University of Groningen

Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium

Tio, RA; Tkebuchava, T; Scheuermann, TH; Lebherz, C; Magner, M; Kearny, M; Esakof, DD; Isner, JM; Symes, JF

Published in:
Human Gene Therapy

DOI:
[10.1089/10430349950016366](https://doi.org/10.1089/10430349950016366)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1999

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Tio, RA., Tkebuchava, T., Scheuermann, TH., Lebherz, C., Magner, M., Kearny, M., Esakof, DD., Isner, JM., & Symes, JF. (1999). Intramyocardial gene therapy with naked DNA encoding vascular endothelial growth factor improves collateral flow to ischemic myocardium. *Human Gene Therapy*, 10(18), 2953-2960. <https://doi.org/10.1089/10430349950016366>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Intramyocardial Gene Therapy with Naked DNA Encoding Vascular Endothelial Growth Factor Improves Collateral Flow to Ischemic Myocardium*

RENE A. TIO, TENGIS TKEBUCHAVA, THOMAS H. SCHEUERMANN, CORINNA LEBHERZ,
MEREDITH MAGNER, MARIANNE KEARNY, DARYLL D. ESAKOF,
JEFFREY M. ISNER, and JAMES F. SYMES

ABSTRACT

Both VEGF protein and VEGF DNA in combination with an adenoviral vector have been shown to enhance collateral formation in a porcine model of chronic myocardial ischemia. We sought to determine whether direct intramyocardial injection of naked DNA encoding for VEGF could similarly improve myocardial perfusion. Initially, 23 nonischemic pigs received either 200 μ g of plasmid DNA encoding β -galactosidase (pCMV β , $n = 11$) or 500 μ g of phVEGF165 ($n = 12$) into four separate sites in the myocardium via a small anterolateral thoracotomy incision in the fourth intercostal space. Two additional groups of pigs received an intramyocardial injection of either phVEGF165 ($n = 6$) or pCMV β ($n = 7$) 3 to 4 weeks after implantation of an ameroid constrictor around the left circumflex coronary artery. The injections caused no change in heart rate or blood pressure, and no ventricular arrhythmias or histologic evidence of inflammation. VEGF protein was detected by Western blot in VEGF-treated animals, with the strongest bands closest to the injection site. Plasma VEGF concentration (ELISA) increased from 3 ± 2 to 27 ± 13 pg/ml ($p = 0.035$) by day 4 after treatment. No increase in VEGF protein was noted in pCMV β -treated animals whereas these did stain positive for β -Gal. Resting myocardial blood flow (colored microspheres) was significantly reduced in the ischemic versus nonischemic territory in control animals (1.07 ± 0.05 versus 1.32 ± 0.05 ; $p < 0.05$) but not VEGF-treated pigs (1.32 ± 0.24 versus 1.13 ± 0.12 ; $p = \text{NS}$). Maximal vasodilatation with adenosine significantly increased flow to the ischemic region in VEGF-treated pigs (2.16 ± 0.57 versus 1.32 ± 0.24 ; $p < 0.05$) but not controls (1.31 ± 0.05 versus 1.17 ± 0.06 ; $p = \text{NS}$). Collateral filling of the occluded circumflex artery improved in five of six VEGF-treated pigs (mean change in Rentrop score, +1.5). We conclude that direct intramyocardial transfection phVEGF165 is safe and capable of producing sufficient VEGF protein to enhance collateral formation and myocardial perfusion. This approach may offer an alternative therapy for patients with intractable myocardial ischemia not amenable to PTCA or CABG.

OVERVIEW SUMMARY

Recombinant vascular endothelial growth factor protein and its gene combined with an adenoviral vector are capable of stimulating collateral formation in animal models of chronic myocardial ischemia. To determine whether direct intramyocardial injection of VEGF DNA without a viral vector could be performed safely and similarly improve myocardial perfusion, we injected plasmid DNA encoding ei-

ther β -galactosidase (controls) or VEGF₁₆₅ directly into the myocardium of pigs who had previously had an ameroid constrictor implanted around the circumflex coronary artery. The injections caused no significant deleterious effects to the animals and VEGF protein was detected both in the myocardial tissue and in the plasma of the animals treated with VEGF₁₆₅. Myocardial blood flow measured by colored microspheres was significantly improved in the ischemic territory of VEGF-treated animals but not con-

Division of Cardiovascular Research and the Department of Surgery and Medicine, St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, MA 02135; and Department of Cardiology, University Hospital, Groningen, The Netherlands.

