Synergistic Effect of Bone Marrow Mobilization and Vascular Endothelial Growth Factor-2 Gene Therapy in Myocardial Ischemia

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Background—We performed a series of investigations to test the hypothesis that combining angiogenic gene therapy and cytokine (CK)-induced endothelial progenitor cell mobilization would be superior to either strategy alone for treatment of chronic myocardial ischemia.

Methods and Results—A swine model of chronic myocardial ischemia and a murine model of acute myocardial infarction were used in this study. In both models, animals were randomly assigned to 1 of 4 treatment groups: Combo group, intramyocardial vascular endothelial growth factor (VEGF)-2 gene transfer plus subcutaneous injection of CKs; VEGF-2, VEGF-2 gene transfer plus saline subcutaneously injected; CK, empty vector transfer plus CKs; and control, empty vector plus subcutaneous saline. Acute myocardial infarction was also induced in wild-type mice 4 weeks after bone marrow transplantation from enhanced green fluorescent protein transgenic mice to permit observation of bone marrow–derived cells in the myocardium after acute myocardial infarction. In chronic myocardial ischemia, combination therapy resulted in superior improvement in all indexes of perfusion and function compared with all other treatment groups. In the bone marrow transplant mice, double immunofluorescent staining revealed that the combination of CK-induced mobilization and local VEGF-2 gene transfer resulted in a significant increase in the number of bone marrow–derived cells incorporating into the neovasculature, indicating that recruitment and/or retention of bone marrow–derived progenitors was enhanced by mobilization and that local VEGF-2 gene transfer can provide signals for recruitment or incorporation of circulating progenitor cells.

Conclusions—Mobilization of endothelial progenitor cells with cytokines potentiates VEGF-2 gene therapy for myocardial ischemia and enhances bone marrow cell incorporation into ischemic myocardium. (Circulation. 2004;110:1398-1405.)

Key Words: cytokines ■ endothelial cells ■ ischemia ■ stem cells ■ vascular endothelial growth factor

Catheter-based, intramyocardial vascular endothelial growth factor (VEGF) gene transfer has been shown to induce therapeutic angiogenesis in preclinical models of myocardial ischemia1,2 and to increase exercise tolerance time and decrease the incidence of anginal episodes in pilot studies in patients with chronic myocardial ischemia.3 These favorable effects of VEGF gene transfer were accompanied by objective evidence of improvement of myocardial perfusion in a recent placebo-controlled, double-blind pilot clinical trial.4 The principal mechanism of these effects was initially thought to be the formation of new blood vessels by sprouting and migration of preexisting endothelial cells in ischemic tissue.5–8 However, extensive preclinical data have suggested that a portion of the effect of angiogenic cytokines (CKs) involves the mobilization and recruitment of precursor cells, capable of differentiation into endothelial cells, from the bone marrow (BM).9,10 Recent clinical data have revealed that VEGF gene transfer is also accompanied by the mobilization of BM-derived endothelial progenitor cells (EPCs).11 Moreover, extensive preclinical and early clinical data have revealed that EPCs may exert a therapeutic effect on ischemic tissue when administered systemically or locally.12–16 Together, these data suggest that administration of exogenous angiogenic growth factors may stimulate both angiogenesis and vasculogenesis for therapeutic neovascularization.

Granulocyte colony stimulating factor (G-CSF) and stem cell factor (SCF) are CKs that have been clinically applied for mobilization of BM-derived hematopoietic stem cells into the systemic circulation.17–19 Administration of G-CSF and SCF...
has been reported to improve left ventricular (LV) function in mice with acute myocardial infarction (MI) through increased homing of mobilized, BM-derived EPCs and cardiomyogenic progenitor cells into ischemic myocardium, providing direct evidence that mobilization of BM progenitors might represent a viable strategy for preserving the integrity and restoring function in ischemic tissue. In the present study, we performed experiments to test the hypothesis that BM mobilization can augment VEGF gene transfer--induced therapeutic neovascularization by enhancing the contribution of BM--derived precursor cells.

Methods

Experimental Animals

All animals were handled in accordance with the guidelines of the Animal Care and Use Committee at St Elizabeth's Medical Center (Boston, Mass).

