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Novel Heparanase-Inhibiting Antibody Reduces Neointima Formation

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Basic fibroblast growth factor (bFGF), stored bound to heparan sulfate proteoglycans in the extracellular matrix (ECM) of the arterial media, may initiate smooth muscle cell (SMC) proliferation after coronary intervention, thus contributing to restenosis. bFGF mobilization from ECM stores after injury may be induced by platelet degranulation products such as heparanase. Therapies aimed at the inhibition of bFGF release and activation may assist in prevention of restenosis. To test this theory, we first examined the mobilization and activation of bFGF in the arterial media by platelet-derived heparanase. Heparanase, locally delivered to the rat carotid artery, was found to release bFGF and induce substantial SMC proliferation in the absence of actual vascular injury. An antibody that neutralizes heparanase was then developed and evaluated in a rat carotid balloon injury model. Local delivery of anti-heparanase IgG was found to inhibit bFGF release by approximately 60% ($p < 0.001$) at 4 d; this correlated with the significant reduction in neointima formation observed at 14 d (intimal area/medial area: control 1.3 ± 0.3, anti-heparanase 0.35 ± 0.12, $p < 0.0001$). Platelet-derived heparanase is therefore likely to be important in initiating events leading to restenosis *via* bFGF mobilization. Furthermore, heparanase neutralization may assist in the prevention of restenosis following vascular injury.

Key words: bFGF, heparanase, restenosis, smooth muscle cells, thrombosis.

Abbreviations: bFGF, basic fibroblast growth factor; ECM, extracellular matrix; PEG-DA, polyethylene glycol diacrylate; SMC, smooth muscle cell.

Restenosis persists as the primary limitation of procedures for coronary revascularization (*1*). Following vascular injury, SMCs undergo proliferation, migration and matrix deposition, contributing to the formation of an occlusive neointimal layer (*1*). Prevention of the initial proliferative response may attenuate the cascade of events leading to restenosis and minimize arterial re-occlusion. bFGF is an important SMC mitogen in the restenosis process and may play an integral role in the initiation of SMC proliferation following vascular injury (*2–6*). For example, exogenous bFGF has been shown to significantly increase SMC proliferation following balloon injury (*4–6*), and treatment with bFGF-neutralizing antibodies immediately following injury has been shown to decrease SMC proliferation by approximately 80% (*3*). However, treatment with bFGF-neutralizing antibodies six days post-injury did not inhibit intimal SMC proliferation (*3*), suggesting that bFGF plays an early role in the restenosis process. Additionally, bFGF protein is readily available, stored at high levels in the medial layer of the arterial wall. Moreover, bFGF content in the arterial wall has been shown to dramatically decrease within four days of injury due to release from extracellular matrix (ECM) stores and consequent, cellular uptake and proteolytic degradation (*7*, *8*). Furthermore, intraluminal blood barriers formed *in situ* immediately after balloon injury were found to completely prevent platelet adhesion and to inhibit bFGF mobilization, suggesting that a luminally-derived factor is required for bFGF release from the ECM and subsequent activation (*8*).

Heparan sulfate proteoglycans (HSPG) in the ECM bind bFGF in inactive form (*9*), protecting it from proteolytic degradation (*10*). ECM sequestration may provide a localized source of heparin-binding growth factors, such as bFGF, that can be released during developmental and wound healing processes. The release of bFGF from the ECM is thought to contribute to the proliferative response of SMCs following vascular injury (*9*). However, the mechanisms of bFGF release from HSPGs in the ECM in response to vascular injury are currently unclear. Heparanase is a heparan sulfate–degrading enzyme that is generated at sites of vascular injury during platelet degranulation (*11*) and that releases complexes of bFGF and HS fragments with increased mitogenic potential (*12–15*). Heparanase can also be derived from inflammatory cells, such as neutrophils (*16*), monocytes (*17*), and activated but not resting T lymphocytes (*18*). Recent studies reporting the crystal structure of the FGF-FGFR-heparin ternary complex, where simultaneous binding of FGF and heparin were found to induce receptor dimerization, have provided a structural basis for the essential role of HS fragments in FGF signal transduction (*19*). bFGF has been shown to be liberated from the ECM of cultured vascular SMCs by heparanase (*14*, *15*). Furthermore,

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heparanase stimulates SMC proliferation and migration *in vitro via* the mobilization of bFGF (*15*).

