CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

MODULATION OF MYOCARDIAL PERFUSION AND VASCULAR REACTIVITY BY PERICARDIAL BASIC FIBROBLAST GROWTH FACTOR: INSIGHT INTO ISCHEMIA-INDUCED REDUCTION IN ENDOTHELIUM-DEPENDENT VASODILATATION

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Objectives: The present study was designed to study the effects of a single intrapericardial injection of basic fibroblast growth factor on myocardial vascular resistance and endothelium-dependent microvascular dilatation in a porcine model of chronic myocardial ischemia and to investigate the mechanism of ischemia-induced impairment of endothelium-dependent vasodilatation. **Methods:** Yorkshire pigs underwent ameroid constrictor placement on the left circumflex coronary artery. At 3 weeks, animals were randomized to a single intrapericardial injection of saline solution (n = 10), 30 µg basic fibroblast growth factor (n = 10), or 2 mg basic fibroblast growth factor (n = 10). Myocardial vascular resistance in the normal (left anterior descending) and ischemic collateral-dependent (left circumflex artery) territories (using colored microspheres) and microvascular reactivity to adenosine diphosphate and sodium nitroprusside were measured before treatment and 4 weeks after treatment. The expression of inducible and endothelial nitric oxide synthase was determined in normal and ischemic myocardium by means of reverse transcriptase-polymerase chain reaction and Western analysis, and the effect of nitric oxide on endothelium-dependent vasodilatation was determined. **Results:** Compared with results in the control group, treatment with basic fibroblast growth factor resulted in significant improvement in left circumflex artery resistance and endothelium-dependent vasodilatation, reflecting increased collaterals. Myocardial ischemia was associated with increased expression of inducible nitric oxide synthase with no change in endothelial nitric oxide synthase. However, the nitric oxide donor sodium nitroprusside did not affect endothelium-dependent vasodilatation to adenosine diphosphate. **Conclusions:** A single intrapericardial bolus of basic fibroblast growth factor may be a useful therapeutic strategy for the treatment of myocardial ischemia in patients with coronary artery disease. Although chronic myocardial ischemia is associated with increased expression of inducible nitric oxide synthase, it does not appear to be the cause of altered endothelial function. (J Thorac Cardiovasc Surg 1998;116:1022-8)

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Therapeutic angiogenesis is a novel approach to the treatment of myocardial ischemia based on the use of proangiogenic growth factors to induce the growth and development of new blood vessels. Angiogenesis is a complex process involving endothelial and smooth muscle cell proliferation and migration, formation of new capillaries, breakdown of existing extracellular matrix, and deposition of new extracellular matrix.¹ We and others have shown that various heparin-binding growth factors, including basic fibroblast growth factor (bFGF),²⁻⁸ acidic fibroblast growth factor, and vascular endothelial growth factor (VEGF),^{2,9-12} induce functionally significant angiogenesis in chronic myocardial ischemia. Given the typically long time course of new collateral vessel development, most attempts to stimulate angiogenesis have used various methods of prolonged growth factor delivery, including gene therapy, continuous infusions, repeated injections, or sustained-release polymers to provide extended exposure to the desired growth factor.^{2-5,7,9,12-15}

Chronic myocardial ischemia is associated with reduced endothelium-dependent microvascular dilatation, which may be restored after growth factor therapy that results in improved perfusion of the ischemic zone.¹⁶⁻²³ With growth factor treatment or reperfusion, endothelium-dependent microvascular dilatation parallels various other measures of angiogenesis, including angiographic collaterals, microsphere flow, myocardial vascularity by morphometric analysis, and restoration of myocardial function.¹⁹⁻²⁵ This recovery of endothelium-dependent vasodilation may, in addition, reflect recovery of endothelial function. Endothelium-independent microvascular relaxation, however, is preserved even in the presence of severe chronic myocardial ischemia.^{16,19,20,26} The purpose of the present study was to determine the effects of a single intrapericardial injection of bFGF in a porcine model of chronic myocardial ischemia and to further investigate the underlying mechanisms of the impairment of endothelium-dependent microvascular dilatation in chronic myocardial ischemia. An *in vitro* approach was used to study vascular responses of coronary microvessels to eliminate metabolic, autoregulatory, and other extravascular influences.

