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A Growth Factor Mixture That Significantly Enhances Angiogenesis in Vivo

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ABSTRACT

Studies of therapeutic angiogenesis have generally focused on single growth factor strategies. However, multiple factors participate in angiogenesis. We evaluated the angiogenic potential of a growth factor mixture (GF_m) derived from bovine bone. The major components of GF_m (SDS-polyacrylamide gel electrophoresis, mass spectrometry, and Western blot) include transforming growth factor-β1–3, bone morphogenic protein-2–7, and fibroblast growth factor-1. GF_m was first shown to induce an angiogenic response in chorioallantoic membranes. Next, myocardial ischemia was induced in 21 dogs (ameroid) that were randomized 3 weeks later to received GF_m 1 mg/ml (I), GF_m 10 mg/ml (II), or placebo (P) (with investigators blinded to conditions) injected in and adjacent to ischemic myocardium. Dogs were assessed 6 weeks later using quantitative and semiquantitative measures. There were GF_m concentration-dependent improvements in distal left anterior descending artery (LAD) opacification by angiography (P: 0.4 $\pm$ 0.2, I: 1.1 $\pm$ 0.14, II: 1.6 $\pm$ 0.3, angiographic score $p = 0.014$). Histologically, there was also concentration-dependent vascular growth response of relatively large vessels (P: 0.21 $\pm$ 0.15, I: 1.00 $\pm$ 0.22, II: 1.71 $\pm$ 0.18, vascular growth score $p = 0.001$). Resting myocardial blood flow (colored microspheres) was not significantly impaired in any group. However, maximum blood flow (adenosine) was reduced in ischemic territories and did not improve in GF_m-treated hearts. GF_m, a multiple growth factor mixture, is a potent angiogenic agent that stimulates large vessel growth. Although blood flow did not improve during maximal vasodilatory stress, large intramyocardial collateral vessels developed and angiographic visualization of the occluded distal LAD improved significantly. The use of multiple growth factors may be an effective strategy for therapeutic angiogenesis provided a more effective delivery strategy is devised that can achieve improved maximum blood flow potential.

Since their discovery over 15 years ago, several angiogenic growth factors have been isolated and purified (Folkman and Klagsbrun, 1987). The concept that such agents could be used therapeutically to induce both small and large vessel growth (angiogenesis and arteriogenesis, respectively) in states of chronic ischemia due to atherosclerosis has driven both experimental and clinical studies. In both ischemic limbs and heart muscle, the majority of studies have focused on the delivery of a single growth factor to stimulate vascular growth. The most extensively studied proteins are members of the VEGF and FGF families (Isner et al., 1996; Mack et al., 1998; Laham et al., 1999; Unger et al., 2000). There is evidence that delivery of these compounds, in the form of protein or gene therapy, can enhance blood flow to ischemic tissue in various experimental models (Unger et al., 1994; Mack et al., 1998). However, for a number of acknowledged reasons, results obtained in experimental models often do not translate directly into clinical practice.

One unanswered question about therapeutic angiogenesis is whether delivery of a single growth factor to an ischemic organ will be sufficient to induce growth of relatively large conduit vessels (arteriogenesis). Since ischemic syndromes resulting from arteriosclerosis affect the conduit vessels and not the small arterioles or capillaries, the growth of large vessels can be considered requisite for successful therapy. Many factors participate in the process of arteriogenesis (Beck and D'Amore, 1997). Recent studies have identified synergistic effects of angiogenic agents in the induction of vascular growth (Gajdusek et al., 1993; Ramoshebi and Ripamonti, 2000). Moreover, while prior published animal studies using single-factor strategies noted above have shown improvements, none has shown normalization of blood flow to treated ischemic myocardium. Identification of the factors involved, clarification of the role each factor plays,

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; HPLC, high-pressure liquid chromatography; IBP, inactive bone protein; PAGE, polyacrylamide gel electrophoresis; CAM, chorioallantoic membranes; GF_m, growth factor mixture; Df, fractal dimension; LAD, left anterior descending artery; BrdU, bromodeoxyuridine; CMS, colored microspheres.

and an understanding of the events involved in the process of arteriogenesis currently constitute areas of active investigation (Carmeliet, 2000).

Recently, a naturally occurring growth factor mixture (GF_m) isolated from bovine long bones was shown to enhance bone formation in several models, including a standard rabbit model of lumbar spinal fusion (Boden et al., 1997). Early in the course of investigating the bone formation stimulation properties of GF_m, it became evident that this mixture also promoted vascular growth. This was particularly evident in a rat ectopic bone development assay in which a collagen disk saturated with GF_m was placed subcutaneously over the chest wall. By 3 weeks after implant, the collagen was replaced with a bony ossicle, and an extensive vascular network (including large vessels) was observed on the surface of the ossicles. In the present study, we follow up on these preliminary findings by formally studying the angiogenic properties of GF_m. We present results indicating that indeed this multiple growth factor strategy effectively induces large vessel growth in chronically ischemic myocardium.

