCE proliferation over the entire concentration range studied (Fig. 3).

FGF Binding to Endothelial Cells. The cell proliferation results suggested to us that the inhibitory effects of heparinases I and III on neovascularization *in vivo*, and BCE proliferation *in vitro*, may reflect their ability to interfere with the action of bFGF. It is known that heparan sulfate (as a low-affinity receptor) is essential for bFGF to bind its high-affinity receptor. The degradation of cell-surface heparan sulfate with heparinase I results in inhibition of bFGF binding to its receptor (6, 15–18). Furthermore, heparinase III treatment substantially reduced receptor binding, mitogenic activity, and blocked terminal differentiation of MM14 skeletal muscle cells (19). These results taken together suggested to us that heparinases could alter the action of bFGF at the level of receptor binding. Therefore, we investigated the effects of treatment with heparinases I, II, and III on ^{125}I-bFGF receptor binding.

BCEs were treated with the three heparinases for 30 min at 37°C, and then equilibrium binding of ^{125}I-bFGF to heparan sulfate and receptors on the cells was evaluated. In the binding assay, heparinase treatment of BCEs decreased ^{125}I-bFGF binding to these cells in a dose-dependent fashion (Fig. 4). There was a significant loss of bFGF binding to BCEs with increasing heparinase concentrations, and there were differences in the effects of the three heparinases. While heparinase I inhibited bFGF binding to heparan sulfate and receptor sites with IC_{50} values of 0.5 and 1.5 nM, respectively, the IC_{50} values for heparinase II were 2 and 8 nM, respectively, for the same sites. On the other hand, heparinase III was most potent in inhibiting bFGF binding to heparan sulfate and its receptors, having IC_{50} values of 0.15 and 0.2 nM, respectively.

Comparing the effects of the three heparinases at different concentration ranges, for heparinase III, over the concentration range 0.1–1 nM, ≈80% of bFGF binding to the heparan sulfate and ≈50% of binding to the receptor was eliminated. Heparinase I in the same concentration range reduced heparan sulfate and receptor binding by ≈60% and ≈20%, respectively. In contrast, for heparinase II, only ≈25% heparan sulfate binding sites and ≈10% of the receptor binding are affected. However, at a higher concentration range of 10–100 nM, almost all the heparan sulfate binding and ≈90%, ≈85%, and ≈80% of the receptor binding are eliminated for heparinases III, I, and II, respectively.

DISCUSSION

Inhibition of Cell Proliferation and FGF Binding to Cells. It has been reported that ≈70% of bFGF, added to BCEs, binds to the extracellular matrix (ECM) heparan sulfate and ≈7% binds to the cell-surface receptor (14). It is possible that lower concentrations of heparinase III are effective in releasing the bFGF–heparin complex bound to the ECM, which aids bFGF binding to its receptor. It was observed that exogenous heparin and heparin oligosaccharides (at least dodecasaccharides) potentiate BCEs in the presence of acidic FGF (20). This observation of the importance of fragment sizes in