Thirty-two male Yorkshire swine (Pine Acre Rabbity Farm, Norton, Mass) weighing 20 to 25 kg were used to induce chronic myocardial ischemia. After left thoracotomy, an aneurysm constrictor (Research Instruments SW) was placed around the proximal portion of the left circumflex coronary artery (LCx) as previously described. 23 Thirty BM transplant (BMT) animal models were also prepared as previously described as a means of documenting the kinetics of BM-derived cells in the ischemic myocardium. In brief, female C57BL/6 mice received BM mononuclear cells from transgenic mice constitutively overexpressing enhanced green fluorescent protein (eGFP mice, C57BL/6-TgN[ACTbEGFP]1Osb, Jackson Laboratory) after sublethal irradiation. Four weeks after BMT, by which time the BM of the recipient mice was reconstituted, BMT mice were used for experiments.

Mice were anesthetized with 2,2,2-Tribromoethanol (200 μL/kg body weight IP), orally intubated with a 22G IV catheter, and ventilated with a respirator (Harvard Apparatus). A left intercostal thoracotomy was performed, and the ribs were retracted with 5-0 polypropylene sutures to open the chest. After the pericardium was opened, the left anterior descending coronary artery (LAD) was ligated distal to the bifurcation between the LAD and diagonal branch with 8-0 polypropylene sutures through a dissecting microscope. After positive end-expiratory pressure was applied to fully inflate the lung, the chest was closed with 7-0 polypropylene sutures. The overall survival ratio after MI was 86% at 4 weeks.

Administration of Plasmid Human VEGF-2 Gene and CKs

In the swine study, NOGA nonfluorescent LV electromechanical mapping was performed to guide injections to foci of myocardial ischemia 5 weeks after constrictor placement. The NOGA system (Cordsis) of catheter-based mapping and navigation and has been previously described in detail. 2,25,26 Ischemic myocardium was identified as a zone with unipolar voltage higher than an automatically defined cutoff and linear local shortening of <3%. 21 This definition was consistent in all examinations throughout this study. Immediately after the ischemic territory was identified by NOGA mapping, 800 μg plasmid human VEGF-2 gene (phVEGF-2) in 3 mL PBS or 800 μg empty vector in 3 mL PBS was injected into 6 sites within the ischemic myocardium (500 μL to each site) using the NOGA injection catheter (MyoSTAR, Cordsis). The rhG-CSF (5 μg · kg⁻¹ · d⁻¹) and rhSCF (20 μg · kg⁻¹ · d⁻¹) or control saline was injected subcutaneously in swine with myocardial ischemia for 7 days starting immediately after the intramyocardial gene transfer. Swine were randomly assigned to 1 of 4 treatment groups: Combo group (n=8), catheter-based intramyocardial gene transfer of 800 μg of phVEGF-2 and subcutaneous rhG-CSF (5 μg · kg⁻¹ · d⁻¹) and rhSCF (20 μg · kg⁻¹ · d⁻¹) for 7 days immediately after the gene transfer; VEGF-2 group (n=8), phVEGF-2 gene transfer and saline injection; CK group (n=8), empty vector transfer and subcutaneous rhG-CSF and rhSCF injection; and control group (n=8), empty vector transfer and saline injection.

In the mouse study, 100 μg phVEGF-2 or empty vector as control (both dissolved in 100 μL saline) was administered intramyocardially with a 30G needle distal to the LAD occlusion site immediately after LAD ligation. The combination of CKs with recombinant human (rh) G-CSF (50 ng/g body weight) and rhSCF (200 ng/g) or control saline was administered (subcutaneously) daily for a week (days 0 to 6) after MI (rhG-CSF and rhSCF were supplied by Amgen, Inc). The mice were randomly assigned to 4 subgroups (n=6 in each group): empty vector plus saline (control group), empty vector plus CKs (CK group), phVEGF-2 plus saline (VEGF-2 group), or phVEGF-2 plus CKs (Combo group).

Physiological Assessment of LV Function and Ischemia

In the swine study, transthoracic echocardiography (SONOS 5500), selective left coronary angiography, and NOGA LV electromechanical mapping were performed 5 weeks after constrictor placement (just before injection of genes) and 4 weeks after gene injection. Echocardiographic fractional shortening (FS) and regional wall motion scores were quantified by use of the LV short-axis view at the midpapillary muscle level. Collateral flow to the LCx territory was graded angiographically in a blinded fashion with the Rentrop scoring system. The area of ischemia was quantified by NOGA mapping as previously described. 2 All data were evaluated by blinded observers (echocardiography by K.K., coronary angiography by S.S., and postprocessing analysis of the NOGA mapping by I.J.).

