

EXPERIMENTAL STUDIES

Evaluation of the Effects of Intramyocardial Injection of DNA Expressing Vascular Endothelial Growth Factor (VEGF) in a Myocardial Infarction Model in the Rat—Angiogenesis and Angioma Formation

Ernst R. Schwarz, MD,*† Mark T. Speakman, MD,*† Mike Patterson, PhD,* Sharon S. Hale, BS,* Jeffrey M. Isner, MD,‡ Laurence H. Kedes, MD,§ Robert A. Kloner, MD, PhD*†

Los Angeles, California and Boston, Massachusetts

- OBJECTIVES** The effects of direct intramyocardial injection of the plasmid encoding vascular endothelial growth factor (phVEGF₁₆₅) in the border zone of myocardial infarct tissue in rat hearts were investigated.
- BACKGROUND** Controversy exists concerning the ability of VEGF to induce angiogenesis and enhance coronary flow in the myocardium.
- METHODS** Sprague-Dawley rats received a ligation of the left coronary artery to induce myocardial infarction (MI). At 33.1 ± 6.5 days, the rats were injected with phVEGF₁₆₅ at one location and control plasmid at a second location (500 μg DNA, n = 24) or saline (n = 16). After 33.1 ± 5.7 days, the hearts were excised for macroscopic and histologic analysis. Regional blood flow ratios were measured in 18 rats by radioactive microspheres.
- RESULTS** phVEGF₁₆₅-treated sites showed macroscopic angioma-like structures at the injection site while control DNA and saline injection sites did not. By histology, 21/24 phVEGF₁₆₅-treated hearts showed increased focal epicardial blood vessel density and angioma-like formation. Quantitative morphometric evaluation in 20 phVEGF₁₆₅-treated hearts revealed 44.4 ± 10.5 vascular structures per field in phVEGF₁₆₅-treated hearts versus 21.4 ± 4.7 in control DNA injection sites (p < 0.05). Regional myocardial blood flow ratios between the injection site and noninfarcted area did not demonstrate any difference between phVEGF₁₆₅-treated hearts (0.9 ± 0.2) and saline-treated hearts (0.7 ± 0.1).
- CONCLUSIONS** Injection of DNA for VEGF in the border zone of MI in rat hearts induced angiogenesis. Angioma formation at the injection sites did not appear to contribute to regional myocardial blood flow, which may be a limitation of gene therapy for this application. (J Am Coll Cardiol 2000;35:1323–30) © 2000 by the American College of Cardiology

Severe and prolonged ischemia results in cell death. Although ischemia is thought to be a stimulus for angiogenesis via vascular growth factors (1,2) (i.e., the production and development of new blood vessels to provide ischemic tissue with oxygen), the production for new vascular cells requires approximately 24 h (2), a time frame during which irreversible cardiomyocyte death has already occurred. One of the

major challenges of cardiovascular research is to reduce the impact of myocardial ischemia by means of thrombolytic or interventional therapy shortly after acute coronary occlusion or by enhancing angiogenesis in the setting of chronic ischemia (3). Recent advances in molecular biology have allowed for the introduction of deoxyribonucleic acid (DNA) encoding angiogenic proteins into tissues, and this has been shown to augment collateralization (4–6). Gene therapy with vascular endothelial growth factor (VEGF), a 45-kD heparin-binding dimeric glycoprotein that is an endothelial cell specific mitogen (7–9), has been shown to be effective in hind limb ischemia models in the rabbit (10–12) and demonstrated striking effects in a single patient with claudication (13). Its effects in the heart are controversial (14,15).

From the *Heart Institute Research, Good Samaritan Hospital and †Division of Cardiology, University of Southern California, Los Angeles, California; and the ‡Division of Cardiovascular Research, St. Elisabeth's Medical Center and Tufts University School of Medicine, Boston, Massachusetts; and the §Department of Biochemistry and Molecular Biology, Department of Medicine, University of Southern California, Los Angeles, California. Supported in part by the Kenneth T. and Eileen L. Norris Foundation.