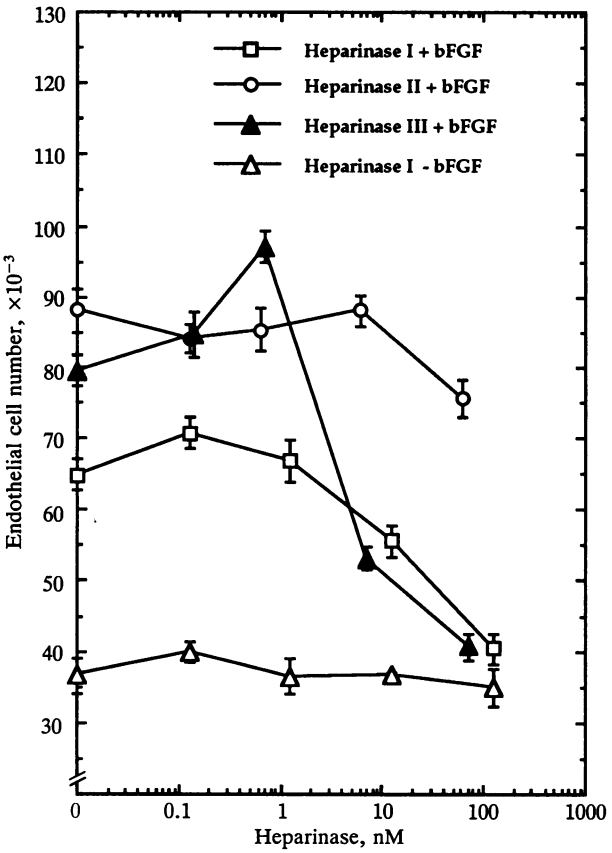

FIG. 3. Effect of heparinases I, II, and III on bFGF-stimulated (12 ng/ml) BCE proliferation. The *x* axis shows the endothelial cell count after bFGF treatment (except in the case of heparinase alone). The *y* axis shows the total concentration of enzymes added. Control wells contained cells in PBS alone. In the absence of bFGF, the data for heparinase I alone are shown.

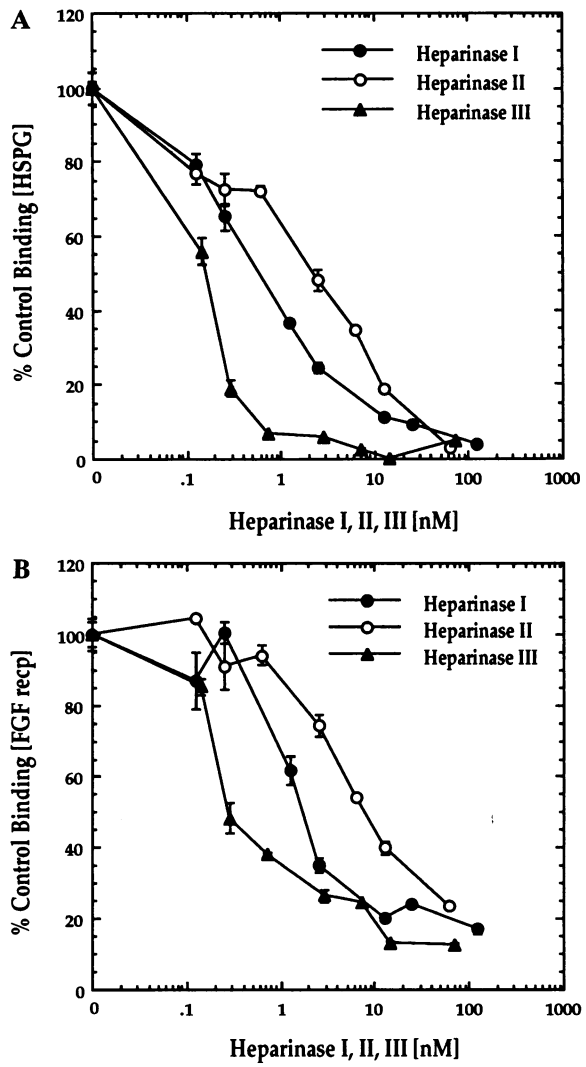

FIG. 4. Effect of treatment with heparinases I, II, and III on bFGF binding to BCE. ^{125}I-bFGF binding to HSPG (*A*) and cell-surface receptor (*B*) on confluent BCEs treated with the indicated concentrations of heparinases I, II, and III.

proliferation is consistent with the effects of heparinase III on BCE proliferation; heparinase III cleaves heparan sulfate into dodeca- and higher-order saccharides having intact bFGF binding sites, while heparinase I cleaves at the bFGF binding site and heparinase II leaves behind disaccharide fragments (21). Exogenous heparin also potentiates the early responses to bFGF but has little effect on the long-term biological effects of bFGF and BCE proliferation (22). The antiproliferative effect of heparinases I and III appears specific to BCE, in that these heparinases did not inhibit platelet-derived growth factor BB stimulated proliferation of bovine aortic smooth muscle cells or bFGF-stimulated NIH 3T3 cells, even when tested at twice the concentration of heparinase in the same assay (data not shown). Interestingly, heparinase II potentiated smooth muscle cell proliferation (data not shown).

