

PRECLINICAL RESEARCH

Cellular, But Not Direct, Adenoviral Delivery of Vascular Endothelial Growth Factor Results in Improved Left Ventricular Function and Neovascularization in Dilated Ischemic Cardiomyopathy

Arman Askari, MD,* Samuel Unzek, MD,† Corey K. Goldman, MD, PhD,†
Stephen G. Ellis, MD, FACC,* James D. Thomas, MD, FACC,* Paul E. DiCorleto, PhD,†
Eric J. Topol, MD, FACC,* Marc S. Penn, MD, PhD, FACC*†

Cleveland, Ohio

OBJECTIVES	We sought to compare the effects on angiogenesis and left ventricular (LV) function of adenoviral vascular endothelial growth factor-165 (AdVEGF-165) gene delivery by direct injection of AdVEGF-165 to the transplantation of skeletal myoblasts (SKMB) transfected with AdVEGF-165 in a rat model of ischemic cardiomyopathy.
BACKGROUND	Angiogenesis offers the potential for treating ischemic cardiomyopathy. However, the optimal method of delivering angiogenic factors for neovascularization remains undetermined. With the increased clinical interest in cell therapy for the treatment of LV dysfunction, SKMB transplantation may serve as a means of gene transfer.
METHODS	Two months after left anterior descending coronary artery ligation, rats received either injection of an adenoviral construct encoding VEGF-165, or 1 million SKMB transfected with AdLuciferase (AdLuc) or AdVEGF-165. Cardiac function was assessed echocardiographically, and neovascularization was assessed histologically four weeks after therapy.
RESULTS	Neovascularization was significantly increased by both AdVEGF delivery strategies (100 ± 7% and 185 ± 33% increase in vascular density compared with SKMB alone, respectively). However, cell-based delivery, but not direct injection of AdVEGF-165, resulted in increased cardiac function (73.5 ± 12.6% and 1.5 ± 8.8% increase in shortening fraction compared with saline control; AdLuc-transfected SKMB: 29.4 ± 15.0%). The improved function was not due to increased engraftment of VEGF expressing SKMB. Rather, improved function correlated with less apoptosis in the border zone in those animals that received AdVEGF-165 expressing SKMB.
CONCLUSION	Our data demonstrate that cell-based delivery of VEGF leads to an improved treatment effect over direct adenoviral injection, and suggest that already developed adenoviral vectors that encode secreted factors could potentially offer greater efficacy in combination with SKMB transplantation. (J Am Coll Cardiol 2004;43:1908–14) © 2004 by the American College of Cardiology Foundation

Therapeutic angiogenesis for the treatment of ischemic heart disease has demonstrated efficacy in several animal models and human pilot trials in patients not suitable for coronary revascularization. Angiogenic factors tested in human trials include fibroblast growth factor (FGF)-1, -2, and -4 and several isoforms of vascular endothelial growth factor (VEGF) (1). The optimal delivery method to induce neovascularization has yet to be determined.

Clinical strategies for delivery of angiogenic factors have included delivery of protein through intravenous (2) or intracoronary injection (2,3), intracoronary injection of adenovirus (4) or direct intramyocardial injection of

protein-coated beads (5), naked deoxyribonucleic acid (6), or adenoviral-based angiogenic gene products (7). Intracoronary delivery strategies of VEGF protein are limited by systemic toxicities including hypotension that develop in response to the high doses required to induce neovascularization (8,9). Naked DNA requires direct myocardial injection due to rapid degradation by circulating nucleases.

We have recently demonstrated the potential efficacy of cell transplantation as a means for reestablishing stem cell homing for the purpose of myocardial regeneration months after myocardial infarction (MI) (10). Similarly, autologous skeletal myoblast (SKMB) cell transplantation has shown some promise for improving left ventricular (LV) function when delivered within a few weeks of acute MI in multiple animal models (11–14), and is now being studied in clinical populations as an adjunct to coronary artery bypass grafting (15). The effects of adenoviral-mediated gene transfer to SKMB prior cell transplantation in the setting of an ischemic cardiomyopathy months after MI has not been studied.

From the Departments of *Cardiovascular Medicine and †Cell Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio. Dr. Askari is a recipient of the Vascular Biology Working Group Research Award and an American Heart Association, Ohio Valley Affiliate, Post-Doctoral Fellowship Award. Dr. Penn is a recipient of a grant from the Ralph Wilson Foundation. Drs. Askari and Unzek contributed equally to this work.