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Abbreviations and Acronyms

CMV	=	cytomegalovirus
DNA	=	deoxyribonucleic acid
ECG	=	electrocardiogram
H + E	=	hematoxylin and eosin
MI	=	myocardial infarction
VEGF	=	vascular endothelial growth factor

An elegant series of experiments defined striated muscle (16–19), including heart (20–22) as a tissue with the unique property of being able to take up and express recombinant proteins after local injection of DNA. Such direct injection technology is capable of delivering exogenous genes into myocardium fully governed by hormone inducible, tissue specific or strong viral promoters (23–25). We hypothesized that ischemic myocardium would be capable of taking up and expressing VEGF delivered by plasmid DNA injections. So far, the effects of VEGF in infarcted tissue in the rat heart have not been investigated. We hypothesized that application of VEGF encoding DNA directly to the healing infarct might augment angiogenesis and increase blood flow in infarcted rat heart. Potential benefits would include enhancing blood flow to a healing infarct, possibly accelerating healing, preventing remodeling and allowing open vessels to provide drugs to the area of scar. In addition, clinical trials are currently underway assessing the effect of this type of gene therapy in patients with chronic ischemia. Whether injection of the gene for VEGF into tissue recently infarcted is safe is not yet known.

METHODS

Preparation of plasmid DNA. The plasmids phVEGF₁₆₅ or pCMVbeta (Clontech Laboratories, Palo Alto, California) were transformed into DH5a cells (Gibco Max Efficiency competent cells), plated on LB plates containing ampicillin (100 µg/ml) and grown overnight at 37°C. Single colonies were picked into 10 ml of LB plus ampicillin and grown for 8 to 10 h at 37°C. Aliquots of 5 ml were used to inoculate 1L of prewarmed LB plus ampicillin, and the culture was incubated overnight with shaking.

DNA was extracted from the cultures with Qiagen Plasmid Giga Kit. Three L of culture usually provided 10 to 12 mg of plasmid DNA. All experiments were conducted in accordance with institutional guidelines and *The Guide for the Care and Use of Laboratory Animals* published by the National Research Council, Washington DC, 1996.

Sprague-Dawley rats, weighing between 200 and 350 g, were used (Charles River, Inc., Wilmington, Massachusetts). Each rat underwent:

- 1) at baseline, coronary occlusion;
- 2) at a mean of 33.1 ± 6.5 days, range of 24 to 50 days, injections at two separate sites of phVEGF₁₆₅ and control DNA or saline; and

- 3) at a mean of 33.1 ± 5.1 days, range of 26 to 44 days, excision of the hearts.

Apart from the final procedure, all surgeries were performed under aseptic conditions. The rats were anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg), intraperitoneally. They were intubated and artificially respirated with a rodent respirator (Harvard) with 15 to 20 ml/kg room air at a respiration rate of 75 breaths/min.

Coronary artery occlusion. The chest was opened in the fourth intercostal space. The pericardium was opened, and a 6.0 prolene suture was placed around the base of the left coronary artery near the left atrial appendage. Coronary occlusion was confirmed by the presence of deep S waves on the electrocardiogram (ECG) and ventricular arrhythmias within the first 20 to 30 min after occlusion. Thereafter, the chest and the skin were closed in layers, and the animals were allowed to recover.

Injection of phVEGF₁₆₅ or saline. At 33.1 ± 6.5 days after coronary artery occlusion, the animals were used for either a treated (phVEGF₁₆₅) group or a control (saline) group in random order (except the first five animals, which all received phVEGF₁₆₅). The feasibility of gene transfer in the rat model has been described before by our group (25–27). This time interval after coronary occlusion was chosen to avoid injection into necrotic debris but chosen to inject into partially healed infarcts. After opening the chest and identifying the infarcted area in the anterolateral wall, two 6.0 prolene sutures were placed at the border zones of the infarcted tissue: one anteriorly and one laterally. These areas were chosen for two reasons: 1) to introduce plasmid DNA into a “watershed” area in terms of blood supply, and 2) to augment the effects of locally expressed VEGF from cells bordering the necrotic area (19,20). The sutures were used to stabilize the myocardium for injection and to close the injection hole so that the agents would not leak out. In the treated group, 500 µg of phVEGF₁₆₅ plasmid DNA in a volume of 0.15 ml was injected intramyocardially at the left anterior border zone of the visible scar tissue in the left anterior free wall by use of a 0.5 ml syringe and a 24G needle. In the same rats, control DNA (500 µg, 0.15 ml) of the reporter gene pCMVbeta (7.2 Kb), a plasmid capable of expressing the *E. coli* beta-galactosidase gene under the control of human cytomegalovirus (CMV) immediate early promoter/enhancer was injected into the left lateral border zone of the scar tissue. Thirty-six animals were used for the phVEGF₁₆₅ group, and 19 animals were used for saline-treated controls (that is, without DNA injection). In a subgroup of eight animals, the injection sites for phVEGF₁₆₅ and pCMVbeta were changed to the lateral and anterior infarct border zones, respectively. In control animals, 0.15 ml saline was injected at both sites. Care was taken to inject the substances within the epicardial layer of the scar, and this was confirmed by the presence of a ‘bubble’ in the epicardium as the syringe was unloaded. In two

