Progressive Attenuation of Myocardial Vascular Endothelial Growth Factor Expression Is a Seminal Event in Diabetic Cardiomyopathy

Restoration of Microvascular Homeostasis and Recovery of Cardiac Function in Diabetic Cardiomyopathy After Replenishment of Local Vascular Endothelial Growth Factor

Young-sup Yoon, MD, PhD; Shigeki Uchida, MD, PhD; Osamu Masuo, MD, PhD; Manfred Cejna, MD; Jong-Seon Park, MD, PhD; Hyeon-cheol Gwon, MD, PhD; Rudolf Kirchmair, MD; Ferdinand Bahlman, MD; Dirk Walter, MD; Cynthia Curry, BS; Allison Hanley, BS; Jeffrey M. Isner, MD†; Douglas W. Losordo, MD

Background—Diabetic cardiomyopathy (DCM) is characterized by microvascular pathology and interstitial fibrosis, which leads to progressive heart failure; however, the pathogenesis of DCM remains uncertain.

Methods and Results—Using the streptozotocin-induced diabetic rat model, we evaluated the natural course of DCM over a period of 1 year by serial echocardiography, Western blot analysis for vascular endothelial growth factor (VEGF), endothelial progenitor cell assays, myocardial blood flow measurements, and histopathologic analysis that included terminal dUTP nick end-labeling (TUNEL), capillary and cardiomyocyte density, and fibrosis area. Downregulation of myocardial VEGF expression preceded all other features of DCM and was followed by increased apoptosis of endothelial cells, decreased numbers of circulating endothelial progenitor cells, decreased capillary density, and impaired myocardial perfusion. Apoptosis and necrosis of cardiomyocytes ensued, along with fibrosis and progressive diastolic and then systolic dysfunction. To provide further evidence of the central role of VEGF in the pathophysiology of DCM, we replenished myocardial VEGF expression using naked DNA gene therapy via direct intramyocardial injection of plasmid DNA encoding VEGF (phVEGF165). VEGF-replenished rats showed increased capillary density, decreased endothelial cell and cardiomyocyte apoptosis, and in situ differentiation of bone marrow–derived endothelial progenitor cells into endothelial cells. These anatomic findings were accompanied by significant improvements in cardiac function.

Conclusions—These findings suggest that downregulation of VEGF may compromise microvascular homeostasis in the myocardium and thereby play a central role in the pathogenesis of DCM. (Circulation. 2005;111:2073-2085.)

Key Words: diabetes ■ cardiomyopathy ■ growth substances ■ gene therapy ■ angiogenesis

Heart disease, often presenting as cardiomyopathy, is the leading cause of death among patients with diabetes mellitus (DM). DM, in turn is the most significant comorbidity of patients with heart failure (HF) and adversely affects outcomes in patients with cardiovascular disease. Both insulin-dependent and non–insulin-dependent DM may result in myocardial dysfunction in the absence of coronary artery disease or systemic hypertension. This condition, diabetic cardiomyopathy (DCM), has been postulated to contribute to both a higher incidence of congestive HF and mortality in diabetic patients. Because clinical studies of DCM have been limited by the influences of other comorbidities, such as coronary artery disease, experimentally induced animal models of DCM have been developed. As in humans, in streptozotocin-induced non–insulin-dependent DM rats, it is well documented that both diastolic and systolic function are impaired independent of epicardial coronary disease. Pathologically, studies from humans and animal models reveal myocardial hypertrophy, microvascular pathology, myocyte death, and varying degrees of fibrosis. Interstitial fibrosis, one of the characteristic
findings of DCM, can act as a barrier to myocardial perfusion that can worsen myocardial ischemia.12 Vascular endothelial growth factor (VEGF; specifically, VEGF-A) is a major mediator of neovascularization in physiological and pathological conditions, playing crucial roles in developmental blood vessel formation and regulation of hypoxia-induced tissue angiogenesis.13 Previously, we showed that attenuated augmentation of VEGF expression in response to hindlimb ischemia was responsible for the impaired recovery observed in diabetic animals.14 In that case, however, the deficit was manifest only on induction of a physiological stress. Recently, expression of the mRNA for cardiac VEGF was shown to be decreased in both diabetic rats and in humans; however, no data on protein expression or the relationship between VEGF expression and any pathological abnormalities or functional impairment were provided.15

The relationship between VEGF expression, the microvasculature, and cardiac function was demonstrated most vividly in experiments in transgenic mice lacking the VEGF isoforms VEGF165 and VEGF189, which exhibited impaired myocardial angiogenesis and subsequently developed severe left ventricular (LV) dysfunction.16 In human idiopathic dilated cardiomyopathy, mRNA transcript levels of VEGF165 and VEGF189 and protein levels of VEGF and VEGF receptor (VEGFR) type 1 have been shown to be downregulated,17 consistent with a role for VEGF in the myocardial microvasculature. Furthermore, experimental animal models and patient studies have also provided evidence that cardiomyopathy can result from microcirculatory defects in the absence of epicardial coronary artery stenosis.18 The above findings suggested a possible role of decreased VEGF expression resulting in microvascular defects and ischemia in the pathogenesis of DCM. Accordingly, we performed a study to determine whether loss of the homeostatic effect of myocardial VEGF expression played a role in the pathogenesis of DCM.

First, we sought to determine the relationship between VEGF/VEGFR receptor expression, microvascular integrity, and cardiac function in the streptozotocin-induced diabetic rat model by serial investigation over 12 months. These studies, as detailed below, documented that downregulation of VEGF expression preceded the onset of microvascular endothelial cell apoptosis and a subsequent cascade of events that culminated in systolic HF. To further elucidate the primary role of VEGF in the advent of DCM, we restored VEGF expression with a single intraperitoneal injection of streptozotocin (65 mg/kg) in 0.9% saline.19 Serum glucose levels were measured 1 week later and before gene therapy, and all animals with levels <300 mg/dL were excluded from the study. Age-matched rats were used as nondiabetic controls.