Materials and Methods

GF_m. The proteins found in GF_m are purified as one mixture from a noncollagenous protein extract of bovine femurs as detailed previously (Poser and Benedict, 1994). Briefly, mid-diaphyseal segments of bovine femurs are cleaned, pulverized, and demineralized. Proteins of apparent molecular weights between 10,000 and 100,000 are extracted by ultrafiltration, lyophilized, and purified by reverse-phase high-pressure liquid chromatography (HPLC). The protein mixtures containing active ingredients for bone formation used in this study have been shown to elute between 35.0 and 37.1% acetonitrile (v/v) (Poser and Benedict, 1994). Fractions eluting at slightly higher volume percentages have been shown to be inactive for bone formation (inactive bone protein, IBP) and were used in the present study as negative controls.

GF_m Content Analysis. One- and two-dimensional polyacrylamide gel electrophoresis (PAGE), HPLC, mass spectrometry, and Western blot analyses were used to investigate the contents of GF_m. PAGE analysis was conducted according to standard techniques. Tropomyosin (33 kDa, pI 5.2) and lysozyme (14 kDa, pI 10.5–11) were added to the samples as internal markers. Gels were stained with Coomassie Blue, and protein spots were excised from dried gels for mass spectrometry and sequence analysis.

For mass spectrometry, individual fractions from the HPLC column used to isolate the GF_m components from all the bone proteins were separated by SDS-PAGE in the Xcell II minigel system (Novex, San Diego, CA). Bands identified by staining with Coomassie Blue were excised and subjected to trypsin digestion in the gel slice as previously described. Proteolytic fragments were extracted in 50% acetonitrile/0.01% trifluoroacetic acid and were dried. Subsequently, the peptides were dissolved in matrix solution (10 mg/ml 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid) containing angiotensin and bovine insulin as internal standards. Samples were spotted onto a sample plate, washed with water to remove buffer salts, and analyzed by a Voyager DE-RP mass spectrometer in the linear mode (Applied Biosystems, Foster City, CA).

For Western analysis, proteins in the bone extract were separated by SDS-PAGE and were electroblotted onto a polyvinylidene difluoride membrane. The blots were probed with either commercially available monoclonal antibodies against various human proteins or with a polyclonal antibody against bovine FGF-1. The bands were visualized with an horseradish peroxidase-conjugated secondary antibody with a chemiluminescent substrate (Pierce Chemical, Rockford, IL) according to standard procedures.

Studies in Chorioallantoic Membranes (CAM). The angiogenic activity of GF_m was compared with that of a range of concentrations of recombinant human bFGF (10 μg/ml) and VEGF (10 μg/ml) or their combination (5 μg/ml each) (proteins obtained from R & D Systems Inc., Minneapolis, MN), as well as their carrier (povidone), in quail CAMs ($n = 6$ per group) (Parsons-Wingerter et al., 2000). Previous studies have shown that vascular growth response in CAMs to either bFGF or VEGF at concentrations of 10 μg/ml are on the plateau of the respective dose-response curves (Parsons-Wingerter et al., 1998, 2000). Fertilized Japanese quail eggs (*Coturnix coturnix japonica*) were opened into Petri dishes on day 3 postfertilization. After 4 days in culture at 37°C (i.e., day 7), the growth factors were solubilized in prewarmed carrier solution and added in a total volume of 0.5 ml to each embryo. The test material was evenly distributed on the surface of the CAM, which was cultured for 24 h at 37°C. Embryos were fixed in 4% paraformaldehyde/2% glutaraldehyde solution in phosphate-buffered saline. The CAMs were dissected from the embryos and were mounted on glass slides. Digital images were acquired at 10× magnification with a computer-supported digital camera attached to a microscope. The fractal dimension (Df) of each image was determined with previously validated software (Parsons-Wingerter et al., 1998). The Df (baseline) for a day 7 CAM is 1.372 (Parsons-Wingerter et al., 1998). The amount of vascular growth in CAMs treated for 24 h was reported as the percentage of change in Df relative to control (povidone-treated) CAMs.

Pilot Study in Canine Myocardium. Six adult mongrel dogs (20–25 kg) were anesthetized with thiopental sodium (15 mg/kg, i.v.) and maintained with 0.5 to 2.0% inhaled isoflurane. Via a left lateral thoracotomy, the proximal left anterior descending artery (LAD) was isolated and an ameroid constrictor was placed to induce ischemia over time. The dogs received intramyocardial growth factor injections of GF_m diluted in povidone (1 mg/ml, $n = 4$) or IBP (1 mg/ml; $n = 2$). All injections were 0.15 ml and were spaced ~1 injection/cm^2. Injections were made in both the anterior (ischemic) and posterior (normal) walls with five to nine injections performed in each region. Animals survived for either 2 or 6 weeks. All animals received postoperative bromodeoxyuridine (BrdU) injections (schedule provided below). Dogs were euthanized with pentobarbital (100 mg/kg), hearts were removed, and transmural tissue blocks were submitted for histologic and immunohistochemical evaluation (Masson's Trichrome stain; BrdU; smooth muscle actin; von Willebrand factor).

Efficacy Study in Chronically Ischemic Canine Myocardium. A randomized, blinded, placebo-controlled study was performed. An ameroid constrictor was placed in 21 adult mongrel dogs (20–30 kg) to create chronic ischemia. To minimize collateral flow in the acute setting, all visible epicardial obtuse marginal or posterior branches seen to connect with the LAD or LAD diagonal vessels were ligated (4–0 polypropylene sutures). One silicon tube (Tygon, Cardiovascular Instrument Corp., Wakefield, MA) was chronically implanted into the left atrium and another into the descending aorta.