While many therapeutic strategies are being developed for the prevention of restenosis, a better understanding of the cascade of events leading to arterial re-occlusion, especially the initial proliferative stimulus, may permit more specific molecular-based approaches. In this study, we investigated whether (i) heparanase releases active bFGF from the arterial wall, inducing SMC proliferation, (ii) mobilized bFGF regulates the actions of additional growth factors, and (iii) heparanase neutralization can inhibit bFGF release following vascular injury and inhibit intimal thickening.

MATERIALS AND METHODS

Materials—Proteins and antibodies were obtained from CalBiochem unless otherwise noted.

Chemicals were obtained from Sigma Chemicals unless otherwise noted. Tissue culture dishes, multi-well plates and glass slides were obtained from Fisher Scientific.

Hydrogel Precursor Synthesis—Polyethylene glycol diacrylate (PEG-DA) was prepared by combining 0·1 mmol/ml dry PEG (10 kDa; Fluka), 0·4 mmol/ml acryloyl chloride, and 0·2 mmol/ml triethylamine in anhydrous dichloromethane and stirring under argon overnight. The resulting PEG-DA was then precipitated with ether, filtered, dried under vacuum, dialyzed against DI water (2,000 MW cut off cellulose ester membrane; Fisher Chemicals), and lyophilized.

Heparanase Treatment of the Arterial Wall—Heparanase was periadventitially delivered to the uninjured arterial wall using PEG-DA hydrogels that provide prolonged protein release (approximately 4 days) to assess whether it could activate bFGF, overcome native growth regulatory mechanisms and induce SMC proliferation. PEG-DA (0·5 g/ml) was dissolved in a solution of heparanase (10 μg/ml) or HEPES-buffered saline (control), each of which also contained 20 μl/ml 2,2-dimethoxy-2-phenylacetophenone in *N*-vinylpyrrolidinone (300 mg/ml stock solution). All solutions were sterilized by filtration (0·2 μm). Male Sprague-Dawley rats (350–450 g) were initially anesthetized with an isoflurane (4 ml/min)/ medical grade oxygen (1 ml/min) mixture (TEC 5 continuous flow anesthesia system; Summit Medical). During the surgical procedure, the gas mixture was reduced to 2·5 ml/min isoflurane and 1 ml/min oxygen. The left carotid artery was surgically exposed, and 200 μl of the appropriate PEG-DA hydrogel precursor solution was added to the perivascular space surrounding the common carotid. The liquid precursor was polymerized *in situ* by exposure to long wavelength ultraviolet light (365 nm, 10 mW/cm^2, 45 s exposure) to form a periadventitial hydrogel for protein delivery. Animals in the sham control group received gels lacking protein incorporation. Additional controls were left untreated. Alternatively, heparanase was periadventitially delivered in conjunction with an anti-bFGF IgG, previously shown to neutralize bFGF activity (*5*), using Pluronic F127 hydrogels. Pluronic F127 is a liquid at low temperatures, but transforms to a gel at physiological temperatures (*20*), allowing prolonged local release of IgG over approximately 48 h (*21*). 200 μl of chilled Pluronic F127 solution (BASF Corporation; 250 mg/ml, pH 7·4) containing 2 μg of heparanase and 200 μg of anti-bFGF IgG was added to the perivascular space surrounding the common carotid. Rats were sacrificed at 4 (n = 6 per group) and 14 (n = 6 per group) days by CO_2 asphyxiation after isoflurane-induced anesthesia; arteries were excised, rinsed with phosphate buffered saline (PBS) and prepared for histological and biochemical analysis.