Methods

Chronic myocardial ischemia model. Yorkshire pigs of either sex weighing 15 to 18 kg were anesthetized with intramuscular ketamine (10 mg/kg) and inhalational halothane. By sterile technique, a right popliteal cutdown was performed and a 4F arterial catheter was inserted for blood sampling and pressure monitoring. A left thoracotomy was performed

through the fourth intercostal space during mechanical ventilation. The pericardium was opened, and an ameroid constrictor of 2.5- to 2.75-mm internal diameter (matched to the diameter of the artery) was placed around the proximal circumflex (LCX) coronary artery.^{4,7,9} The pericardium was closed with 6-0 Prolene suture (Ethicon, Inc, Somerville, NJ), and the chest was closed. A single dose of intravenous cefazolin (70 mg/kg) was given and intramuscular narcotic analgesics were administered as needed. Animals were then allowed to recover for 3 weeks (time sufficient for ameroid closure) before growth factor delivery. Animals were treated according to National Institutes of Health guidelines and the protocol was approved by the Institutional Animal Care and Utilization Committee of the Beth Israel Deaconess Medical Center.

Growth factor delivery. Three weeks after ameroid placement, animals were anesthetized with intramuscular ketamine (10 mg/kg) and inhalational halothane. By sterile technique, a right femoral cutdown was performed and an 8F arterial sheath was inserted for blood sampling, pressure monitoring, and left heart catheterization. Coronary angiography was then performed in multiple views with the use of a 7F JR4 diagnostic catheter (Cordis Corporation, Opa Locka, Fla) to confirm the status of the LCX. After LCX occlusion was documented, percutaneous subxiphoid pericardial access was undertaken.

The animals were then randomized to three treatment groups: (1) control: intrapericardial saline solution ($n = 10$); (2) bFGF 30 μ g: intrapericardial bFGF ($n = 10$); and (3) bFGF 2 mg: intrapericardial bFGF ($n = 10$).

Heparin (3 mg) was used in the vehicle for fibroblast growth factor administration. At the end of the procedure, a set of colored microspheres (blue) was injected into the left atrium (via retrograde cannulation of the left atrium) to obtain baseline (pre-treatment) myocardial vascular resistance. The animals were then allowed to recover for 4 weeks.

Final study. Four weeks after intrapericardial treatment, all animals underwent final evaluation. Pigs were anesthetized with intramuscular ketamine (10 mg/kg) and inhalational halothane. By sterile technique, a left femoral cutdown was performed and an 8F arterial sheath was inserted for blood sampling, pressure monitoring, and left heart catheterization. Myocardial vascular resistance at rest was determined with colored microspheres (yellow). Animals were then put to death while anesthetized and the heart was obtained for further analysis.

Coronary vascular resistance. Colored microspheres spheres (15 ± 0.1 μ m diameter, Triton Technology Inc, San Diego, Calif) were used to determine coronary vascular resistance before the start of treatment (blue) and at the time of final study. For determination of coronary vascular resistance 3 and 7 weeks after ameroid placement, an angiographic catheter was advanced into the left atrium through the mitral valve. Catheter position was verified by injection of contrast material into the left atrium. In addition, mean left atrial pressure was recorded. A set of microspheres (6×10^6) was diluted in 10 mL of saline solution and injected into the left atrium over 30 seconds. Reference blood samples were withdrawn

Fig 1. Myocardial vascular resistance in the normal (LAD) and collateral-dependent (LCX) territories at 3 weeks. Four weeks after intrapericardial drug delivery, vascular resistance in the collateral-dependent LCX territory was lower in bFGF-treated animals than in control animals. Data are shown as mean $\pm$ standard deviation. *Statistical significance ($P < .0017$) compared with LAD at 3 weeks and LCX final compared with LCX at 3 weeks.

with the use of a syringe pump at a constant rate of 5 mL/min through the femoral artery. After study completion, the heart was excised and regional myocardial vascular resistance was determined by means of the following formula:

$$\text{Resistance (mm Hg/mL/min/gm)} = \frac{\text{MAP}}{(\text{Sample AU} \times \text{Withdrawal rate}) / (\text{Reference AU} \times \text{sample weight})}$$

where MAP is mean arterial pressure and AU is optical absorbance units.

