

Inhibition of the Cardiac Angiogenic Response to Surgical FGF-2 Therapy in a Swine Endothelial Dysfunction Model

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Background—Discrepancy exists between the potent effects of therapeutic angiogenesis in laboratory animals and the marginal results observed in patients with advanced coronary artery disease. In vitro and small animal data suggest that angiogenesis may depend on locally available nitric oxide (NO), but the impact of endothelial dysfunction on therapeutic angiogenesis in the myocardium has been unclear. We compared the effects of clinically applicable angiogenesis methods in swine in which endothelial dysfunction was experimentally induced to that observed in normal swine.

Methods and Results—Miniswine were fed either a regular (N=13) or hypercholesterolemic diet (N=13) for 20 weeks. Hypercholesterolemic swine showed coronary endothelial dysfunction on videomicroscopy. Animals from both groups received 100 μ g of perivascular sustained-release fibroblast growth factor (FGF)-2 in the lateral myocardial territory, previously made ischemic by placement of an ameroid constrictor around the circumflex artery. After 4 weeks of FGF-2 therapy, lateral myocardial perfusion was significantly lower in hypercholesterolemic than in normocholesterolemic swine, both at rest and during pacing (0.44 ± 0.04 versus 0.81 ± 0.15 mL/min/g at rest, respectively; $P=0.006$; and 0.50 ± 0.06 versus 0.71 ± 0.10 mL/min/g during pacing; $P=0.02$). Hypercholesterolemic swine showed no net increase in perfusion from FGF-2 treatment. Endothelial cell density and FGF receptor-1 expression were significantly lower in the lateral territory of hypercholesterolemic versus normocholesterolemic animals.

Conclusions—The cardiac angiogenic response to FGF-2 treatment using clinically applicable methods was markedly inhibited in hypercholesterolemic swine with coronary endothelial dysfunction. These findings suggest that coronary endothelial dysfunction is major obstacle to the efficacy of clinical angiogenesis protocols and constitutes a target toward making angiogenesis more effective in patients with advanced coronary disease. (*Circulation*. 2003;108[suppl II]:II-335-II-340.)

Key Words: angiogenesis ■ coronary disease ■ endothelium-derived factors

Therapeutic angiogenesis is a promising modality for patients with advanced, diffuse coronary artery disease (CAD) who have failed or who are not eligible for conventional revascularization interventions, such as coronary angioplasty, stenting, or bypass grafting. Angiogenesis is a physiologically potent process involved in growth and development and that may one day translate into a first-line approach for the treatment of chronic ischemic heart disease.¹ Despite a large number of studies demonstrating impressive effects of therapeutic angiogenesis in animals, results of clinical trials have shown modest if any benefit,¹⁻³ and two large phase II/III trials have been completely negative.^{4,5}

Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast-growth factor (FGF)-2 operate through the release of endothelial-derived nitric oxide (NO) via the activation of tyrosine kinase receptors.⁶ Patients with advanced CAD have significant endothelial dysfunction

and diminished coronary NO release, which constitute key pathologic features of their disease.⁷ Although in vitro data and murine studies in peripheral organs have indicated that the effects of VEGF and FGF-2 may depend on local NO availability,^{8,9} the impact of endothelial dysfunction on angiogenesis in the heart or using clinically relevant methods has not been elucidated. We examined these issues by comparing the effects of surgical angiogenic therapy using clinically applicable methods in a hypercholesterolemic swine model of endothelial dysfunction to that observed in swine fed a normal diet and concomitantly undergoing the same experimental regimen.

Materials and Methods

General Experimental Sequence

Twenty-six Yucatan miniswine of either sex (Charles River Laboratories, Cambridge, MA) were used for the studies. After weaning

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at 5 weeks of age, animals were randomized to be fed either a hypercholesterolemic diet (Purina Modified Mini-Pig Grower Diet 5081, Richmond, IN) composed of 20% lard, 4% cholesterol, and 1.5% sodium cholate (HICHOL group; N=13) or a regular pig chow (NORMAL group; N=13) for the duration of the study (total 20 weeks).