Depletion of bFGF from the Arterial Wall—Heparanase releases bFGF from the ECM *in vitro* and may play an important role in inducing bFGF release *in vivo* (*15*, *22–24*). Prior studies have shown that bFGF levels in the arterial wall significantly decrease 4 d after injury (*7*, *8*). Using similar techniques, arteries exposed to heparanase were examined for bFGF content after 4 d. Tissue samples were homogenized, and total protein concentration in each sample was determined by the bicinchoninic acid assay (Pierce). bFGF levels relative to total protein were measured *via* ELISA. Tissue homogenate was coated onto 96-well plates and incubated at 4°C overnight. Wells were blocked with incubation buffer (PBS; pH 7·4, 5 mg/ml bovine serum albumin, 0·05% tween 20, 0·02% NaN_3) for 1 h at 37°C, incubated with rabbit anti–human bFGF IgG (cross-reacts with rat bFGF; Biodesign) diluted 1 : 250 in incubation buffer for 1 h at 37°C, washed 4 times with PBS, incubated with HRP-conjugated goat anti-rabbit IgG (Biodesign) diluted 1 : 250 in incubation buffer for 1 h at 37°C, washed 4 times with PBS, and reacted with 3,3′,5,5′-tetramethylbenzidine as the HRP substrate. Absorbance readings were taken on a microplate reader (450 nm; Bio-tek Instruments).

Histology—Arterial segments were fixed in 10% buffered formalin overnight, embedded in Histoprep freezing medium (Fisher Scientific) and cryo-sectioned (10 μm; MICROM). Immunohistochemical staining was carried out for proliferating cell nuclear antigen (PCNA). Samples were permeabilized with methanol for 2 min, treated with 3% H_2O_2 for 10 min, incubated with rabbit anti-mouse PCNA IgG (Zymed) diluted 1:100 in PBS containing 3% fetal bovine serum (FBS) for 2 h, washed 3 times with PBS, incubated with goat anti–rabbit IgG HRP (Zymed) diluted 1:100 in PBS containing 3% FBS for 1 h and washed 3 times with PBS. Sections were then stained with the HRP substrate diaminobenzidine (DAB; Vector Laboratories) for 15 min and counter-stained with eosin. Negative controls were exposed to the secondary antibody only. To investigate intimal thickening after injury, samples were stained with van Gieson's elastin stain. The total number of nuclei in each region was quantified following staining with hematoxylin and eosin.

Preparation of Anti-Heparanase IgG—To more closely examine the role of heparanase in the restenosis process, we developed an antibody that neutralizes heparanase (*15*). An antibody against a 20-amino-acid peptide corresponding to the active site of platelet-derived heparanase (*25*), G^{215} through D^{234}, was raised in rabbits (Sigma Genosys) as previously described (*15*). IgG was purified by serum filtration through a Hi Trap protein A column (Pharmacia Amersham); neutralizing activity was confirmed by its ability to prevent heparanase-mediated degradation of heparan sulfate (*15*).

Anti-Heparanase IgG Treatment after Balloon Injury—Male Sprague-Dawley rats (400–450 g) were anesthetized as described above, the left carotid artery was surgically

exposed, microsurgical clamps were applied to isolate a zone in the common carotid, and a balloon injury was created by passing an inflated 2F Fogarty catheter (Edwards Life Sciences) through the common carotid three times. 200 μl of chilled Pluronic F127 solution (250 mg/ml, pH 7·4) containing 200 μg of anti-heparanase rabbit IgG was applied periadventitially. A sham group received gels lacking protein incorporation. A second control group received gels containing 200 μg of non-specific rabbit IgG. Additional control animals were left untreated. Rats were sacrificed at 4 ($n = 4$ per group) and 14 ($n = 7$ per group) days by CO_2 asphyxiation; arteries were excised and prepared for histological or biochemical analysis as described above.

Statistical Analysis—All experiments were performed minimally in triplicate. Error bars reflect standard deviations, and p-values were assessed using two-tailed, unpaired t-tests. P-values less than 0·05 were determined to be significant.

RESULTS

Heparanase Releases bFGF from Uninjured Arteries—We examined bFGF release from the arterial wall upon exposure to heparanase by periadventitial delivery to uninjured rat carotid arteries. bFGF content and SMC proliferation were assessed 4 d after treatment with heparanase alone or in conjunction with anti-bFGF IgG. Arteries treated with heparanase ($p < 0·00004$) or heparanase and anti-bFGF IgG ($p < 0·0003$) were shown to have decreased bFGF content compared to shams and controls (Fig. 1), suggesting that heparanase liberates bFGF from sequestration in the arterial media. Furthermore, heparanase treatment ($p < 0·0001$) increased medial SMC proliferation at 4 d (Fig. 2, A–E) while simultaneous treatment with heparanase and a bFGF-neutralizing antibody did not, suggesting that heparanase increases proliferation through bFGF activation. Heparanase-induced proliferation largely subsided by 14 d (Fig. 2F; $p < 0·002$). While heparanase was not found to induce intimal thickening in the absence of vascular injury, it did contribute to luminal narrowing (Fig. 3D; $p < 0·01$) by increasing medial area (Fig 3E; $p < 0·0006$) and medial thickness (Fig 3F; $p < 0·0001$). This was due to the increased number of medial SMCs after heparanase treatment (control, 571 ± 45; sham, 581 ± 32; heparanase, 813 ± 72; number of