animals (one from each group), the sutures were removed a few minutes after injection to assess the effects of the suture material. Thereafter, the chest was closed and the animals were allowed to recover.

Expression of VEGF. To ensure that the phVEGF₁₆₅ plasmid expresses VEGF, an *in vitro* analysis using mammalian cells was performed. Therefore, 293 and cos 7 cells were transfected with plasmid DNA using the calcium phosphate precipitation method. After 72 hours, the media was harvested and washed cells lysed. The media was concentrated ten-fold with 75% ammonium sulfate. Aliquots of boiled media and lysate were separated by denaturing polyacrylamide gel electrophoresis and examined by Western blotting with A-20, an anti-VEGF antibody (Santa Cruz Biotechnology) and antirabbit IgG (Amersham, Sunnyvale, California). Furthermore, VEGF protein and aliquots of media and cell lysate from cells transfected with the plasmid vector backbone were examined.

Radioactive microsphere injection and excision of the hearts. 33.1 ± 5.7 days after injection, all animals were reanesthetized, and the chest was opened. In a subgroup of 18 animals, radioactive microspheres (NEN Research Products, Boston, Massachusetts), approximately 100,000 in number, labeled with ¹⁴¹Ce, were injected into the left ventricular cavity. The microspheres were allowed to circulate for one min. Thereafter, the animals were euthanized, and the hearts were excised. Tissue specimens from the anterior and the lateral injection sites within the infarcted border zone and from the septum were excised for measurements of radioactivity. All infarcts were fairly large, and they were composed of mainly fibroblasts and collagen. The activities in the VEGF-injected and the saline-injected regions were expressed per weight of heart tissue as a ratio relative to the activity in the septum and the control infarcted tissue.

Morphologic analysis. After excision of the hearts at 33.1 ± 5.7 days after injection, the injection sites were identified and examined grossly in all hearts. Short axis slices were made from apex to base. In hearts that did not receive microspheres, the transmural slices were then processed for histology. In hearts used for radioactive microsphere injection, hearts were dissected, and the samples used for radioactivity measurements were examined for later histologic analysis. The slices were processed for hematoxylin-eosin (n = 39) and smooth muscle alpha-actin antibody staining (n = 20), as previously reported (28).

In addition, tissues from four VEGF-treated hearts and one control heart were incubated with antihuman antibody to VEGF (R&D Systems, Minneapolis, Minnesota) at a concentration of 50 µg/ml for 20 min at 37°C, in humidity chambers. After rinsing in PBS, the tissues were incubated in horse radish peroxidase-tagged labeling reagent (BioGenex, San Ramon, California) for an additional 20 min at 37°C. The specimens were rinsed in PBS and

reacted with metal enhanced diaminobenzidine (Sigma) for 2 to 5 min at room temperature, with periodic monitoring by microscopic examination. After development, the samples were rinsed and stained with either hematoxylin and eosin or nuclear fast red and metanil yellow. Sections were dehydrated through xylene and mounted with permount.

In a subgroup of 30 animals, a morphometric analysis of blood vessels was conducted. The hematoxylin and eosin (H + E) slides were examined and marked by one investigator on each side of the infarction. This corresponded to the injection sites of phVEGF₁₆₅ or pCMVbeta or saline (or noninjection). The slides were then examined for evidence of vascular proliferation by another investigator unaware of the treatment groups. The tissue sections were projected through a 10× objective and a 4× projection lens to a 46 × 61 cm paper palate and the tissue perimeter and vascular structures identified, outlined and planimeted.

