Angiogenic potential of perivascularly delivered aFGF in a porcine model of chronic myocardial ischemia

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1Angiogenesis Research Center, Cardiovascular Division, 2Department of Medicine, 3Department of Radiology, and 4Department of Surgery, Beth Israel Deaconess Medical Center, and 5Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston 02215; 6Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and 7Merck Research Laboratories, Rahway, New Jersey 07065-0900

Lopez, John J., Elazer R. Edelman, Alon Stamler, Mark G. Hibberd, Pottumarthi Prasad, Kenneth A. Thomas, Jerry DiSalvo, Ronald P. Caputo, Joseph P. Carrozza, Pamela S. Douglas, Frank W. Sellke, and Michael Simons. Angiogenic potential of perivascularly delivered aFGF in a porcine model of chronic myocardial ischemia. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H930–H936, 1998.—A number of heparin-binding growth factors, including basic (bFGF) and acidic (aFGF) fibroblast growth factors have been shown to promote angiogenesis in vivo. In this study, we employed a sustained-release polymer extravascular delivery system to evaluate the angiogenic efficacy of a novel form of genetically modified aFGF in the setting of chronic myocardial ischemia. Fifteen Yorkshire pigs subjected to Ameroid occluder placement on the left circumflex (LCX) artery were treated with perivascularly administered aFGF in ethylene vinyl acetate (EVAc) polymer (10 µg, n = 7) or EVAc alone (controls, n = 8). Seven to nine weeks later, after coronary angiography to document Ameroid-induced coronary occlusion, all animals underwent studies of coronary flow and global and regional left ventricular function. Microsphere-determined coronary flow in the Ameroid-compromised territory was significantly increased in aFGF-treated compared with control animals, and this improvement in perfusion was maintained during ventricular pacing. Left ventricular function studies demonstrated improved global and regional function in aFGF-treated animals. We conclude that local perivascular delivery of genetically modified aFGF results in significant improvement in myocardial flow and regional and global left ventricular function.

METHODS

Growth factor and delivery system preparation. A stabilized site-directed mutant of human aFGF, denoted Ser-117, in which Cys-117 was converted to a Ser residue by site-directed mutagenesis, was expressed in Escherichia coli and purified to apparent homogeneity by a method that eliminated detectable endotoxin (23, 29). Ethylene vinyl acetate (EVAc; Dupont, Wilmington, DE) matrices were made as specified (10, 11) by adding 1.5 g of EVAc dissolved in 0.5 g of porcine serum albumin to a lyophilized solution of 5.5 ml phosphate-buffered saline; 140 µg of human aFGF either with or without 140 µg of purified recombinant Ser-117 aFGF were then added to this solution. The mixture was poured into a precooled mold, allowed to harden, and then put under a 600-mTorr house vacuum at 20°C for 2 days. The procedure resulted in porous EVAc matrix containing a diffusely distributed growth factor. Sections of 160 mg of matrix containing ~14 µg of heparin with or without 14 µg of Ser-117 aFGF were cut and activated with sterile water before perivascular placement.

brain but is comparably abundant in heart (7, 32), where it is localized within cardiac myocytes (47). Embryonic cardiac myocytes also express FGF receptors that are required for proliferation in vivo (26) and that respond mitogenically to aFGF in vitro (31) but lose these receptors and FGF responsiveness as they terminally differentiate. In contrast, vascular endothelial cells from vessels throughout fetal and adult tissues, including the heart, retain FGF receptors and their responsiveness to aFGF in vitro. aFGF is a potent endothelial cell mitogen and chemotactic factor that is active in animal models of angiogenesis (see review, Ref. 42), dermal repair and capillary growth (25), and large vessel reendothelialization (5). Purified aFGF, however, has not been demonstrated to be efficacious in models of peripheral or coronary ischemia and, in fact, has been reported not to promote angiogenesis in ischemic dog myocardium (2). A possible explanation for this latter observation is that wild-type aFGF, which was delivered in the absence of heparin that is typically used to stabilize it, is rapidly inactivated in vivo. Therefore, to resolve whether aFGF is active in cardiac ischemia, we tested a genetically stabilized mutant of human aFGF bound to heparin in a clinically relevant porcine model. We report herein that this aFGF significantly enhanced coronary flow and improved cardiac function.

RECENT DISCOVERY and characterization of heparin-binding growth factors has led to a growing appreciation of their role in normal and pathological angiogenesis (14, 15, 43). Acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and their receptors have been isolated within the cardiovascular system and found to be upregulated in the setting of myocardial ischemia (7, 8, 12, 20). These findings have brought about interest in utilizing these agents to promote therapeutic angiogenesis.