Clinicopathological Evaluation of Diabetic Hearts
To assess cardiac function, we performed echocardiography every month in 50 DM and 15 normal rats followed up for a minimum of 12 months after induction of diabetes, and 7 rats from each group were euthanized at 12 months. In a separate series of experiments, 5 DM and paired control rats were euthanized every 2 months for 10 months to harvest cardiac samples. Histological studies, which included staining with hematoxylin and eosin, periodic acid Schiff, Masson’s trichrome, CD31, desmin, α-sarcomeric actinin, and terminal dUTP nick end-labeling (TUNEL), were performed to investigate serial pathological changes in the diabetic myocardium. The endothelial progenitor cell (EPC) culture assay20 was performed at 2, 4, 8, and 12 months to determine circulating EPC numbers (n = 6 at each time point). Myocardial VEGF protein content and VEGFR type 2 (VEGFR-2) phosphorylation was determined with Western blot (WB) and immunoprecipitation on the 2- and 10-month samples. As a separate experiment, myocardial blood flow (MBF) was measured at baseline and after 10 months in control rats and in DM rats with and without HF (n = 5 each). Procedural details are described below.

Bone Marrow Transplantation
Inbred female Fisher 344 rats with 8 to 10 months of DM with HF were used as recipients of BMT (n = 10). Donor rats were male Fisher 344 rats aged 12 to 14 weeks. BMT was performed as described elsewhere.21 The radiation dose used was 10.0 Gy, and the number of transplanted cells administered was 1.0 × 10^7 in 200 μL volume. Before injection into the tail vein, bone marrow cells were stained with CellTracker CM-DiI (Molecular Probes).22 Four weeks after BMT, pVEGF165 or LacZ plasmid was injected into the myocardium as described above (n = 5 in each group). Two weeks after gene therapy, hearts were harvested for histological analysis.

Echocardiography
Transhorracic echocardiography was performed with a 6.0- to 15.0-MHz transducer (SONOS 5500, Agilent) as described previously.23 A 2D directed M-mode image of the LV short axis was taken just below the level of the papillary muscles. LV end-diastolic (LVEDD) and end-systolic (LVESD) dimensions were measured according to the modified American Society of Echocardiography–recommended guidelines. Fractional shortening (FS) was calculated according to the formula FS = ([LVEDD − LVESD]/LVEDD) × 100%. Color flow-guided pulsed-wave Doppler echocardiography was performed with the sample volume at the LV outflow tract in apical 5-chamber view to measure the time-velocity integral (Figure 1f). LV outflow tract diameter was measured in the parasternal long-axis view. Stroke volume (SV) and cardiac output (CO) were calculated by the following formulas: SV = (LV outflow tract diameter/2)^2 × time-velocity integral (Figures 1g and 1h); CO (mL/min) = SV (mL)/heart rate (bpm).24 Pulsed-wave Doppler spectra of mitral inflow were recorded from an apical 4-chamber view, with the sample volume placed at the tips of the mitral valve leaflets in diastole. The following measurements were performed: mitral peak flow velocity of the early filling wave (E), peak flow velocity of late filling wave (A), and the E/A-wave ratio (E/A; Figure 1d). All measurements represent the mean of at least 3 consecutive cardiac cycles.

Pressure Measurements
After the chest wall was opened, a small incision was made in the apex of the LV, and a 1.4F high-fidelity pressure transducer (Micro-tip catheter, Millar Instrument) was introduced into the LV. After hemodynamics were stabilized, LV systolic pressure, LV end-diastolic pressure, and the maximal rates of pressure rise (+dP/dt) and of pressure fall (−dP/dt) were recorded with a polygraph (model 7P, Grass Instrument).25

Methods

Induction of Diabetes
St. Elizabeth’s Institutional Animal Care and Use Committee approved all protocols. Male Sprague-Dawley rats and female Fisher 344 rats (Charles River, Wilmington, Mass) aged 8 weeks were used. Except for bone marrow transplantation (BMT) experiments, all studies were performed with Sprague-Dawley rats. DM was induced with a single intraperitoneal injection of streptozotocin (65 mg/kg in 0.9% saline).19 Serum glucose levels were measured 1 week later and before gene therapy, and all animals with levels <300 mg/dL were
MBF Measurement
Nonradioactive gold (198Au)-labeled microsphere (15 μm; BioPAL) combined with a neutron activation technique was used to determine MBF. The detailed principles and procedures are described elsewhere.25 Briefly, after left thoracotomy, a 24-gauge angiocatheter was positioned in the left atrium through its appendage. After laparotomy, a 21-gauge angiocatheter was advanced into the abdominal aorta through the right femoral artery up to the level of the renal arteries to withdraw reference blood samples. Immediately after 5×10⁶ microspheres in 200 μL volume were injected into the left atrium, a 1-mL blood sample was withdrawn over 1 minute. Thereafter, hearts were harvested and weighed. Both heart and blood samples were dried in 70°C overnight, and all samples were sent for analysis (BioPAL), where they were exposed to a field of neutrons. Spectroscopic analysis was performed on each sample to obtain disintegrations per minute, and MBF was calculated.25

Histological Examination and Morphometry
At the time of rat euthanasia, the aortas were perfused with saline. Hearts were sliced into 3 transverse sections from apex to base, fixed with 4% paraformaldehyde or methanol, or frozen in OCT compound and sectioned at 5-μm intervals.

Immunohistochemistry for CD31 and VEGF, and Analysis of Capillary-Myocyte Density
Endothelial cells (ECs) were identified by immunohistochemical staining with a mouse monoclonal antibody against rat CD31 (Serotec) followed by a biotinylated horse anti-mouse IgG and horse radish peroxidase (Signet).23 In BMT cardiac samples, ECs were identified with biotinylated isolectin B4 (1:200; Vector) as the primary antibody and FITC-conjugated goat anti-mouse IgG (1:200) as the secondary antibody.23 Cardiomyocytes (CMCs) were identified by mouse α-sarcemeric actinin antibody (Sigma, 1:200) and a biotinylated rabbit anti-mouse IgG and horse radish peroxidase (Signet). A total of 12 visual fields from the inner and outer third of the myocardium (6 fields each) at which a cross section of capillaries and CMCs was clearly visible were randomly selected, and the number of capillaries or CMCs was counted at 200× magnification (n=6 in each group). Immunohistochemical localization of VEGF was performed with a rabbit polyclonal antibody against VEGF (1:500; Santa Cruz) and a biotinylated goat anti-rabbit IgG and horse radish peroxidase (Signet).