*Presented at the Surgical Forum, American College of Surgeons, October 27, 1998, Orlando, FL.

trols. Coronary angiography demonstrated improved collateral filling of the occluded circumflex artery in five of six VEGF-treated pigs but in only one of the control animals. We conclude from this study that direct intramyocardial administration of VEGF₁₆₅ can be performed safely and is capable of producing sufficient VEGF protein to enhance collateral development and improve myocardial perfusion.

INTRODUCTION

THE TREATMENT of chronic stable angina is aimed at maintaining a positive balance between myocardial oxygen supply and demand. The first-line treatment in most patients will be pharmacologic therapy, followed by angioplasty or bypass surgery. In some patients who have diffuse coronary artery disease, or have had multiple previous interventions, repeat angioplasty or coronary artery bypass graft (CABG) may not be feasible because of poor target vessels, lack of conduits, or unacceptable operative risk. Despite maximal pharmacological therapy many of these “inoperable” patients are incapacitated by frequent anginal attacks.

Vascular endothelial growth factor (VEGF) is a potent angiogenic mitogen. Administration of the protein itself and transfection with VEGF DNA have been shown to improve collateral blood flow to ischemic limbs both in animal models and, in the case of VEGF DNA, in a phase I clinical trial (Takeshita *et al.*, 1994a; Tsurumi *et al.*, 1996; Baumgartner *et al.*, 1998). The present study was conducted, first, to evaluate the feasibility and optimal technique of intramyocardial gene transfer, and second, to assess the efficacy and safety of direct intramyocardial gene therapy with VEGF in a porcine model of myocardial ischemia.

MATERIALS AND METHODS

Animal model

Thirty-six Yorkshire swine weighing 25–35 kg were studied under protocols approved by the Animal Care and Use Committee of St. Elizabeth’s Medical Center (Boston, MA) and in accordance with the *Guide for the Care and Use of Laboratory Animals* [Department of Health and Human Services, publication no. (NIH)86-23, revised 1985].

Animals were retained on a normal diet. All procedures were performed under anesthesia and using sterile techniques. Pigs were anesthetized with an intramuscular injection of ketamine (25 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA), acepromazine (0.2 mg/kg; Fermenta Animal Health Company, Kansas City, MO), and atropine (0.05 mg/kg; Fujisawa, Deerfield, IL). Ventilation with 2% isoflurane (Fort Dodge Laboratories) was used to ensure adequate anesthesia throughout the experiment. Pancuronium (0.08 mg/kg, administered intravenously; Elkins-Sinn, Cherry Hill, NJ) was given as a muscle relaxant.

Levodromaron (2 mg, subcutaneous; Roche Laboratories, Nutley, NJ) was given as analgesic at the beginning of the procedure and supplemented as necessary. Lidocaine (50 mg; Abbott Laboratories, North Chicago, IL) was given prophylactically. On completion of each procedure animals received an

additional injection of levodromaron, and were observed during recovery until fully conscious. All animals received 500 mg of cefazolin (Kefzol; Eli Lilly International, Indianapolis, IN) subcutaneously at the beginning of the procedure and two 500-mg doses for 5 days postoperatively.

At the end of the study period the animals were again anesthetized in the manner described above. Hemodynamic parameters and left ventricular function were evaluated as indicated below. Afterward the animals were killed with euthanasia solution, and the heart, and part of the lungs, liver, and mediastinal lymph nodes, were excised for further analysis.

Study groups

The experimental protocol, including a breakdown of study groups, time of sacrifice, and the number of animals in each section, is illustrated in Table 1.

Nonischemic pigs. Twenty-three pigs were treated with either pCMV β ($n = 11$) or phVEGF₁₆₅ ($n = 12$) and sacrificed after 3–5 days (pCMV β) or 1–8 weeks (phVEGF).

After stable anesthesia was achieved as described above, the left carotid artery was cannulated with a 7F sheath for blood pressure monitoring and catheterization. The pigs were placed in a lateral position, to enable an incision in the left fourth intercostal space. After opening the pericardium the gene solution was injected directly into the myocardium with a 25-gauge needle. To prevent penetration of the left ventricular (LV) wall the needle was covered with a rubber stopper, leaving only 5 mm of the distal end free for injection. In this way the solution was always injected at the same depth. To be sure that no blood vessel or the LV cavity was reached, a check was performed before each injection to determine if blood could be aspirated. If this was the case the needle was readjusted. During introduction of the needle and injection, the heart rhythm was monitored carefully. With the needle in the myocardium the gene solution was slowly injected under visual guidance to assure that no fluid leaked back along the needle. Injection of the DNA was monitored in several pigs with direct epicardial echocardiography. This demonstrated that injection of a volume of 2 ml resulted in distribution of the injectate over an area of 1–2 cm of adjacent myocardium.