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Abbreviations and Acronyms

AdLuc	= adenoviral Luciferase
AdVEGF-165	= adenoviral vascular endothelial growth factor-165
LAD	= left anterior descending coronary artery
LV	= left ventricle/ventricular
MI	= myocardial infarction
SKMB	= skeletal myoblasts

In this study we directly compare the efficacy of two delivery strategies of adenoviral vascular endothelial growth factor-165 (AdVEGF-165) in a model of ischemic cardiomyopathy. Because untoward biological effects of adenoviral therapy are dose-dependent, we chose to compare equal doses of adenovirus in each of two arms: direct injection of AdVEGF-165 versus injection of SKMB transfected with AdVEGF-165. Our data demonstrate that efficacy of current adenoviral constructs encoding angiogenic factors can be significantly enhanced by combining gene transfer with cell transplantation due to a significant decrease in the adverse effects of adenovirus with this strategy.

METHODS

In our studies, ischemic cardiomyopathy was induced by anterior wall MI in 60 150- to 175-g male Lewis rats (Harlan Labs, Indianapolis, Indiana) via ligation of the left anterior descending (LAD) artery. Ischemic cardiomyopathy developed for two months before randomization to treatment strategy. There were three treatment groups in our study and two control groups. The control groups were injection of saline or intramyocardial injection of 10^7 pfu of adenoviral Luciferase (AdLuc) in five divided doses. Two groups were transplanted with 1 million SKMB that were transfected with 10^7 pfu of either AdVEGF-165 or AdLuc. A fourth group received five intramyocardial injections of AdVEGF-165 totaling 10^7 pfu.

LAD ligation. The animal research committee approved all animal protocols. Animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care animal facility of the Cleveland Clinic Foundation. Animals were anesthetized with sodium pentobarbital, 50 mg/kg, intubated, and ventilated with room air at 80 breaths/min using a pressure cycled rodent ventilator (Kent Scientific Corp., RSP1002, Torrington, Connecticut). Anterior wall MI was induced by direct ligation of the LAD with the aid of a surgical microscope (Leica M500, Heerbrugg, Switzerland).

Two-dimensional echocardiography. Two-dimensional echocardiography was performed five to seven days and eight weeks after LAD ligation using a 15-MHz linear array transducer interfaced with a Sequoia C256 (Siemens Medical Solutions, Malvern, Pennsylvania). Two-dimensional and M-Mode echocardiography were also performed four weeks after SKMB transplantation. Rats were lightly sedated with ketamine (50 mg/kg) for each echocardiogram.

Left ventricular dimensions and wall thickness were assessed using digitally recorded short-axis two-dimensional clips and M-Mode images from the mid-LV just below the papillary muscles. Measurements were made offline using ProSolve (Problem Solving Concepts, Indianapolis, Indiana) by two blinded observers to treatment groups (16). Each measurement in each animal was made six times, from three randomly chosen M-Mode clips out of five recorded. Shortening fraction (shortening fraction [%] = (LVEDD - LVESD)/LVEDD \times 100, where LVEDD = LV end-diastolic dimension and LVESD = LV end-systolic dimension) was calculated from M-Mode recordings.

Cell preparation and cell and viral delivery. Skeletal myoblasts were harvested from the hind limbs of several Lewis rats (Harlan Labs), plated in 175 ml culture flask, and grown in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum, 300 mg/l endothelial cell growth supplement, and the antibiotics penicillin, streptomycin, and ofloxacin. Cells were passed once 75% confluence was achieved to avoid differentiation.

On the day before cell transplantation, purified myoblasts were transfected with 1×10^7 pfu/ml of replication deficient, E1-, E3-deleted adenovirus-expressing VEGF-165 or adenovirus-expressing luciferase (control), both under the control of a cytomegalovirus promoter. Eight weeks after LAD ligation, myoblasts were harvested with trypsin, washed extensively in PBS to remove any free viral particles, and reconstituted immediately before transplantation. Animals were then anesthetized, ventilated, and subjected to a lateral thoracotomy for direct visualization of the infarct zone. Approximately 1×10^6 cells were injected per animal in five locations in the peri-infarct zone. The volume of each injection was 40 to 50 μ l using the same spatial distribution for cell injections in all animals: two injections each along the infarct border zones of the anterior-septal and posterior-lateral walls and one injection near the apex of the infarct zone. Similarly, direct injection of AdVEGF-165 or AdLuc into the peri-infarct was accomplished through five injections of 0.2×10^7 pfu each. No injections were made into the infarct zone. Volume of each injection was 100 μ l. In all experiments, two injections were made along the left and two along the right border of the peri-infarct zone; the fifth injection was in the peri-infarct zone at the LV apex.