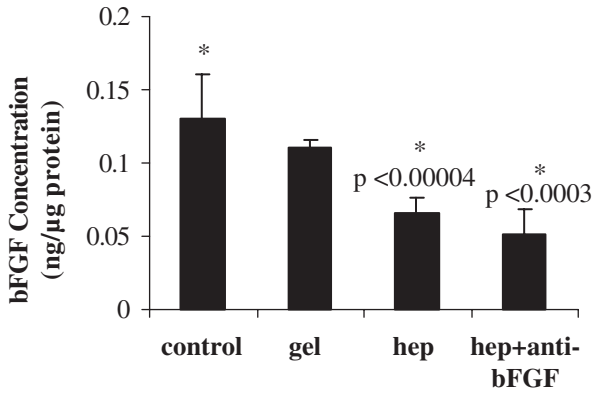

Fig. 1. **bFGF depletion following treatment of uninjured arteries with sham hydrogel (gel), hydrogel containing 2 μg heparanase (hep) or hydrogel containing 2 μg heparanase and 0·2 mg anti-bFGF IgG (hep + anti-bFGF) compared to control (untreated arteries).** bFGF content was assessed *via* ELISA 4 days after treatment.

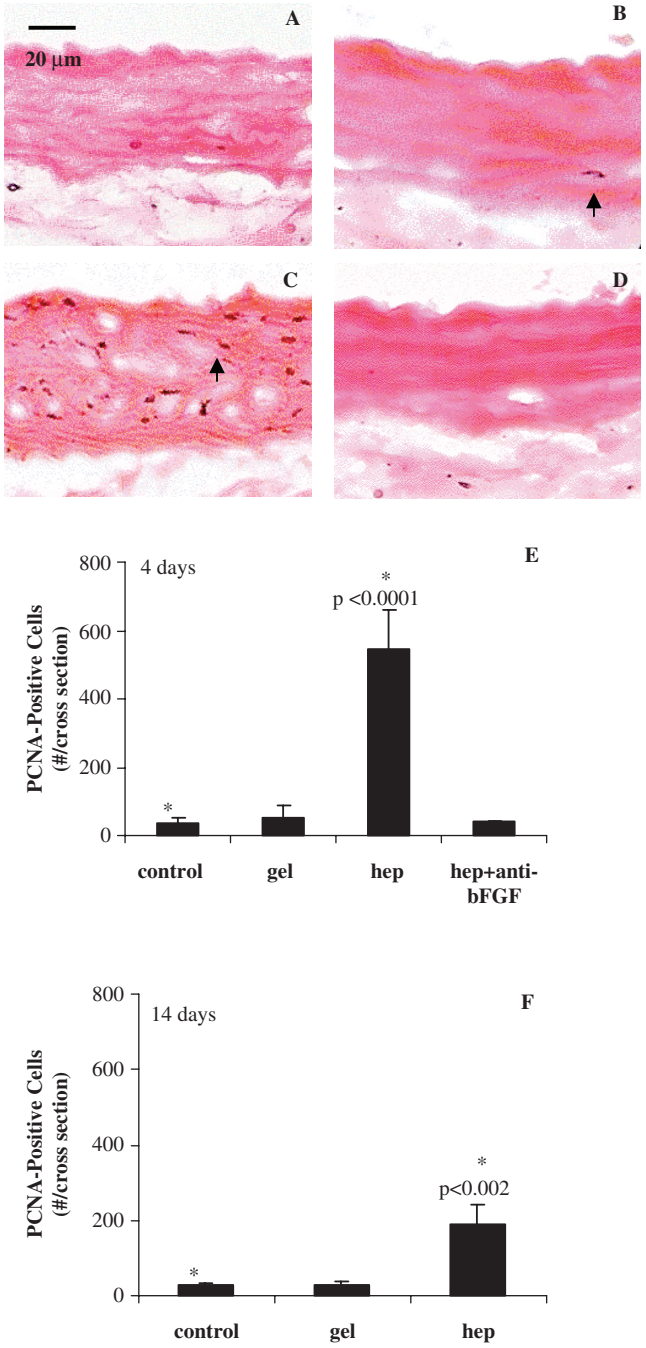

Fig. 2. **Medial SMC proliferation following treatment of uninjured arteries with sham hydrogel (gel), hydrogel containing 2 μg heparanase (hep) or hydrogel containing 2 μg heparanase and 0·2 mg anti bFGF IgG (hep + anti-bFGF) compared to control (untreated arteries).