VEGF Gene Delivery to Myocardium
Deleterious Effects of Unregulated Expression

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**Background**—Vascular endothelial growth factor (VEGF) is being investigated for therapeutic angiogenesis in ischemic myocardium. Primarily, transient delivery systems have been tested. The goal of this study was to investigate the effects of continuous expression of VEGF in myocardium by use of myoblast-mediated delivery.

**Methods and Results**—Primary murine myoblasts (5 × 10⁵ cells in 10 μL of PBS with 0.5% BSA) expressing both the murine VEGF gene and the β-galactosidase (β-gal) gene from a retroviral promoter were implanted in the ventricular wall of immunodeficient mice (n = 11) via a subdiaphragmatic approach. Control immunodeficient mice (n = 12) were injected with the same number of myoblasts expressing only the β-gal gene. Between days 14 and 16, surviving mice were euthanized and the hearts processed for histology. In the experimental group, 11 of 11 mice demonstrated failure to thrive by day 13; 5 deaths occurred between days 8 and 15. There were no complications in the control mice. Histochemistry documented successful implantation of myoblasts (positive β-gal reaction product) in 6 of 6 surviving experimental mice and 12 of 12 controls. Histology disclosed intramural vascular tumors resembling hemangiomas in the VEGF-myoblast-injected myocardium in 6 of 6 surviving mice. β-Gal–expressing cells were present at the site of the vascular tumors. Immunohistochemistry localized abundant endothelial nitric oxide synthase and CD31 (platelet and endothelial cell adhesion molecule) within the lesion, consistent with the presence of endothelial cells.

**Conclusions**—In this model, unregulated continuous expression of VEGF is associated with (1) a high rate of failure to thrive/death and (2) formation of endothelial cell–derived intramural vascular tumors in the implantation site. These results underscore the importance of regulating VEGF expression for therapeutic angiogenesis. (Circulation. 2000;102:898-901.)

**Key Words:** angiogenesis ■ genes ■ coronary disease

Over the past decade, the field of cardiovascular tissue engineering has advanced to the point of consideration as a potential therapy. The challenge has been to deliver a biologically active substance to myocardium or blood vessels globally, or to a particular site, with a high enough efficiency to alter expression of a protein or set of proteins. One strategy is direct delivery of the substance into the desired site. For example, exogenous vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis,1,2 has been delivered directly in vivo as a purified protein, as a DNA plasmid, or via viral vectors to induce angiogenesis.3–5 These initial studies have led to VEGF gene delivery trials for the improvement of blood flow in ischemic myocardium.6,7

Another strategy is to introduce cells genetically manipulated ex vivo, such as skeletal myoblasts, that on injection secrete proteins that can increase tissue concentrations of a biologically active substance locally.8–12 In this case, the efficiency of delivery and concentration of the factor may be enhanced by the duration of production, which is long-term, whereas direct gene introduction methods are often transient. As a result, myoblasts engineered to deliver VEGF have been found to be unusually potent in their ability to induce vascular growth12 and have also been shown to be angiogenic.12a This method shows promise for delivery of therapeutic proteins, because the angiogenesis may facilitate delivery of other proteins or even growth of the transplanted muscle. In the setting of myocardial ischemia, for example, the transplanted muscle may be effective not only in restoring contractility/conduction properties to the infarct area but also in increasing circulation locally. Thus, cardiovascular tissue engineering conceivably could be used to modulate myocardial injury for treatment of ischemic heart disease.

If tissue engineering is to be used for repair of injured myocardium, the effects of high-level constitutive expression of the genes delivered must be fully understood. In this regard, myoblast-mediated expression of VEGF of long
duration in skeletal muscle leads to formation of vascular tumors. This deleterious response to VEGF was observed in nonischemic skeletal muscle and does not appear to occur via angiogenesis but rather may involve a mechanism related to vasculogenesis. These studies suggest that VEGF may have different effects depending on concentration; at low concentrations, angiogenesis may prevail, whereas at high concentrations, vasculogenesis dominates.

In this study, we investigated the effects of high-level, localized expression of VEGF in the murine myocardium. We show here that implantation of VEGF-expressing skeletal muscle myoblasts in the myocardium results in the formation of hemangiomas.