Adenoviral construct. The adenoviral construct encoding VEGF-165 and luciferase was a generous gift from Gen Vec Inc. (Gaithersburg, Maryland). Briefly, 293 cells were obtained from American Type Culture Collection (ATCC CRL 1573, Manassas, Virginia) and were maintained in DMEM supplemented with 10% calf serum. The E1-, E3-deleted adenovirus-vector-encoding AdVEGF-165 or luciferase was generated by linearizing the shuttle vector plasmid at a unique restriction site adjacent to the left-end-inverted-terminal repeat and co-transfected into 293 cells with ClaI-digested H5dl324 DNA (17). After two sequential plaque purifications, vector stocks were propagated on 293 cells and purified through three sequential bandings on

Table 1. LV Wall Thickness, Cavity Dimensions, and Shortening Fraction Four Weeks After the Designated Treatment

Treatment	Wall Thickness (mm)		LV Dimensions (mm)		Shortening Fraction (%)	Infarct Size (% LV Cavity Circumference)
	Anterior	Posterior	End-Systolic	End-Diastolic		
Saline (n = 7)	0.85 ± 0.07	2.30 ± 0.1	9.6 ± 0.6	10.3 ± 0.6	6.8 ± 2.1	46.2 ± 5.1
AdLUC (n = 4)	0.87 ± 0.10	2.78 ± 0.25	10.8 ± 1.0	11.6 ± 1.3	6.2 ± 2.0	44.9 ± 6.3
AdVEGF (n = 4)	0.93 ± 0.09	2.67 ± 0.18	10.4 ± 1.0	11.2 ± 1.1	6.9 ± 1.2	43.5 ± 8.1
SKMB (n = 7)	0.84 ± 0.07	2.33 ± 0.14	9.0 ± 1.1	9.9 ± 0.8	8.9 ± 2.7*	45.1 ± 3.3
SKMB + AdVEGF (n = 6)	0.85 ± 0.15	2.47 ± 0.27	8.8 ± 1.2	10.1 ± 1.2	11.8 ± 2.1*†	48.7 ± 6.9

Treatment was begun eight weeks after LAD ligation. *p < 0.05 compared with saline group; †p < 0.05 compared with AdVEGF group. Data represent mean ± SD. LAD = left anterior descending coronary artery; LV = left ventricular; SKMB = skeletal myocyte.

cesium chloride gradients. The purified virus was dialyzed against a buffer containing 10 mM Tris, pH 7.8, 150 mM NaCl, 10 mM MgCl₂, and 3% sucrose and stored at -80°C until use. The transgene expression is under the control of the cytomegalovirus immediate early promoter.

Histologic analysis. Rats were euthanized, their hearts harvested for analysis four weeks after cell transplantation following perfusion fixation with HistoChoice (Amresco Inc., Solon, Ohio), and sectioned into three equal divisions perpendicular to the LV long axis. The mid-ventricular section was paraffin-embedded, and several sections 6-μm thick were obtained just below the papillary muscle for analysis. Sections were stained with hematoxylin and eosin for histologic analysis. To assist with blood vessel identification, sections were stained using an antibody to von Willebrand Factor (DAKO, Carpinteria, California) and an horseradish peroxidase-labeled goat anti-mouse secondary antibody. These sections were counterstained with hematoxylin. Blood vessels were counted throughout the infarct zone from four sections from each animal by a trained observer blinded to the identity of each animal.

Infarct size was quantified from hematoxylin-and-eosin-stained cross-sections from the mid-LV. Infarct size was quantified by % LV area containing infarcted tissue; however, because these hearts were 12 weeks after MI and the anterior wall had significantly thinned, the % area containing infarcted tissue did not adequately reflect the size of the MI induced by LAD ligation. Therefore, we also quantified the percent of the LV cavity circumference that had infarcted tissue.

Measurement of apoptosis by TUNEL assay. Apoptotic cells were identified by direct staining of the condensed nuclei or fragmented DNA with TUNEL-based staining (In Situ Cell Death Detection Kit, AP, Roche, Basel, Switzerland) (18). Tissues were deparaffinized, and the TUNEL reaction mixture comprised by a label and an enzyme solution was placed over the sections and incubated in a humidified chamber at 37°C for 1 h. The slides were rinsed and subsequently incubated with the Converter-AP (anti-fluorescein antibody Fab fragments from sheep, conjugated with alkaline phosphatase) for 30 min also in a humidifying chamber. After treatment with lavamisole to inhibit endogenous alkaline phosphatase activity, a substrate solution (alkaline phosphatase substrate kit, Vector Laboratories, Burlingame, California) was added for 45 min, and

tissues were counterstained with hematoxylin. In parallel, a positive control was done using DNAase I before labeling procedures to verify specificity of staining. Observers blinded to treatment quantified the number of TUNEL-positive cells per field from sections obtained from the mid-ventricle. Cardiac myocytes were identified by their distinct histologic appearance. Five high power fields (~120 nuclei/field) were assessed from each animal. The apoptotic index is the number of TUNEL-positive cells per 100 cells. **Statistical analysis.** Data are presented as mean ± SD. Comparisons between groups were by Student *t* test, or by analysis of variance.

