Construction and biological characterization of an HB-GAM/FGF-1 chimera for vascular tissue engineering

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Objective: **Cardiovascular tissue engineering approaches to vessel wall restoration have focused on the potent but relatively nonspecific and heparin-dependent mesenchymal cell mitogen fibroblast growth factor 1 (FGF-1). We hypothesized that linking FGF-1 to a sequence likely to bind to cell surface receptors relatively more abundant on endothelial cells (ECs) might induce a relative greater EC bioavailability of the FGF-1. We constructed a heparin-binding growth-associated molecule (HB-GAM)/FGF-1 chimera by linking full-length human HB-GAM to the amino-terminus of human FGF-1β (21-154) and tested its activities on smooth muscle cells (SMCs) and ECs.**

Methods: **Primary canine carotid SMCs and jugular vein ECs were plated in 96-well plates in media containing 10% fetal bovine serum and grown to approximately 80% confluence. After being growth arrested in serum-free media for 24 hours, the cells were exposed to concentration ranges of cytokines and heparin, and proliferation was measured with tritiated-thymidine incorporation. Twenty percent fetal bovine serum was used as positive control, and phosphate-buffered saline was used as negative control.**

Results: **In the presence of heparin the HB-GAM/FGF-1 chimera stimulated less SMC proliferation than did the wild-type FGF-1 with a median effective dose of approximately 0.3 nmol versus approximately 0.1 nmol ($P < .001$). By contrast, the chimera retained full stimulating activity on EC proliferation with a median effective dose of 0.06 nmol for both cytokines. Unlike the wild-type protein, the chimera possessed heparin-independent activity. In the absence of heparin, the chimera induced dose-dependent EC and SMC proliferation at 0.06 nmol or more compared with the wild-type FGF-1, which stimulated minimal DNA synthesis at 6.0-nmol concentrations.**

Conclusions: **The HB-GAM/FGF-1 chimera displays significantly greater and uniquely heparin-independent mitogenic activity for both cell types, and in the presence of heparin it displays a significantly greater EC specificity. (J Vasc Surg 2001;33:554-60.)**

The development of intimal hyperplasia is a consequence of smooth muscle cell (SMC) migration and proliferation along with accumulation of extracellular matrix and represents a primary failure mode after vascular intervention. It has been suggested that the endothelium plays an important role in maintaining the homeostasis of the normal arterial wall and that the reestablishment of endothelial integrity can inhibit SMC proliferation and thus prevent intimal hyperplasia. An effective means to induce endothelialization of vascular grafts or injured vessels is likely to improve long-term patency. It could also provide an alternative approach to the tissue engineering strategy of in vitro generation of all layers of arterial wall, which may be limited by the immunogenicity of endothelial cells (ECs).

We have developed techniques to locally deliver cytokines to vascular grafts and injured arteries with a hydrogel-based delivery system. In previous work, we applied fibroblast growth factor–1 (FGF-1) with heparin onto canine aortoiliac and thoracoabdominal aortic expanded polytetrafluoroethylene grafts with fibrin glue (FG).[1,2] The grafts impregnated with FG containing FGF-1 and heparin exhibited enhanced endothelialization when compared with FG-treated or untreated grafts. However, a significant increase in intimal thickening was observed in the FGF-1 treated specimens explanted after 20 weeks.

To date, an ideal cytokine for vascular application has not been reported. Vascular endothelial growth factor

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Competition of interest: nil.