Determination of Fibrosis
The extent of myocardial fibrosis was quantified after Masson’s trichrome staining (Sigma). After scanning and recording the blue

Figure 1. pHVEGF165 gene therapy improves cardiac function in D-HF rats. a–f, Echocardiographic assessment of FS, E/A, and cardiac output (CO). a, c, e, Four weeks after each treatment, FS, E/A, and CO were improved in VEGF group. Other groups showed worsening FS, E/A, and CO after treatment. b, Representative M-mode echocardiogram showing improved wall motion after VEGF gene therapy. Both EDD and ESD and therefore FS were improved. d, Representative pulsed-wave Doppler echocardiogram of mitral inflow showing improved E-wave velocity and therefore E/A after VEGF gene therapy. f, Pulsed-wave Doppler and 2D echocardiogram showing measurement of time-velocity integral (TVI) at aortic annulus and LV outflow tract (LVOT) in parasternal long-axis view. g–i, Pressure transducer measurement of cardiac function. Four weeks after each treatment, +dp/dt (g) and −dp/dt (h) were improved only in VEGF group. i, Representative dp/dt tracing showing improvement of both +dp/dt and −dp/dt after VEGF gene therapy. **P<0.01 vs sham and LacZ.
area from each field, which represents the fibrosis area, with Adobe Photoshop 5.0 (Adobe Systems), we converted the images into a 256 gray scale. These images were opened with National Institutes of Health (NIH) image 1.62 software, and the area of fibrosis was calculated with a threshold function. For quantification, 4 visual fields were randomly selected under 40× magnification.

Determination of Apoptosis by TUNEL
Myocardial apoptosis was detected in paraffin-embedded cardiac sections. In situ labeling of fragmented DNA was performed by the TUNEL method. DNA fragments were identified with an in situ cell detection kit (Roche) according to the manufacturer’s instructions. Briefly, sections were treated with 20 μg of proteinase K. After 3 washes with PBS, sections were incubated in a solution of TdT with fluorescein dUTP mixture at 37°C for 1 hour. Next, sections were stained with mouse monoclonal antibody against CD31 or α-sarcomeric actinin followed by rhodamine-labeled anti-mouse IgG to identify ECs or CMCs, respectively. Sections were then counterstained with DAPI for localization of nuclei. Histochemical fluorescent signal was detected by fluorescent microscopy. A total of 12 visual fields (total ~10,000 nuclei) in which a cross section of capillaries and CMCs was clearly visible were randomly selected, and the number of TUNEL-positive nuclei costained with EC or CMC marker, which was regarded as EC or CMC apoptosis, was counted at 400× magnification.

Circulating EPC Culture Assay
Mononuclear cells isolated from 500 μL of peripheral blood from each rat were cultured in EPC media on 4-well glass slides coated with rat vitronectin (Sigma). Two days after culture, EPCs recognized as attaching cells, costained with Dil-labeled acetylated LDL (Biomedical Tech) and FITC-conjugated BS-1 lectin (Sigma), were counted with fluorescent microscopy at 200× magnification. Although these 2 markers are not independent proof of endothelial identity, the concurrence of these markers has been shown to identify a population of cells that is primarily of endothelial lineage and correlates with quantification of circulating EPCs by fluorescence-activated cell sorter analysis for Flk-1 and Sca-1 coexpression.

WB of VEGF and Immunoprecipitation of VEGFR-2 Protein
To investigate VEGF protein expression, WB analysis was performed as described previously. Briefly, after culture, EPCs were homogenized in lysis buffer, protein extracts (100 μg per sample) were separated on a 12% SDS-PAGE (Bio-Rad) and electrotransferred onto PVDF membranes (Amersham Pharmacia). Samples were probed with anti-VEGF rabbit polyclonal antibody (1:500; Santa Cruz) overnight at 4°C. Equal protein loading was confirmed by reprobing the membranes with a mouse monoclonal antibody to α-tubulin (1:1000; Calbiochem). To investigate the effect of VEGF overexpression on phosphorylation of VEGFR-2 (Flk-1), immunoprecipitation-WB analysis was performed as described previously. Briefly, aliquots of protein extracts (1 ng) were incubated for 2 hours at 4°C with 3 μg of monoclonal antibody against phosphotyrosine (Upstate Biotechnology), followed by incubation with 40 μL of protein G-agarose beads (Roche) overnight at 4°C. Immunoprecipitates of tyrosine-phosphorylated proteins were separated by 7.5% SDS-PAGE and electrotransferred onto PVDF membranes. The membranes were immunoblotted overnight at 4°C with a rabbit polyclonal antibody against VEGFR-2 (1:500; Santa Cruz). Antigen-antibody complexes were visualized with ECL+ chemiluminescence reagent (Amersham), followed by exposure to Hyperfilm ECL (Amersham). Densitometric analysis was performed with NIH image 1.62 software.

Restoration of Myocardial VEGF Expression via Gene Therapy
Beginning 5 months after induction of DM, we screened HF rats by serial echocardiography every 2 weeks. HF was defined as FS <34%. (This cutoff was derived from the average FS [38.4%] of 8-12-month-old control rats minus 3×SEM [1.3%]; <99.0% of the CI of the control). All of the HF rats had significant diastolic dysfunction.

The time point for inclusion into the gene therapy protocol was immediately after FS dropped to <34%. The FS value was confirmed by at least 2 independent examinations separated by a >3-day interval. A total of 42 HF rats were selected after screening and randomized to treatment with phVEGF165, LacZ plasmid, or sham operation (n=14 in each group). The same numbers of non-DM rats (ND) and DM rats without HF (D-NF) were used as nonfailure control groups. From each group, 11 rats were used to measure cardiac function and were euthanized at 4 weeks to obtain cardiac samples. Three rats were euthanized at week 1.