** Immunohistochemical staining for PCNA: A, control; B, sham hydrogel; C, heparanase; or D, heparanase and 0·2 mg anti bFGF IgG. Examples of PCNA-positive cells are indicated by arrows. The number of cells in S-phase (PCNA+) was determined by PCNA-HRP staining 4 days (E) and 14 days (F) after treatment.

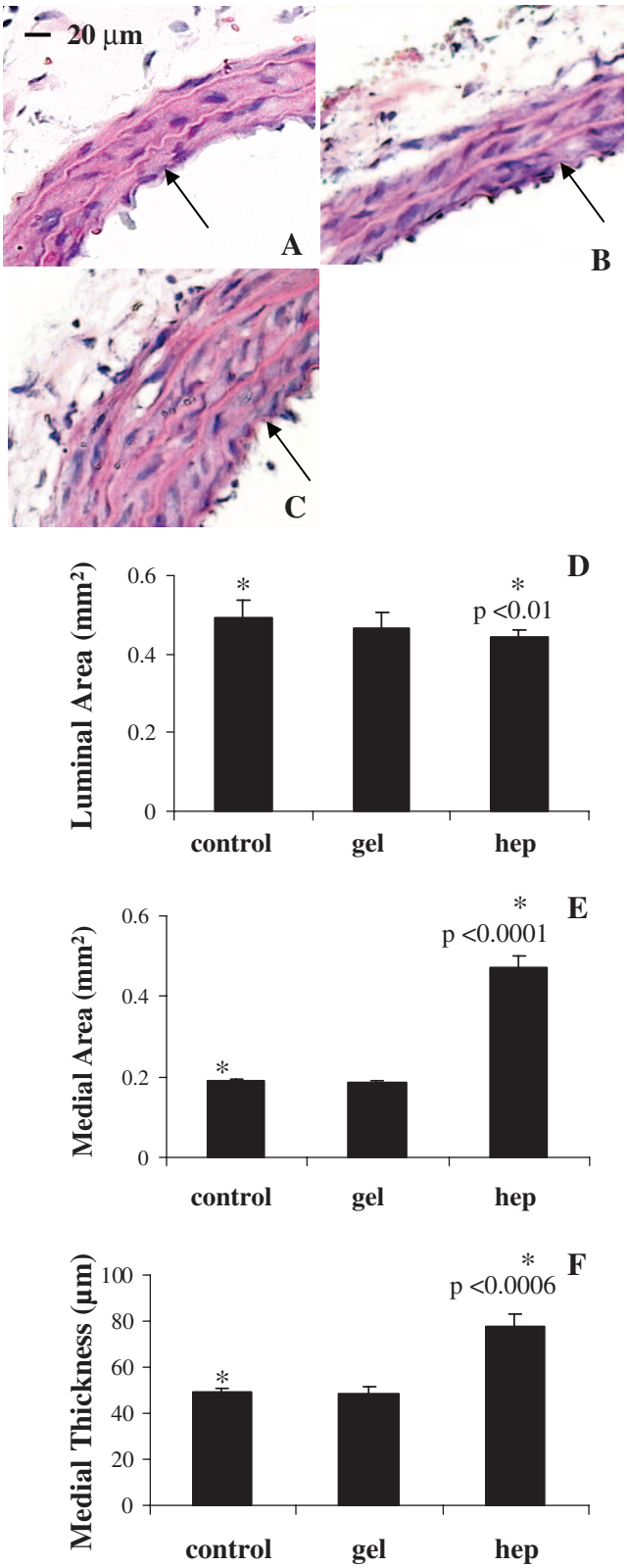

Fig. 3. **Histological sections of uninjured arteries after treatment with: A, control (untreated); B, sham hydrogel (gel); or C, 2 μg heparanase (hep).** Arrows indicate the internal elastic lamina, which separates the intimal and medial layers of the vessel. Luminal area (D), medial area (E) and medial thickness (F) were measured 14 days after treatment.