Microvessel analysis. Coronary arterial microvessels (60–180 μm in internal diameter) were dissected from the subepicardial region of the LAD (normal) and collateral-dependent LCX (ischemic) territories. Microvessels were placed in a Plexiglas acrylic microvessel chamber (University of Iowa Medical Instruments, Iowa City, Iowa), cannulated with dual glass micropipettes measuring 30 to 80 μm in diameter, and secured with 10-0 nylon monofilament suture (Ethicon, Somerville, NJ). Oxygenated (95% oxygen, 5% carbon dioxide) Krebs buffer solution warmed to 37°C was continuously circulated through the microvessel chamber with a reservoir containing 100 mL. The vessels were pressurized to 40 mm Hg in a no-flow state by means of a biuret manometer filled with Krebs buffer solution.^{19–25,27} With an inverted microscope (40 $\times$ –200 $\times$, Olympus, Tokyo, Japan) connected to a video camera, the vessel image was projected onto a black and white television monitor (Hitachi Medical Corp, Tokyo, Japan). An electronic dimension analyzer (Living System Instrumentation, Burlington, Vt) was used to measure internal lumen diameter. Measurements were recorded with a strip-chart recorder (Gravtec, Irvine, Calif). Vessels were allowed to equilibrate for 30 minutes in Krebs buffer solution

before an intervention and for 15 minutes between applications of each drug. Microvessels were precontracted by 30% to 60% of baseline with acetylcholine (10^{-7} to 10^{-6} mol/L). Microvascular responses to adenosine diphosphate (ADP, an endothelium-dependent vasodilator) and sodium nitroprusside (SNP, an endothelium-independent vasodilator) were recorded. In addition, to investigate the potential effect of basal nitric oxide (NO) production on endothelium-dependent vasodilation, we measured the response of microvessels to ADP in the presence of different concentrations of SNP (10^{-6} and 10^{-7} mol/L) that would be associated with physiologic concentrations of NO.

Endothelial and inducible nitric oxide synthase expression. Endothelial (eNOS) and inducible nitric oxide synthase (iNOS) expression were assessed in the normal and ischemic myocardium to explore their role in the reduced endothelium-dependent vasodilation seen with ischemia.

Reverse transcriptase–polymerase chain reaction. RNA was isolated from normal (LAD) and ischemic (LCX) myocardium by means of a commercially available kit (Trizol kit, Life Technologies, Rockville, Md). RNA (0.5 μg per reaction) was reverse transcribed with 20 U of Moloney murine leukemia virus reverse transcriptase in 50 mmol/L Tris-HCl (pH 8.3), 3 mmol/L MgCl_2 , 75 mmol/L KCl, 1 mmol/L deoxynucleotide triphosphates, and 0.05 nmol 3' primer. The reaction was carried out at 37°C for 1 hour and then stopped by heating the mixture to 70°C for 15 minutes to destroy reverse transcriptase activity before the polymerase chain reaction. Specific primers for eNOS and iNOS were designed on the basis of previously published sequences (eNOS: 5' [sense] primer: 5'-GCCTCGCTCTGAAAGA-3', 3' [anti-

Fig 2. Endothelium-independent (SNP, **A**) and endothelium-dependent (ADP, **B**) vasodilation in the normal (LAD) myocardium. Responses are expressed as percent relaxation of acetylcholine-induced precontraction.