The studies involved three separate procedures on each animal. With the exception of their diet, HICHOL and NORMAL, swine underwent the same experimental sequence. Anesthesia was performed as reported previously,¹⁰ and all animals received humane care in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and the National Research Council's *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animals and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985).

The first procedure, performed via small left anterolateral thoracotomy at 18 weeks of age, consisted in the placement of a 1.75-mm ameroid constrictor around the proximal circumflex artery and the injection of 1.5×10^7 red microspheres into the left atrium during temporary circumflex coronary occlusion, to subsequently allow for identification, by shadow labeling, of the myocardial territory at risk.¹¹

The second procedure, also performed via left anterolateral thoracotomy 3 weeks after ameroid placement, consisted of the implantation of 10 sterile heparin-alginate sustained-release beads each containing 10 μg of FGF-2 (Chiron, Emeryville, CA) in the subepicardium and myocardium surrounding the proximal and mid-circumflex coronary artery.¹¹ Concomitantly, 1.5×10^7 black microspheres were injected in the left atrium during rest conditions, to allow for determination of baseline perfusion after ameroid closure and prior to the effects of FGF-2.

The third procedure was carried out at 25 weeks of age (4 weeks after FGF-2 implantation and 7 weeks after ameroid placement). Sternotomy was performed, 1.5×10^7 yellow microspheres were injected into the left atrium during rest conditions, and 1.5×10^7 orange microspheres were injected during pacing. Euthanasia was then performed with 10 mL/kg of a saturated KCl solution administered intravenously. Cardiac samples were harvested and snap frozen for molecular studies, sectioned, weighed, and refrigerated for myocardial microsphere analyses, and put in 4°C 3-[N-morpholino] propane sulfonic acid (MOPS) solution for in vitro assessment of coronary microvascular reactivity. Ameroid constrictors were resected along with a segment of circumflex artery and examined under low power magnification.

In Vitro Assessment of Coronary Microvessel Reactivity

After cardiac harvest, epicardial coronary arterioles (70 to 150 μm in diameter and 1 to 2 mm in length) originating from branches of the left anterior descending and circumflex arteries were dissected from the surrounding tissue with a $\times 40$ dissecting microscope and examined in isolated organ chambers, as described previously.¹² The responses to sodium nitroprusside (SNP) (1 nM to 100 μM), an endothelium-independent cGMP-mediated vasodilator, and adenosine 5'-diphosphate (ADP) (1 nM to 10 μM), VEGF (1 fM to 1 nM), and FGF-2 (1 fM to 1 nM), three endothelium-dependent receptor-mediated vasodilators that act via bioavailable NO, were studied.¹³

Myocardial Blood Flow Determinations

Myocardial perfusion was assessed during each procedure with isotope-labeled microspheres (ILM) (BioPAL, Worcester, MA) using methods previously reported.¹⁰ Isotope-labeled microspheres of different isotopic mass were used at each experimental stage. Red (gold-labeled) microspheres were injected during temporary circumflex occlusion at the time of ameroid placement to identify myocardial samples that originated from the circumflex coronary distribution (those with the lowest count of gold-labeled microspheres). Black (samarium-labeled) ILM were used during the second procedure to determine baseline blood flow in the lateral territory 3 weeks after ameroid placement, at the time of FGF-2 implantation. Yellow

(iridium-labeled) and orange (rhenium-labeled) ILM were injected at rest and during right ventricular epicardial pacing at 145 beats per minute during the third procedure, 4 weeks after FGF-2 implantation. Following euthanasia, seven circumferential, transmural left ventricular sections were collected for ILM assays in each animal, weighed, and dried. Each sample was exposed to neutron beams and microsphere densities measured in a gamma counter. Adjusted myocardial blood flows, reflecting changes in lateral myocardial perfusion during FGF-2 treatment, were determined from the 2 myocardial samples, which showed the lowest count of red microspheres by using the following equation: *Adjusted blood flow at rest* = *crude blood flow at rest* (yellow) – *crude blood flow at baseline* (black), and *adjusted blood flow during pacing* = *crude blood flow during pacing* (orange) – *crude blood flow at baseline* (black).