Three weeks after ameroid constrictor placement, myocardial blood flow was measured at rest and during adenosine infusion. The chronically implanted aortic line was connected to a transducer (Statham Instruments, Inc., Oxnar, CA) for instantaneous and mean aortic pressure measurement. Colored microspheres (CMS; Dye-Trak, Triton Technology Inc., San Diego, CA) were infused rapidly into the left atrium (2 ml, 6×10^6 spheres) through the left atrium catheter. Withdrawal of a reference blood sample from the aortic line was begun just prior to CMS infusion (7 ml/min for 2 min). Adenosine was then infused at a dose titrated to induce ~20% decrease in mean aortic pressure, followed by infusion (and aortic reference sample withdrawal) of a second set of CMS.

Dogs were then anesthetized as above for a second surgical procedure 3 weeks later. Following anesthesia, a baseline coronary angiogram was performed to confirm ameroid closure and to define the degree of collateral filling of the distal LAD. The ameroid completely occluded flow to the distal LAD in every case. A thoracotomy was then performed and animals were randomized into one of three groups (seven dogs per group): placebo (povidone only), low concentration GF_m (1 mg/ml), or

high concentration GF_m (10 mg/ml). Randomization occurred on the morning of the surgery prior to knowledge of the angiogram and all investigators were blinded to treatment group until the final analyses of all data were complete. Animals received a total of 15 to 20 intramyocardial injections to the LAD area with the test solution (0.15 ml/injection, one injection/cm^2). Shallow 4–0 Prolene stitches were placed over each injection site to allow their identification at sacrifice. Animals received subcutaneous injections of BrdU starting the day before surgery (25 mg/kg), on the day of surgery, and days 1, 3, 5, 7, 9, 13, and 20 after surgery (15 mg/kg).

Six weeks after treatment, blood flow assessment (CMS) and coronary angiography were repeated. Animals were sacrificed and the heart removed. Three transmural tissue blocks, each containing one or two injection sites, were isolated, cut into epicardial, midwall, and endocardial sections, and evaluated histologically (Masson's Trichrome, BrdU, von Willebrand factor, and smooth muscle actin). The remainder of the heart was divided into 36 transmural blocks; each block was divided further into epicardial and endocardial sections (~1 g each). Blood flow was calculated from the CMS data according to standard techniques (Kowallik et al., 1991).

Statistics. Data were expressed as mean ± S.E.M. Between-group comparisons of ordinal data were performed with a Kruskal-Wallis test followed by repeated Mann-Whitney *U* tests to determine individual differences. Within-group differences were tested by the Friedman test; if significant, this was followed by repeated Wilcoxon tests. Between-group comparisons of continuous variable were done by one-way analysis of variance followed by Scheffe post hoc test. Within-group comparisons were done by paired samples *t* test. $p < 0.05$ was considered statistically significant.

Results

GF_m Composition. PAGE analysis of GF_m included 13 major bands, representing 65% of the protein by weight. These major bands have been identified by mass spectrometry. Two major components are BMP-3 and TGF-β-2. Other identified proteins that are probably not contributors to angiogenic activity include three that are related to histone H1.1 and three that matched with the ribosomal proteins S20, L6, and L32. Also identified were cathepsin L and proteins related to α-2-macroglobulin receptor-associated protein, retinoic acid receptor responder protein 2, secreted phosphoprotein 24, and lysyl oxidase-related protein.

By immunoblotting we have confirmed the presence of BMP-2 through 7, TGF-β-1 through 3, and FGF-1. With the exception of BMP-3 and TGF-β-2, these components are present at less than 1% of the total protein. VEGF and FGF-2 have not been detected.

Quail CAM Assay. Compared with povidone alone, CAMs exposed to GF_m for 24 h showed a greater vascular density and more vessel branchings (Fig. 1, A and B). The rate of vascular growth in CAMs subjected to povidone alone was similar to that of control CAMs (data not shown) and was set at 100% (Fig. 1C). The rate of vascular growth was approximately doubled with bFGF, VEGF, or their combination. GF_m at concentrations of 1 or 10 mg/ml elicited a slightly greater (although not statistically significant) vascular growth relative to that stimulated by bFGF, VEGF, or their combination.

Pilot Study. In GF_m (1.0 mg/ml)-treated animals allowed to survive for 2 weeks, large, BrdU-positive, conduit-sized vessels with diameters up to 300 μm could be detected in areas surrounding the injection sites, both in ischemic and nonischemic areas of the heart (Fig. 2, A–D). Six weeks after treatment with GF_m, numerous well organized large vessels as well as smaller arterioles and capillaries in the surrounding myocardium exhibited abundant BrdU incorporation (Fig. 2, E–H). Compared with nontreated ischemic tissue, GF_m-treated ischemic myocardium exhibited a greater than 5-fold increase in BrdU-stained, newly formed arterioles, with diameters ≥50 μm containing ≥2 BrdU-positive cells (6.6 ± 4.0 versus 1.3 ± 1.8 vessels/cm^2, $p < 0.05$). In contrast, IBP (1.0 mg/ml, an inactive protein fraction derived from bovine bones) failed to induce any significant growth of vessels larger than capillaries either close to the injection sites or in remote areas after 2 weeks (Fig. 3, a and b), although the inflammatory response at the injection site appeared similar with all the factors. These observations suggest that GF_m can induce large vessel growth and the major component is not due to inflammation.