Fig 3. Endothelium-independent (SNP, **A**) and endothelium-dependent (ADP, **B**) vasodilation in the collateral-dependent LCX myocardium showing preserved response to SNP with impaired response to ADP, restored by bFGF treatment. Responses are expressed as percent relaxation of acetylcholine-induced precontraction.

sense] primer: 5'-TAAAGGTCTTCTTCTGGTGATGCC-3'-iNOS 5' [sense] primer: 5'-GCCTCGCTCTGGAAAGA-3',3' [antisense] primer: 5'-TCCATGCAGACAACCTT-3'). Complementary DNA (cDNA) 5 μ L was amplified for 35 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute (melting, annealing, and extension temperatures, respectively). Calibration was performed by co-amplification of the same cDNA samples with primers for glyceraldehyde-3-phosphate dehydrogenase as internal standard (5' [sense] primer: 5'-CCATGGAGGAAGGCTGGGG-3' and 3' [antisense] primer: 5'-CAAAGTTGTCATGGATGACC-3'). After amplification, polymerase chain reaction mixtures were electrophoresed on 1.5% agarose gel containing ethidium bromide, and polymerase chain reaction products were visualized on an ultraviolet transilluminator and photographed. The images were scanned and analyzed with the Molecular Dynamics densitometric quantitative software (Molecular Dynamics, Sunnyvale, Calif).

Western analysis. Proteins were extracted with a buffer containing Tris-HCl (50 mmol/L, pH 7.5), NaCl (0.15 mol/L), ethyleneglycoltetraacetic acid (0.1 mmol/L), ethylenediaminetetraacetic acid (0.1 mmol/L), Nonidet P-40 (1%, v/v), β -mercaptoethanol (0.1%, vol/vol), and protease in-

hibitors (10 μ g/mL leupeptin, 10 μ g/mL pepstatin A, 10 μ g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Proteins were solubilized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with the use of 8% polyacrylamide gels, and the proteins were then transferred to a nitrocellulose membrane overnight at 4°C. After being blocked, membranes were incubated for 1 hour with the NOS-specific antibody (eNOS: polyclonal [rabbit] anti-eNOS antibody; iNOS: polyclonal [rabbit] anti-iNOS antibody (Affinity BioReagents, Inc, Golden, Colo) at room temperature. Membranes were then washed 3 times in TBS-0.1% Tween 20 and incubated with a horseradish-peroxidase-conjugated anti-rabbit immunoglobulin G. Immuno-reactive protein bands were visualized by means of the enhanced chemiluminescence system (Amersham Corp, Arlington Heights, Ill). Equal protein loading was then confirmed by membrane coumassie staining.

Statistical analysis. Data are expressed as mean $\pm$ standard deviation. Vascular responses are expressed as percent relaxation of the acetylcholine-induced contraction. Continuous variables were compared by unpaired or paired Student *t* test and analysis of variance (Bonferroni-corrected *t* tests were used for multiple group comparisons).

Fig 4. Expression of iNOS and eNOS in ischemic and normal myocardium by reverse transcriptase–polymerase chain reaction (RT-PCR) and Western analysis.

Results

Thirty animals had placement of an ameroid occluder over the LCX coronary artery, randomized to each of the 3 treatment groups with 10 animals in each group. No significant hemodynamic effect of intrapericardial bFGF administration was noted, with no change in blood pressure, heart rate, or left atrial pressure.

Myocardial vascular resistance. To evaluate the angiogenic potential of intrapericardial bFGF in chronic myocardial ischemia, we measured regional myocardial vascular resistance at different time points using colored microspheres. Three weeks after implantation of ameroid occluders, at the time of intrapericardial drug delivery, myocardial vascular resistance in the collateral-dependent LCX territory was similar in all treatment groups (Fig 1, $P = .8$) and was significantly higher than resistance in the LAD territory (LCX resistance: 92.84 ± 34.9 mm Hg/mL per minute per gram; LAD resistance: 64.84 ± 19.5 mm Hg/mL per minute per gram; $P < .0001$). Four weeks after intrapericardial drug delivery, LCX resistance was significantly lower in bFGF-treated animals than in control animals [Fig 1, LCX vascular resistance 82.54 ± 16.4 mm Hg/mL per minute per gram in control animals vs 72.24 ± 13.8 mm Hg/mL per minute per gram in the 30- μ g bFGF group [$P = .08$] and 67.84 ± 20.0 and 1.25 ± 0.15 mm Hg/mL per minute per gram in the 2-mg bFGF group [$P = .03$]].