Immunohistochemistry

Myocardial samples from the circumflex territory of HICHOL and NORMAL swine were stained with biotin-labeled lectin from *Bandeiraea simplicifolia* BS-1, counterstained with methyl green, and examined for capillary endothelial cell density in a triplicate, blinded fashion from $700 \times 550 \mu\text{m}$ (0.385 mm^2) randomly selected left ventricular cross-sectional fields, according to methods reported previously.¹⁰

RT-PCR Analysis of eNOS Expression

Endothelial nitric-oxide synthase (eNOS) mRNA was analyzed by reverse transcription polymerase chain reaction (RT-PCR) in light of the potentially low levels of expression that can make Northern blot insensitive. A reverse transcription reaction was performed on 2 μg of total RNA isolated from the anterior and lateral myocardial territories using an oligo dT primer (Gibco BRL custom primer, Carlsbad, CA) at 37°C for 90 minutes. Specific gene primers of constitutional nitric oxide synthase-3 (eNOS) for polymerase chain reaction (PCR) analysis were designed with the Biology WorkBench 3.2 software (SCC, San Diego, CA) based on the published sequences as follows: sense 5'-AGCCTCACTCTGTCTTCCA-3' (bases 1373 to 1393); antisense 5'-CTGTTGTAGGGGCC-AGACGT-3' (bases 1786 to 1806). Primers for 18S RNA producing a 300-base pair fragment (Amicon, Beverly, MA) were used for amplification of each sample in proportion to the amount of template control. PCR conditions were created with an automated program of 94°C denaturation for 1 minute, 58°C annealing, and 72°C extending for a total of 32 cycles. PCR products were subjected to electrophoresis on 1% agarose gel, visualized by ultraviolet activation of incorporated ethidium bromide, and photographed. All experiments were repeated.

Northern Analysis of FGFR1 mRNA Expression

Myocardial samples from the anterior and lateral myocardial territories were homogenized for 30 seconds on ice and total RNA was isolated with a Tri-Reagent solution (Sigma, St. Louis, MO). A 10- μg RNA pellet was dissolved in RNase-free water, fractionated on a 1.3% formaldehyde-agarose gel, and transferred to a Gene-Screen Plus filter (Perkin Elmer, Boston, MA). cDNA probes of FGF Receptor-1 (FGFR1) were labeled with $\alpha^{32}\text{P}$ -dCTP (New England Nuclear, Boston, MA) using a random-priming labeling kit (Boehringer, Indianapolis, IN), and purified from unincorporated nucleotides with G-50 Quick Spin Columns (Boehringer). The specific activity of the probes used was 1 to 2×10^9 cpm/ μg . The blots were hybridized at 68°C for 3 hours in QuikHyb solutions (Stratagene, La Jolla, CA). After hybridization, the blots were washed twice in 2X saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) for 15 minutes at room temperature, and then rewashed twice in 0.1X SSC, 0.1% SDS for 15 minutes at 60°C. Autoradiography was carried out at -80°C for 16 to 20 hours.

Western Blot of FGFR1 Protein Levels

Whole-cell lysates were isolated from the homogenized anterior and lateral myocardial samples with a RIPA solution (Biotech, Ashland, MA) and centrifuged at $12\,000 \times g$ for 10 minutes at 4°C to separate