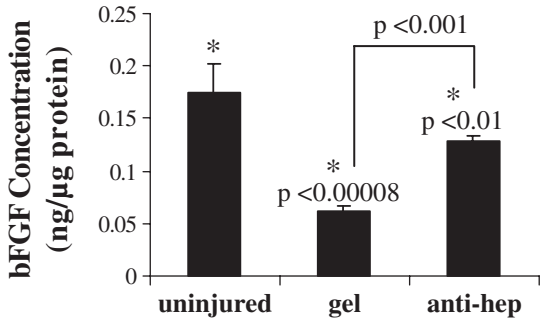

Fig. 4. **bFGF depletion 4 days after injury and treatment with sham hydrogel (gel) or hydrogel with 200 μg anti-heparanase IgG (anti-hep) compared to uninjured arteries.** bFGF content was assessed *via* ELISA.

medial SMCs compared to control, $p < 0{\cdot}0001$). The lack of intimal SMCs at this time may be due to the lack of endothelial denudation where the endothelial lining serves as a physical and/or chemical barrier to SMC migration (*26*).

Heparanase Neutralization Inhibits bFGF Activation and Decreases Intimal Thickening—We have developed an antibody that neutralizes heparanase (*15*) and evaluated its efficacy in the rat carotid balloon injury model. An antibody against a peptide corresponding to the active site of platelet-derived heparanase (G^{215}–D^{234}) (*25*) was raised in rabbits and the neutralizing activity of the purified IgG was confirmed by its ability to prevent heparanase-mediated heparan sulfate degradation (*15*). The left common carotid artery was injured by passage of a 2F Fogarty catheter through the vessel 3 times. In the anti-heparanase treatment group, a Pluronic F127 gel containing anti-heparanase IgG was applied periadventitially; this method enables prolonged and local delivery similar to the PEG-DA gel used for heparanase delivery but more appropriate for release of high molecular weight IgG. Arterial bFGF content was determined 4 d after injury via ELISA. Anti-heparanase IgG was found to inhibit bFGF depletion from the vessel wall by approximately 60% compared to sham controls (Fig. 4; $p < 0{\cdot}001$). Heparanase neutralization was shown to completely inhibit medial SMC proliferation 4 d after injury (Fig. 5D) and to significantly reduce intimal SMC proliferation at 14 d compared to sham controls (Fig. 5E; $p < 0{\cdot}00005$). We also examined neointima formation 14 d after injury. Treatment with anti-heparanase IgG was found to significantly inhibit neointimal hyperplasia as shown by decreases in intimal thickness (Fig. 6C; sham 81 ± 17 μm, anti-heparanase 34 ± 9 μm, $p < 0{\cdot}0002$), the I/M ratio (Fig. 6D; sham 1·3 ± 0·3, anti-heparanase 0·35 ± 0·12, $p < 0{\cdot}0001$), and the number of intimal SMCs (sham 633 ± 56, anti-heparanase 229 ± 39, $p < 0{\cdot}00001$) compared to sham controls.

DISCUSSION

bFGF has been shown to play a key role in the initiation of SMC proliferation following vascular injury. For example, anti-bFGF IgG treatment after balloon injury has been found to inhibit SMC proliferation and reduce restenosis