Microvessel analysis. Coronary arterial microvessels were studied for response to ADP (endothelium-dependent vasodilator) and SNP (endothelium-independent vasodilator). Similar concentrations of acetylcholine were required in LCX and LAD microvessels to attain similar ($45\% \pm 5\%$ of baseline) degrees of precontraction values. Microvascular response to SNP was similar in the LAD and LCX territories for all groups (Figs 2 and 3). However, endothelium-dependent (ADP) vasodilation was normal in the LAD (Fig 2) but not LCX (Fig

Fig 5. Endothelium-dependent vasodilation (ADP) was not affected by the presence of 2 different concentrations (10^{-6} and 10^{-7} mol/L) of SNP, an NO donor. Responses are expressed as percent relaxation of acetylcholine-induced precontraction.

3) distribution in control animals, indicating dysfunctional endothelium in the ischemic zone. Administration of bFGF 30 μ g (analysis of variance, $P = .03$) and bFGF 2 mg ($P = .0005$) improved endothelium-dependent vasodilation in the LCX epicardium with almost normal relaxation in the 2-mg bFGF group (Fig 3).

Endothelium-dependent vasodilation is NO-dependent and is inhibited by *N*^w-nitro-L-arginine methyl ester.^{19-22,24} To investigate the mechanism of ischemia-induced impairment of endothelium-dependent relaxation, we studied the expression of iNOS and eNOS in normal and ischemic myocardium. By Western analysis, the ischemic myocardium had a 2.74 ± 0.7 -fold increase in the level of iNOS protein over normal myocardium, with no change in eNOS level (Fig 4). The expression of iNOS mRNA was also increased in ischemic myocardium (2.3 ± 0.6 times) as compared with normal myocardium according to reverse transcriptase–polymerase chain reaction. However, eNOS mRNA level was unchanged. To determine whether the increased iNOS expression in ischemic tissue and the resultant increase in basal NO production may inhibit eNOS activity²⁸ and reduce endothelium-dependent vasodilation, we measured the microvascular response to ADP in the presence of 2 different concentrations (10^{-6} and 10^{-7} mol/L) of SNP, an NO donor (Fig 5). SNP did not have any significant effect on the response of microvessels to ADP, nor did these concentrations affect the response of vessels to additional SNP if the degree of precontraction was normalized.

Discussion

Previous studies have demonstrated that administration of bFGF^{3-8,29,30} or VEGF^{2,9-12} results in significant

angiogenesis in several models of myocardial ischemia. However, most attempts to stimulate angiogenesis have used various methods of prolonged growth factor delivery, including gene therapy, continuous infusions, repeated injections, or sustained-release polymers to provide extended exposure to the desired cytokine. The applicability of these measures to patients is limited.

Intrapericardial bFGF treatment resulted in improvement (reduction) in myocardial vascular resistance, which is collateral dependent in the ameroid constrictor model with an occluded LCX. This reduction in vascular resistance reflects growth of new collaterals. In addition, intrapericardial bFGF restored endothelium-dependent microvascular vasodilatation. These effects are similar to the previously reported perivascular sustained-release bFGF and VEGF effects^{4,21,22} in this animal model.

The present study demonstrates a reduction in myocardial vascular resistance in the ischemic collateral-dependent LCX distribution and normalization of LCX endothelium-dependent microvascular relaxation in a model of chronic myocardial ischemia in which a single intrapericardial injection of bFGF was used. We have previously shown that bFGF (10-100 µg) incorporated into heparin-alginate microspheres (for sustained delivery) and implanted on the epicardial surface of the occluded LCX results in significant improvement in myocardial perfusion and endothelium-dependent vasodilation in the setting of chronic myocardial ischemia.^{4,30} This relatively invasive delivery method, however, may not be applicable to the majority of patients with coronary artery disease. Thus the single intrapericardial approach is a more attractive strategy for therapeutic angiogenesis.