TABLE 1. STUDY GROUPS

	<i>Injection volume (ml)</i>	<i>Sacrifice</i>	<i>Number</i>
Nonischemic pigs			
pCMV β	4 $\times$ 0.5	3–5 days	4
	4 $\times$ 2	3–5 days	4
	4 $\times$ 5	3–5 days	3
phVEGF165	4 $\times$ 2	1 week	5
		4 weeks	5
		8 weeks	2
Ischemic pigs			
pCMV β	4 $\times$ 2	4 weeks after treatment	7
phVEGF165	4 $\times$ 2	4 weeks after treatment	6

Ischemic pigs. In a total of 13 pigs chronic ischemia was induced by placing an ameroid constrictor (Research Instruments, Corvallis, OR) around the left circumflex coronary artery. Three weeks later, pigs underwent repeat thoracotomy and were treated by intramyocardial injection of either phVEGF165 ($n = 6$) or pCMV β ($n = 7$).

Plasmids and injectate volume

A nuclear-specific cytomegalovirus (CMV) promoter–enhancer–driven β -galactosidase-carrying plasmid (pCMV β) was used for evaluation of the appropriate injection volume for the intramyocardial injection method. To establish the optimal volume each pig received four intramyocardial injections containing 50 μ g of plasmid DNA. The injection sites were chosen at the corners of a 1 $\times$ 1 cm square. Various volumes per injection site were used: 0.5 ml (total volume, 2 ml; $n = 4$), 2.0 ml (total volume, 8.0 ml; $n = 4$), and 5 ml (total volume, 20 ml; $n = 3$). The β -galactosidase activity in the myocardium was measured after sacrifice. Treatment with the lower volume (4×0.5 ml) in four pigs resulted in a mean β -galactosidase activity of 1578 ± 566 RLU/mg protein, treatment with 4×2 ml ($n = 4$) resulted in 1328 ± 436 RLU/mg protein, and treatment with 4×5 ml ($n = 3$) resulted in 1366 ± 373 RLU/mg protein, all significantly higher than untreated control hearts ($n = 4$; 660 ± 103 RLU/mg). On the basis of these experiments a volume of 4×2.0 ml was chosen for treatment with the plasmid containing DNA for VEGF (phVEGF165). For evaluation of protein production blood samples were taken before and after treatment to measure plasma VEGF protein levels with a commercially available ELISA kit (R&D Systems, Minneapolis, MN). After treatment with the plasmid, pigs were sacrificed after either 1 week ($n = 5$), 4 weeks ($n = 5$), or 8 weeks ($n = 2$).

Evaluation of gene expression

In the pCMV β -treated pigs the β -galactosidase activity in the myocardial tissue was evaluated with a chemiluminescence assay (Tropix, Bedford, MA). Before measuring β -galactosidase activity, tissue homogenates were pretreated with Chelex 100 to inactivate a natural inhibitor of the enzyme (Oswald *et al.*, 1997).

In the phVEGF165-treated pigs gene expression of the plasmid DNA was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Western blotting for VEGF protein as previously described. In short, tissue specimens were stored at -80°C until assayed. The tissue was homogenized in lysis buffer spun down twice at high speed. Equal amounts (100 μ g) of protein were loaded on a 12% gel under nonreducing conditions. After blotting, the membrane was exposed to a rabbit anti-VEGF antibody (sc152; Santa Cruz Biotechnology, Santa Cruz, CA) followed by the secondary (anti-rabbit) antibody labeled with horseradish peroxidase. Last, the membrane was put in enhanced chemiluminescence (ECL) solution and exposed to X-ray film.

Study parameters

Hemodynamics. Throughout the experiments, the condition of the animals was carefully monitored by means of continu-

ous transcutaneous oximetry, intraarterial blood pressure measurement, and electrocardiographic registration of the limb leads and one modified precordial lead.

Myocardial enzymes. Myocardial enzymes were measured from serum samples at the beginning of the procedure, at the end of the procedure, and the following day for creatine phosphokinase (CPK), and CPK-MB isoenzyme determination.

Left ventricular function. To assess the effect of the injectate volume and procedure on LV function, left ventricular angiograms were obtained at baseline (before thoracotomy) and at the end of the study period, in the nonischemic pigs. Angiograms (6 frames per second) were taken from the 55° left anterior oblique view and from the 35° right anterior oblique view. A planimetric program (MACmeasure 1.9) was used to calculate from these two views left ventricular ejection fractions from end systolic and end diastolic frames. The average of these two measurements was taken for each time point.