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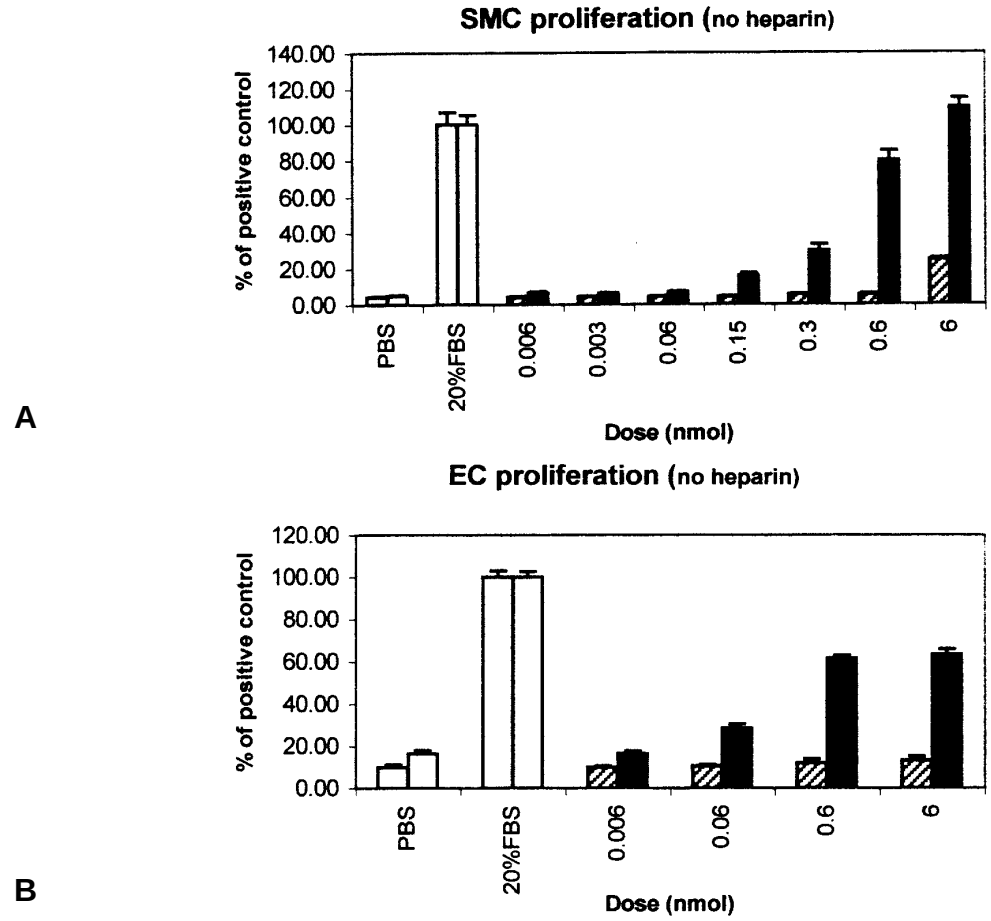

Fig 1. Proliferation of SMCs **(A)** and ECs **(B)** in response to various concentrations of wild-type FGF-1 (*striped bar*) and the HB-GAM/FGF-1 chimera (*solid bar*) in absence of heparin expressed relative to 20% FBS positive control (mean ± SD). *EC,* Endothelial cell; *SMC,* smooth muscle cell.

(VEGF) has caused attention in the past because of its EC-specific mitogenic and chemoattractant activities. However, the results are controversial when applied to animal models.[3-7] Most of the naturally occurring growth factors are nonspecific mesenchymal cell mitogens such as FGF-1, which has a potent proliferative effect on both SMCs and ECs.[8] In the attempt to achieve a potent mitogen with relative EC specificity, a series of FGF-1 mutants have been established and tested in our laboratories.

Heparin-binding growth-associated molecule (HB-GAM), also called *pleiotrophin,* is an 18-kd secreted protein of 136 amino acids with a high affinity to heparin.[9] It also binds to transmembrane heparan sulfate-proteoglycan (HS-PG) including syndecan-1, syndecan-3 (N-syndecan), and syndecan-4 (ryudocan) with high affinity.[10-12] We hypothesized that syndecans are likely in relatively greatest abundance on ECs, and therefore, an HB-GAM/FGF-1 chimeric construct would preferentially alter FGF-1 bioavailability to ECs versus SMCs by virtue of this altered high affinity binding site density. Thus, we constructed an HB-GAM/FGF-1 chimera by linking full-length human HB-GAM to the amino-terminus of human FGF-1β (21-154) and tested its activities on SMCs and ECs.