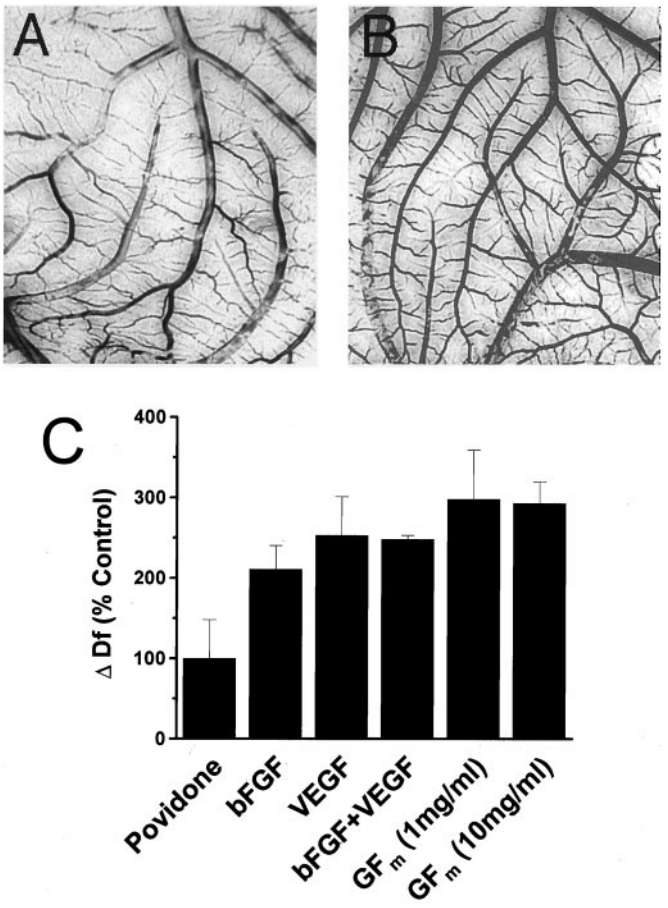

Fig. 1. Effects of growth factors on vascular growth on CAMs of fertilized Japanese quail eggs. Representative digitized pictures of CAMs exposed to either placebo (A) or GF_m 10 mg/ml (B). C, summary of quantitative image analysis of percentage of change in fractal dimension in response to different growth factors (Df, mean ± S.D., $n = 6$ in each group).

Effects in Chronic Myocardial Ischemia. Histologic samples were examined and graded for vascular growth *outside the scar observed at the injection site* according to a semiquantitative overall vascular growth index (0, no angiogenic response; 1+, mild-to-moderate angiogenic response; 2+, significant angiogenic response). According to this analysis, as summarized in Table 1, GF_m induced a robust concentration-dependent neovascular response in ischemic canine myocardium. The size of the scar was also concentration-dependent.

Baseline and follow-up angiograms were also compared by a three-point semiquantitative scale (0, no change in distal LAD opacification; 1+, mildly improved distal LAD opacifi-