Gene therapy was performed as follows. After the rats were anesthetized with an intraperitoneal injection of ketamine (65 mg/kg) and xylazine (9 mg/kg), detailed baseline echocardiography and pressure transducer measurements were performed. With mechanical ventilation, the chest was opened via a left parasternal thoracotomy, and the heart was exposed. In the VEGF group, a total of 500 μg of phVEGF165 in a volume of 250 μL was injected directly into the myocardium with a custom-designed 30-gauge needle. Injections were performed at 5 sites: right ventricular free wall, basal and midanteri or wall, lateral wall, and posterior wall. phVEGF165 is a naked plasmid DNA that encodes human VEGF165. Complementary DNA clones for recombinant human VEGF165, isolated from cDNA libraries prepared from HL-60 leukemia cells, were assembled into a simple eukaryotic expression plasmid that uses the 736-bp cytomegalovirus promoter/enhancer to drive VEGF expression. In the LacZ group, the same dose (500 μg in 250 μL) of pCMV- nlsLacZ, a nuclear-targeted LacZ gene plasmid that encodes the protein β-galactosidase, was injected in an identical fashion. The sham, ND, and D-NF groups underwent only open thoracotomy without any injection. In all experiments, investigators performing the examinations and assessments were blinded to treatment assignment.

Detection of phVEGF165 by Polymerase Chain Reaction
Tissues were processed with a genomic DNA isolation kit (DNeasy tissue kit; Qiagen). Polymerase chain reaction (PCR) was performed with primer sets unique to the promoter and VEGF-encoding region of phVEGF165, as described previously.

Statistical Analysis
All results were expressed as mean±SEM. Statistical analysis was performed with an unpaired Student’s t test for comparisons between 2 groups and ANOVA followed by Scheffé’s procedure for more than 2 groups. Probability values <0.05 were considered to denote statistical significance.

Results
The present studies consisted of 2 separate investigations. First, we detailed the natural history of DCM at the functional, anatomic, and molecular level. We then performed intervention studies, replenishing myocardial VEGF by a gene therapy approach. Accordingly, results are divided into 2 sections.

Natural History of DCM: Reduced VEGF Expression Precedes Decreased Capillary Density and LV Dysfunction
Temporal Changes of Cardiac Function in Diabetic Rats
FS and E wave to A wave ratio (E/A) were used to investigate the temporal changes of systolic and diastolic function, respectively. Both systolic and diastolic dysfunction developed and progressed in proportion to the duration of DM (Figure 2). Diastolic dysfunction preceded systolic dysfunction, similar to the pattern observed in humans, beginning...
from 2 to 3 months after induction of DM (Figure 2). Although diastolic function deteriorated in all DM rats, significant systolic dysfunction (defined by FS $<34\%$) developed only in 24\% (12/50) of DM rats, with the onset generally after 6 months of DM (Figures 2b through 2g). The average time from induction of DM to development of HF, defined by both systolic and diastolic dysfunction, was 9.2±1.4 months. In addition, whereas diastolic function deteriorated gradually, in most cases of HF, systolic function showed a relatively abrupt deterioration, after 8 to 10 months, which suggests that there exists a certain threshold beyond which compensatory mechanisms are unable to sustain systolic function (Figure 2c). Compared with control rats, DM rats showed decreased heart rate and widening of QRS complexes on ECG (Figures 2h and 2i).

**Pathological Characteristics of Diabetic Rats**

In normal rats, CMCs were well organized, and capillaries were organized symmetrically around CMCs (Figure 3a). Diabetic myocardium demonstrated architectural distortion along with the progressive destruction of CMCs and ECs (Figures 3b through 3d). Quantitatively, capillary density evaluated by CD31 staining was significantly reduced in DM rats compared with controls beginning at 6 months (Figure 3e). The decrease in capillary density was also reflected by a significant decrease in MBF, which was lower after 9.5±1.4 months of DM in both the D-NF (0.88±0.07 mL·min$^{-1}$·g$^{-1}$) and HF (D-HF; 0.52±0.09 mL·min$^{-1}$·g$^{-1}$) rats than in the 10-month control rats (1.18±0.11 mL·min$^{-1}$·g$^{-1}$), respectively (control versus D-NF, 25\% reduction, $P<0.05$; control versus D-HF, 56\% reduction, $P<0.01$; Figure 2j). MBF of D-HF rats was significantly lower than that of diabetic D-NF rats ($P<0.05$). Thus, diabetes alone resulted in a decrease in MBF, with the most significant reductions in MBF associated with the development of HF.

CMC density was evaluated by hematoxylin and eosin staining and was also found to be significantly decreased in
DM rats compared with the controls at 12 months (Figure 3f). Immunohistochemistry with anti-desmin antibody demonstrated disordered and irregular patterns of myofibrillar arrangement of CMCs in long-standing DM. DM rats developed progressive intracardiac fibrosis in the absence of epicardial coronary artery stenosis. Interstitial and perivascular fibrosis was observed in early phase of DM (Figures 3i and 3j), followed by more widespread interstitial fibrosis (k), and finally, replacement fibrosis occurred along with myocardial death (l). Quantification of fibrosis area showed significant increase of fibrosis in DM rats compared with control rats starting at 8 months. n=5 for each group. Unpaired Student’s t test was used for statistical analysis.

We investigated myocardial apoptosis using double staining with TUNEL and CD31 or α-sarcomeric actinin. In the early phases (2 and 4 months) of DM, apoptosis was observed predominantly in ECs, and the number of TUNEL-positive ECs was significantly higher in DM rats than in control rats beginning at 2 months and continuing to the end of the study period (2 months: 8±0.9 versus 2±0.3/10⁴ nuclei, P<0.001; 4 months: 10±1.3 versus 3±0.5/10⁴ nuclei, P<0.001; 8 months: 13±1.6 versus 4±0.5/10⁴ nuclei, P<0.001; 12 months: 14±2.1 versus 6±0.7/10⁴ nuclei, P<0.01; Figures 4a through 4d and 4i). CMC apoptosis was not prominent in the early phase of DM; however, beginning at 8 months TUNEL-positive CMCs were found in significantly higher numbers in the DM rats than in the control rats (8 months: 11±2.2 versus 4±0.8/10⁴ nuclei, P<0.05; 12 months: 15±2.9 versus 4±0.7/10⁴ nuclei, P<0.01; Figures 4e through 4h and 4j).
We next determined myocardial VEGF protein content and VEGFR-2 phosphorylation using WB and immunoprecipitation. Normal rats showed similar levels of VEGF and VEGFR-2 phosphorylation between 2 and 10 months; however, DM rats revealed significantly decreased VEGF expression and VEGFR-2 phosphorylation at both 2 and 10 months after the onset of DM compared with the respective control rats, with the most profound decrease noted at the last time point studied (Figure 4k).