RESULTS

Effect of SKMB transplantation on LV function. The goal of our study was to determine if SKMB expressing VEGF-165 led to significant neovascularization of the infarct zone and improved LV function. Therefore, our initial experiments focused on the effects of syngeneic SKMB transplantation on vascular density and LV function. Transplantation of SKMB transfected with AdLuc into the peri-infarct zone eight weeks after LAD ligation did not result in neovascularization of the infarct zone (data not shown). Skeletal myoblast transplantation did, however, produce a 30% increase in LV function as measured by shortening fraction at the level of the papillary muscle (Table 1).

Neovascularization in response to cell-based and direct adenoviral VEGF delivery. We compared the angiogenic response between direct injection of AdVEGF-165 to transplantation of SKMB transfected with AdVEGF-165 or AdLuc. We held constant the biologic dose of control (AdLuc) or AdVEGF-165 adenovirus used in each experimental arm to 1×10^7 pfu. To verify that we had similarly sized infarcts in each of the treatment groups, infarct size was quantified from hematoxylin-and-eosin-stained cross-sections from the mid-ventricle 12 weeks after acute MI (four weeks after treatment). The infarct size, whether measured as % of LV cavity circumference bordering the infarct zone (Table 1) or % of LV area of infarcted tissue (data not shown), was not significantly different between those animals that received direct injection of saline, AdVEGF, AdLuc, transplantation of SKMB transfected with AdLuc, or AdVEGF.

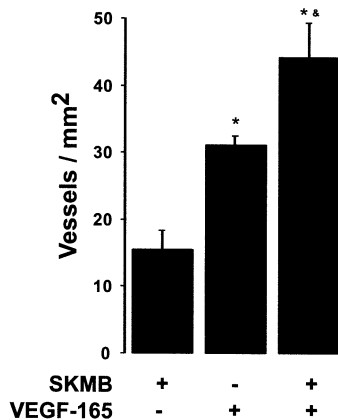


Figure 1. Direct adenoviral injection or transplantation of cells expressing vascular endothelial growth factor (VEGF)-165 induces neovascularization. Eight weeks after myocardial infarction induced by left anterior descending coronary artery ligation, the peri-infarct zone was injected with either 1×10^7 pfu of adenoviral VEGF-165 (AdVEGF-165) (n = 4) or 1 million skeletal myoblast (SKMB) transfected with AdLuciferase (n = 7) or AdVEGF-165 (n = 6) in five equally divided injections. The vasculature was identified by von Willebrand factor immunohistochemistry, and vascular density was quantified throughout the infarct zone. Data represent mean \pm SD. *p < 0.01 compared with SKMB transplantation alone. **p < 0.05 compared with AdVEGF injection.

The vascular density within the infarct zone was quantified by counting von Willebrand factor-stained vessels in tissue sections obtained just below the level of the papillary muscles. We found a significant increase in vascular density in those animals that received either injection of AdVEGF-165 or cell transplantation with VEGF-165 expressing SKMB (Fig. 1) compared with hearts that were transplanted with AdLuc-transfected SKMB. There was no gross or histologic evidence of hemangioma formation with any treatment strategy. A significantly greater increase in vascular density was seen in those animals treated with transplantation of SKMB expressing VEGF compared with direct AdVEGF injection (Fig. 1).

The representative photomicrographs in Figure 2 show that the infarct zone following AdLuc-transfected SKMB transplantation is relatively avascular (Fig. 2A). The neovascularization after VEGF-165 therapy by either modality results in the development of increased vascular density as characterized by an increase in the number of capillaries and small arterioles (Figs. 2B and 2C).

Cell-based delivery of VEGF results in improved LV function. To determine if either direct viral injection or cell-based expression of VEGF-165 leads to improved LV function, echocardiography was performed 12 weeks after MI (four weeks after treatment). The LAD ligation model used for these studies resulted in a significant decrease in shortening fraction. Improved LV function was seen in the hearts that underwent transplantation with SKMB expressing VEGF-165 compared with hearts that received direct injection of AdVEGF-165 (Table 1, Fig. 3). Furthermore, the improvement in LV function seen with transplantation of VEGF-165 expressing SKMB was significantly greater than that seen with transplantation of AdLuc-transfected