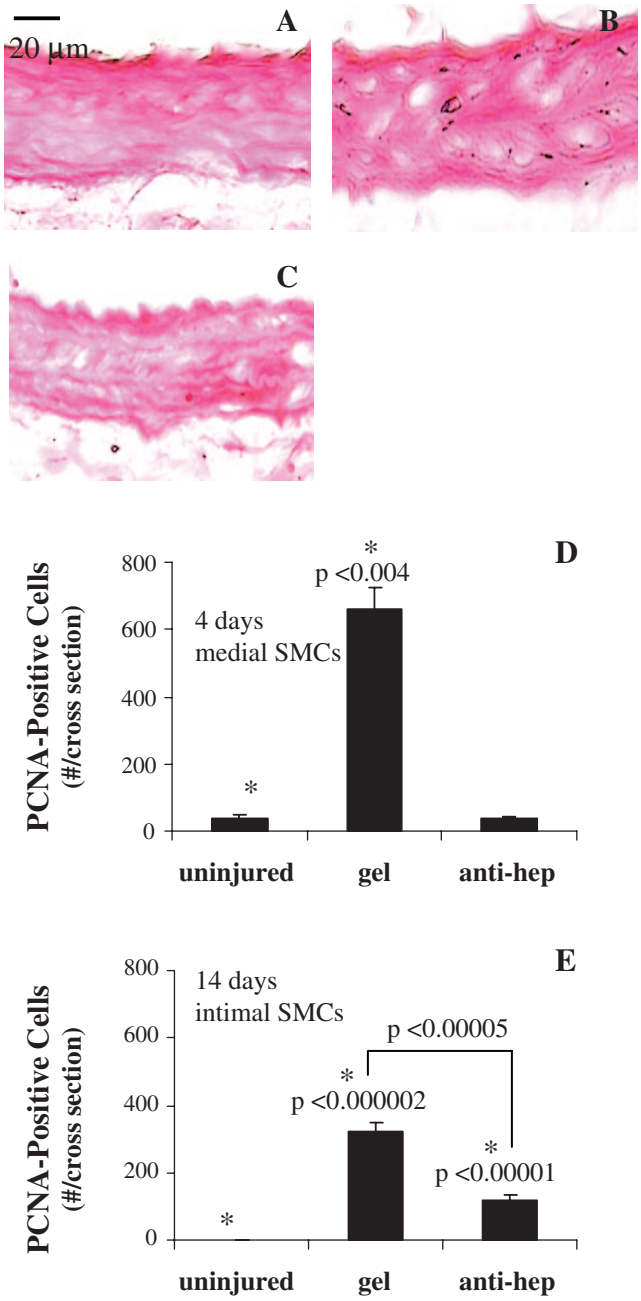

Fig. 5. **Medial SMC proliferation of uninjured arteries (A) or 4 days after balloon injury and treatment with sham hydrogel (gel; B) or hydrogel with 200 μg anti-heparanase IgG (anti-hep; C).** The number of medial SMCs in S-phase 4 days after injury (D) or the number of intimal SMC in S-phase 14 days after injury (E) was determined by PCNA-HRP staining.

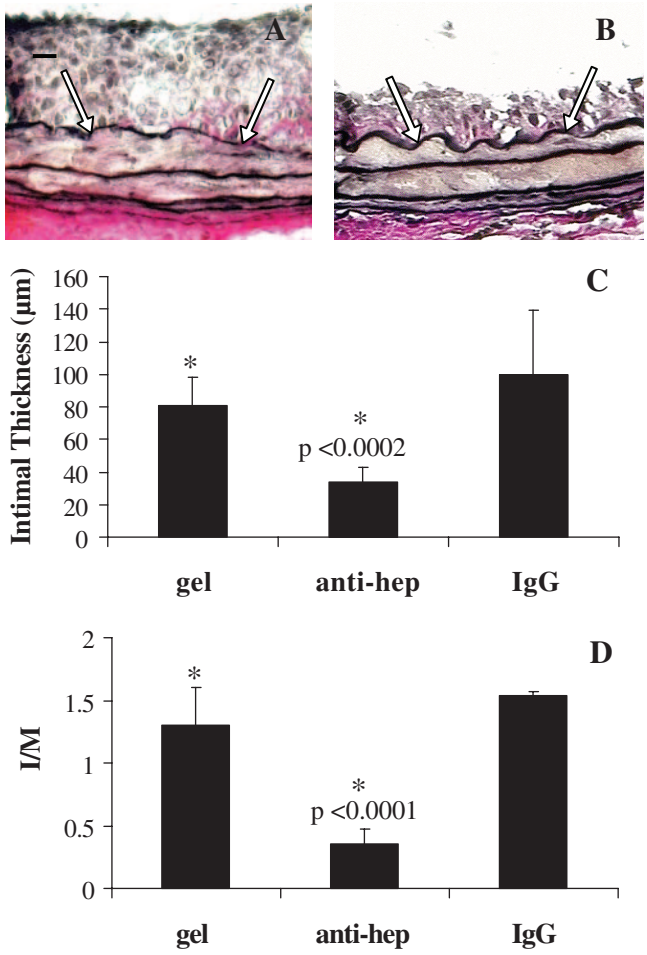

Fig. 6. **Histological sections of rat carotid arteries 14 days after injury and treatment with sham hydrogel (gel; A) or hydrogel with 200 μg anti-heparanase IgG (anti-hep; B).** Arrows indicate the internal elastic lamina, which separates the intimal and medial layers of the vessel. Intimal thickness (C) and the ratio of intimal area/medial area (D) 14 days after treatment.