The heparin-binding fibroblast growth factor (FGF) family is composed of 10 currently identified mitogens (20, 27, 42, 48) and 4 additional homologs of unknown function (39). aFGF (or FGF-1), one of the best characterized family members, was originally isolated from...
Evaluation of regional coronary flow. Colored microspheres (15-μm diameter; Triton Technology, San Diego, CA) were used to determine coronary flow at the time of final study at rest and during rapid (180 beats/min) ventricular pacing. To determine the extent of LCX territory, a set of colored microspheres was injected before the Ameroid constrictor placement with the LCX artery held transiently occluded. Thus all sections of the myocardium containing <10% of microspheres from this set at the time of final study were considered to belong to the LCX territory. For determination of coronary flow at the time of final study, a left atrial line was placed under direct vision and a set of microspheres (6 × 10^6) was forcefully injected after verification of catheter placement. Reference blood samples were withdrawn using a syringe pump at a constant rate of 4 ml/min through the femoral artery.

After the study was completed, the heart was excised and the left ventricle was dissected free of other structures, and then an 1-cm-thick transaxial slice was cut at the midventricular level. From this slice, eight radial samples were made as previously described (18). The tissue samples and the reference blood samples were digested with potassium hydroxide, microspheres were reclaimed using a vacuum filter, and the dyes from the microspheres were extracted using N, N-dimethylformamide. The dye samples were analyzed in a spectrophotometer (HP-8452A, Hewlett-Packard, Palo Alto, CA). From the optical density (OD) measurements, the myocardial flow was calculated as blood flow (tissue sample X; ml·min⁻¹·g⁻¹) = [withdrawal rate (ml/min)]/[weight (tissue sample X; g)] × [OD (tissue sample X)/OD (reference blood sample)].

Statistics. All data are expressed as means ± SD. A P value ≤ 0.05 was considered significant. Continuous variables including echocardiographic comparison of regional and global left ventricular function and regional coronary flow were compared using two-tailed t-tests. Serial data for the same group were compared using paired t-tests.

RESULTS

Study groups. All 15 animals survived the initial surgery. Of this total, one animal (aFGF group) did not demonstrate angiographic evidence of vessel occlusion and was excluded from data analysis. In addition, one animal in the aFGF group died after coronary angiography before coronary blood flow studies and echocardiography could be completed.

Implantation of heparin-alginate or EVAc pellets was not associated with any evidence of histologically apparent inflammatory response at the site of implantation. Furthermore, serial histological sections of the LCX and LAD coronary arteries did not show any neointimal formation or myocardial inflammatory reaction at the site of growth factor implantation (Fig. 1).

Coronary angiography documented LCX occlusion at the site of Ameroid constrictor implantation in all study animals. Placement of occluders resulted in small areas of akinetic myocardium in both aFGF and control groups. Echocardiographic determination of the size of these areas (defined as %akineti, or contractile, endocardial circumference from the short-axis plane) showed no significant differences between the groups (17.5 ± 2.6% for aFGF vs. 16.0 ± 2.7% for control, P = not
significant (NS)]. The size of the LCX perfusion area in all groups was determined at the time of Ameroid application by injection of a set of colored microspheres with the circumflex artery kept transiently occluded. Analysis of microsphere density at the time of death demonstrated similar LCX perfusion territories in both groups (data not shown).

There were no significant differences in hemodynamic state between the groups with regard to either blood pressure (122 ± 12.2/77.2 ± 10.0 for aFGF vs. 110.7 ± 6.0/73.3 ± 7.7 mmHg for control, P = NS) or heart rate (109 ± 8.3 for aFGF vs. 106.9 ± 8.9 beats/min for control, P = NS) at the time of the final study.

Assessment of regional myocardial flow. At the time of the final study, there was no significant difference in the LAD (nonischemic) territory blood flow between the two groups of animals (coronary blood flow: 0.79 ± 0.15 for aFGF vs. 0.80 ± 0.24 ml·min⁻¹·g⁻¹ for control, P = NS). However, coronary flow in the LCX territory was significantly higher in the aFGF-treated than in control animals both at rest (coronary blood flow: 0.71 ± 0.05 for aFGF vs. 0.49 ± 0.06 ml·min⁻¹·g⁻¹ for control, P = 0.001) and during rapid (180 beats/min) pacing (coronary blood flow: 0.94 ± 0.17 for aFGF vs. 0.58 ± 0.22 ml·min⁻¹·g⁻¹ for control, P = 0.01). Furthermore, pacing resulted in a significant increase in coronary flow in the LCX territory in aFGF-treated but not control animals (Fig. 2).