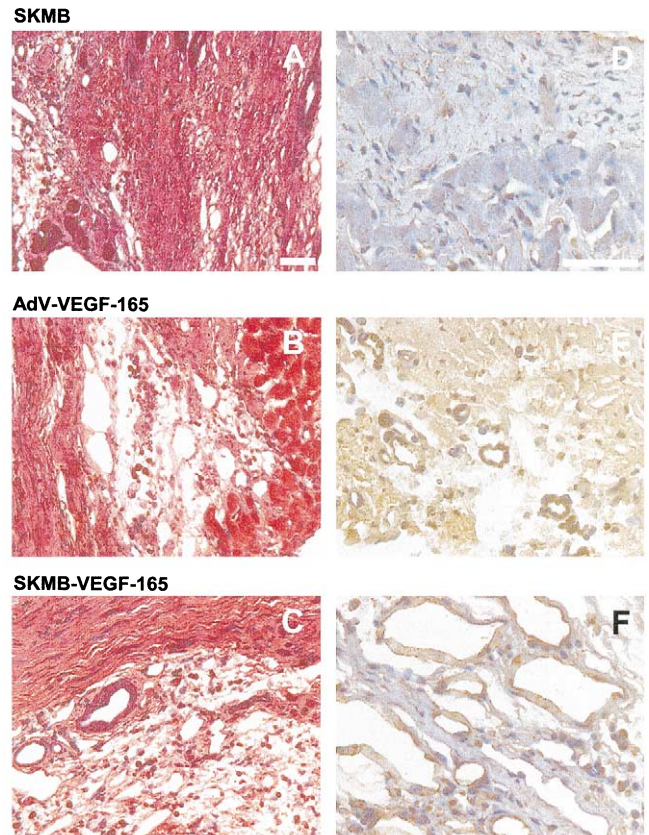


Figure 2. Vascular endothelial growth factor (VEGF)-165 induces neovascularization in the infarct zone. Representative sections of the infarct zone four weeks after injection into the peri-infarct zone of either 1 million skeletal myoblasts (SKMB) transfected with AdLuciferase (A, D), 1×10^7 pfu adenoviral VEGF-165 (AdVEGF-165) (B, E), or 1 million SKMB transfected with AdVEGF-165 (C, F) in five equally divided injections. (A, B, C) H & E staining and (D, E, F) immunohistochemistry for von Willebrand factor.

SKMB. Despite a significant increase in vascular density with direct injection of AdVEGF-165, no improvement in LV function compared with saline injection alone was seen. Direct injection of 10^7 pfu of AdLuc did not result in a statistically significant decrease in LV function (Table 1).

Effect of cell-based delivery of VEGF-165 on SKMB engraftment. To determine if the improved LV function with transplantation of VEGF-165 expressing SKMB was due to greater engraftment and survival of the SKMB into the infarct border zone, total protein extracts from pulverized LVs were assessed for SKMB content by Western blot analysis using an antibody (MY-32, ~200 kD) specific for skeletal myocyte heavy chain myosin (Fig. 4A). Five days after sham operation or transplantation with 1×10^6 SKMB transfected with AdLuc or AdVEGF-165, MY-32 was seen only in animals that received SKMB transplantation (Fig. 4A). Using this approach, we demonstrated that transfecting cells with AdVEGF-165 or AdLuc before transplantation did not result in any difference in skeletal myosin heavy chain content in the transplanted heart (Fig. 4B), suggesting similar levels of SKMB engraftment and survival between treatment groups. These data are consis-

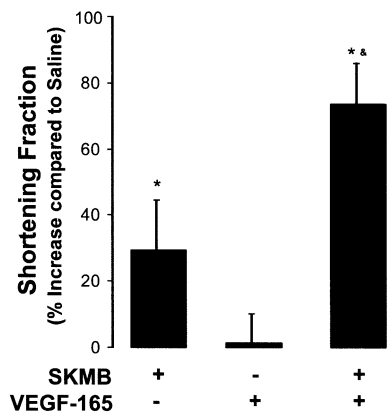


Figure 3. Transplantation of vascular endothelial growth factor (VEGF)-165 expressing skeletal myoblasts (SKMB) significantly increases LV function. Eight weeks after myocardial infarction, the peri-infarct zone was injected with either saline (n = 7), 1×10^7 pfu of adenoviral VEGF-165 (AdVEGF-165) (n = 4), or 1 million SKMB transfected with AdLuciferase (n = 7) or AdVEGF-165 (n = 6) in five equally divided injections. Left ventricular function was quantified four weeks later by echocardiogram. Left ventricular function is presented as shortening fraction (%) relative to saline control. Data represent mean \pm SEM. *p < 0.03 compared with saline control. **p < 0.05 compared with SKMB.

tent with hypothesis that the augmented LV function seen with transplantation of SKMB-expressing VEGF was not due to increased SKMB engraftment.

Cell-based AdVEGF delivery attenuates the inflammatory response and reduces apoptosis in the infarct border zone. To better understand plausible mechanisms leading to augmented LV function in animals receiving SKMB-based VEGF, we assessed hearts histologically for the inflammatory response to AdLuc or AdVEGF delivery and immunohistochemically for apoptosis. Four weeks after treatment, the peri-infarct zone in animals injected with adenovirus consistently revealed a robust inflammatory infiltrate (data not shown) that was not present in any of the animals transplanted with VEGF-165 expressing SKMB. In addition, a paucity of apoptotic cells were visualized in animals that received either SKMB alone or VEGF-expressing SKMB (Figs. 5A, 5C, and 5D); however, direct injection of AdVEGF induced a significantly greater number of apoptotic cells within the peri-infarct border zone at four weeks after treatment (Fig. 5B). The direct injection of AdLuc resulted in similar levels of apoptosis as seen in response to AdVEGF (Fig. 5D). Importantly, some of the TUNEL-positive cells within the infarct border zone were cardiac myocytes (Figs. 5B and 5D).