Histologic evaluation. The heart of each animal was removed, examined for gross pathologic changes, weighed, and rinsed in cold phosphate-buffered saline (PBS). The left ventricle was dissected free and sliced transversally, perpendicular to the long axis of the heart, into slices 1 cm in thickness at five different levels from the apex to the base of the heart. From each slice sections were taken from anterior, lateral, posterior, and septal regions. In addition, biopsies from right ventricle, right atrium, left atrium, lung, liver, mediastinal lymph nodes, and pericardium were taken.

At all levels one slide was stained with hematoxylin–eosin for evaluation of infiltrative cells.

From all tissues one part was snap-frozen and stored at -80°C for quantitative assay of β -galactosidase, or for Western blotting in the case of phVEGF165 treatment. The other part was fixed in 4% formaldehyde in PBS for 2 hr at 4°C . The tissues were then divided into two parts again. One part was incubated in 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) solution for 20 hr and location of β -galactosidase activity was determined as previously described (Couffignal *et al.*, 1997).

Myocardial perfusion. Myocardial blood flow was determined as described previously (Hariawala *et al.*, 1996). In short, colored microspheres (Triton Technology, San Diego, CA) were used to determine myocardial blood flow in ischemic (circumflex territory) and nonischemic tissue (left anterior descending [LAD] territory). Tissue was taken at two levels—proximally (near the base of the heart) and distally (closer to the apex). Flow was determined at rest and after intravenous administration of 6 mg of adenosine. Microspheres (15 ± 0.1 μ m in diameter) with two different colors (yellow and blue, or yellow and red) were used. Flow was quantified by measuring the dye content of the tissue with a spectrophotometer (HP8452; Hewlett Packard, Palo Alto, CA). Reference blood samples were taken from the femoral artery at a rate of 6 mg/min. Flow was calculated in milliliters per minute per gram of tissue.

Coronary angiography. Via an arterial sheath in the femoral or carotid artery, a 6 or 7F left Judkins coronary diagnostic

catheter was advanced to the ostium of the left coronary artery. Cineangiograms (6 frames per second) were made from the 30° left anterior oblique position, after injection of 500 μ g of nitroglycerin into the left main stem. Contrast medium (10 ml) was injected with a power infusion pump (Medrad Technologies, Philadelphia, PA) at a rate of 3 ml/sec. These angiograms were repeated from the same angles, using the same injection protocol at follow-up. Collateral filling of the occluded circumflex (Cx) artery and its branches was scored according to Rentrop: 0, no filling; 1, filling of small side branches; 2, partial filling of epicardial segments of the circumflex or obtuse marginal (OM) branches; 3, complete filling of epicardial CX or OM branches (Rentrop *et al.*, 1998). Angiograms were presented to a panel of eight observers blinded to therapy and pre- or posttreatment information. The consensus score for each angiogram was taken to evaluate changes in Rentrop score.

RESULTS

Safety issues

The intramyocardial injections of pCMV β did not cause changes in heart rate regardless of the volume injected: 4×0.5 ml (preinjection, 145 ± 23 bpm; postinjection, 127 ± 20 bpm); 4×2 ml (preinjection, 143 ± 4 bpm; postinjection, 142 ± 4 bpm); 4×5 ml (preinjection, 143 ± 5 bpm; postinjection: 145 ± 3 bpm). Diastolic blood pressure did not change after injection of 4×0.5 ml (preinjection, 45 ± 4 mmHg; postinjection, 47 ± 4 mmHg), or after 4×2 ml (pre, 42 ± 2 mmHg; post, 43 ± 2 mmHg), or after 4×5 ml (pre, 44 ± 2 mmHg; post, 44 ± 2 mmHg). The same was found for the systolic blood pressure: 4×0.5 ml (pre, 83 ± 4 mmHg; post, 82 ± 4 mmHg), 4×2 ml (pre, 81 ± 3 mmHg; post, 81 ± 2 mmHg). In the pigs treated with phVEGF165 (4×2 ml), the heart rate did not change (pre, 136 ± 8 bpm; post, 141 ± 5 bpm). Systolic blood pressure decreased slightly (pre, 95 ± 3 mmHg; post, 89 ± 3 mmHg; $p = 0.01$) as did the diastolic blood pressure (pre, 62 ± 3 mmHg; post, 54 ± 2 mmHg; $p = 0.007$). This recovered within 5 min, without major hemodynamic consequences. During injection no malignant ventricular arrhythmias were encountered in any animal. In three pigs nonsustained ventricular tachycardia without hemodynamic deterioration occurred during injection. In the majority of the pigs sporadic premature ventricular contractions (PVCs) were present at the moment of entering the myocardium with the needle. The electrocardiogram did not show evidence of myocardial injury in any of the pigs. In the phVEGF165-treated pigs the CPK level increased from 381 ± 55 IU/liter at the beginning of the procedure, to 766 ± 125 IU/liter at the end of the procedure, and to 5274 ± 1245 IU/liter after 1 day. The myocardial-specific MB isoenzyme, however, did not increase (1.5 ± 0.2 , 2.2 ± 0.5 , and 2.2 ± 0.4 ng/ml). None of the pigs had an MB fraction higher than the normal limit (5.0 ng/ml). The heart weights at sacrifice were determined to detect evidence of myocardial interstitial, but were not different between the groups: pCMV β injections of 4×0.5 ml (6.56 ± 0.73 g/kg), 4×2 ml (5.64 ± 0.29 g/kg), and 4×5 ml (5.84 ± 0.26 g/kg); phVEGF-injected pigs sacrificed after 1 week (6.07 ± 0.32 g/kg), 4 weeks (5.70 ± 0.84 g/kg), and 8 weeks (5.96 ± 0.30 g/kg). On histologic examination no more