MATERIALS AND METHODS

Materials. Chemicals and biologicals were obtained as follows: oligonucleotide primer used for polymerase chain reaction (PCR) was purchased from Bioserve (Laurel, Md); TAQ polymerase from Perkin Elmer (Foster City, Calif); Bluescript SK+ from Stratagene (La Jolla, Calif); pET 3C and the *Escherichia coli* strain BL21(pLysS) from Novagen (Madison, Wis); heparin Sepharose and S-Sepharose from Pharmacia Biotech (Piscataway, NJ); Vydac C_{18} 4.6 × 220 mm from The Separations Group (Hesperia, Calif); 0.05% trypsin/0.53 mmol/L EDTA and collagenase from Gibco (Grand Island, NY); fibronectin, human thrombin, FGF-1 from the American Red Cross (Rockville, Md); thiopental sodium from Abbott Laboratories (Morris Plains, NJ); bovine lung heparin from Pharmacia and Upjohn (Kalamazoo, Mich); anti–von Willebrand factor (vWF) antibody from Dako Corp (Carpenteria, Calif); anti–α-actin antibody from

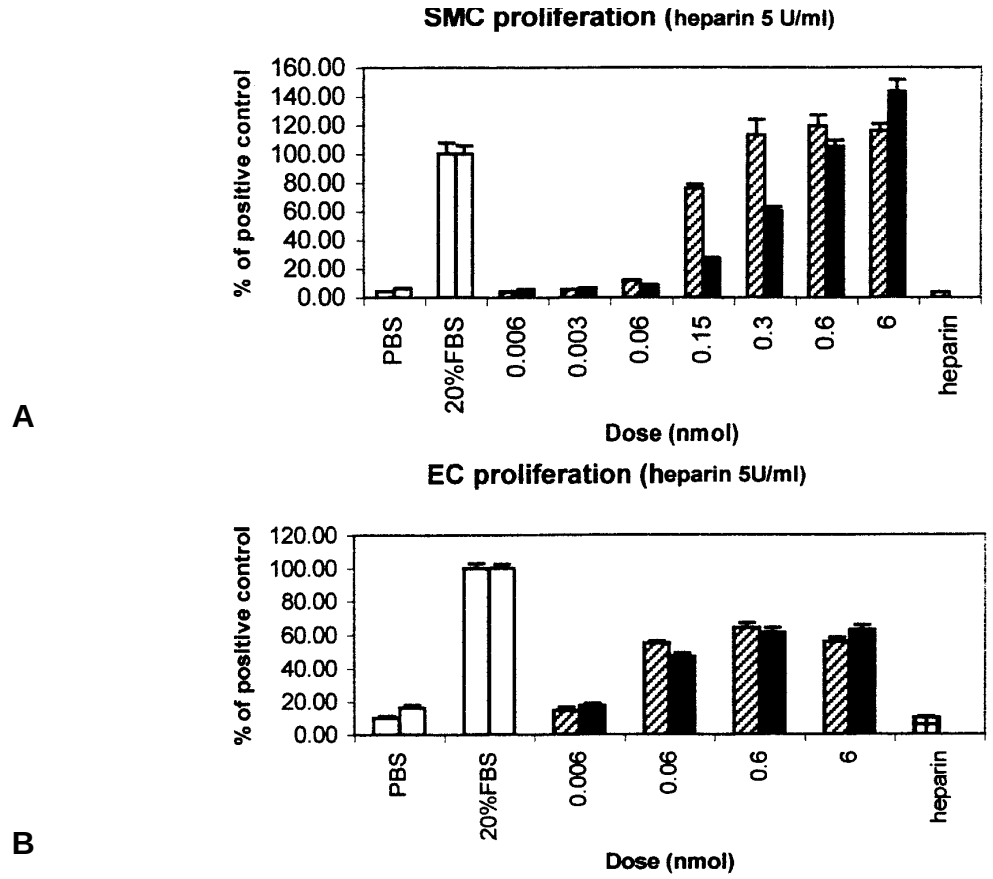

Fig 2. Proliferation of SMCs **(A)** and ECs **(B)** in response to various concentrations of wild-type FGF-1 (*striped bar*) and HB-GAM/FGF-1 chimera (*solid bar*) in presence of 5 U/mL of heparin expressed relative to 20% FBS positive control (mean ± SD). *EC,* Endothelial cell; *SMC,* smooth muscle cell.

Sigma Chemical Company (St Louis, Mo); tritiated thymidine from NEN Life Science Products (Boston, Mass); and methanol, trichloroacetic acid, acetic acid, and scintillation fluid from Fisher Scientific (Fair Lawn, NJ).