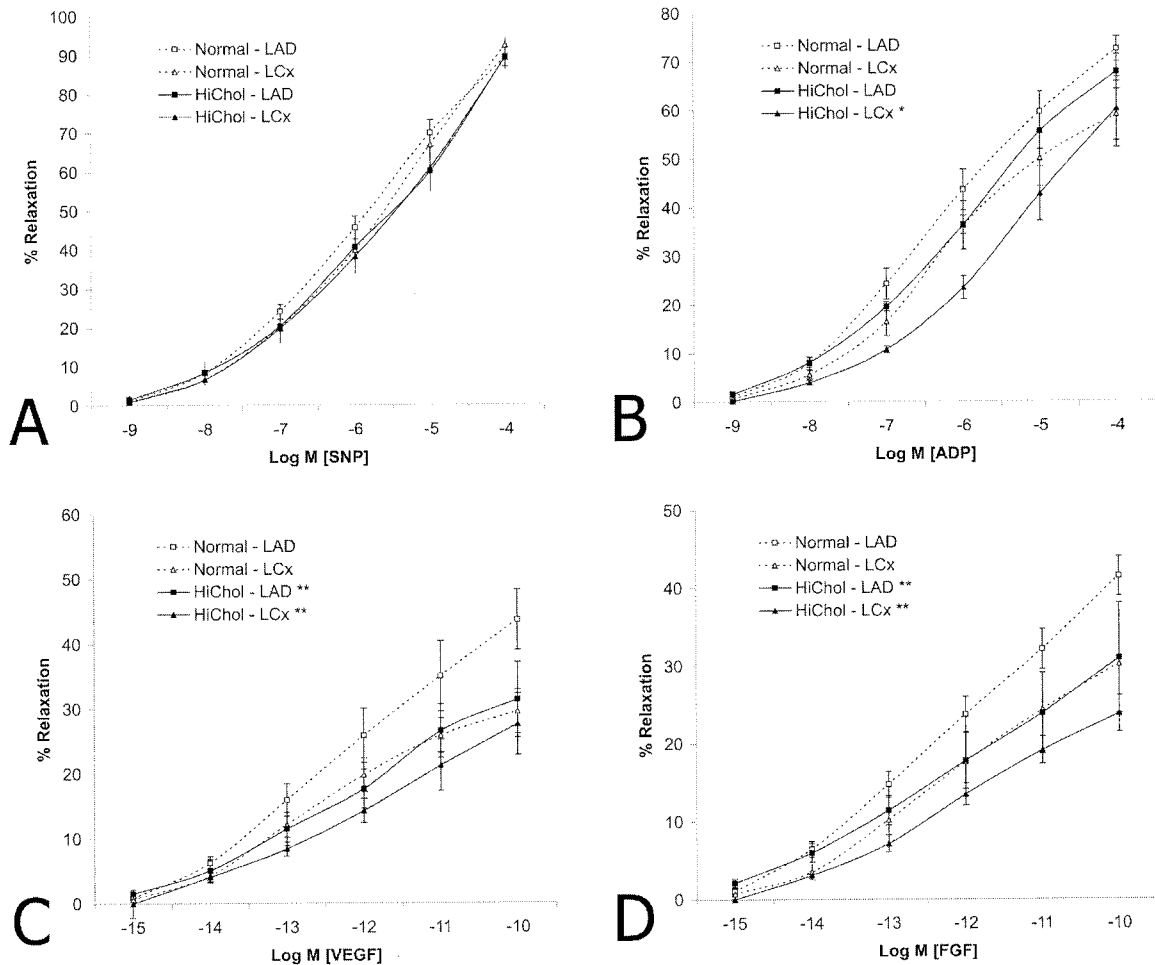


Figure 1. Coronary microvessels responses, expressed in percent relaxation following U44619-induced constriction. (A) Endothelium-independent responses to the vasodilator sodium nitroprusside (SNP). (B) Endothelium-dependent response to the vasodilator adenosine 5' diphosphate (ADP). (C) Endothelium-dependent response to the vasodilator vascular endothelial growth factor (VEGF). (D) Endothelium-dependent response to the vasodilator fibroblast growth factor (FGF)-2. * $P < 0.001$, HICHOL - LCx versus other groups. ** $P \leq 0.04$, HICHOL - LCx and HICHOL - LAD versus NORMAL - LCx and NORMAL - LAD groups.

soluble from insoluble proteins. The supernatant protein concentration was measured spectrophotometrically at 595-nm wavelength with a DC protein assay kit (Bio-Rad, Hercules, CA). Forty micrograms of total protein were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Bedford, MA). FGFR1 was detected by incubating the membrane with a 1:500 dilution of polyclonal anti-human FGFR1 antibody (Oncogene, Boston, MA) for 2 hours, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000) for 1 hour. Immune complexes were visualized using the ECL detection system (Amersham, Piscataway, NJ). Bands were measured by densitometric analysis of autoradiograph films.