DISCUSSION

Therapeutic gene transfer for the induction of neovascularization may be beneficial for patients who are not candidates for coronary revascularization for the treatment of their myocardial ischemia (2-5,7), or as an adjunct to coronary revascularization. Local delivery of angiogenic factors minimizes untoward systemic effects of angiogenesis, such as tumor growth and retinopathy, and permits precise target-

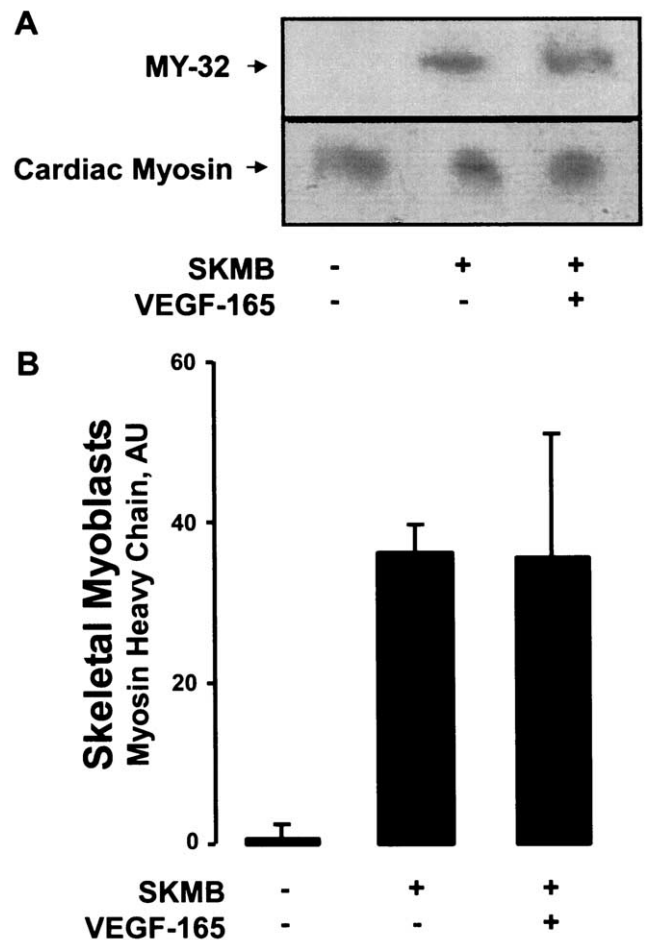


Figure 4. Adenoviral vascular endothelial growth factor (AdVEGF-165) transfection does not alter skeletal myoblast (SKMB) engraftment. (A) Representative lanes from Western blot analyses of 50 μ g of protein extract from whole hearts five days after sham operation (n = 3), or transplantation with 1×10^6 SKMB transfected with either AdLuciferase (n = 3) or AdVEGF-165 (n = 4) 18 h before transplantation using antibodies specific to either skeletal myocyte myosin heavy chain (MY-32) or the light chain of cardiac myosin. (B) Quantification of MY-32 bands from Western blot analysis in arbitrary units. Data represent mean \pm SD. p = NS SKMB vs. SKMB + AdVEGF-165.

ing of desired neovascularization. Furthermore, transient expression of angiogenic factors appears to be sufficient to induce neovascularization and minimize systemic effects and hemangioma formation (19). Multiple strategies have been studied for delivering angiogenic growth factors including direct injection of purified angiogenic proteins, plasmid, or viral constructs that encode for these proteins.

Autologous SKMB transplantation is a potential treatment modality for the treatment of ischemic cardiomyopathy. Several preclinical studies have demonstrated augmented LV systolic and diastolic function with SKMB transplantation (11,12). In addition, preliminary clinical studies have demonstrated feasibility and safety of SKMB transplantation, prompting active clinical trials (15,20). The potential for utilizing skeletal myoblasts as vehicles of gene delivery has been suggested in recent animal studies (21-