In a subgroup of animals, the epicardial and endocardial circumferences of the infarcted ventricle (scar) and the total left ventricle were measured by planimetry and expressed as the ratio of scar circumference to left ventricular circumference.

Statistical analysis. All values are given as means ± standard error of the mean (SEM). Comparisons between the groups or within the groups were made using paired or group *t* test, as appropriate. Data regarding histology of the angiomatous structures were tested by Fisher exact test. P values <0.05 were considered statistically significant.

RESULTS

A total of 56 rats that survived the coronary occlusion procedure were used for either the treatment (phVEGF₁₆₅) or control (saline) group. One rat died during anesthesia before injection, 15 rats (27%) died within 24 h after the surgery (12 rats after injection of phVEGF₁₆₅, 3 rats after injection of saline). Forty surviving rats (24 phVEGF₁₆₅-treated rats, 16 control rats) were analyzed.

Macroscopic evaluation (Figure 1, A and B). Macroscopic inspection of the hearts at the time of euthanasia typically revealed the presence of broad-based angioma-like formations at the site of phVEGF₁₆₅ injection, whether injected anteriorly or laterally, but not at the site of pCMVbeta injection. The angioma-like structure was also found in the heart in which the suture material was removed after injection. In contrast, saline injected control hearts demonstrated small adhesions in some cases but no angioma-like structures.

Qualitative histologic analysis. Light microscopy of the H + E slices was performed in 24 phVEGF₁₆₅-treated hearts and in 15 saline-treated control hearts. Angioma-like structures, which appeared to be vascularized formations originating from a broad base upon the epicardial surface, were found in 21/24 phVEGF₁₆₅-treated hearts. In one of the control hearts, there was increased vascularity in the

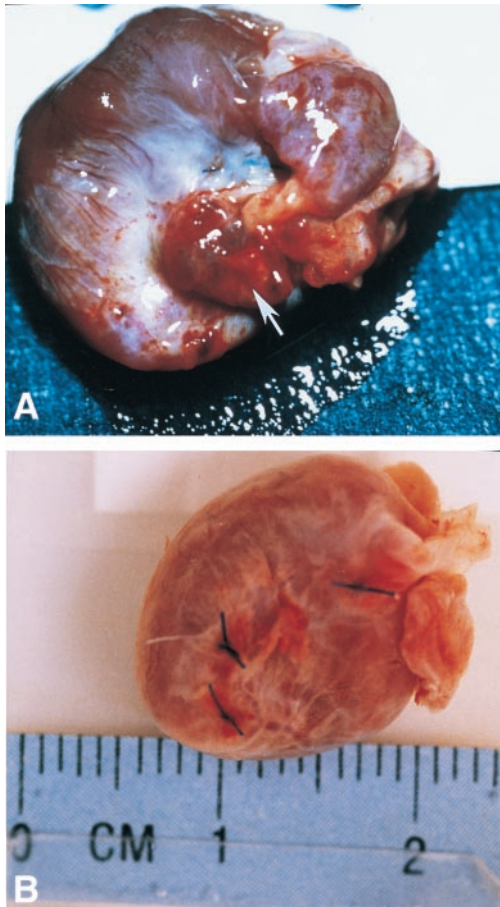


Figure 1. (A) Rat heart with infarct (white scar) four weeks after injection of plasmid for phVEGF₁₆₅. Note prominent red angioma-like structure on the anterior surface of the scar (arrow). (B) Rat heart with infarct four weeks after injection of saline. A few threadlike adhesions are present near site of saline injection (two lower sutures) but no angioma.

region of the saline injection site with minimal protuberance over the scar ($p < 0.0001$, Fisher exact test).

Cross sections of H + E slices of phVEGF₁₆₅-treated hearts containing these angioma-like structures showed them protruding from the surface of the scars often several mm. Within these structures most of the small vessels consisted only of simple endothelial cell tubes, but some vessels with smooth muscle cells in the media also were identified. Erythrocytes often could be visualized within the vessels. Lymphocytes were occasionally found in treated and control hearts at the injection sites and tended to be more prominent in hearts injected with phVEGF₁₆₅. Both compact and loose fibrous tissue were also visualized in these structures as well as occasional fat cells. Figures 2 and 3 illustrate typical H + E examples for phVEGF₁₆₅-treated and control hearts. Within the angioma-like structures, most vascular structures were negative for alpha-actin staining, again suggesting that they were primarily simple endothelial tubes without smooth muscle.