Histological Assessment of Neovascularization and LV Remodeling

All swine were killed 4 weeks after gene transfer. At necropsy, swine hearts were sliced in a bread-loaf fashion into 4 transverse sections from apex to base, and each section was separated to anterior, lateral, posterior LV free wall, interventricular septum, and right ventricular free wall. All tissues obtained from each segment were fixed in 100% methanol. Immunohistochemistry for isoclectin B4 was also performed to evaluate capillary density in the ischemic myocardium identified by NOGA mapping.

Double Immunofluorescence Histochemistry

The hearts of GFP-BM transplanted mice were harvested at predetermined time points after surgery and prepared for frozen tissue sections. Frozen cross sections (6-μm thickness) were air dried and fixed with 4% paraformaldehyde for 5 minutes. After washing with PBS, double immunohistochemistry was performed with antibodies against GFP and isoclectin B4. Nonspecific protein binding was blocked with 10% normal goat serum. Rabbit polyclonal anti-GFP antibody (1:200 dilution; Molecular Probe) was used at 4°C overnight, followed by goat anti-rabbit IgG conjugated with Cy2 (1:500 dilution; Jackson ImmunoResearch) as a secondary antibody for 30 minutes at room temperature. The endothelial cell--specific marker, biotinylated isoclectin-B4 (1:100 dilution; Vector Laboratories), was used as a secondary primary antibody for capillary staining and visualized by binding with rhodamine-conjugated streptavadin (1:500 dilution; Jackson Immunoresearch) for 30 minutes at room temperature. Normal rabbit IgG served as a negative control for GFP detection. Nuclei were counterstained with DAPI (1:5000) and mounted in aqueous mounting medium. Images were examined with a fluorescent microscope (Nikon ECLIPSE TE2000).

Double-positive cells were quantified in 5 randomly selected fields from 5 sections from each heart. All morphometric studies were performed by 3 examiners (H.M., A.H., and M.L.) who were blinded to treatment assignment.

Statistical Analysis

All values are expressed as mean±SE. Student’s paired t test was used to compare data before and after treatment. ANOVA was
performed to compare data among the 4 treatment groups. A value of
\( P < 0.05 \) was considered statistically significant.

**Results**

Two pigs died in the control and CK groups before the final assessment. There were no deaths in the VEGF-2 or Combo groups.

**BM Mobilization Augments the Effects of VEGF-2 Gene Transfer for Attenuation of Chronic Myocardial Ischemia**

In the swine study, selective left coronary angiography was performed to evaluate collateral development before and after treatment. The mean Rentrop score of collateral development was performed to identify capillaries in the ischemic myocardium 4 weeks after treatment. Capillary density was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (879.9 \pm 44.8, 717.0 \pm 75.7, 326.4 \pm 14.1, and 345.0 \pm 20.4/mm², respectively; \( P < 0.03 \) versus VEGF-2, \( P < 0.0001 \) versus CK and control). Capillary density was also significantly greater in the VEGF-2 group than in the CK and control groups (\( P < 0.0001 \)). Capillary density in the Combo group was similar to that in the control group (Figure 3). These data reveal that in addition to augmenting the angiographically visible collateral supply, combined VEGF-2 myocardial gene therapy plus CK-induced mobilization of BM progenitors resulted in a significant increase in microvascular capillary density compared with monotherapy.

**Cytokine-Induced BM Mobilization Enhances Therapeutic Neovascularization by VEGF-2 Gene Transfer**

In the swine study, selective left coronary angiography was performed to evaluate collateral development before and after treatment. The mean Rentrop score of collateral development to the LCx territory at baseline was 1.3 \pm 0.3 in the Combo group, 1.4 \pm 0.3 in the VEGF-2 group, 2.0 \pm 0.4 in the CK group, and 1.0 \pm 0.3 in the control group (\( P = \text{NS} \)). The change in the Rentrop score after treatment was significantly greater in the Combo group than in the control group (\( P < 0.01 \)). The change in the Rentrop score in the Combo group was similar to those in VEGF-2 and CK groups; however, neither the VEGF-2 nor the CK group showed an improvement in Rentrop scores that was significantly greater than the controls (Figure 2). These data indicated that there was anatomic evidence of improved collateral formation in the Combo therapy group compared with all other treatment groups.