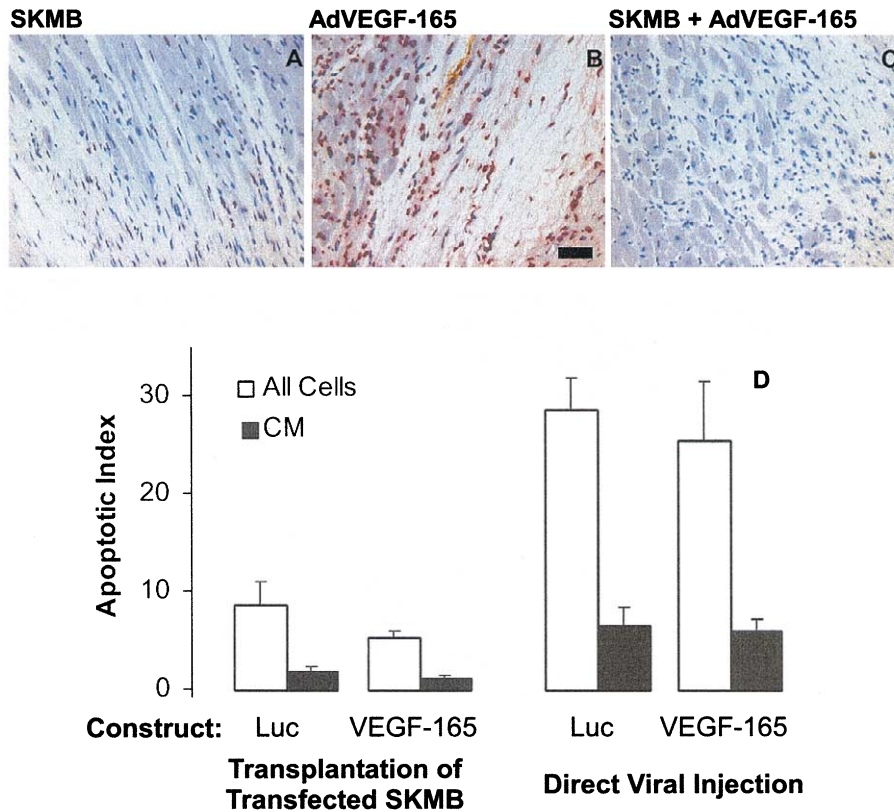


Figure 5. Adenoviral vascular endothelial growth factor-165 (AdVEGF-165) injection results in increased TUNEL-positive cardiac myocytes in the infarct border zone. Representative TUNEL-stained (red) nuclei in sections from the peri-infarct zone four weeks after injection of (A) 1 million skeletal myoblasts (SKMB), (B) 1×10^7 pfu AdVEGF-165, or (C) 1 million SKMB transfected with 1×10^7 pfu AdVEGF-165 each in five equally divided injections. (D) Number of TUNEL-positive cells (white bars) and TUNEL-positive cardiac myocytes (black bars) per 100 nuclei as quantified in five high power fields (~ 600 nuclei) from the infarct border zone per animal. Data represent mean \pm SD. $n = 4$ animals per group. $p < 0.01$ direct viral injection versus transfected SKMB for both Luc and VEGF-165. No significant differences were observed between VEGF-165 and Luc within each delivery strategy.

23); however, it has never been directly compared to the treatment effects of direct viral injection.

We tested the hypothesis that viral transfection of SKMB before transplantation with an adenoviral construct encoding for VEGF-165 would be more efficacious at inducing neovascularization and improving LV function than direct AdVEGF delivery or SKMB transplantation alone, in a model of ischemic cardiomyopathy. Transplantation of SKMB after adenoviral-mediated gene transfer has the advantage over the transplantation of SKMB following retroviral, lentiviral, adeno-associated viral, or stable transfection because gene expression after adenoviral-mediated transfection is transient. In the case of VEGF gene transfer, chronic VEGF expression could theoretically lead to hemangioma formation or retinopathy. Consistent with prior studies, transplantation of 1 million SKMB did not induce significant neovascularization but resulted in improved LV function.

The same amount of AdVEGF-165 resulted in significant increases in neovascularization by both delivery strategies studied (Fig. 1). However, transplantation of VEGF-165 expressing SKMB resulted in an $\sim 70\%$ increase in LV function, and no improvement in LV function was observed with direct injection of AdVEGF-165 (Fig. 3). The in-

crease in LV function was not due to greater SKMB survival within days after transplantation (Fig. 4).

Plausible mechanisms for the synergistic improvement in LV function observed with transplantation of VEGF-expressing SKMB and a lack of improvement in LV function observed in AdVEGF-treated animals despite increased neovascularization may relate to the induced inflammatory response to therapy and to apoptosis. Adenoviral-mediated gene transfer to the myocardium has been shown to cause robust myocardial inflammation and cardiac myocytes apoptosis (24). We found a significantly greater inflammatory response in the myocardial infarct border zone four weeks after direct AdVEGF injection compared with an absence of inflammation in the myocardium injected with AdVEGF-transfected SKMB. Furthermore, TUNEL staining revealed a substantial proportion of apoptotic cells, including apoptotic cardiac myocytes, within the infarct border zone of animals that received direct AdVEGF compared with SKMB-based VEGF delivery. Similar differences in apoptotic index of cardiac myocytes as we observed between SKMB and direct AdVEGF injection have been shown to result in significant differences in LV function (18).