Staining for VEGF in a subgroup of VEGF-treated

hearts demonstrated brownish-black positive stain, which was observed to be concentrated around angioma-like structures. The cells that appeared to express VEGF were most likely fibroblasts.

Morphometric analysis of vascular structures. Morphometric analysis was performed in 20 phVEGF₁₆₅-treated hearts and in 10 saline-treated control hearts. In phVEGF₁₆₅-treated hearts, the slices containing angioma-like structures were analyzed. In addition, in five phVEGF₁₆₅-treated hearts, slices that did not contain the angioma-like structures were also analyzed. The number of vascular structures within the projected field was significantly higher in phVEGF₁₆₅-injected sites at 44.4 ± 10.5 per field as compared with pCMVbeta-injected areas at 21.4 ± 4.7 , $p < 0.05$, and significantly higher compared with the equivalent saline injection sites in control animals (21.1 ± 3.0 , $p < 0.05$ vs. phVEGF₁₆₅ injection site). There was a nonsignificant trend toward higher numbers of vascular structures in phVEGF₁₆₅ injection sites if compared with all saline injection sites in control animals (30.1 ± 4.6). In contrast, in those slices not containing angioma-like structures in the phVEGF₁₆₅-treated hearts, there were 12.6 ± 2.2 vascular structures ($p < 0.05$ vs. phVEGF₁₆₅-treated slices with angioma-like structures [44.4 ± 10.5]). The percent of the area examined that contained vascular structures was greater within those left ventricular slices containing angioma-like structures in phVEGF₁₆₅-treated animals ($4.5 \pm 1.3\%$) versus those slices in phVEGF₁₆₅-treated animals that received control DNA ($1.1 \pm 0.1\%$, $p < 0.05$) versus equivalent control saline-treated areas (2.4 ± 0.6 , $p = 0.08$) versus all saline injected areas ($2.8 \pm 0.6\%$).

Expression of VEGF. We insured that phVEGF₁₆₅ was capable of producing VEGF polypeptide in mammalian cells. Bands at 21 kD corresponding to the reported size of phVEGF₁₆₅ were present in both cell lysate and media of both 293 and cos 7 cells. To insure further that the bands were not due to interaction between VEGF and secondary antibody, primary antibody was neutralized by a blocking peptide (Santa Cruz Biotechnology, Santa Cruz, California) before incubation with the membrane. The 21 kD bands and the positive control band were no longer detected. Thus, mammalian cells carrying the phVEGF₁₆₅ plasmid DNA we delivered to the myocardium was capable of synthesizing and secreting VEGF protein.

Measurement of infarct and left ventricular circumferences. Measurements of the epicardial and endocardial infarct scar and total circumferences of the left ventricle in 22 hearts (12 phVEGF₁₆₅-treated hearts and 10 controls) revealed an epicardial scar/left ventricular circumference ratio of 0.26 ± 0.02 in phVEGF₁₆₅-treated animals versus 0.32 ± 0.03 in controls ($p = 0.09$). The endocardial scar/left ventricular circumference ratio was 0.34 ± 0.04 in