Data Analysis

Data are reported as means \pm SEM. Microvessel responses are expressed as percent relaxation of the precontracted diameter and were analyzed by fitting a linear regression model examining the relationship between vessel relaxation, log concentration of the vasoactive agent of interest, and the experimental group or myocardial territory of origin (SAS Version 8, Cary, NC). PCR products and blots are expressed as a ratio of mRNA to loading band density and were analyzed after digitization and quantification of x-ray films with Image-Quant (Molecular Dynamic, Sunnyvale, CA). PCR products, blots, and ILM data were analyzed with 2-tailed analyses of variance. Bonferroni corrections were applied to multiple tests and

probability values of less than 0.05 were considered statistically significant.

Results

Feasibility

There was no procedural mortality. One swine in the normal diet group and three swine in the hypercholesterolemic group died during the diet modification period prior to the first procedure. Serum cholesterol levels were significantly higher in the HICHOL group than in the NORMAL group at each procedure. For example, mean serum cholesterol at the time of the third procedure was 790 ± 71 mg/dL in the HICHOL group versus 83 ± 6 mg/dL in the NORMAL group ($P < 0.001$). Ameroid constrictors had completely occluded the circumflex artery by the time of the third procedure in all animals.

Coronary Microvessel Reactivity

Results of microvessel assays are summarized in Figure 1. Relaxation to the endothelium-independent vasodilator sodium nitroprusside was identical between study groups and

myocardial territories (Figure 1A). Endothelium-dependent vasorelaxation, a measure of endothelial bioavailable NO, was significantly impaired in HICHOL versus NORMAL swine in the circumflex distribution in response to ADP ($P<0.001$; Figure 1B), and in both left anterior descending (LAD) and circumflex (LCx) distributions in response to VEGF ($P=0.005$ and $P=0.03$, LAD and LCx distributions, respectively, HICHOL versus NORMAL swine; Figure 1C) and FGF-2 ($P=0.004$ and $P=0.04$, LAD and LCx distributions, respectively, HICHOL versus NORMAL swine; Figure 1D).

eNOS Expression

Myocardial eNOS mRNA expression was not significantly different between the 2 groups in either myocardial territory ($125\pm 40\%$ and $80\pm 20\%$, eNOS densitometry ratio of HICHOL to NORMAL animals in the anterior and circumflex territory, respectively; $P\geq 0.13$).

Myocardial Perfusion

Baseline lateral myocardial blood flow at the time of FGF-2 implantation was similar between HICHOL and NORMAL swine (0.44 ± 0.06 versus 0.53 ± 0.07 mL/min/g, NORMAL versus HICHOL animals, respectively; $P=0.19$) (Figure 2A). However, lateral myocardial blood flow at both rest and pacing 4 weeks after initiation of FGF-2 treatment was markedly lower in HICHOL than in NORMAL swine (0.44 ± 0.04 versus 0.81 ± 0.15 mL/min/g, rest perfusion of HICHOL versus NORMAL animals, respectively; $P=0.006$; and 0.50 ± 0.06 versus 0.71 ± 0.10 mL/min/g, perfusion of HICHOL versus NORMAL animals during pacing; $P=0.02$). After adjusting for baseline, only NORMAL swine demonstrated an net increase in perfusion as a result of FGF-2 therapy, whereas HICHOL animals had no augmentation at rest and during pacing (0.36 ± 0.13 versus -0.10 ± 0.07 mL/min/g, adjusted rest perfusion increase of NORMAL versus HICHOL animals, respectively; $P<0.001$; and 0.26 ± 0.09 versus -0.03 ± 0.06 mL/min/g, adjusted pacing perfusion increase of NORMAL versus HICHOL animals; $P=0.001$; Figure 2B).

Capillary Endothelial Cell Density

The density of lectin-positive capillary endothelial cells after 4 weeks of FGF-2 therapy was significantly higher in the lateral myocardial territory of NORMAL compared with HICHOL animals (71.3 ± 16.4 versus 54.1 ± 10.9 cells per 0.385 mm², NORMAL versus HICHOL animals; $P=0.04$; Figure 3).