than five inflammatory cells per high-power field were present. No arteriovenous anomalies were found in the heart or in other tissues (Fig. 1).

Myocardial and systemic VEGF production

Plasma samples for VEGF protein measurements were taken on a daily basis during the first week and again at 2 and 3 weeks after injection in all pigs treated with phVEGF165. In individual pigs a pronounced increase in VEGF protein concentration was noted. Although individual VEGF increases were variable, the mean VEGF concentration increased from 3.0 ± 2.0 to 27.4 ± 10.9 pg/ml ($p = 0.041$) (Fig. 2). No detectable levels were found in pCMV β -treated pigs. In ischemic pigs the pretreatment VEGF concentrations were 18.6 ± 2.6 and 18.5 ± 6.7 pg/ml in the control and phVEGF groups, respectively. In both groups an increase was found after intramyocardial injection to 50.2 ± 14.3 and 40.1 ± 7.8 pg/ml ($p = \text{NS}$), respectively.

Western blotting of tissue obtained from nonischemic pigs sacrificed 1 week after intramyocardial VEGF gene transfer showed strong bands in the region closest to the injection site (Fig. 3). In the hearts from pigs sacrificed 4 and 8 weeks after treatment fainter but clear bands were still present. No such bands were found in pigs injected with pCMV β . Similar evidence of VEGF protein production was also detected on Western blots of myocardial tissue from all VEGF-treated pigs in the ischemic group.

Ventricular function

Global left ventricular ejection fraction (LVEF) was assessed by ventriculography in VEGF-treated pigs in the nonischemic

FIG. 1. Histologic section taken from the anterior wall of the left ventricle of a pig treated with phVEGF165, showing no evidence of inflammatory reaction or abnormal vascular development.

FIG. 2. The plasma concentrations of VEGF protein (ELISA) after treatment with phVEGF165, showing peak levels between day 2 and day 5 after intramyocardial injection. VEGF was no longer detectable in the plasma of any pig after 2 weeks.

group only to look for evidence of any adverse effects of the injection procedures. Mean LVEF at baseline prior to VEGF injection was $39 \pm 6\%$ ($n = 10$) compared with a mean value of $32 \pm 6\%$ at sacrifice in pigs monitored for 1 week ($n = 5$), $46 \pm 6\%$ at 4 weeks ($n = 5$), and $39 \pm 8\%$ at 8 weeks ($n = 2$). No significant difference was detected between any of the post-injection values and the baseline LVEF.

Myocardial blood flow

Myocardial blood flow was measured by the colored microsphere method at rest and after intravenous administration of 6 mg of adenosine (maximal vasodilation) in the ischemic pigs only (Table 2). Resting flow to ischemic myocardium was lower than flow to nonischemic myocardium in the control group but not in the phVEGF165-treated pigs. After maximal vasodilation flow to the nonischemic myocardium increased in

both groups. No increase in flow to ischemic myocardium was found in the control group, whereas in the phVEGF165-treated pigs a significant increase in flow to ischemic myocardium was noted. The maximal flow was lower than the maximal flow to nonischemic myocardium in both groups.

Coronary angiography

Collateral filling of the occluded circumflex artery was evaluated by angiography. On the basis of a blinded comparison of the angiograms from each animal obtained 3 weeks after constrictor placement (at time of gene transfer) with those done 4 weeks after treatment, a collateral score according to Rentrop was determined. In all but two pigs in the control group, a lower Rentrop score was achieved 4 weeks after therapy compared with 3 weeks after constrictor placement. In the phVEGF165-treated animals collateral flow improved in all but one animal as reflected in a higher Rentrop score at follow-up (Fig. 4). The overall change in Rentrop score was significantly greater in the phVEGF165 group (Fig. 5).