The number of circulating EPCs was also evaluated at 2, 4, 8, and 12 months by the standard EPC culture method. EPC counts revealed a progressive decrement in accordance with the duration of DM, which became significantly lower beginning at 4 months (Figures 4l through 4n).

To summarize the serial pathophysiological changes in the DM heart, downregulation of VEGF is the first observed pathological change, followed by EC apoptosis within the myocardium and decreases of circulating EPCs. At this point, diastolic dysfunction had ensued. Subsequently, a significant decrease of capillary density was observed, followed by apoptosis and necrosis of CMCs and a reduction of CMC density with replacement fibrosis. These latter changes were accompanied by the onset of systolic dysfunction.

**Restitution of VEGF Expression in the Diabetic Heart Reverses DCM**

To further define the primary pathophysiological role of decreased VEGF expression in the advent of DCM, we used

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**Figure 4.** Diabetes induces downregulation of VEGF, EC apoptosis, and decreases of circulating EPCs. a–d, Myocardial sample 2 months after DM shows predominant EC apoptosis demonstrated by TUNEL (green) and CD31 (red) double staining (white arrows). Original magnification, ×400. e–h, Twelve-month sample shows increased apoptosis including noticeable CMC apoptosis (yellow arrows) disclosed by TUNEL (green) and α-sarcomeric actinin (red; α-sar actinin or α-sarcom actinin) double staining. Original magnification, ×400. i and j, Quantitatively, TUNEL-positive ECs (i) and CMCs (j) were significantly increased beginning 2 and 8 months after onset of DM, respectively. *P<0.05, **P<0.01, ***P<0.001. n=5 for each group. k, VEGF WB shows decreased VEGF protein in DM rats compared with normal (NL) rats and shows that decrease was more profound in 10-month-old DM rats. Tubulin control shows equal protein loading of each sample. VEGFR-2 immunoprecipitation shows decreased phosphorylation of VEGFR-2 in DM rats compared with control rats and shows that decrease was more severe in 10-month-old DM rats. n=5 in each group. l and m, Number of circulating EPCs, identified as double positive (yellow) by BS-1 lectin (green) and Dil-acLDL (red) uptake, was decreased at 12 months after DM compared with baseline (0 months). n, EPC numbers started to decrease between 2 and 4 months after onset of DM and progressively decreased over 12 months. n=6 in each group. *P<0.05, **P<0.001. For statistical analysis, unpaired Student’s t test was used (i, j, n).
Figure 5. Intramyocardial phVEGF165 gene therapy increases VEGF expression and capillary density and decreases myocardial apoptosis and fibrosis. 

a, PCR of myocardial tissue with primers specific for VEGF plasmid 1 and 4 weeks after gene therapy revealed presence of phVEGF165 DNA in phVEGF165-transfected rats but not in LacZ-transfected controls. b, WB for VEGF showed that phVEGF165-transfected myocardium showed 2.2 and 2.3 times higher expression of VEGF than sham and LacZ-treated hearts, respectively. c, phVEGF165-transfected myocardium demonstrated 2.5 and 2.6 times increase in phosphorylated VEGFR-2 compared with sham and LacZ controls. d–h, Immunohistochemistry with anti-VEGF antibody demonstrated that compared with ND and D-NF samples, D-HF control (sham [f] and LacZ [g]) hearts showed significant decreases of VEGF expression. However, phVEGF165-transfected myocardium (h) showed increased VEGF expression. Original magnification, ×200. i–n, CD31 staining reveals that compared with ND and D-NF samples, D-HF control (sham [f] and LacZ [g]) hearts showed significant decreases of VEGF expression. However, phVEGF165-transfected myocardium (h) showed increased VEGF expression. Original magnification, ×200.
a gene therapy approach to transiently restore VEGF expression after the onset of HF.

**Detection of phVEGF165 DNA by PCR and Immunoblot Analysis of VEGF and VEGFR-2 After VEGF Gene Therapy**

To document successful transgene delivery to the myocardium, we performed PCR to evaluate the persistence of injected phVEGF165 DNA. The PCR primers of phVEGF165 were so designed to detect the promoter and VEGF165-encoding region, and they do not host VEGF DNA. PCR of myocardial samples at 1 and 4 weeks after gene delivery revealed the presence of phVEGF165 DNA in the myocardium of the phVEGF165-transfected samples (Figure 5a). Myocardial tissues from control treated hearts showed no PCR bands, as expected.

To assess transgene expression of injected phVEGF165 in the myocardial tissues, we performed WB for VEGF, immunoprecipitation/WB for VEGFR-2 phosphorylation, and VEGF immunohistochemistry on 1-week samples. Compared with ND and D-NF rats, D-HF rats (sham and LacZ) showed profound downregulation of VEGF expression (Figure 5b). Compared with sham-operated or LacZ plasmid–injected samples, phVEGF165-transfected samples showed 2.2 and 2.3 times higher expression of VEGF, respectively (P<0.05, respectively; n=5 in each group). We next investigated the effect of phVEGF165 gene transfer on the tyrosyl phosphorylation of VEGFR-2 by immunoprecipitation with an anti-phosphotyrosine antibody followed by WB with an anti-VEGFR-2 antibody (Figure 5c). D-HF rats showed a decrease in phosphorylated VEGFR-2 compared with ND rats. D-HF rats transfected with phVEGF165 showed a 2.5- and 2.6-fold increase in phosphorylated VEGFR-2 compared with sham-operated or LacZ plasmid–injected rats (P<0.05, respectively; n=5 in each group). Immunohistochemistry with anti-VEGF antibody again disclosed that phVEGF165 gene therapy resulted in local VEGF expression, cardiac sections were stained with anti-VEGF antibody followed by WB with an anti-VEGFR-2 antibody (Figure 5c). D-HF rats showed a decrease in phosphorylated VEGFR-2 compared with ND rats. D-HF rats transfected with phVEGF165 showed a 2.5- and 2.6-fold increase in phosphorylated VEGFR-2 compared with sham-operated or LacZ plasmid–injected rats (P<0.05, respectively; n=5 in each group). Immunohistochemistry with anti-VEGF antibody again disclosed that phVEGF165-transfected hearts exhibited a widespread increase in VEGF expression compared with LacZ-transfected or sham samples (Figures 5d through 5h).