FGFR1 mRNA Expression and Protein Levels

FGFR1 mRNA expression was significantly lower in the circumflex territory of HICHOL swine and was $47\pm 10\%$ that of NORMAL swine ($P=0.03$; Figure 4). Furthermore, FGFR1 expression in the lateral myocardial territory was $200\pm 30\%$ that of the anterior territory in NORMAL swine ($P=0.001$), but only $78\pm 27\%$ in HICHOL animals ($P=0.003$; NORMAL versus HICHOL). FGFR1 protein levels were significantly lower in the circumflex territory of

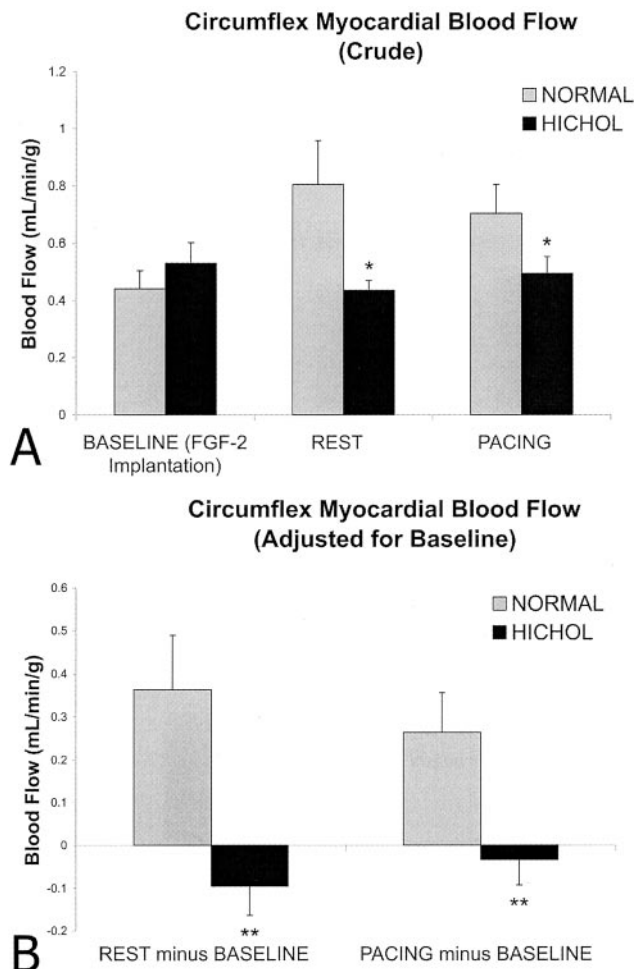


Figure 2. Crude and adjusted circumflex territory myocardial blood flow in normocholesterolemic (NORMAL) and hypercholesterolemic (HICHOL) swine after circumflex ameroid occlusion and FGF-2 treatment. (A) Both groups had equal perfusion at the time of FGF-2 implantation (BASELINE), but only NORMAL swine developed an angiogenic response demonstrated by increased rest and pacing blood flows 4 weeks later ($*P<0.025$, NORMAL versus HICHOL swine). (B) Rest and pacing myocardial blood flows adjusted for baseline perfusion revealed no net angiogenic effect in hypercholesterolemic animals ($**P<0.002$, versus NORMAL).

HICHOL versus NORMAL swine ($50\pm 8\%$; $P<0.001$; Figure 5).

Discussion

In this study, swine in which a 20-week hypercholesterolemic diet led to endothelial dysfunction had a markedly decreased functional response to surgical FGF-2 protein therapy when compared with normal swine that underwent a similar experimental protocol. This was observed as a lack of myocardial perfusion increase during FGF-2 treatment in hypercholesterolemic swine, decreased capillary endothelial cell density, and a decrease in FGF-2 receptor expression and protein levels in the circumflex territory of hypercholesterolemic swine, suggestive of inhibition in the signal transduction of FGF-2 in the presence of endothelial dysfunction. These findings have important implications for clinical therapeutic angiogenesis research by indicating that endothelial dysfunction