FIG. 3. Western blot of tissue extracts from nonischemic pigs sacrificed 1 week after intramyocardial VEGF gene transfer, showing strong bands in the region closest to the injection site (4) and weaker but demonstrable VEGF protein in sections taken further from the injection site (level 3, 1 cm; level 2, 2 cm; level 1, 3 cm from injection site).

DISCUSSION

VEGF has been shown to be a potent endothelial cell mitogen *in vitro*, with the capability of stimulating neovascularization *in vivo*. The VEGF₁₆₅ protein has been demonstrated to induce collateral formation and augment perfusion in ischemia limbs, as well as in ischemic myocardium (Takeshita *et al.*, 1994b; Harada *et al.*, 1996). Transfection with plasmid DNA encoding VEGF₁₆₅ has been shown to be effective in ischemic limbs in various experimental studies (Takeshita *et al.*, 1996;

TABLE 2. MYOCARDIAL BLOOD FLOW^a

	Nonischemic zone		Ischemic zone	
	Pre	Post	Pre	Post
Control (pCMVβ)				
Proximal	1.32 ± 0.05	1.78 ± 0.04 ^b	1.07 ± 0.05 ^c	1.01 ± 0.06
Distal	1.35 ± 0.02	1.78 ± 0.06 ^b	1.17 ± 0.06 ^c	1.31 ± 0.053
phVEGF165				
Proximal	1.25 ± 0.11	1.96 ± 0.02 ^b	0.96 ± 0.16	1.37 ± 0.14 ^{b,c}
Distal	1.13 ± 0.12	1.67 ± 0.05 ^b	1.32 ± 0.24	2.16 ± 0.57 ^b

^aMyocardial blood flow (ml/g/min) as measured with colored microspheres at baseline (pre) and after 6 mg of adenosine was administered intravenously (Post). Nonischemic zone, LAD territory; ischemic zone, circumflex territory. Proximal samples near base of heart, distal near apex.

^b*p* < 0.05 versus Pre.

^c*p* < 0.05 versus nonischemic value.

Tsurumi *et al.*, 1996). It has also been shown that this treatment strategy is effective in certain patients with end-stage critical limb ischemia (Baumgartner *et al.*, 1998). To extend this form of therapy to patients with inoperable coronary artery disease (CAD) an acceptable method of administering the plasmid to the ischemic myocardium is required. We considered that in patients with advanced CAD, intractable but stable angina, and no other revascularization option, a small thoracotomy might be considered acceptable for this purpose. Furthermore, after plasmid injection, the production of the protein, accompanied by a beneficial effect, should be demonstrated. We performed this study to investigate the feasibility and efficacy of intramyocardial gene transfer with phVEGF165.

Initially the effect of differences in injection volume were considered using pCMVβ. We have shown in a previous study that increasing injection volumes from 50 to 300 μl in rabbit hearts caused increasing transfection efficiency (Gal *et al.*, 1993). Theoretically, increasing injection volumes may facilitate the spread of the DNA into the tissue. However, in the present study, we did not detect a difference in β-galactosidase (β-Gal) activity between injection volumes of 0.5, 2, and 5 ml. Indeed, with the highest volume, a slight decrease in β-Gal activity was observed.

Previous studies investigating experimental angiogenic strategies have utilized either the recombinant protein or DNA plus an adenoviral vector. When the recombinant protein was used it was administered either into the heart as a single injection (Hariawala *et al.*, 1996) or by long-term infusion (Banai *et al.*, 1994). Systemic side effects such as hypotension were of concern after intracoronary administration (Horowitz *et al.*, 1997) but were avoided by longer term infusion and were not seen after intramyocardial injection of phVEGF165 in the present study. The feasibility of adenovirus-mediated gene transfer with angiogenic factors has been demonstrated by a number of studies (Giordana *et al.*, 1996; Mack *et al.*, 1998). The use of adenovirus-mediated gene therapy has a major potential limitation: it may elicit a cellular immune response to viral or transgene antigens resulting in destruction of transfected cells. Therefore, repeated administration of the recombinant virus is typically ineffective. Such immunologic problems can be avoided by using plasmid-mediated transfection.