**Restoration of Myocardial VEGF Expression Reverses Pathological Features of Diabetic HF**

Myocardial tissues were analyzed for capillary and CMC density by CD31 and hematoxylin and eosin staining, respectively. Compared with ND rats, D-HF rats (sham and LacZ) showed a significant decrease in capillary and CMC density (P<0.01; Figures 5i through 5l). Compared with the sham and LacZ groups, the VEGF group showed significantly increased capillary density (VEGF=8236±531/mm² versus sham=6653±479/mm², LacZ=6701±485/mm²; P<0.05; Figures 5m and 5n). CMC density showed a similar trend but was not statistically different (VEGF=6632±397/mm² versus sham=5617±352/mm², LacZ=5584±344/mm²; P=0.07; Figure 5o).

TUNEL was used to compare intramyocardial apoptosis between treatment groups. EC apoptosis determined by TUNEL and CD31 double-staining was significantly lower in the VEGF group than in the sham and LacZ groups (13±2.1 versus 22±2.3 and 21±2.3/10⁴ nuclei, respectively; P<0.05; Figures 5p through 5s). CMC apoptosis determined by TUNEL and α-sarcomeric actinin double-staining was significantly lower in the VEGF group than in the sham and LacZ groups (16±2.2 versus 25±2.7 and 26±2.4/10⁴ nuclei, respectively; P<0.05; Figure 5s). Next, we quantified intramyocardial fibrosis by Masson’s trichrome staining. The D-HF (sham and LacZ) groups showed significantly greater fibrosis area than the ND and D-NF groups (Figure 5w). In the VEGF group, the fibrosis area was smaller but was not statistically different from the saline and LacZ groups (7.8±1.0% versus 10.9±1.2% and 11.2±1.3%, respectively; P=0.09; Figures 5t through 5w).

**Restoration of Myocardial VEGF Expression Preserves Circulating EPC Counts and Contribution of EPCs to Homeostasis of the Myocardial Microvasculature**

Before gene therapy, EPC counts were significantly lower in D-HF rats than in ND or D-NF rats but were not different between the VEGF, sham, and LacZ groups. Two weeks after gene therapy, the VEGF group showed significantly higher EPC numbers than the sham and LacZ groups (537±51 versus 279±39 and 302±41/mm², respectively; P<0.01; n=6 in each group; Figures 6a through 6c).

To investigate the EPC contribution to the myocardial microvasculature, we performed BMT using bone marrow cells labeled with DiI after harvesting them from syngeneic donor rats. Four weeks after BMT and another 2 weeks after phVEGF165 gene therapy, cardiac sections were stained with isolecitin B4. Bone marrow–derived DiI-labeled cells were localized with isolecitin B4–positive cells in the myocardium, which indicates that bone marrow–derived cells had been recruited to the myocardium and differentiated into ECs, implying that recruitment of bone marrow–derived cells played a role in the recovery of myocardial function that resulted from restored local VEGF expression (Figures 6d through 6g). We could not detect any evidence for bone marrow–derived EPC incorporation into the microvasculature in the myocardial samples of diabetic LacZ or sham-operated rats.

**Recovery of Cardiac Function in DCM After Restoration of VEGF Expression**

To evaluate the impact of restoration of VEGF expression on global cardiac function, we used echocardiography and mi
crotransducer LV pressure measurements. During the 4-week follow-up period, 1 rat died in the sham and LacZ groups, respectively. At baseline, compared with the ND and D-NF groups, D-HF groups showed significantly lower FS, E/A ratio, and cardiac output (all \( P<0.01 \); Table). Four weeks after each treatment, FS, E/A ratio, and cardiac output were improved in the VEGF group (VEGF versus sham and LacZ; FS 1.8 ± 0.3% versus −1.7 ± 0.2% and −1.9 ± 0.2%, \( P<0.01 \); E/A 0.14 ± 0.02 versus −0.07 ± 0.01 and −0.05 ± 0.01, \( P<0.01 \); cardiac output, 7.8 ± 1.4 versus −3.8 ± 0.6 and −3.1 ± 0.7 mL/min, \( P<0.01 \); Figures 1a through 1f). The decrement of FS, E/A, and cardiac output in the control groups (sham and LacZ) can be explained by the additive effects of the natural progression of HF in DM and open chest surgery. Invasive hemodynamic measurements demonstrated that LV systolic pressure, \( +dP/dt \), and \( −dP/dt \) were all significantly lower in D-HF rats than in ND and D-NF rats (all \( P<0.01 \); Table). Four weeks after each treatment, \( +dP/dt \), and \( −dP/dt \) were also improved in the VEGF group (VEGF versus sham and LacZ, respectively: \( +dP/dt \) 236 ± 33 versus −201 ± 27 and −175 ± 29, \( P<0.01 \); \( −dP/dt \) 289 ± 46 versus −188 ± 22 and −167 ± 24, \( P<0.01 \); Figures 1g through 1i).

**Discussion**

DCM poses a potentially increasing major public health problem as the incidence of DM rises and medical management prolongs the life span of diabetic patients. Although diabetes is a major risk factor for the development of congestive HF, the pathophysiology of DCM remains uncertain. Here, by serial clinicopathological investigation of streptozotocin-induced diabetic rats over 12 months, we document a progressive decrease of myocardial VEGF expression, followed in sequence by decreased numbers of circulating EPCs, increased EC apoptosis, and reduced capillary density in the myocardial microcirculation, which leads to reduced myocardial perfusion and ultimately to overt cardiac failure.