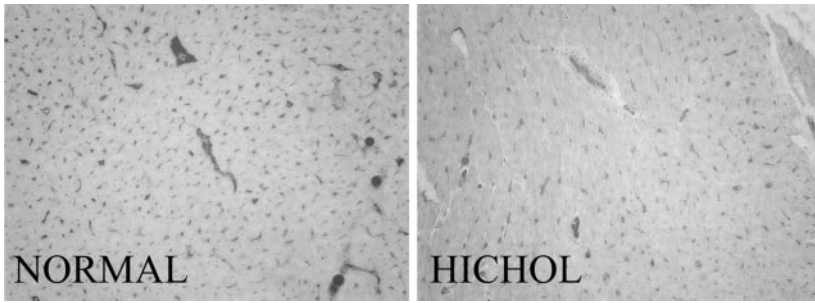


Figure 3. Left circumflex territory myocardium at 4 weeks after FGF-2 treatment. The density of capillaries endothelial cells was lower in hypercholesterolemic (HICHOL) than in normocholesterolemic (NORMAL) animals.

tion may inhibit the effects of angiogenic growth factors in patients with advanced coronary artery disease. This may explain the marginal clinical effects observed to date with therapeutic angiogenesis and suggest that the treatment of coronary endothelial dysfunction with agents such as the NO substrate L-arginine in patients eligible for angiogenic therapy could constitute a new concomitant approach.

Previous Work on the Role of NO in Angiogenesis

Previous in vitro and murine work indirectly supports the notion that the angiogenic effects of the two most studied growth factors, VEGF and FGF-2, depend on local NO availability. Work from our laboratory has shown that the stimulated synthesis and release of endothelium-derived NO by VEGF and FGF-2 are largely regulated by tyrosine kinases.⁶ The proliferative effects of VEGF have been shown to be decreased in the presence of NOS inhibitors.^{14,15} Jang et al demonstrated in murine studies that apoE- hypercholesterolemic mice exhibited attenuated collateral vessel formation in response to a FGF-2 disk angiogenesis system, and that the

inhibition was fully reversed by the oral administration of L-arginine.⁹

Hypercholesterolemia and Endothelial Dysfunction

Several groups have indicated that endothelial dysfunction can be achieved in pigs by way of hypercholesterolemic diet modification alone. Cohen et al¹⁷ have demonstrated that coronary ring segments of swine fed a high-cholesterol diet for as little as 9 weeks have attenuated endothelium-dependent relaxation to serotonin despite the absence of intimal proliferative changes on light or electron microscopy. Similarly, Hasdai et al have shown that pigs fed a hypercholesterolemic diet for 10 weeks had reduced vasorelaxation to bradykinin,¹⁸ abnormal responses to the endothelin B receptor agonist sarafotoxin 6c,¹⁸ and impaired arteriolar relaxation to insulin-like growth factor.¹⁹ The mechanism for endothelial dysfunction in experimental hypercholesterolemia may be related, like the pathogenesis of coronary disease in humans, to increased oxidant stress resulting from the induction of xanthine oxidase, NADH/NADPH oxidase, and uncoupling of endothelial nitric oxide synthase with inactivation of NO from reactive oxygen species.²⁰ Expression of eNOS mRNA in hypercholesterolemic models has previously been found to be unpredictable and associated either with compensatory

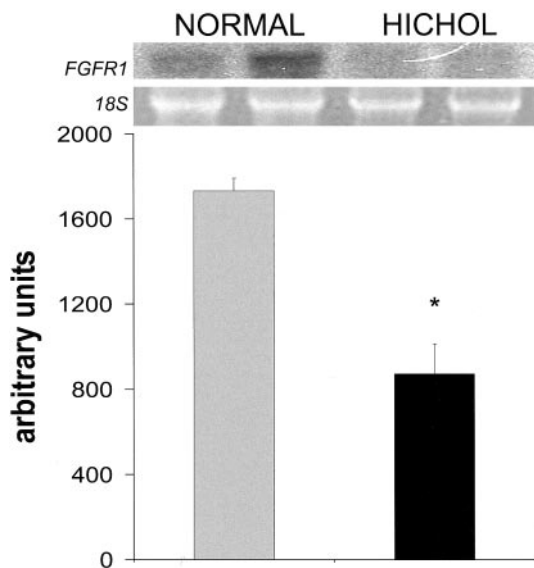


Figure 4. Northern blot analysis of FGFR1 mRNA expression in normocholesterolemic (NORMAL) and hypercholesterolemic (HICHOL) swine after circumflex ameroid occlusion and FGF-2 treatment. Representative images and bar graph summarizing expression in the lateral myocardial territory are shown. Ribosomal 18S subunit was used as a correction factor for quantification. Lateral myocardial FGFR1 expression was significantly lower in hypercholesterolemic animals ($47 \pm 10\%$ that of NORMAL swine; $*P=0.03$).