The media used were as follows: EC complete medium consisted of M199 (Gibco), 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco), FGF-1 5 ng/mL, and heparin 5 U/mL; EC growth medium consisted of M199, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin; EC quiescent medium consisted of Iscove's modified Dulbecco medium (Gibco), 2 mmol/L L-glutamine (Gibco), 5.5 × 10-5 mmol/L 2-mecaptoethanol (Gibco), 5% bovine serum albumin (Sigma), insulin-transferrin-selenium (insulin 10 μg/mL, transferrin 5.5 μg/mL, selenium 0.0067 μg/mL; Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin; SMC growth medium consisted of Dulbecco modified Eagle medium (DMEM) (Gibco), 10% FBS, 10 mmol/L L-nonessential amino acids (Gibco), 100 mmol/L sodium pyruvate (Gibco), 50 μg/mL gentamicin (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin; and SMC quiescent medium consisted of DMEM:F12 (Gibco), insulin 1 μmol/L (Sigma), L-ascorbic acid 0.2 mmol/L (Sigma), transferrin 5 μg/mL (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin.

Construction, expression, and purification of the HB-GAM/FGF-1 chimera. The chimera was constructed with an antisense primer (primer A: 5′-GAA GGT GGT GAT TTC CCC TTC AGC ATC CAG CAT CCT CTC CTG TTT CTT GCC-3′) designed to span the 5′ end of the FGF-1 open reading frame (ORF), and the 3′ end of the HB-GAM ORF. A hybrid complementary DNA (cDNA) fragment was generated from the human HB-GAM cDNA (gift of M. Jaye) by PCR with primer A and a sense primer (primer B: 5′-AAT TCC ATA TGG GGA AGA AAG AGA AAC CAG AAA AAA AAG TG-3′). This PCR product consists of the ORF of HB-GAM without the signal peptide sequence, and the first 24 nucleotides of the FGF-1 ORF are at its 3′ end. Another fragment was generated by PCR with primers C: (5′-GCT GAA GGG GAA ATC ACC ACC TTC-3′) and D: (5′-CAA CAG AGA TCT TTA ATC AGA GGA GAC-3′) that generated the ORF of FGF-1, excluding the initiator