We previously documented the feasibility and efficacy of plasmid-mediated gene transfer in nonischemic hearts of rabbits and microswine (Gal *et al.*, 1993). The present report is the first to describe the use of nonviral, plasmid-based gene transfer to induce angiogenesis in a model of chronic myocardial is-

FIG. 4. Left coronary angiograms taken from an ischemic pig treated with phVEGF165 (**A**) 3 weeks after constrictor placement at the time of VEGF administration and (**B**) 4 weeks after VEGF administration. Rentrop grade III filling of the distal circumflex vessel by ipsilateral collaterals as well as collaterals from the LAD and its branches is evident.

FIG. 5. Change in Rentrop score average for the ischemic pigs in each of the control (pCMV β) and phVEGF165-treated groups ($p = 0.001$).

chemia. Although transfection efficiency with plasmids is lower than with adenoviral vectors the level of protein production, at least in the case of VEGF₁₆₅, appears sufficient to obtain meaningful bioactivity as demonstrated by the results of this and other studies (Isner *et al.*, 1998). We believe this is to a large extent because VEGF₁₆₅ contains an NH₂-terminal signal sequence that allows the protein to be secreted from the cell, thus enhancing its potential bioactivity. In addition, VEGF is endothelial cell specific, with its primary receptor (Kdr) being located exclusively on endothelial cells, thus allowing essentially all the VEGF activity to be focused on the cellular element most involved in angiogenesis.

The effect of phVEGF on plasma VEGF protein production was evident within 2 days and peaked at about 4–5 days after gene transfer. However, individual values were highly variable. At the tissue level, however, the greatest concentration of VEGF was consistently seen closest to the injection site. Remarkably, protein was detectable after transfection in nonischemic pig hearts as long as 8 weeks after treatment. This is consistent with intramyocardial injection studies performed in rodents (Buttrick *et al.*, 1992; Gal *et al.*, 1993). VEGF was also detectable in the plasma and myocardium of ischemic pigs in the control group.

Comparison of myocardial blood flow to the ischemic circumflex territory demonstrated similar findings at baseline in both the control and VEGF-treated animals. While vasodilation with adenosine did not augment flow to the ischemic territory in control animals a significant improvement was seen in the VEGF group. Interestingly, the magnitude of this augmentation of collateral flow to the territory subtended by the occluded circumflex coronary artery was in fact superior to that achieved in previous experiments from our laboratory in which VEGF₁₆₅ protein was injected into the left coronary artery of the identical animal model (Hariawala *et al.*, 1996).

Alternative techniques for myocardial gene delivery include intracoronary administration and percutaneous catheter-based myocardial injection. While both of these approaches would have the advantage of avoiding the need for a thoracotomy and general anesthesia, intracoronary injection has been shown to

be far less effective in delivering the gene to the myocardium (Lee *et al.*, 1999) and a catheter-based intramyocardial delivery system is still under development.

In conclusion, this study demonstrates that intramyocardial injection of phVEGF165 via a minimally invasive thoracotomy is feasible. It results in sufficient VEGF production to significantly enhance myocardial perfusion in ischemic pig hearts. These data suggest that future clinical trials should utilize direct intramyocardial gene transfer via a minithoracotomy to evaluate the potential benefits of gene therapy with VEGF in patients with advanced coronary disease.

REFERENCES

- BANAI, S., JAKLITSCH, M.T., SHOU, M., LAZAROUS, D.F., SCHEINOWITZ, M., BIRO, S., EPSTEIN, S.E., and UNGER, E.F. (1994). Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* **89**, 2183–2189.
- BAUMGARTNER, I., PIECZEK, A., MANOR, O., BLAIR, R., KEARNEY, M., WALSH, K., and ISNER, J.M. (1998). Constitutive expression of phVEGF₁₆₅ following intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97**, 1114–1123.
- BUTTRICK, P.M., KASS, A., KITSIS, R.N., KAPLAN, M.L., and LEINWAND, L.A. (1992). Behavior of genes directly injected into the rat heart in vivo. *Circ. Res.* **70**, 193–198.
- COUFFINHAL, T., KEARNEY, M., SULLIVAN, A., SILVER, M., TSURUMI, Y., and ISNER, J.M. (1997). Histochemical staining following LacZ gene transfer underestimates transfection efficiency. *Hum. Gene Ther.* **8**, 929–934.
- GAL, D., WEIR, L., LECLERC, G., PICKERING, J.G., HOGAN, J., and ISNER, J.M. (1993). Direct myocardial transfection in two animal models: Evaluation of parameters affecting gene expression and percutaneous gene delivery. *Lab. Invest.* **68**, 18–25.
- GIORDANA, F.J., PING, P., MCKIRNAN, D., NOZAKI, S., DEMARIA, A.N., DILLAMNN, W.H., MATHIEU-COSTELLO, O., and HAMMOND, H.K. (1996). Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile

- function in an ischemic region of the heart. *Nature Med.* **2**, 534–539.
- HARADA, K., FRIEDMAN, M., LOPES, J.J., WANG, S.Y., LI, J., PEARLMAN, J.D., EDELMAN, E.R., PRASAD, P.V., SELKE, F.W., and SIMONS, M. (1996). Vascular endothelial growth factor administration in chronic myocardial ischemia. *Am. J. Physiol.* **270**, H1791–1802.
- HARIAWALA, M., HOROWITZ, J.R., ESAKOF, D., SHERIFF, D.D., WALTER, D.H., CHAUDHRY, G.M., DESAI, V., KEYT, B., ISNER, J.M., and SYMES, J.F. (1996). VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J. Surg. Res.* **63**, 77–82.
- HOROWITZ, J.R., RIVARD, A., VAN DER ZEE, R., HARIAWALA, M.D., SHERIFF, D.D., ESAKOF, D.D., CHAUDHRY, M., SYMES, J.F., and ISNER, J.M. (1997). Vascular endothelial growth factor/vascular permeability factor produces nitric oxide-dependent hypotension. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2793–2800.
- ISNER, J.M., BAUMGARTNER, I., RAUH, G., SCHAINFELD, R., BLAIR, R., MANOR, O., RAZVI, S., and SYMES, J.F. (1998). Treatment of thromboangiitis obliterans (Buerger's disease) by intramuscular gene transfer of vascular endothelial growth factor: Preliminary clinical results. *J. Vasc. Surg.* **28**, 964–975.
- LEE, L., HACKETT, N., POLCE, D., EL-SAWY, T., ISOM, W., CRYSTAL, R., and ROSENGART, T. (1999). Focal angiogenic therapy: A comparison of intracoronary versus intramyocardial delivery of vascular endothelial growth factor utilizing adenovirus vectors. In *Society of Thoracic Surgeons 35th Annual Meeting, San Antonio, TX*. [Abstract]
- MACK, C.A., PATEL, S.R., SCHWARZ, E.A., ZANZONICO, P., HAHN, R.T., ILERCIL, A., DEVEREUX, R.B., GOLDSMITH, S.J., CHRISTIAN, T.F., SANBORN, T.A., KOVESDI, I., HACKETT, N., ISOM, O.W., CRYSTAL, R.G., and ROSENGART, T.K. (1998). Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J. Thorac. Cardiovasc. Surg.* **115**, 168–177.
- OSWALD, H., HEINEMANN, F., NIKOL, S., SALMONS, B., and GUNZBURG, W.H. (1997). Removal of an inhibitor of marker enzyme activity in artery extracts by chelating agents. *BioTechniques* **22**, 78–81.
- RENTROP, K.P., THORNTON, J.C., FEIT, F., and VAN, B.M. (1998). Determinants and protective potential of coronary arterial collaterals as assessed by an angioplasty model. *Am. J. Cardiol.* **61**, 677–684.
- TAKESHITA, S., ZHENG, L.P., BROGI, E., KEARNEY, M., PU, L.Q., BUNTING, S., FERRARA, N., SYMES, J., and ISNER, J. (1994a). Therapeutic angiogenesis: A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J. Clin. Invest.* **93**, 662–670.
- TAKESHITA, S., PU, L.Q., ZHENG, L., FERRARA, N., STEIN, L.A., SNIDERMAN, A.D., ISNER, J.M., and SYMES, J.F. (1994b). Vascular endothelial growth factor induces dose-dependent revascularization in a rabbit model of persistent limb ischemia. *Circulation* **90**, II-228–234.
- TAKESHITA, S., WEIR, L., CHEN, D., ZHENG, L.P., RIESSEN, R., BAUTERS, C., SYMES, J.F., FERRARA, N., and ISNER, J.M. (1996). Therapeutic angiogenesis following arterial gene transfer of vascular endothelial growth factor in a rabbit model of hindlimb ischemia. *Biochem. Biophys. Res. Commun.* **227**, 628–635.
- TSURUMI, Y., TAKESHITA, S., CHEN, D., KEARNEY, M., ROSSOW, S.T., PASSERI, J., HOROWITZ, J.R., and SYMES, J.F. (1996). Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral development and tissue perfusion. *Circulation* **94**, 3281–3290.

Address reprint requests to:

Dr. James F. Symes
11 Nevins Street, Suite 306
Boston, MA 02135

E-mail: jsymes@semc.org

Received for publication April 22, 1999; accepted after revision September 21, 1999.