Echocardiographic evaluation of regional and global myocardial function. Two-dimensional and M-mode echocardiography were used to measure left ventricular global (Fig. 3) and regional function (Fig. 4) in open-chest animals. Treatment with aFGF resulted in highly significant improvement of global ejection fraction measured both at rest (P = 0.003) and during pacing (P = 0.002). Likewise, aFGF treatment also resulted in significant preservation of regional LCX myocardial function during pacing (P = 0.02 vs. control).
DISCUSSION

Ischemia is characterized by inadequate blood flow and tissue oxygenation that is typically the consequence of decreased patency of atherosclerotic vessels. Clinical strategies to increase blood flow in ischemic peripheral and cardiac muscle have largely depended on vasodilation, angioplasty, and surgical revascularization. The uses of these approaches are restricted by the physiological limit of vasodilation and its side effects, the inaccessibility of angioplasty sites, and the limitations of surgery including graft failure and the absence of healthy patent vessels for autologous vascular transplants. Improvement in blood flow and muscle function can also be achieved by neovascularization, or angiogenesis, providing new collateral vessels.

The therapeutic use of angiogenic growth factors has been the subject of intensive investigation over the last several years (for review, see Ref. 46) and has demonstrated promise as a potential modality to treat both acute and chronic myocardial ischemia. Thus a number of studies have shown that bFGF, whether delivered systemically at high dose or locally at lower doses, results in improvement in collateral vessel flow, histological evidence of new vessel formation, and improvements in myocardial function in models of both acute and chronic myocardial or peripheral limb ischemia (1, 4, 18, 22, 44). Similarly, administration of vascular endothelial growth factor (VEGF) (3, 17, 19, 41) and FGF-5 (16) has been reported to produce functionally meaningful angiogenesis. However, unlike these growth factors, there are limited data regarding aFGF angiogenic efficacy in myocardial ischemia. Acidic FGF delivery via a soaked sponge placed between an internal mammary artery and the LAD myocardium (2) failed to demonstrate angiographic evidence of new vessel formation, and although aFGF delivery using fibrin glue applied between the aorta and the heart produced evidence of local extramyocardial collateral formation, it did not demonstrate any physiological significance of these collaterals (13, 33). It remains unclear, however, whether this lack of angiogenic effect was due to lack of ischemic stimulus, inadequate method of local drug delivery, rapid degradation of aFGF in vivo, or inherent failure of aFGF to promote angiogenesis in the myocardium.