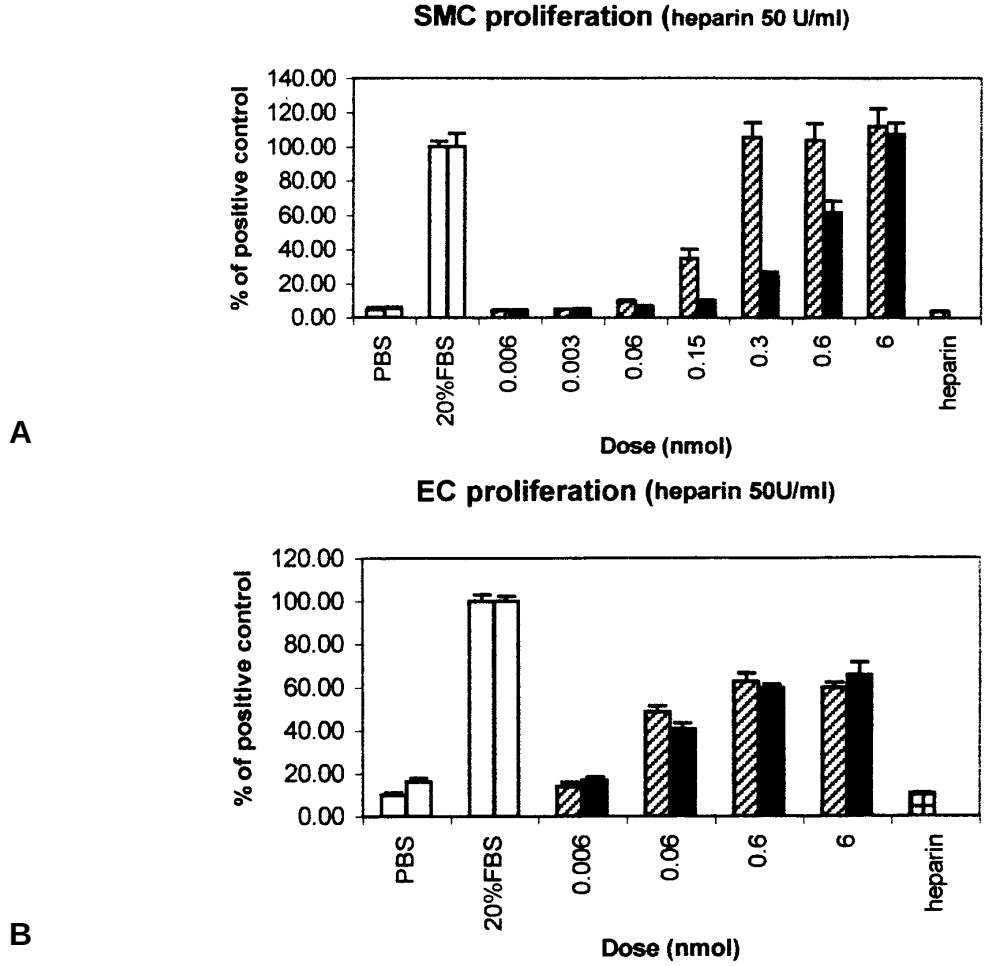

Fig 3. Proliferation of SMCs **(A)** and ECs **(B)** in response to various concentrations of wild-type FGF-1 (*striped bar*) and HB-GAM/FGF-1 chimera (*solid bar*) in presence of 50 U/mL of heparin expressed relative to 20% FBS positive control (mean ± SD). *EC,* Endothelial cell; *SMC,* smooth muscle cell.

methionine. These two fragments were combined in a single PCR reaction with primers A and D. The resulting cDNA product is a chimera of HB-GAM and FGF-1. The chimera was subcloned into Bluescript SK+, and the cDNA sequence was confirmed by dideoxy sequencing. The chimera was excised from this plasmid and subcloned into pET-3C for high-level protein expression.

The BL21-pLysS strain of *E coli* containing the pET-3C/chimera construct was grown at 37°C to late log phase, induced with 50 nmol isopropyl thiogalactose, and incubated for an additional 16 hours at room temperature. The cells were collected by centrifugation at 5000*g* for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was frozen at –70°C. The cell pellet was thawed and resuspended in 100 mmol/L Tris pH 7.4, 10 mmol/L EDTA, 100 mmol/L glucose, and DNA sheared by sonication. The suspension was clarified of insoluble debris by centrifugation at 10,000*g* for 10 minutes. The chimera protein was purified by heparin-Sepharose affinity chromatography, ion-exchange chromatography on S-Sepharose fast flow, and finally by reversed-phase chromatography on a Vydac C18 column.