Previous studies identified microvascular abnormalities in the diabetic myocardium, evidenced by qualitative descriptions of arteriolar pathologies, microaneurysms, and interstitial fibrosis or by showing perfusion defects by radiological studies. Others focused on the role of VEGF in diabetic hearts or other organs as a secondary responder to gross ischemic insults such as ischemic cardiovascular diseases.9,15 In contrast, the present study has for the first time uncovered the primary pathophysiological role of VEGF in the initiation and progression of microvascular deficiency in diabetic hearts in the absence of gross arterial occlusion (ie, DCM) and how this deficiency of VEGF can lead to HF. Moreover, the present study discloses that DCM is a unique form of ischemic heart disease by quantitative demonstration of decreased microcirculation and myocardial perfusion.

Restoration of VEGF expression with a local gene therapy approach restores the myocardial microcirculation and cardiac function. These studies invoke a pivotal role for VEGF in homeostasis of myocardial perfusion and also imply that circulating EPCs play a role, both pathologically and, potentially, therapeutically. The fact that transient restoration of myocardial VEGF expression could improve cardiac function underscores the significance of decreased VEGF expression and microvascular pathogenesis that precedes development of overt DCM.

In the DM rat model, cardiac function deteriorated in proportion to the duration of diabetes. Although diastolic dysfunction developed in all rats and preceded systolic
dysfunction, systolic function was impaired only in one fourth of rats during 1-year follow-up. These findings are well correlated with those of human studies that indicate that asymptomatic diabetic patients generally had diastolic dysfunction and only a portion of subjects later developed systolic dysfunction.9,32 Serial clinicopathological investigation of DM rats revealed that the decrease of VEGF expression in the myocardium was the initial event, followed by EC apoptosis, decreases in capillary density, and decreases in circulating EPC counts. These changes were followed by diastolic dysfunction, CMC apoptosis, myocardial degeneration, replacement fibrosis, and finally systolic dysfunction. This temporal sequence and its reversal by VEGF gene therapy provide evidence that implicates disordered VEGF homeostasis in the pathophysiology of DCM.

DM has been associated with protein manifestations of macrovascular and microvascular pathology in multiple vascular beds. Early studies characterized the pathological changes in the diabetic myocardium, including microangiopathy; however, the pathological role played by the apparent attrition of the microvasculature remained uncertain.9,11,33 In the present study, serial quantification of capillary density in the diabetic myocardium with an EC-specific marker, CD31, discloses a progressive attenuation of the microvasculature in accordance with the duration of DM and in proportion to the degree of cardiac dysfunction. The decrease in capillary density is accompanied by decreased myocardial perfusion, a direct indicator of myocardial ischemia, and by progressive LV dysfunction. This constellation of findings is consistent with a clinical study that demonstrated that diabetic subjects with abnormal stress thallium scans had lower ejection fractions than subjects with normal scans.34

The present data reveal that downregulation of VEGF in DM hearts is the earliest event detected. VEGF is well known for its role in vascular permeability and angiogenesis and is also important for preserving EC integrity by preventing apoptosis.35,36 VEGFR-2 (KDR/Flk-1) is the predominant VEGF receptor that mediates angiogenesis and antiapoptotic activities of VEGF.37 The upstream regulator of VEGF in myocardium has yet to be investigated in detail. Streptozotocin-induced diabetes is well characterized by insulin insufficiency. One potential mechanism for decreased VEGF expression is loss of insulin-induced VEGF expression. The major cell types in myocardium include cardiomyocytes, ECs and fibroblasts, all of which have been shown to produce VEGF. Studies have demonstrated that insulin can increase VEGF expression in these cell types by activating the PI3-kinase/Akt pathway.38 Furthermore, mice with a vascular EC-specific knockout of the insulin receptor have been shown to have defective neovascularization.39 Whether other molecules are involved in the insulin-VEGF activation pathway needs to be assessed in future studies.

The findings of the present study indicate that early downregulation of VEGF and VEGFR-2 could play a key role for initiating the pathophysiological cascade that leads to the onset of DCM. This hypothesis is further supported by reports demonstrating that VEGF expression is constitutively depressed in both diabetic skeletal muscle and nerves19 and that pVEGF165 gene therapy can attenuate diabetic neuropathy by restoring the vasa nervorum,19 which is depleted in diabetes. Recently, CMC-derived VEGF was shown to play an important paracrine role in the maintenance of cardiac function,40 which underscores the homeostatic role of VEGF. CMCs, a major component of myocardium, synthesize and secrete VEGF40,41 and also possess VEGF receptors,20 which are important for CMC survival.40 Therefore, progressive loss of CMCs in DM might further reduce already decreased myocardial VEGF content and thereby induce a vicious circle of CMC degeneration, ultimately leading to HF. This pathophysiological insight about the role of VEGF in the development and progression of DCM led us to attempt restoration of local VEGF expression as a means of verifying the pathological role of VEGF in developing DCM. This hypothesis is further supported by experiments in VEGF120/120

### Baseline Characteristics of Diabetic Rats

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>D-NF</th>
<th>Sham</th>
<th>LacZ</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>282±17</td>
<td>193±25</td>
<td>188±27</td>
<td>189±25</td>
<td>187±24</td>
</tr>
<tr>
<td>FS, %</td>
<td>38±2</td>
<td>37±2</td>
<td>29±2†‡</td>
<td>30±2†‡</td>
<td>30±2†‡</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>9.8±0.2</td>
<td>10.1±0.2</td>
<td>10.4±0.3</td>
<td>10.5±0.2</td>
<td>10.4±0.3</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>6.3±0.2</td>
<td>6.4±0.2</td>
<td>7.2±0.2†‡</td>
<td>7.3±0.2†‡</td>
<td>7.3±0.2†‡</td>
</tr>
<tr>
<td>E wave, cm/s</td>
<td>84±4</td>
<td>74±4</td>
<td>69±4*</td>
<td>70±3*</td>
<td>69±4*</td>
</tr>
<tr>
<td>A wave, cm/s</td>
<td>44±3</td>
<td>53±3</td>
<td>54±3*</td>
<td>55±3*</td>
<td>54±2*</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.81±0.2</td>
<td>1.40±0.2</td>
<td>1.27±0.1*</td>
<td>1.26±0.1*</td>
<td>1.28±0.1*</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>102±5</td>
<td>98±6</td>
<td>58±4§</td>
<td>57±3§</td>
<td>59±4§</td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>108±5</td>
<td>92±4</td>
<td>81±4§</td>
<td>83±4§</td>
<td>82±4§</td>
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<td>LVEDP, mm Hg</td>
<td>2±0.4</td>
<td>4±1</td>
<td>7±2</td>
<td>6±2</td>
<td>6±2</td>
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<td>+dP/dt, mm Hg/s</td>
<td>4621±236</td>
<td>3816±299</td>
<td>2922±284§</td>
<td>2839±264§</td>
<td>2940±251§</td>
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<tr>
<td>−dP/dt, mm Hg/s</td>
<td>3106±172</td>
<td>2512±156</td>
<td>1787±161§</td>
<td>1801±136§</td>
<td>1825±178§</td>
</tr>
</tbody>
</table>