Because endothelium-dependent vasodilatation is an NO-dependent mechanism, we studied the expression of 2 isoforms of NOS in normal and ischemic myocardium. Myocardial ischemia results in increased expression of iNOS protein and mRNA without any change in eNOS expression. Therefore ischemia-induced impairment of endothelium-dependent vasodilatation cannot be attributed to changes in the expression of eNOS in ischemic tissue. The increased expression of iNOS may result in increased basal NO production, which has been shown to inhibit partially purified eNOS.²⁸ However, endothelium-dependent vasodilatation was not affected by SNP (an NO donor), negating this hypothesis. Therefore the mechanism of ischemia-induced impairment of endothelium-dependent vasodilatation may be unrelated to the increased expression of iNOS. Intrapericardial bFGF restores endothelium-dependent vasodilatation, which may

indicate restoration of normal endothelial function owing to restoration of normal myocardial blood flow, coronary perfusion pressure, or other mechanisms not examined in this study.

Conclusion

We conclude that a single intrapericardial injection of bFGF in a porcine model of chronic myocardial ischemia results in an improvement in myocardial vascular resistance (reflecting increased collateralization of the ischemic myocardium) and endothelium-dependent microvascular dilatation. Thus single-bolus intrapericardial bFGF administration may be a useful therapeutic strategy for the treatment of myocardial ischemia in patients with coronary artery disease. Although chronic myocardial ischemia is associated with increased expression of iNOS, its relationship to impaired endothelium-dependent relaxation is uncertain.

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Discussion

Dr Edward D. Verrier (*Seattle, Wash*). This is an important study, because so many of the studies with angiogenic factors show proliferation of small vessels, but the relationship between anatomic changes showing angiogenesis and the functional recovery of muscle have been lacking. Combining that information with the ease of administration of this growth factor into the pericardium makes this study particularly important.

Dr Verdi J. DiSesa (*Chicago, Ill*). You have obviously done a lot of nice work. Unfortunately, you tried to present a great deal of data very quickly, and I found myself having difficulty being convinced of an argument I wanted to believe. You did not define collateral index. You did not explain how you measured blood flow in the LCX distribution. Those points are crucial to what Dr Verrier was just saying, that is, demonstrating functional importance of these new capillaries that you hypothesize the growth factor was producing.

Dr Laham. The collateral index has been published previously and is a measure of the angiographic distribution and the angiographic filling of collaterals. Colored microspheres are used to determine flow, as well as resistance. The functional improvement and perfusion are determined by magnetic resonance imaging. All of these methods have been previously published extensively in the literature.

Dr Jacob Kolff (*Johnstown, Pa*). I would like to use angiotensin somehow or see what effect it would have. What if our philosophy is that the act of producing ischemia already maximally stimulates neoangiogenesis? Perhaps that can be stopped by applying some kind of an antivasculature or neovascularization drug or enzyme. That way you would stop the collateralization, and perhaps you could show that these factors are in fact important if they are on the positive side of producing this neovascularization.

Dr Laham. It does seem from all our previous work that supplementation of these growth factors is necessary in most of these studies. It is probable that the ischemic response of the normal myocardium produces bFGF and VEGF. This has been demonstrated, but not to an extent that is sufficient for the growth of collaterals that would lead to significant improvement in function. The supplementation of these growth factors has been extensively studied in a sustained-release form. This is now an established technique, and phase 1 and phase 2 clinical trials in patients are now being conducted. The preliminary results are very promising. However, this study was designed to evaluate a single intrapericardial delivery, which was shown to be comparable with the sustained-release formulations that we and others had previously used. That is really the novelty of the study.