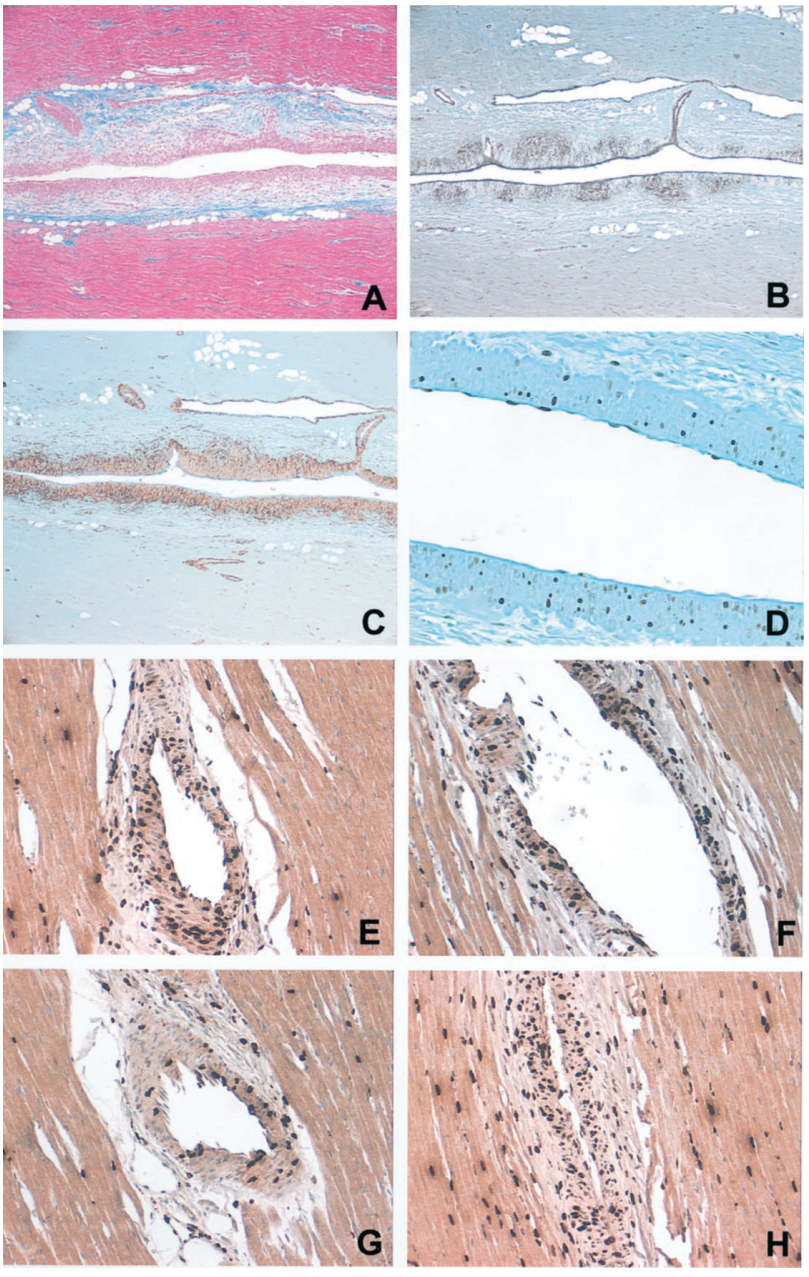

Fig. 2. Masson's Trichrome-stained section (A, 40× original magnification) showing large vessel with several side branches 2 weeks after GF_m injection into ischemic myocardium. As shown in adjacent sections, immunostained with antibodies against von Willebrand factor (B) and smooth muscle actin (C), the vessel is lined by endothelium surrounded by poorly organized layers of smooth muscle cells. That von Willebrand factor staining was not limited to the endothelial layer provides another indication that this vessel was not fully organized. Nuclei staining for proliferating cell nuclear antigen (200×, D) is indicative of neovascularization. Similar vessels were identified in both the ischemic and nonischemic areas of these hearts. Six weeks after ameroid placement and GF_m injection, relatively large but apparently mature vessels were seen (E–H). Evidence of vascular growth is based on the abundant BrdU incorporation seen in the endothelial and smooth muscle cells.

cation; 2+, significantly improved distal LAD opacification). Examples of in vivo angiograms from a high-concentration GF_m animal are shown in Fig. 4. Early during contrast injection at the baseline study (Fig. 4A), the ameroid completely occluded antegrade flow through the LAD; there was only faint LAD opacification late in the injection (Fig. 4B). Six weeks after GF_m treatment in the same animal, a new collateral vessel was seen early during the contrast injection (Fig. 4C, C1) that became more prominent along with more prominent LAD opacification seen later in the injection (Fig. 4D). These findings were not seen in the placebo group. Post-mortem ex vivo angiography on the same heart (Fig. 5, A and B) illustrates the ameroid closure; early during the contrast injection (Fig. 5A), two collateral vessels (C1, C2) were identified as responsible for distal LAD opacification late during the injection (Fig. 5B). Figure 5D shows an ex vivo angiogram from a placebo animal late during a direct left main injection. Here, "late" means that the picture is obtained with maximum dye injection to ensure visualization of an image obtained with maximal filling of all vessels that are present. Even under these extreme conditions, the LAD is completely missing. Although the image of Fig. 5B (from a treatment animal) is taken later than the image of Fig. 5A from the same animal, it is not as late as in the comparable injection in Fig. 5D as suggested by the relatively little filling of the circumflex vessel. Although this is an "earlier" image than in Fig. 5D, the LAD is readily seen. These findings indicate collateralization to the fully occluded distal LAD is significantly increased in the treatment compared with the placebo animal. The results of the semiquantitative blinded angiographic analysis (Table 1) confirmed a statistically significant improvement in the angiographic score at both high and low concentrations, compared with the placebo group.