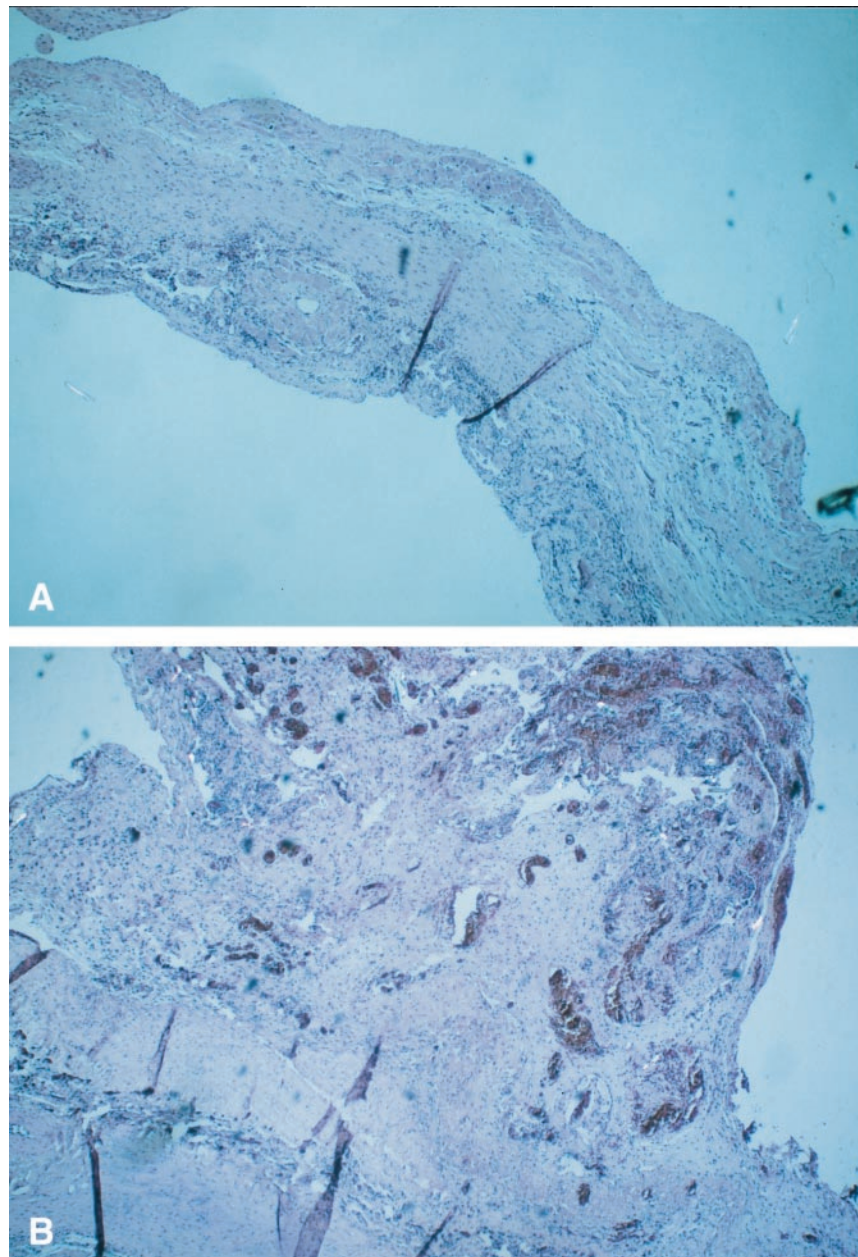


Figure 2. (A) Hematoxylin-eosin staining of cross section of rat infarct scar from a control heart after eight weeks. Most of the anterior free wall is replaced with fibrous tissue. There are a few islands of viable myocytes in the subepicardium and around vessels in the subepicardium. The LV cavity is at the lower left. (B) phVEGF₁₆₅ treated scar. The infarct is at the lower left (same orientation as (A)). There is a broad-based mass protruding from the surface of the scar that contains numerous vascular structures as well as fibrous tissue.

VEGF-treated hearts versus 0.40 ± 0.04 in controls ($p = \text{NS}$).

Measurements of regional blood flow. Radioactive microspheres were used to measure relative blood flow in 12 phVEGF₁₆₅-treated hearts and in six saline-treated controls. The septum, which was noninfarcted in this model, served as the reference region. The pCMVbeta injection site served as the control infarct region in treated animals. The ratios between phVEGF₁₆₅ injection site and pCMVbeta injection site in treated and between the two saline injection

sites in control animals were used for group comparison. The ratio of counts of the injection site and in the septum between treated and control hearts were analyzed (Table 1). There were no significant differences in relative blood flow between the two groups.