In the swine study, histochemical staining for isolecit B4 was performed to identify capillaries in the ischemic myocardium 4 weeks after treatment. Capillary density was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (879.9 \pm 44.8, 717.0 \pm 75.7, 326.4 \pm 14.1, and 345.0 \pm 20.4/mm², respectively; \( P < 0.03 \) versus VEGF-2, \( P < 0.0001 \) versus CK and control). Capillary density was also significantly greater in the VEGF-2 group than in the CK and control groups (\( P < 0.0001 \)). Capillary density in the Combo group was similar to that in the control group (Figure 3). These data reveal that in addition to augmenting the angiographically visible collateral supply, combined VEGF-2 myocardial gene therapy plus CK-induced mobilization of BM progenitors resulted in a significant increase in microvascular capillary density compared with monotherapy.

**BM Mobilization Augments the Effects of VEGF-2 Gene Transfer on LV Function in Chronic Myocardial Ischemia**

In the swine study, echocardiographic FS and regional wall motion score before treatment were similar in all groups (FS: Combo, 27.6 \pm 1.3%; VEGF-2, 29.6 \pm 0.9%; CK, 30.5 \pm 1.4%; control, 29.4 \pm 1.2%; regional wall motion score: Combo, 22.4 \pm 1.0; VEGF-2, 20.8 \pm 0.7; CK, 20.4 \pm 0.5; control, 20.5 \pm 0.8). The improvement in FS after treatment was significantly greater in the Combo group than in the VEGF-2, CK, and control groups (5.3 \pm 0.9%, 1.0 \pm 1.2%, 1.1 \pm 0.8%, and 0.8 \pm 1.3%, respectively; \( P = 0.03 \) versus VEGF-2, \( P = 0.01 \) versus CK, \( P = 0.001 \) versus control). Changes in FS were similar in the VEGF-2, CK, and control groups. Regional wall motion score after treatment was significantly improved in the Combo group compared with the VEGF-2, CK, and control groups (\( \Delta 3.9 \pm 1.0, -1.3 \pm 0.9, 0.4 \pm 0.5 \), and 1.2 \pm 0.9, respectively; \( P = 0.04 \) versus VEGF-2, \( P = 0.009 \) versus CK, and \( P = 0.0004 \) versus control; Figure 4a and 4b).

**Cytokine Mobilization Increases Recruitment and Incorporation of BM Cells Into Myocardial Neovascularure**

Immunohistostiochemistry was performed on the hearts from BMT mice 1 week after MI to assess BM-derived cell incorporation into the neovascularure. Double immunofluorescent staining for eGFP and isolecit B4 permitted identification of BM-derived cells that also expressed a marker of endothelial cell identity (Figure 5A). The double-positive cells were quantified and were found to be most abundant in the border zones between ischemic and nons ischemic tissue in the Combo group (50.7 \pm 5.8), followed by the VEGF-2 group (19.8 \pm 3.7). Both groups had significantly greater numbers of double-positive cells than the control group (\( P < 0.001 \)), and the number of double-positive cells in the Combo group was significantly greater than in the VEGF group (\( P < 0.01 \)); Figure 5B. As shown in Figure 5A, some of the double-positive cells were incorporated into tubular structures, consistent with vasculogenesis. These data provide evidence that VEGF gene therapy stimulates vasculogenesis in the myocardium and that this effect can be augmented by BM mobilization.

**Discussion**

The concept of therapeutic angiogenesis by administration of angiogenic genes or proteins has been established in numerous preclinical models.1,5,8 Recently, pilot clinical trials of therapeutic angiogenesis using some of these growth factors have been reported in patients with coronary artery disease.4,29–31 Although subjective symptoms have been significantly improved in these phase I and II trials, some studies have failed to demonstrate significant improvement in objective findings such as myocardial perfusion and exercise tolerance. Analysis of the data generated in all these pilot studies reveals at least 2 common features: (1) In each study, the effect of a single agent was evaluated, and (2) certain patients are “nonresponders.” The absence of a response in certain individuals is a consistent feature of all therapies and is the basis for the concept of pharmacogenomics, the science of designing drugs based on genetic features of individual
patients. Lacking this tailored approach to drug development, physicians have traditionally tried combining drugs to achieve therapeutic effects in patients with conditions refractory to single agents.