EC and SMC harvest. Animal care complied with *The Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) and the *Principles of Laboratory Animal Care* (National Institutes of Health publication no. 85-23, revised 1985).

Adult, mongrel dogs were anesthetized with thiopental sodium, intubated, and ventilated. Anesthesia was maintained with nitrous oxide and halothane. Bilateral neck incisions were made. For ECs, bilateral external jugular veins were removed, inverted, and processed per our previously reported protocol.[13] The veins were sequentially placed into 0.05% trypsin/EDTA 0.53 mmol/L and collagenase 100 U/mL at 37°C for 10 minutes each. After the veins were discarded, the trypsin and collagenase solutions were centrifuged, and the pellets were resuspended

in EC complete medium. The ECs were plated onto a fibronectin-coated T-25 culture flask (2.5 μg/cm^2) and incubated in a 37°C, 5% carbon dioxide chamber. The media were changed every 2 to 3 days, and confluent cells were passaged with trypsin-EDTA. EC identity was confirmed with immunofluorescent dual staining with vWF and α-actin antibodies. Only ECs exhibiting 95% of positive vWF staining and 2% or less of α-actin staining were used for the proliferation assays. ECs were used within passages 1 through 3.

SMCs were obtained from canine carotid arteries by means of our previously published explant technique.[14] The carotid arteries were opened longitudinally, and the intima and adventitia were removed by scraping and dissecting with a scalpel. The medial layer was minced and placed into SMC growth media. Primary SMCs migrating from the explants were used in all experiments. SMC identity was confirmed with immunofluorescent staining with α-actin antibodies, and only cultures exhibiting 95% positive staining were used in the proliferation assays.

EC and SMC proliferation assays. ECs, at 10,000 cells per well, were plated into 96-well polystyrene plastic plates (Becton Dickinson, Lincoln Park, NJ) in 200 μL of EC growth medium and allowed to grow for 3 days until the wells were approximately 80% confluent. The medium was removed, the cells were washed with phosphate-buffered saline (PBS), and 200 μl/mL of serum-free EC quiescent media were placed on each well. After 24 hours quiescence, 50 μL of test solution containing various concentrations of either FGF-1 or HB-GAM/FGF-1 chimera plus various concentrations of heparin, diluted in PBS such that the final concentration in the well was the stated value, was placed on the wells. Fifty microliters per well of PBS was used as negative control, and 50 μL per well of FBS was used as positive control. After 48 hours, 1 μCi per well of tritiated thymidine was placed on the wells, and the plates were processed 24 hours later. To process, the medium was removed, and the wells were washed with 0.9% saline. The cells were fixed in 100% methanol for 10 minutes, lysed with distilled water, and precipitated with 5% trichloroacetic acid. The cell lysates were washed with distilled water, and the DNA was solubilized by adding 100 μL of 0.3 mol/L sodium hyroxide. This solution was placed into 10 mL of scintillation fluid that contained 20 μL of acetic acid to avoid opacification of the scintillation fluid with an accompanying dampening of the counts per minute.

For SMCs, the same protocol was used but with different growth and quiescent media. FGF-1 and HB-GAM/FGF-1 chimera at 0.006 to 6 nmol ± heparin at 0, 5, or 50 U/mL was tested. There were five replicate wells per each treatment. All experiments were repeated at least three times with consistent results.

Statistics. Normalized data (percent of positive control = [counts per minute treatment/counts per minute positive control] × 100%) were used to compare the difference between treatments. Because both positive and negative control wells were repeated on all plates, this normalization corrects for potential differences caused by the sequence of plate analysis or by the placement of the plates within the incubator. At the level of $\alpha = .05$, one-way analysis of variance and Student-Newman-Keuls methods (Sigmastat software 1.0, Chicago, Ill) were used to determine significance. General linear models procedure (SAS system, Mountainview, Calif) was used to analyze the dose-response curves.