by approximately 80% (*3*). HSPGs in the ECM and basement membrane bind heparin-binding growth factors, such as bFGF, sequestering them and inhibiting proliferation under normal conditions (*9*). Liberation of these growth factors directly into the extracellular space may provide a mechanism for the rapid induction of cell proliferation and migration following vascular injury (*9*).

Tissue injury has been found to trigger the rapid, localized release of bFGF, facilitating cellular responses such as proliferation and migration (*3–8*, *27*), though the mechanisms responsible for this release have been poorly understood. We have previously shown that heparanase, released during platelet degranulation, can release bFGF from ECM sequestration *in vitro*, mobilizing bFGF and inducing the proliferation and migration of cultured SMCs (*15*). We have also shown that low numbers of degranulating platelets seeded on top of SMCs are similarly able to release bFGF from the ECM (*15*). Furthermore, increases in proliferation under these conditions were found to be largely inhibited by bFGF neutralization (*15*).

The present study has shown that heparanase releases bFGF from sequestration in the arterial wall, mobilizing the growth factor and inducing SMC proliferation. After 4 d, uninjured arteries treated with heparanase contained significantly less bFGF than controls, suggesting its release from ECM sequestration. Furthermore, heparanase was found to substantially increase medial SMC proliferation at 4 d with proliferation declining at 2 wk. These data agree with previous findings that bFGF maximally

stimulated medial SMC proliferation 2 d after injury, and proliferation subsided after 2 wk (*7*). Heparanase was also shown to decrease luminal area by increasing the number of medial SMCs and medial area. No intimal SMCs were found following heparanase treatment. However, these studies were carried out in the absence of injury or endothelial denudation. The lack of a biochemical gradient formed by platelet degranulation products from the intima to the media may contribute to the lack of intimal SMCs. In addition, the arterial endothelium produces factors that limit SMC proliferation and migration and may serve as a physical barrier to SMC migration into the intima. It has been well documented that endothelial cells maintain the integrity of the vascular wall and inhibit ongoing SMC proliferation (*26*). In fact, perivascular endothelial implants have been found to substantially decrease intimal thickening following vascular injury (*26*).

We further investigated whether heparanase neutralization following balloon injury could inhibit bFGF activation and decrease intimal thickening. Our studies have shown heparanase neutralization to inhibit bFGF release from the arterial wall and reduce bFGF-mediated SMC proliferation and intimal thickening. However, the anti-heparanase antibody did not completely inhibit bFGF release, and this may contribute to the small degree of neointima formation observed under these conditions. It is possible that bFGF released by other platelet-derived factors, such as platelet factor-4 (*15*), or directly from injured SMCs stimulated the slight proliferative response seen in these studies. Additional growth factors released by platelets after injury may have also played a role in the proliferative response.

Our present studies have also indicated a role for heparanase and bFGF in the progression of restenosis *via* up-regulation of the expression of receptors for growth factors released from platelets and thought to be involved in vascular healing and restenosis. bFGF release during the initial wave of restenosis may stimulate later events, such as intimal SMC proliferation, migration and ECM deposition by enhancing the signal transduction of several growth factors released from platelets or circulating in plasma.

Sequestration of heparin-binding growth factors in the ECM provides a local source of mitogenic and chemotactic agents that may be mobilized by heparan sulfate–degrading enzymes, such as heparanase, in response to vascular injury. We have found heparanase to release bFGF from sequestration in the vascular wall and to increase SMC proliferation, contributing to luminal narrowing even in the absence of vascular injury. Furthermore, mobilized bFGF may prepare SMCs for mitogenic signaling by additional growth factors during the healing process by regulating growth factor expression levels. Heparanase neutralization, for instance using antibodies directed against the active site of heparanase, may be therapeutically advantageous in the prevention of restenosis due to its early role in the cascade of events initiating the process.

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