In this study we found that local EVAc-based delivery of a genetically modified form of aFGF resulted in significant improvement in resting collateral blood flow that was maintained during rapid pacing with parallel improvements in global and regional left ventricular function. Improvements in flow both during rest and pacing were previously seen with VEGF (17), but only during pacing with bFGF (18). Flow during pacing reveals functional reserve vascular capacity that, when decreased, can contribute to exercise-related pain expe-
tution of this single Cys residue increases the activity
published observation) or mitogenic potency (29) and,
either the conformational unfolding temperature (un-
Cys residue unique to human aFGF does not alter
single sulfur-to-oxygen atom substitution in the one
(6) with an isomorphous Ser residue (Ser-117). This
culture, with a half-life of only 15 min (29). The
potency or even complete inactivation. In the absence of
heparin or other equivalent polyanions, wild-type hu-
angiogenesis assays (42), its reported lack of angiogenic
comprehensive FGF receptor subtype binding exhib-
activities exhibited by bFGF and FGF-5 would also be
work efficiently through both forms (28). Therefore,
function during both rest and pacing.
These activities might be largely attributable to
neovascularization induced by aFGF, because other
angiogenic proteins have also been reported to enhance
and flow in models of ischemia. Endothelial
cells within ischemic cardiac muscle can become selec-
tively responsive to angiogenic mitogens. Hypoxic cap-
illary endothelial cells exhibit threefold-enhanced expres-
ion of high-affinity FGF receptors in culture along
with an increased mitogenic and chemotactic respons-
siveness to bFGF (37). aFGF functions efficiently
through all seven ligand-selective FGF receptor sub-
types generated by alternative splicing of the four
known FGF receptor genes (37). bFGF and FGF-5
appear to act principally through subsets of five and
two of these receptor subtypes, respectively (28). Expres-
sion of mRNA encoding one or both alternatively spliced
versions of FGF-1 is increased 2.4-fold in ischemic
porcine myocardium (36). FGF-5 functions through one
of the forms of this receptor, whereas bFGF and aFGF
work efficiently through both forms (28). Therefore,
activities exhibited by bFGF and FGF-5 would also be
expected to be exhibited by aFGF. In light of the
comprehensive FGF receptor subtype binding exhib-
ited by FGF along with its activity in several in vivo
angiogenesis assays (42), its reported lack of angiogenic
activity in ischemic dog myocardium as discussed above
is surprising.
The instability of aFGF, especially in the absence of
heparin to which it binds (29), can lead to diminished
potency or even complete inactivation. In the absence of
heparin or other equivalent polyanions, wild-type hu-
man aFGF unfolds at or slightly below physiological
temperature (45) and inactivates at 37°C in tissue
culture, with a half-life of only 15 min (29). The
stabilized mutant used in this study was generated (23)
by replacement of one of the three buried Cys residues
(6) with an isomorphous Ser residue (Ser-117). This
single sulfur-to-oxygen atom substitution in the one
Cys residue unique to human aFGF does not alter
either the conformational unfolding temperature (un-
published observation) or mitogenic potency (29) and,
because of its lack of exposure, should not present a
distinct immunologic surface epitope. However, substi-
tution of this single Cys residue increases the activity
half-life to 1.4 h (29), apparently reflecting a dimin-
ished rate of formation of inactivating disulfide bonds
(unpublished observations) promoted by air oxidation
(29) and, perhaps, catalyzed by trace metals (23).
Wild-type aFGF binds tightly to heparin, which
increases its thermal stability to ~60°C (45) and its
mitogenic half-life at 37°C by nearly 100-fold to 24 h
(29). However, even a 1-day half-life can correspond to
nearly complete loss of activity well before the end of
long-duration slow-release dosing. In the presence of
heparin, the Ser-117 mutant exhibits a 10-fold greater
activity half-life of 240 h (29).
To deliver aFGF, we employed EVAc matrices. This
choice was dictated by the relatively lesser affinity of
aFGF for heparin-alginate than that of bFGF and the
lesser stability of aFGF protein. EVAc matrices have
previously been used to deliver a number of biologically
active materials including heparin (10) and antisense
oligonucleotides (11). These studies have demonstrated
that the copolymer is stable at body temperature and
produces no untoward effects at the site of implanta-
ion. Indeed, in this study we have not observed any
inflammatory reaction at sites of polymer placement.
The previously reported (2) lack of efficacy of aFGF in
a dog model of cardiac ischemia might have been either
a species-related effect, a consequence of the method
of delivery, or the result of relatively rapid inactivation of
wild-type aFGF, especially in the absence of heparin.
The current observation of the efficacy of a stabilized
form of aFGF in the presence of heparin in a clinically
relevant porcine model of cardiac ischemia is consistent
with the known angiogenic activity of the wild-type
protein. Moreover, whatever the reason for the lack of
efficacy in this previous study, treatment with stabi-
ized aFGF clearly results in therapeutically significant
improvements in blood flow, myocardial perfusion, and
function in cardiac ischemia.
Several issues need to be considered in evaluating
the results of the present study. Although all animals
had completely occluded LCX, we cannot rule out the
possibility that aFGF may have influenced the rate of
Ameroid closure compared with that in control ani-
mals. This possibility is potentially relevant given
known, aforementioned vasoactive properties of FGF
and their ability to induce vasodilation in coronary bed
as well as their potential cardioprotective activity. Thus
growth factor-mediated delay in the time of Ameroid
closure may have influenced results in the treatment
groups. In addition, in contrast to previous studies (17,
18) employing bFGF that have shown improvement in
stress-induced flow in growth factor-treated animals,
we have observed improved flow in the compromised
territory in aFGF-treated animals both at rest and
during rapid pacing. This improvement in rest coro-
nary flow may be secondary to superior angiogenic
qualities of aFGF or may be an unintended conse-
quence of the relatively small numbers of animals in
the study.

This work was supported in part by American Heart Association
Massachusetts Affiliate Grant 501-912 (to F. W. Sellke); National
Institutes of Health Grants HL-46716 (to F. W. Sellke), HL-53793 (to


M. Simons, and GM-40039 (to E. R. Edelman); the Whittaker Foundation; and the Burroughs-Wellcome Fund in Experimental Therapeutics (to E. R. Edelman). J. J. Lopez and M. Simons were also supported by the Clinical Investigator Training Program, Beth Israel Deaconess Medical Center-Harvard/Massachusetts Institute of Technology Health Science and Technology, Boston, MA, in collaboration with Pfizer, Inc., Groton, CT.

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Received 9 July 1997; accepted in final form 13 November 1997.

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