In parallel with studies attempting neovascularization by administration of angiogenic CKs, the use of progenitor or stem cells as therapeutic agents in ischemic diseases has emerged. These studies are based on observations indicating that circulating cells, some of which appear to originate in the BM, are capable of homing to and augmenting neovascularization of ischemic tissue. More recent data have indicated that at least part of the effect of locally administered angiogenic CKs results from recruitment of progenitor cells and that the failure of native or therapeutic
neovascularization might result in part from a deficiency in the quantity or quality of these cells.\textsuperscript{11,36–38}

This constellation of findings raised an important fundamental question regarding VEGF gene therapy for therapeutic neovascularization: Is the mechanism of local VEGF predominantly via local effects, enhancing the proliferation and migration of EC in pre-existing blood vessels, or is it possible that VEGF, expressed after gene transfer in the local tissue environment, is acting as a chemokine, recruiting progenitor cells from remote sites to deliver a more varied repertoire of CKs\textsuperscript{39} in addition to providing parent cells for the neovascularure?\textsuperscript{33} The latter possibility is well illustrated in studies by Orlic et al\textsuperscript{40} in the setting of acute ischemia in which the local homing signals for circulating cells are apparently robust, obviating the need for induction of local CK expression.

Accordingly, we hypothesized that the effect of transient local expression of VEGF, mediated by gene transfer of naked plasmid DNA, might be amplified by increasing the circulating supply of progenitor cells by systemically administered hematopoietic stem cell mobilization using GCSF and SCF. This is consistent with a report demonstrating superiority of a combination of growth factor therapy and cell transplantation. In this previous study,\textsuperscript{41} the combination of hepatocyte growth factor gene transfer and neonatal rat cardiomyocyte transplantation had more potent therapeutic efficacy in a model of rat MI compared with either single treatment.

Although the therapeutic potential of systemically administered, mobilizing CKs has been reported in the setting of acute MI,\textsuperscript{20} the efficacy of the same approach in chronic myocardial ischemia has not been defined in animal models. Interestingly, this approach has been attempted in a single human pilot study of granulocyte-macrophage CSF administration.\textsuperscript{42} This study revealed potential benefit by a novel

Figure 2. Change in Rentrop grade of collateral development 4 weeks after gene transfer in porcine model of chronic myocardial ischemia. *$P<0.05$.

Figure 3. a, Representative immunohistochemistry for isoelecon B4 in specimens of ischemic porcine myocardium from 4 treatment groups. These specimens were obtained 4 weeks after gene transfer. b, Capillary density 4 weeks after gene transfer. *$P<0.05$; ***$P<0.001$. 
method of coronary flow measurement, but no change in symptoms or physiologically induced ischemia was reported, and these initial findings have not yet been repeated or extended in further studies.

In our swine study, monotherapy with CKs failed to attenuate chronic myocardial ischemia, to increase vascularity in the ischemic myocardium, or to improve LV function. In contrast, as documented previously, monotherapy with VEGF-2 gene transfer significantly improved chronic myocardial ischemia as documented by NOGA mapping, improved capillary density, and resulted in a favorable trend in LV functional improvement. The results of VEGF-2 gene transfer were consistent with previous reports in preclinical and pilot clinical trials. Most notably, however, the combination of VEGF-2 gene transfer plus CKs was superior to the monotherapies in terms of neovascularization and LV functional recovery. These favorable outcomes support the notion that progenitor cells play a key role in VEGF-induced local tissue revascularization and that the combination of BM mobilization and gene therapy can achieve superior therapeutic neovascularization.

To provide additional evidence for the enhanced contribution of BM-derived cells after combination therapy, BMT from eGFP mice into wild-type mice was performed. Histological examination revealed greater numbers of BM-derived cells in the myocardial neovascular area in mice receiving combination therapy than in those receiving monotherapy. These findings are consistent with prior observations. VEGF-1 has previously been shown to enhance mobilization of BM-derived EPCs into the circulation and to increase the incorporation of EPCs into sites of neovascularization. Intramyocardial VEGF-2 gene transfer also increased circulating EPC counts. These and other prior studies suggested that progenitor cells were an integral component of ischemia-induced neovascularization. The present findings provide additional evidence to support a fundamental role for EPCs in ischemia-induced neovascularization and suggest that therapies directed at enhancing the supply of these cells may be helpful in addressing the failure of native or CK-induced collateral vessel formation. Moreover, the failure of CK-induced EPC mobilization as a monotherapy in the setting of chronic ischemia indicates that
a local signal, in this case provided by VEGF gene therapy, is required for recruitment and incorporation of circulating progenitors. The precise mechanisms governing the recruitment, retention, and incorporation of BM-derived progenitors into the myocardial tissue and the relative roles of each in the enhanced functional recovery documented remain to be elucidated.

Together, these findings underscore the likelihood that progenitor cells must be considered not only as a part of the native mechanisms that govern vascular biology but also as entities whose failure may play a fundamental role in the advent of vascular pathology. Modulation of progenitor cell function therefore represents a reasonable therapeutic target for treatment of ischemic diseases.

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