Baseline microsphere-derived resting blood flow (3 weeks

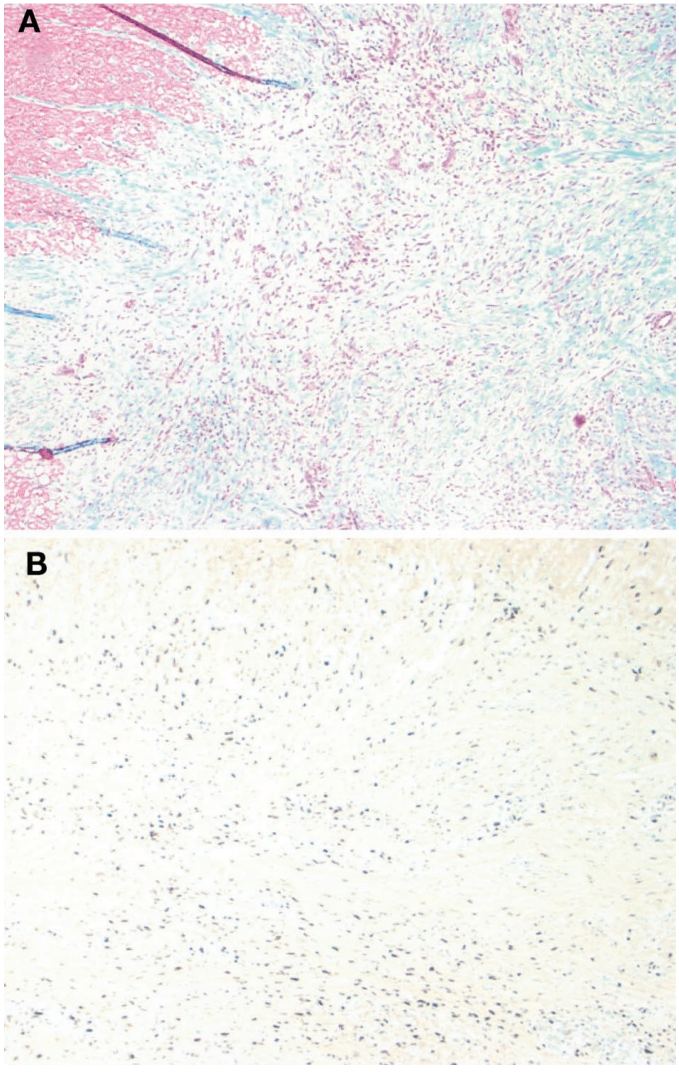

Fig. 3. Reactive inflammatory response after treatment with inactive bone protein (IBP, 1 mg/ml) shows scarring with no significant growth of vessels larger than capillaries (a and b, original magnification 40×).

after ameroid placement) was relatively normal in all myocardial segments, with only mild decreases in the anterior wall (Fig. 6; since results from endocardial and epicardial samples were not significantly different, they were pooled for this analysis). During adenosine vasodilation, however, blood flow increased ~4 fold in most segments, but not in those in the anterior region supplied by the LAD distal to the ameroid constrictor, which increased by less than a factor of 2. These features were similar in all treatment groups, as indicated in Table 1. Thus, despite the histologic and angiographic evidence of large vessel growth, maximal vasodilatory blood flow did not improve following GF_m treatment.

Discussion

GF_m, a protein mixture derived from bovine long bones, is an effective angiogenic agent in quail CAMs and canine myocardium. Several of the identified ingredients of GF_m are among the list of known angiogenic factors, and synergism between some GF_m components (e.g., BMP-7, bFGF, and TGF-β-1) has already been demonstrated in the CAM assay (Ramoshebi and Ripamonti, 2000). In ischemic myocardium, GF_m treatment was associated with growth of vessels with diameters as large as 300 μm with abundant BrdU incorporation. Analysis of the composition of GF_m confirmed the presence of several known angiogenic growth factors, such as bone morphogenic proteins BMP-2 through 7, TGF-β-1 through 3, as well as FGF-1. However, approximately 30% of the protein mass is unidentified. Thus, the observed effects could be related to any one or combination of the known growth factors, or they could reflect activity of as yet unidentified and possibly unknown factors. However, an inflammatory response is not the predominant mechanism underlying the effects of GF_m since similar vascular growth responses were not observed with IBP, which did incite an inflammatory response similar (histologically) to GF_m. However, although not sufficient, this does not exclude the possibility that an inflammatory response is necessary for the angiogenic response of GF_m to occur.

Angiographic findings in the chronically ischemic dog hearts provided additional evidence of large vessel growth. All ameroid constrictors were completely occluded so that at the baseline ischemic evaluation, there was only faint visualization of the distal LAD during contrast agent injections. A GF_m concentration-dependent increase in distal LAD opacification (blinded analysis) was observed, suggesting that collateralization to the distal LAD was improved. This was associated with the appearance of new collateral vessels either bridging around the ameroid constrictor or connecting marginal branches of the circumflex artery to diagonal vessels of the LAD. These vessels, which were particularly evident on ex vivo angiograms, took circuitous courses that are typical of new vessels.

As identified in prior studies of dogs (Unger et al., 1993)

TABLE 1

Summary of quantitative and semiquantitative parameters of vascular growth from histology, angiography, and microsphere analysis of blood flow (mean ± S.E.M., $n = 7$ for each group for each measurement)

	Placebo	GF_m (1 mg/ml)	GF_m (10 mg/ml)	p
Overall vascular growth index	0.21 ± 0.15	1.00 ± 0.22*	1.71 ± 0.18*, **	0.001
Scar (mm^2)	6.5 ± 1.9***	8.7 ± 2.2	15.9 ± 3.4	0.047
Angiographic score	0.4 ± 0.2	1.1 ± 0.14*	1.6 ± 0.3*	0.014
Baseline resting blood flow (ml/min/g)[a]	0.76 ± 0.06****	0.91 ± 0.17	0.90 ± 0.10	
Baseline maximal blood flow (ml/min/g)[a,b]	1.66 ± 0.18****	1.83 ± 0.19****	1.79 ± 0.15****	
Resting blood flow 6 wk after treatment (ml/min/g)[a]	0.70 ± 0.05****	1.07 ± 0.25****	0.90 ± 0.12****	
Maximal blood flow 6 wk after treatment (ml/min/g)[a,b]	1.90 ± 0.18****	2.21 ± 0.12****	1.93 ± 0.19****	

* $p < 0.05$ vs. placebo; ** $p = 0.051$ vs. GF_m 1 mg/ml; *** $p < 0.05$ vs. GF_m 1 mg/ml; **** $p < 0.05$ vs. control area.
[a] Blood flow measurements are from the anterior ischemic region.
[b] Maximal blood flow achieved by intravenous adenosine.