Methods
Myocardial Transplantation of Myoblasts in Mice

The study protocol was approved by the Committee for Animal Research of the University of California at San Francisco and was performed in accordance with the recommendations of the American Association for Accreditation of Laboratory Animal Care. SCID C.B-17 mice, 8-week-old males (Taconic, Germantown, NY), were anesthetized with sodium pentobarbital (40 to 50 mg/kg IP), positioned supine on an animal surgery table, and held in a stable position by paw restraints. A midline laparotomy was made, and primary murine myoblasts expressing both the murine VEGF gene and the β-galactosidase gene from a retroviral promoter were implanted in the ventricular wall (n=11) via a subdiaphragmatic approach. Control mice (n=12) were injected with myoblasts expressing only the β-galactosidase gene. C57BL/6 mice were the source of the primary murine myoblast for both treatment groups. LacZ-expressing cells were further transduced with VEGF virus to generate the VEGF/LacZ-expressing cells. The murine VEGF used is the homologue to human VEGF-165. Each mouse was injected with 5×10^6 cells in 10 μL of PBS with 0.5% BSA. Mice were euthanized between 14 and 16 days.

Histology

After thoracotomy, the hearts were rapidly excised and rinsed in cold saline. The hearts were immersed in fresh buffered 4% paraformaldehyde (pH 7.4) for 24 hours. Under a dissecting microscope, excess tissue at the base of each heart was trimmed. The tissue was cryoprotected in buffered 30% sucrose. The specimen was frozen, and 10-μm sections were cut on a Reichert-Jung cryostat.

The frozen sections were stained with hematoxylin-eosin (HE) or Masson’s trichrome–stained sections. However, histochemical localization of endothelial cells– derived intramural vascular tumors. 12 This deleterious response to VEGF was observed in nonischemic skeletal muscle. Previously, VEGF was thought to

Results

Cell Transplantation and Survival

Myoblasts (positive β-gal reaction product in the ventricle) were successfully implanted in 6 of 6 surviving experimental mice and 12 of 12 controls. In the experimental group that received VEGF (n=11), 5 deaths occurred between days 8 and 13. By day 12, 4 of the 6 surviving mice were cachectic and lethargic. There were no complications in the control mice.

Formation of Vascular Tumors

At 14 to 16 days after myoblast injection, histological sections showed that 6 of 6 murine hearts had lesions either replacing the myocardium or extending through the endocardium to protrude into the cavity (Figure 1). Infiltrative at their margins, these irregular lesions were composed of numerous spindle-shaped cells, some densely packed, defining tiny vascular slits and others forming large vascular spaces filled with red blood cells. There was virtually no inflammation within the lesions. By HE or Masson’s trichrome staining, these lesions closely resembled hemangiomas, with features of both capillary and cavernous types.

Immunoperoxidase studies using antibody to CD31 (PECAM-1) confirmed the endothelial nature of the spindle cells forming the lesions (Figure 1D). Identical staining was seen with endothelial nitric oxide synthase (data not shown). Skeletal myoblasts could not be identified in the HE- or Masson’s trichrome–stained sections. However, histochemical localization of β-galactosidase activity disclosed numerous muscle cells throughout the lesions (Figure 1B).

Control animals injected with myoblasts expressing only the β-gal gene demonstrated normal-appearing myocardium without any hemangioma-like structures (Figure 2).

Discussion

This study demonstrates that constitutive overexpression of VEGF in nonischemic murine hearts can lead to the formation of endothelial cell–derived intramural vascular tumors near the implantation site. Our results are consistent with previously reported observations that hemangiomas result from implantation of VEGF-expressing myoblasts into nonischemic skeletal muscle. Previously, VEGF was thought to