Based on these findings, it is plausible that both delivery

strategies induce beneficial angiogenic effects on the myocardium, but that the deleterious inflammatory effects induced by the direct injection of AdVEGF-165 negates these beneficial effects. Thus, while increasing the dose of AdVEGF-165 or re-administering AdVEGF-165 into the infarct border zone at a later time may result in similar levels of angiogenesis as transplantation of AdVEGF-165-transfected SKMB, it is unlikely that this would result in improved LV function given the potential for further exacerbating the inflammatory response.

The current study confirms and extends existing data that autologous differentiated cell transplantation can augment LV function in a model of ischemic cardiomyopathy at a time remote from the MI. We further demonstrate a simple strategy for potentially improving the clinical efficacy of already developed adenoviral constructs that encode secreted molecules, and we suggest a testable strategy for future trials.

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Reprint requests and correspondence: Dr. Marc S. Penn, Experimental Animal Laboratory, Departments of Cardiovascular Medicine and Cell Biology, Cleveland Clinic Foundation, NC10, Cleveland, Ohio 44195. E-mail: pennm@ccf.org.

REFERENCES

1. Freedman SB, Isner JM. Therapeutic angiogenesis for coronary artery disease. *Ann Intern Med* 2002;136:54-71.
2. Udelson JE, Dilsizian V, Laham RJ, et al. Therapeutic angiogenesis with recombinant fibroblast growth factor-2 improves stress and rest myocardial perfusion abnormalities in patients with severe symptomatic chronic coronary artery disease. *Circulation* 2000;102:1605-10.
3. Simons M, Annex BH, Laham RJ, et al. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. *Circulation* 2002;105:788-93.
4. Grines CL, Watkins MW, Helmer G, et al. Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris. *Circulation* 2002;105:1291-7.
5. Laham RJ, Sellke FW, Edelman ER, et al. Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial. *Circulation* 1999;100:1865-71.
6. Vale PR, Losordo DW, Milliken CE, et al. Randomized, single-blind, placebo-controlled pilot study of catheter-based myocardial gene transfer for therapeutic angiogenesis using left ventricular electromechanical mapping in patients with chronic myocardial ischemia. *Circulation* 2001;103:2138-43.
7. Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* 1999;100:468-74.
8. Hariawala MD, Horowitz JR, Esakof D, et al. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res* 1996;63:77-82.
9. Lopez JJ, Laham RJ, Carrozza JP, et al. Hemodynamic effects of intracoronary VEGF delivery: evidence of tachyphylaxis and NO dependence of response. *Am J Physiol* 1997;273:H1317-23.
10. Askari A, Unzek S, Popovic ZB, et al. Effect of stromal-cell-derived factor-1 on stem cell homing and tissue regeneration in ischemic cardiomyopathy. *Lancet* 2003;362:697-703.
11. Scorsin M, Hagege A, Vilquin JT, et al. Comparison of the effects of fetal cardiomyocyte and skeletal myoblast transplantation on postinfarction left ventricular function. *J Thorac Cardiovasc Surg* 2000;119:1169-75.
12. Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 1998;4:929-33.
13. Koh GY, Klug MG, Soonpaa MH, Field LJ. Differentiation and long-term survival of C2C12 myoblast grafts in heart. *J Clin Invest* 1993;92:1548-54.
14. Penn MS, Francis GS, Ellis SG, Young JB, McCarthy PM, Topol EJ. Autologous cell therapy for the treatment of damaged myocardium. *Prog Cardiovasc Dis* 2002;45:21-32.
15. Menasche P, Hagege AA, Scorsin M, et al. Myoblast transplantation for heart failure. *Lancet* 2001;357:279-80.
16. Askari A, Brennan ML, Zhou X, et al. Myeloperoxidase and plasminogen activator inhibitor-1 play a central role in ventricular remodeling after myocardial infarction. *J Exp Med* 2003;197:615-24.
17. Chinnadurai G, Chinnadurai S, Brusca J. Physical mapping of a large-plaque mutation of adenovirus type 2. *J Virol* 1979;32:623-8.
18. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001;7:430-6.
19. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. *Circulation* 2000;102:898-901.
20. Menasche P, Hagege AA, Vilquin JT, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol* 2003;41:1078-83.
21. Koh GY, Kim SJ, Klug MG, Park K, Soonpaa MH, Field LJ. Targeted expression of transforming growth factor-beta 1 in intracardiac grafts promotes vascular endothelial cell DNA synthesis. *J Clin Invest* 1995;95:114-21.
22. Suzuki K, Murtuza B, Smolenski RT, et al. Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor-expressing skeletal myoblasts. *Circulation* 2001;104:1207-12.
23. Yau TM, Fung K, Weisel RD, Fujii T, Mickle DA, Li RK. Enhanced myocardial angiogenesis by gene transfer with transplanted cells. *Circulation* 2001;104:1218-22.
24. Wright MJ, Wightman LM, Lilley C, et al. In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. *Basic Res Cardiol* 2001;96:227-36.