DISCUSSION

This is the first study investigating the effects of direct phVEGF₁₆₅ plasmid DNA injection in myocardial infarct tissue in the rat heart. The main findings are: 1)

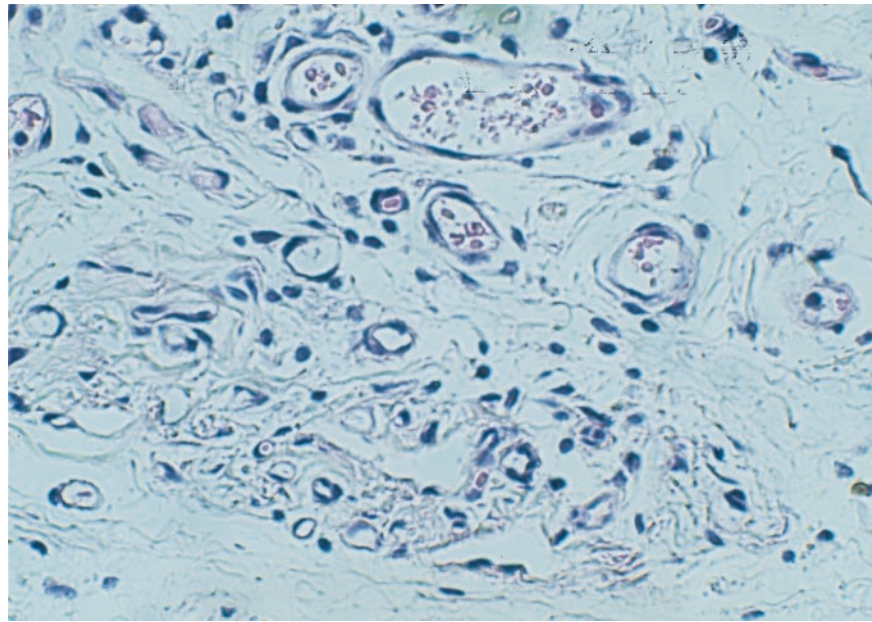


Figure 3. Various sized new blood vessels within angiomatous structure of phVEGF₁₆₅-treated animal. The cells are primarily composed of a few endothelial cells forming a ring.

phVEGF₁₆₅ injection caused angiogenesis, i.e., new vessel formation at the injection site compared with pCMVbeta or saline injection or noninjected tissue; 2) new vessels were located within angioma-like formations, i.e., richly vascular structures protruding from the surface of the heart; and 3) there was no difference in relative regional myocardial blood flow between the phVEGF₁₆₅-treated and the control groups; that is, the angiomatous structures did not contribute to improved regional flow within the myocardium. These data are primarily observational but might be important for future use of vascular growth factors.

The development of angiomatous structures. The development of angioma-like structures was found only within the phVEGF₁₆₅-injected areas and was independent of its location or the presence of a suture. The development of these angioma-like structures is probably analogous to the role of VEGF in tumor angiogenesis (29,30). The clinical significance of these angiomas over time remains unknown. They did not worsen infarct expansion, and, in fact, there was a nonsignificant trend to reduce scar circumference in phVEGF₁₆₅-treated hearts.

It has been shown in dogs that infarcted or chronically ischemic myocardial tissue is more sensitive to the mitogenic effects of another growth factor—exogenous acidic

fibroblast growth factor—because its epicardial application induced a tumor-like smooth muscle cell hyperplasia (31). In our study, tumor-like angioma occurred within infarcted tissue; this phenomenon might limit the clinical application of VEGF by plasmid DNA injection in the treatment of myocardial ischemia in human patients and suggests that such injection perhaps should be avoided in patients with recent infarcts. Of note, one patient who received the plasmid for VEGF in the peripheral arteries developed transient cutaneous angiomas (13). A recently published report of direct injection of phVEGF₁₆₅ into human myocardium in five patients demonstrated a slight reduction of symptoms and improvement in myocardial perfusion (as assessed by nuclear imaging [32]). However, a macroscopic or histologic evaluation of the injection sites was not feasible, and a clinical follow-up is not yet available, but these patients had no recent myocardial infarction, which might play a role in the development of angiomatous structures.