RESULTS

There was approximately a sevenfold spread of tritium-thymidine incorporation between the PBS (negative control) and the 20% FBS (positive control) on ECs with cpm 5809.54 ± 1368.35 versus 39932.78 ± 2718.94 and a 20-fold spread on SMC (cpm 945.28 ± 126.39 vs 19399.95 ± 2475.46) data consistent in these proliferation assays in our laboratory. The FGF-1 exhibited a heparin-dependent property and a striking synergism with heparin, consistent with our previous published studies.[15,16]

Unlike the wild-type protein, the HB-GAM/FGF-1 chimera displayed heparin-independent activity. In the absence of heparin, the wild-type FGF-1 induced only minimal DNA synthesis at concentrations as high as 6 nmol, whereas the HB-GAM/FGF-1 chimera elicited dose-dependent DNA synthesis starting at 0.006 nmol on both ECs and SMCs. At 6 nmol, the HB-GAM/FGF-1 chimera induced fourfold more proliferation than wild-type FGF-1 on both ECs (63.16% ± 2.69% vs 13.14% ± 1.96%, $P < .01$) and SMCs (109.68% ± 5.11% vs 25.08% ± 0.71%, $P < .01$) (Fig 1).

The proliferative activities of both FGF-1 and the chimera were potentiated by the addition of heparin. In the presence of 5 U/mL of heparin, FGF-1 stimulated dose-dependent cell proliferation maximally at 0.6 nmol, giving 63.74% ± 3.08% of positive control on ECs and 119.35% ± 7.06% on SMCs (Fig 2). The chimera exhibited less activity on SMCs with a median effective dose (ED_{50}) of approximately 0.3 nmol compared with approximately 0.1 nmol for FGF-1 ($P < .001$). By contrast, the chimera retained full stimulating activity on EC proliferation with a median effective dose of 0.06 nmol for both cytokines. As the concentration of heparin increased to 50 U/mL, the overall response to both cytokines decreased in SMCs but not in ECs (Fig 3).

DISCUSSION

Although a number of cytokines and growth factors have been experimentally used to manipulate the healing process after vascular procedures, an ideal factor is not currently available. FGFs have been intensively studied in part because of their mitogenic potency. However, the mitogenic activities of FGFs are relatively nonspecific, potentially causing concomitant intimal hyperplasia. To manipulate the biological function of FGF-1, we have constructed a series of FGF-1 mutants, the HB-GAM/FGF-1 chimera among them.

HB-GAM is a neurotrophic factor functioning during neuronal growth and development. The mitogenic activity of this protein is controversial. We and others found that HB-GAM purified from bovine brain was unable to stim-

ulate DNA synthesis or proliferation of BALB/c 3T3, BALB/MK, NRK, and human umbilical vein ECs.[9] The recombinant protein expressed in a prokaryotic or baculovirus system was not mitogenic,[9,17,18] but transfection of human cells with HB-GAM resulted in the appearance of mitogenic activity and phenotypic transformation of the transfected cells.[17,19] The amino acid sequence of HB-GAM is highly conserved among species (approximately 98% identity). Among the 136 amino acids after the cleavage of a 32 amino acid signal peptide, 21% is lysine resulting in an extremely basic molecule, and 10 disulfide-linked cysteine residues form the backbone for the three-dimensional structure.[9] HB-GAM exhibits high affinity to heparin and heparan sulfate and thus can be sequestered in the extracellular compartment.[20,21] By linking HB-GAM to the FGF-1 molecule, we were able to change the heparin dependency of FGF-1 and to obtain a relatively EC-favored proliferative activity.