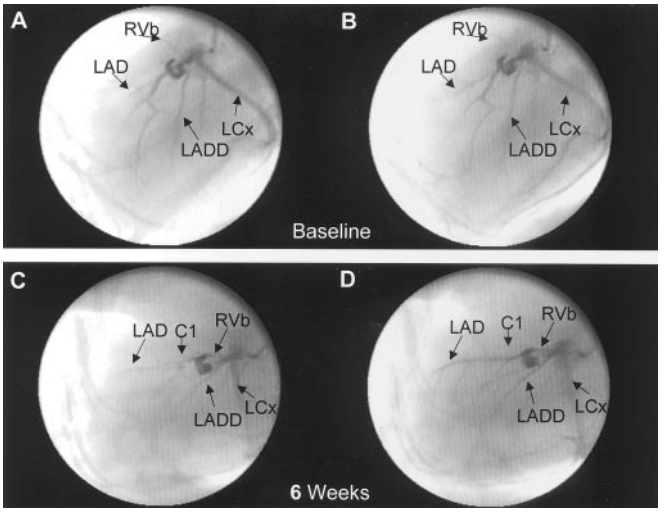

Fig. 4. Sequence of in vivo angiograms from a dog's heart at baseline (3 weeks after ameroid constrictor placement; A and B) and 6 weeks after treatment (C and D) with GF_m (10 mg/ml). At baseline, the ameroid was completely occluded and the LAD demonstrates minimal filling, both early (A) and late (B) during the contrast injection. Six weeks after treatment, the ameroid was again seen to completely occlude the LAD (C), but the LAD showed complete reconstitution late in the injection (D). A newly formed collateral (C1) was detected surrounding the experimentally occluded segment. LADD, diagonal branch; LCx, circumflex artery; RVb, right ventricular branch.

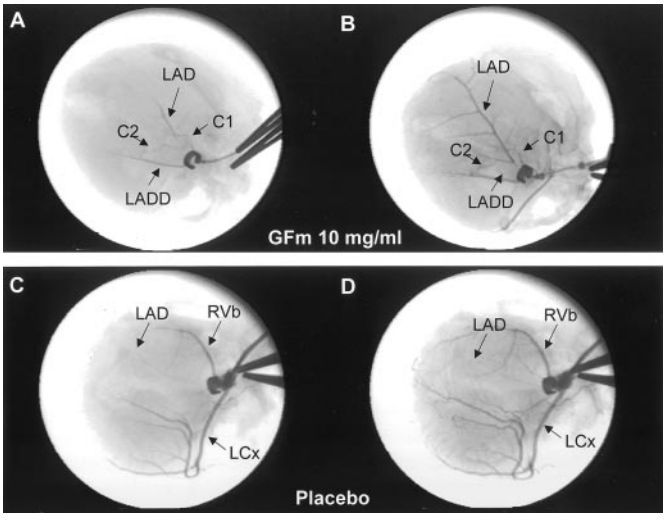

Fig. 5. Sequence of representative ex vivo angiograms taken at the terminal experiment 6 weeks after treatment with GF_m (10 mg/ml; A and B) or povidone (placebo; C and D). The contrast agent was injected via a catheter engaged in the left main coronary artery, revealing the left circumflex coronary artery (LCx) and a right ventricular branch (RVb). Ameroid constrictors completely occluded the LAD in both cases. After GF_m treatment, the LAD opacified early during the injection (A) via two collateral vessels (C1 and C2). Later during the injection (B), the entire LAD was seen. With placebo, the distal LAD is not seen early (C) and is barely evident late during the injection (D).

and pigs (Giordano et al., 1996), resting blood flow 3 weeks after ameroid constrictor placement was not significantly decreased compared with the normally perfused region, the result of natural collateralization. Since distal LAD opacification was poor at this time point, it is evident that normalization of resting flow is due to natural angiogenesis that occurs in the setting of chronic ischemia (Ware and Simons, 1997). However, despite the histologic and angiographic findings indicating the presence of large vessel growth, blood flow to the anterior wall was not improved during vasodilatory stress induced by adenosine. Distal LAD opacification on resting angiography does not in any way indicate the maximal blood flow capacity into the vascular bed; it merely indicates that new anatomic connections exist. This is completely analogous to the common clinical experience where, although a totally occluded epicardial vessel can frequently be visualized angiographically by flow of contrast agent from