In the context of BCE proliferation and the *in vitro* binding results it should be noted that, while all three heparinases show similarities in their dose-dependent inhibition of bFGF binding, there are notable differences in their effects on BCE proliferation. First, in the concentration range 0.1–1 nM, while potentiation of BCE proliferation occurs for heparinase III, the binding assays show inhibition of bFGF binding to BCEs. On the other hand, for heparinase I, while there is no significant effect on BCE proliferation, there is inhibition of bFGF binding to these cells. Surprisingly for heparinase II, there is no such potentiation of BCEs and also only marginal inhibition of bFGF binding to these cells. A possible explanation for the inverse correlation observed between the proliferation and binding assays for heparinases III and I at these concentrations could involve heparan sulfate fragments generated upon heparinase treatment of BCEs. In the binding experiments, after heparinase treatment, the cells were washed to remove degraded heparan sulfate before adding bFGF; while in the BCE proliferation assay, degraded heparan sulfate was not removed over the time period the assay was performed. It is possible that the oligosaccharides generated by enzymatic cleavage play a role in modulating bFGF activity in the BCE proliferation assay. Moreover, in the BCE proliferation assay heparinase III, and not heparinases I and II at low concentrations, potentially release exogenous bFGF that is sequestered by matrix heparan sulfate and aid in the bFGF-mediated BCE potentiation. Second, in the concentration range 10–100 nM, heparinases III and I dramatically inhibit BCE proliferation and also show nearly complete inhibition of bFGF binding to BCEs. However, heparinase II in the same concentration range had no significant effect on BCEs even though bFGF binding results were comparable to those for heparinases III and I. At higher concentrations, heparinases probably are in saturating amounts, which are sufficient to eliminate low-affinity sites. Under these conditions, bFGF binding to the high-affinity sites is impaired by all three enzyme treatments.

Inhibition of Neovascularization *in Vivo*. This work demonstrates direct inhibition of *in vivo* neovascularization by heparinases. Heparinases III and I appear to be among the potent antiangiogenic factors reported to date (23, 24). The above results were initially surprising since an anticipated role of heparinases was to degrade basement membrane, causing bFGF release and hence neovascularization (5, 31). Furthermore, mammalian heparinases are implicated in tumor angiogenesis (25). We observe not only inhibition of neovascularization but also different specificities for the three heparinases. As a consequence of the specificity of heparinases for sites on heparin, they may control either the availability or the removal of unique heparin fragments essential for neovascularization. Heparinase III acts at the more "heparan sulfate-like regions" of the endothelial cell polysaccharide, leaving behind intact bFGF binding sites; heparinase I cleaves the "heparin-like regions" containing the bFGF binding sites; thus, both enzymes modulate, but differently, the availability of specific bFGF binding sites that are required for bFGF-mediated BCE proliferation (26–28). Heparinase II, however, acts nonspecifically to cleave the polysaccharide primarily into disaccharides (10). As a consequence, this enzyme probably does not alter enough bFGF binding sites to affect bFGF binding and activity under the conditions studied. This is consistent with the higher K_m value (an order of magnitude) for heparinase II (10, 12). The events described above are also consistent with the potent inhibitory effect of heparinases, which as enzymes are expected to act catalytically and not stoichiometrically as might other inhibitors of neovascularization.

Central to capillary endothelial cell proliferation is the trimolecular interaction of a growth factor (like FGF), a high-affinity receptor, and a low-affinity heparan sulfate receptor that initiate events leading to neovascularization (5, 6). We suggest that heparinases inhibit neovascularization through depletion of the low-affinity receptors that are critical for bFGF-mediated endothelial cell proliferation. Furthermore, in addition to bFGF, several endothelial cell cytokines have been found to require heparin-like molecules for activity (29, 30). Thus, the mechanism described here for specific heparinase-mediated inhibition of bFGF binding and activity on BCEs might be reflective of a general system for control of cytokines involved in capillary endothelial cell proliferation and neovascularization. A better understanding of these events may offer opportunities for therapeutic intervention in the treatment of pathologies ranging from abnormal wound healing to tumor angiogenesis.

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