Figure 1. At low magnification (A), tumor (blue appearance with arrow) replaces large area of left ventricular free wall. Corresponding adjacent section stained for β-galactosidase activity (B) shows abundant blue reaction product in lesion exclusively. At high magnification (C), tumor is composed primarily of dense aggregates of dark-staining spindle-shaped cells, similar histologically to endothelial cells. Although there are numerous slit-like vascular channels throughout cellular portion of tumor, some neoplastic cells enclose large blood-filled spaces (arrows designate same blood pool in A, C, and D). In a third adjacent section of lesion stained with antibody to CD 31 (D), brown reaction product localizes neoplastic cells, confirming that they are endothelial in nature. A and C, HE; B, X-gal solution; D, antibody to CD 31/Immunoperoxidase methods. Bars=300 μm.
have effects only in ischemic tissues. The previously reported structures that arose in skeletal muscle were proposed to derive at least in part from the recruitment of circulating endothelial precursor cells initiating an unprecedented form of adult vasculogenesis. Similarly, the hemangiomas that resulted from VEGF expression in the nonischemic myocardium may be due to vasculogenesis resulting from the high levels of VEGF at the implantation site. In addition, the extreme response observed may also have occurred because the expression of VEGF was constant, which has not been reported to be the case in transient modes of VEGF delivery based on injection of adenovirus or plasmid DNA.6,7,14 Primary myoblasts transduced with retroviruses revealed unforeseen results because of their ability to express recombinant genes for months to years after implantation into muscle,15,16 and long-term production of the VEGF protein by myoblast-mediated gene transfer results in high local concentrations of VEGF.12

The vascular tumors that we observed in the heart were much smaller than those previously reported in skeletal muscle,12 presumably because the animals did not survive long enough for a larger effect to occur (14 days for the heart versus 44 days for the skeletal muscle). Indeed, several of the VEGF-treated animals died before analysis was scheduled to begin. In all of the surviving VEGF-treated animals, the space-occupying vascular structures could have resulted in significant mechanical dysfunction, high-output cardiac failure due to shunting through these lesions, or cardiac arrhythmias. Any one or a combination could have contributed to the high morbidity and mortality of the VEGF-treated group.

A recent report of limited clinical effect of intracoronary administration of VEGF recombinant protein provided support for a gene therapy approach for therapeutic angiogenesis.17 Because of the short circulating half-life of VEGF recombinant protein and its reduced bioavailability and hypotensive effects, enthusiasm for the use of gene therapy for prolonged exposure to growth factors has been advocated. The delivery of plasmid VEGF or adenoviral expression of VEGF leads to the transient production of VEGF protein and has been shown to augment collateral development and tissue perfusion in ischemic muscle.18–21 Isner et al18 also noted small hemangiomas in a patient treated with intravascular VEGF plasmid that resolved after the presumed temporary expression of VEGF.

The formation of myocardial tumors by growth factors is not unique to VEGF. Banai et al22 observed bizarre, tumor-like whorls of smooth muscle cells in the myocardium of dogs treated with an acidic fibroblast growth factor–saturated sponge applied to the epicardium. The tumors were a result of smooth muscle hyperplasia. Although other growth factors can trigger cell growth, the production of vascular tumors may be specific to VEGF.

A potential advantage in the use of retrovirally transduced myoblast implantation in the treatment of ischemic myocardium is the ability to sustain therapeutic levels of VEGF in combination with cellular transplantation. The concept of cellular myocardial reconstruction is being investigated as an alternative to heart transplantation. Independent investigators have begun to explore the utility of fetal myocardial tissue,23–25 genetically modified cardiac myocytes,26,27 and the use of skeletal muscle cell transplantation in the repair of myocardial infarcts.28 The concept of combining angiogenesis and cellular transplantation for the repair of ischemic/infarcted myocardium is attractive and could serve as the basis for an effective mode of tissue engineering.

In summary, our results in murine hearts demonstrate a potential toxicity of unregulated myoblast-mediated VEGF expression. In our studies, the formation of vascular tumors was limited to the site of myoblast implantation. Similarly, constitutive delivery of VEGF from myoblasts that have been encapsulated in alginate and implanted into nonmuscle sites has led to uncontrolled angiogenesis and inflammation.12a Together, these studies highlight the need for regulated expression of a gene encoding such a potent product in the clinic. Efforts are under way to use regulatable vector systems29–32 that would allow for optimized VEGF levels and therapeutic angiogenesis without the risk of hemangiomas or uncontrolled cellular proliferation.

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