ND indicates age-matched control rats; D-NF, diabetic rats with no HF; Sham, diabetic HF rats with sham operation; LacZ and VEGF, diabetic HF rats injected with psv-LacZ and phVEGF165, respectively; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVSP, LV systolic pressure; and LVEDP, LV end-diastolic pressure.

*P<0.05 vs ND; †P<0.01 vs ND; ‡P<0.01 vs D-NF; §P<0.001 vs ND; ‡P<0.001 vs D-NF.
transgenic mice,\textsuperscript{16} which revealed that absence of the VEGF\textsubscript{164} and VEGF\textsubscript{188} isoforms resulted in impaired angiogenesis, reduced myocardial perfusion, and ischemic cardiomyopathy despite the absence of disease in the epicardial coronary arteries. In fact, DCM has striking similarities to the VEGF\textsuperscript{2/0120} mice, which include decreases in VEGF and VEGFR-2 expression, reduced heart rate, increased QT interval, decreased LV diastolic and systolic function, reduced capillary density, decreased MBF, cardiac dilatation, hypertrophy of CMCs, myocardial disorganization, myocardial collagen deposition, progressive degeneration and atrophy of CMCs, and fibrosis. Together, these similarities reveal that DCM can replicate the spectrum of abnormalities characteristic of primary VEGF deficiency-induced cardiomyopathy.

There is increasing evidence that vascular homeostasis in adults is not solely dependent on regulation of mature ECs but also involves bone marrow–derived EPCs.\textsuperscript{22,27} EPCs are known to contribute up to 25% of ECs in newly formed vessels in animal models.\textsuperscript{42} The present data show that circulating numbers of EPCs are progressively decreased in diabetic rats and suggest that impaired neovascularization, reflected by progressive loss of capillary density in diabetic hearts, can be attributed in part to impaired vasculogenesis that results from decreased EPC availability. This hypothesis is further supported by the fact that DM rats without significant HF (FS $\geq$34\%) showed a lesser degree of impairment of VEGF expression, higher numbers of circulating EPCs, and higher capillary density than those animals that developed significant HF. Most notably, restoration of myocardial VEGF expression replenishes the circulating EPC population and results in significant EPC contribution to the reconstituted myocardial microvasculature.

In HF, the progressive loss of CMCs is one of the key pathological findings. Although the rate of apoptosis in HF is relatively low in absolute numbers (<1% TUNEL-positive cells), and there are controversies about the interpretation of TUNEL staining of CMCs, there has been accumulating evidence in both human and animal models to suggest that apoptosis is an important mode of cell death in HF.\textsuperscript{44} In DM, myocardial ischemia and direct effects of reduced VEGF expression could lead to myocardial apoptosis and necrosis and finally result in cardiac dysfunction.

Our observations suggest that the diabetic myocardium is a mixture of hibernating and degenerative CMCs. This is supported by the fact that the functional improvement observed after VEGF gene therapy is not entirely explained by the magnitude of increase in the number of CMCs. Because the essential criterion of hibernation is functional improvement after revascularization, the present data suggest that DCM includes ischemic hibernation, which is reversed by restoration of VEGF expression. Reconstitution of the myocardial microvasculature by local VEGF expression may have another potential advantage for treatment of DCM by increasing capillary density in the more avascular fibrosis area. The role of interstitial fibrosis in progression of HF has been suggested in a canine model of chronic HF, which indicates that cardiac interstitial fibrosis can act as a barrier to the intimate access that CMCs typically have to adjacent capillaries and thereby increase the oxygen diffusion distance.

This can aggravate local hypoxia, which can ultimately compromise the viability of collagen encircling CMCs.\textsuperscript{12} Although this animal model does not precisely represent the more prevalent type II DM in humans, when we consider that DCM in humans occurs primarily in long-standing diabetic patients and that patients with advanced type II DM ultimately have insulin deficiency, this model appears relevant for these patients and may therefore have therapeutic implications.

The impact of replenishing myocardial VEGF expression in the setting of chronic diabetes can be summarized as follows. First, the restoration of VEGF expression resulted in increased capillary density and thus improved myocardial function. Second, the numbers of circulating, bone marrow–derived EPCs increased, as did the recruitment and incorporation of these progenitors into the reconstituted microvasculature. Thus, replenishment of deficient VEGF via gene therapy reestablished VEGF homeostasis in the myocardium, breaking the vicious circle of EC/CMC apoptosis, myocardial degeneration, and worsening VEGF deficiency.

Currently, the pathogenesis and treatment of diabetic HF are elusive. The present data reveal that downregulation and deficiency of myocardial VEGF play a key role in the initiation and aggravation of DCM and suggest the possibility that strategies targeted at preserving the myocardial microvasculature in DM may retard or prevent the development of this major complication.

Acknowledgments

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Progressive Attenuation of Myocardial Vascular Endothelial Growth Factor Expression Is a Seminal Event in Diabetic Cardiomyopathy: Restoration of Microvascular Homeostasis and Recovery of Cardiac Function in Diabetic Cardiomyopathy After Replenishment of Local Vascular Endothelial Growth Factor

Young-sup Yoon, Shigeki Uchida, Osamu Masuo, Manfred Cejna, Jong-Seon Park, Hyeon-cheol Gwon, Rudolf Kirchmair, Ferdinand Bahlman, Dirk Walter, Cynthia Curry, Allison Hanley, Jeffrey M. Isner and Douglas W. Losordo

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