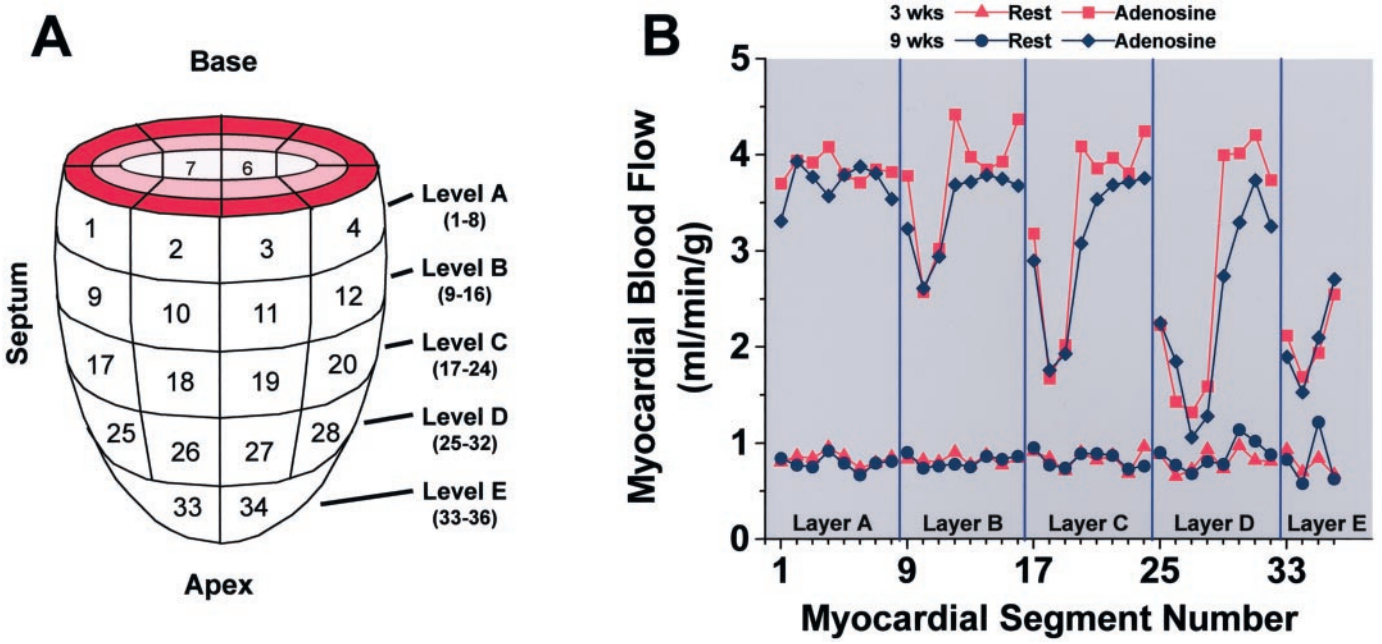

Fig. 6. Hearts were cut into 5 layers (A–E, base to apex) to produce 32 transmural blocks (A). Blood flow at each time point at rest and during vasodilation (adenosine) of each segment was plotted as a function of segment number to reveal ischemic and normally perfused segments (B). This result, obtained from a dog treated with GF_m 10 mg/ml, shows no significant difference between baseline and 6 weeks after treatment. Results from endo- and epicardial layers (analyzed separately) were subsequently pooled because they were highly similar.

collateral vessels, a myocardial perfusion defect is typically observed during stress on a nuclear perfusion imaging study.

Thus, while GF_m effectively induces large vessel growth, it could be that the delivery strategy (direct mid-myocardial injections at ~1-cm interinjection spacing) may not be optimal for improving maximal blood flow to ischemic myocardium. Use of a delivery method to ensure that new vessels grow from well perfused normal epicardial vessels neighboring the ischemic territory [e.g., an intra-arterial injection strategy (Giordano et al., 1996) or preferential subepicardial delivery] might be more effective.

The application of single exogenous growth factors either as protein or gene therapy has thus far been the standard in clinical trials of therapeutic angiogenesis. However, angiogenesis is not a process induced, sustained, or completed by a single molecule (Folkman and Klagsbrun, 1987; Beck and D'Amore, 1997; Coussens et al., 1999; Carmeliet, 2000; Carmeliet and Jain, 2000). Published preclinical studies of single growth factor strategies, whether with proteins or gene therapies, whether with FGF or VEGF, whether injected into the muscle or into an artery, have never demonstrated complete normalization of blood flow to the ischemic territory; such results are summarized in recent reviews (Ware and Simons, 1997; Simons et al., 2000). Clinically, although results of a few small (some unblinded) studies of single growth factor strategies have provided encouraging results (Schumacher et al., 1998; Vale et al., 2001; Laham et al., 1999), results from two relatively large-scale multicenter, double-blind studies now available have been negative; specifically, the VIVA trial of intracoronary and intravenous VEGF (Henry et al., 1999) and the FIRST trial of intracoronary bFGF (M. Simons, unpublished observations). Even in the unblinded clinical study, the extent of revascularization was incomplete, as has been observed in the animal studies. There are multiple possible explanations for variable results between animal studies and for the negative results reported in the earlier noted clinical trials. The fact that single growth factor strategies were used is among the possibilities (mode of delivery being another important factor), but it would be premature to conclude at this time that this is the decisive factor.

Conclusions. The choice of growth factor(s) and delivery strategies for therapeutic angiogenesis are currently the topic of intensive research. Some clinical trials based upon positive preclinical studies have provided negative results (see for example, Henry et al., 1999; Simons et al., 2000) warning about the potentially limited power of preclinical studies to predict clinical utility. Nevertheless, the present results indicate that GF_m, a mixture of growth factors, induces the formation of relatively large vessel. Lack of improved blood flow in treated animals does not diminish the importance of the histologic images of large new vessels seen in response to GF_m treatment. This does highlight the fact, however, that there are limitations to the overall strategy used in the present study. This could relate to the use of an intramyocardial route of administration, the density of injections, the dose of GF_m, or other unidentified factors that may also have impacted the results. As such, the present results serve to emphasize the point that, although necessary, it is not sufficient for an angiogenic therapy to induce vascular growth. The overall strategy, which includes the substance and its mode of delivery, must ensure that the new vessels become part of an effective vascular bed.

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