The effects on myocardial perfusion. Relative, rather than absolute, blood flow measurements were performed in our model because of the difficulty of withdrawing reference blood samples in the rat. The phVEGF₁₆₅ injection site/control area ratios did not show any significant difference compared with controls. Potential explanations for this include the possibility that the new blood vessels were simply too small to allow microsphere penetration, that the spheres may have shunted around the blood vessels in the angioma or that the possibility that flow within these angiomatous structures simply does not differ from flow in the control border zone—that is, these structures may not

Table 1. Regional Blood Flow Ratios

Group I (VEGF) n = 12	VEGF inj./contr. inf.	1.5 ± 0.5
Group II (saline) n = 6	saline inj./contr. inf.	1.4 ± 0.4
Group I (VEGF) n = 12	VEGF inj./septum	0.9 ± 0.2
Group II (saline) n = 6	saline inj./septum	0.7 ± 0.1

contr. = control injection site; inf. = infarct; inj. = injection site.

contribute to overall increase in regional myocardial blood flow.

A variety of studies have investigated the effects of VEGF to induce angiogenesis in peripheral arteries of animals (10-12,27,33,34) and, recently, humans (13,35-37), with some degree of success. Furthermore, in dogs subjected to chronic left circumflex artery stenosis, intracoronary administration of VEGF-peptide increased collateral blood flow (14). In contrast, in another study in dogs subjected to chronic left circumflex artery occlusion, VEGF-protein injected into the left atrial cavity had no effect on collateral flow or blood vessel development (while basic fibroblast growth factor did) (15). The differences between this and the other studies might be explained by the difference in the models, species, route of protein delivery or severity of the ischemic stimulus.

Preliminary results are available from intracoronary application of VEGF-protein in the human heart, which demonstrates improvement in myocardial perfusion as assessed by nuclear imaging (38). However, it remains to be seen whether application of VEGF has long-term beneficial effects in the heart. Notably, it has not been shown whether angiogenesis augments contractile function. In the setting of a healed myocardial infarction, if angiogenesis is induced, it may either improve perfusion of scar tissue or border zone tissue, or, alternatively, the gene and its effect may be distributed all over the heart ("A little VEGF goes a long way," [39]) with improvement in perfusion and salvage of jeopardized cells (40,41). The beneficial effects of VEGF-induced angiogenesis may be masked by the induction of vascular abnormalities, which have been described recently after intravitreal injection in monkeys (42). Furthermore, it has been shown that expression of VEGF is not only essential for embryonic angiogenesis but also can induce the formation of angioblasts, hemangioblasts and vascular and cardiovascular malformation (43,44). This is not surprising given the involvement of VEGF in tumorigenesis (29,30) and neonatal hemangiomas (45).

Study limitations. The exact reason that more rats died within 24 h in the VEGF group is unknown, but:

- 1) more rats were used for phVEGF₁₆₅ injection than for saline, and
- 2) severe hypotension as a side-effect of VEGF in pigs (46,47) and humans (38) has been reported.

One explanation for our findings might be that a high transfection efficiency of gene expression caused angioma formation in our study. The present data confirm that injection of the plasmid causes expression of VEGF, and we demonstrated that labeling appeared most intense around the angioma, indicating an effect directly caused by VEGF plasmid injection. However, we used a dose of phVEGF₁₆₅ identical to that used in rabbits before. The much smaller body weight of rats might have influenced the results. Therefore, in a recent study, smaller doses of phVEGF₁₆₅

were used (125 μ g), similar to doses used in clinical trials, and this did not result in gross angiomas in the rat model (48). These findings support, in part, a dose-dependence that previously has been reported after VEGF gene application in muscle tissue (49). In that study, Spinger et al. (49) reported that high levels of VEGF expression caused hemangiomas in adult skeletal muscles, whereas muscle exposed to low serum levels did not develop vascular malformations.

Conclusions. It seems that a potential therapeutic application of VEGF in a setting of myocardial ischemia or infarction is a double-edged sword: on one hand it has the ability to induce angiogenesis and enhance coronary flow (50), while on the other hand it may also induce vascular malformation. Whether the dose that was used here or the location, mode or time of VEGF injection or other factors play a role in the development of angiomas in our model has not yet been clarified. There is a need for further experimental studies using VEGF to identify the circumstances under which collateral flow can be enhanced without development of vascular malformations.

Reprint requests and correspondence: Dr. Robert A. Kloner, Heart Institute, Good Samaritan Hospital & Division of Cardiology, University of Southern California, 1225 Wilshire Boulevard, Los Angeles, California 90017-2395. E-mail: RKloner@goodsam.org.

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