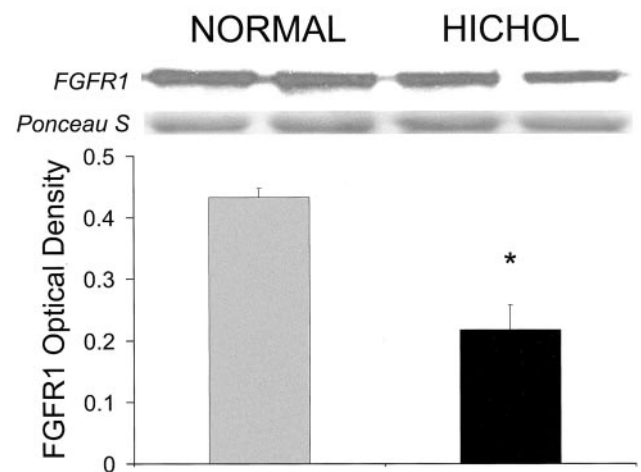


Figure 5. Western blot analysis of FGFR1 protein levels in normocholesterolemic (NORMAL) and hypercholesterolemic (HICHOL) swine after circumflex ameroid occlusion and FGF-2 treatment. Representative images and bar graph summarizing expression in the lateral myocardial territory are shown. Ponceau S optical density was used as a correction factor for quantification. Lateral myocardial FGFR1 protein levels were significantly lower in hypercholesterolemic animals ($50 \pm 8\%$ that of NORMAL swine; $*P<0.001$).

upregulation,²¹ lack of change,²² or downregulation.²³ Other potential mechanisms for hypercholesterolemia-induced endothelial dysfunction include the recently discovered role of caveolin on eNOS inhibition by preventing calmodulin activation,²⁴ and increased circulating levels of asymmetric dimethylarginine, which is an endogenous competitive antagonist of NO synthase.⁹ Although no significant change in eNOS regulation was demonstrated in hypercholesterolemic versus normal swine in the present study, decreased myocardial NO bioavailability was indicated by the presence of markedly impaired endothelium-dependent vasodilation in hypercholesterolemic animals.^{25,26}

Limitations of the Study

Varying degrees of myocardial inflammation secondary to surgery may have influenced the angiogenic response in this study. Another possible source of variation relates to the use of ameroid constrictors. Although all swine had ameroid occlusion confirmed microscopically, the time-course of ameroid closure, which typically occurs over a period of 2 to 3 weeks, could have differed between animals. In light of the study design, differences in the severity of myocardial inflammation and time-course of ameroid closure should however have distributed randomly between the hypercholesterolemic and normal groups. Microsphere-based derivations of myocardial perfusion are also associated with an intrinsic degree of variability, however minimized by the use of 10 or more animals per group.^{27,28}

Conclusions

We demonstrated that hypercholesterolemia-induced endothelial dysfunction led to a marked impairment of the myocardial angiogenic response in chronically ischemic swine treated with perivascular FGF-2. Because endothelial dysfunction and diminished coronary NO release constitute a key feature of coronary disease, and that interventions such as chronic oral L-arginine supplementation exist that can clinically improve coronary endothelial function,^{7,29,30} these findings may bear implications for clinical trials of angiogenesis and propose the evaluation of concomitant endothelial modulation approaches as an adjunct to therapeutic angiogenesis modalities.

Acknowledgments

This work was funded by grant 03247 from the Harvard Center for Minimally Invasive Surgery (M.R.) and by Grants R01 HL69024 and R01 HL46716 from the National Institutes of Health (F.W.S.). Mark Ruel is a research fellow of the Heart and Stroke Foundation of Canada and of the Canadian Institutes of Health Research.

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