The proliferation effect of wild-type FGF-1 is heparin dependent. It is suggested that heparin can stabilize the FGF-1 molecular tertiary structure, prolong its biological life, protect it from heat and acid inactivation, and prevent proteolysis.[22-24] Heparin or heparan sulfate functions as a coreceptor facilitating FGF-1 receptor dimerization, which is required for tyrosine kinase activation, c-fos messenger RNA transcription, and cell proliferation.[25] It appears that the construction of the HB-GAM/FGF-1 chimera diminished the requirement for exogenous heparin. The mechanisms responsible for this modification of FGF-1 function remain to be elucidated. It is unlikely that HB-GAM will trigger the signaling pathway for cell proliferation because HB-GAM itself or combined separately with FGF-1 did not show any proliferation effects on both cell types (data not shown). It was demonstrated that HB-GAM could bind to transmembrane HS-PGs on the cell surface such as syndecan-1, syndecan-4, and especially N-syndecan, which has been suggested to be an HB-GAM receptor/coreceptor.[10] Unlike FGF-2, FGF-1 itself does not bind to N-syndecan,[26] indicating that N-syndecan is not involved in the conventional FGF-1 ligand/receptor interactions. The HB-GAM/FGF-1 chimera structure might bring syndecans into the FGF-1 ligand/receptor interaction thus changing its heparin dependency. However, heparin was able to potentiate the proliferation effect of the HB-GAM/FGF-1 chimera at the low-dose range.

In the presence of heparin, the HB-GAM/FGF-1 chimera retained full proliferative activity of FGF-1 on ECs but exhibited less potency on SMCs. It was found that the affinity of HB-GAM for heparin is higher than that of FGF-2.[20] In addition, the HB-GAM showed a 100-fold stronger binding affinity to immobilized heparin than to soluble heparin.[20] It is possible that the binding of HB-GAM in the chimera to HS-PGs in the extracellular matrix changed the availability of FGF-1 to ECs and SMCs. Evidence suggests that proteoglycans on the cell surface and in the extracellular matrix might differ quantitatively and qualitatively between ECs and SMCs.[27,28] HB-GAM–guided distribution of the chimera might account for the shifting of the proliferative activity of FGF-1 between cell types.

VEGF is an EC-specific growth factor with chemoattractant and angiogenic activities. Theoretically, it may be more likely to induce endothelialization without causing intimal hyperplasia. However, documented results have been controversial. We have locally applied VEGF to balloon-injured canine carotid arteries with an FG delivery system. It was found that FG/VEGF/heparin treatment induced a VEGF-dependent intimal hyperplasia despite non–VEGF-specific augmentation of reendothelialization of the injured arteries, resulting from the delivery vehicle itself.[29] Although the effectiveness of the administration of a bioactive protein needs to be carefully evaluated, the secondary effects induced by the protein in vivo should also be taken into account. VEGF has no direct stimulating effect on SMC proliferation, but it could induce ECs to express platelet-derived growth factor and heparin-binding epidermal growth factor–like growth factor. VEGF-stimulated EC condition medium reportedly stimulates SMC proliferation.[30] The downstream events after the application of a growth factor could be as important as its intrinsic activities for its ultimate effect when used in vivo. In the vascular wall, ECs and SMCs interact with each other either through direct contact or through paracrine mechanisms. To try to manipulate one cell type might inevitably affect the other. Potency and EC specificity would be critical for an in vivo–applied mitogen. We have focused on FGF-1 because of its strong proliferative effect on ECs that is 50 times that of the VEGF (unpublished data).

It is recognized that arterial and venous ECs may respond somewhat differently to some agonists. In our experiment we used venous ECs and arterial SMCs because of their direct clinical relevance.

In conclusion, the HB-GAM/FGF-1 chimera displays uniquely heparin-independent mitogenic activity for both ECs and SMCs, and in the presence of heparin it displays a relative EC specificity. This chimeric construct may provide a novel approach to engineering endothelialized surfaces without the concurrent fibroplastic reaction elicited by wild-type FGF-1.

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