Rescue of Diabetes-Related Impairment of Angiogenesis by Intramuscular Gene Therapy with Adeno-VEGF

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**Diabetes is a major risk factor for coronary and peripheral artery diseases. Although diabetic patients often present with advanced forms of these diseases, it is not known whether the compensatory mechanisms to vascular ischemia are affected in this condition. Accordingly, we sought to determine whether diabetes could: 1) impair the development of new collateral vessel formation in response to tissue ischemia and 2) inhibit cytokine-induced therapeutic neovascularization. Hindlimb ischemia was created by femoral artery ligation in nonobese diabetic mice (NOD mice, $n = 20$) and in control C57 mice ($n = 20$). Hindlimb perfusion was evaluated by serial laser Doppler studies after the surgery. In NOD mice, measurement of the Doppler flow ratio between the ischemic and the normal limb indicated that restoration of perfusion in the ischemic hindlimb was significantly impaired. At day 14 after surgery, Doppler flow ratio in the NOD mice was 0.49 ± 0.04 *versus* 0.73 ± 0.06 for the C57 mice ($P \leq 0.005$). This impairment in blood flow recovery persisted throughout the duration of the study with Doppler flow ratio values at day 35 of 0.50 ± 0.05 *versus* 0.90 ± 0.07 in the NOD and C57 mice, respectively (*P*

limb ischemia. The impact of diabetes in these experimental models, however, was not tested. In the present study, we show that diabetes impairs angiogenesis in a murine model of unilateral limb ischemia. We also demonstrate that this impairment of neovascularization is caused by reduced expression of vascular endothelial growth factor (VEGF) that can be successfully addressed by intramuscular gene transfer.

Materials and Methods

Murine Ischemic Hindlimb Model

All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. The development of angiogenesis in response to regional ischemia was investigated in nonobese diabetic (NOD) mice. These mice have previously been shown to develop a form of diabetes with clinical features similar to those of the human type-I, insulin-dependent diabetes mellitus.[18,19] The NOD mice were treated with bovine insulin (2 units subcutanously three times a week) for the duration of the study. Despite insulin replacement, the mice remained hyperglycemic with urine glucose values >2000 mg/dl. The outcomes observed in the NOD mice were compared with C57BL/6 mice used previously as controls for NOD mice.[18,19]

Surgery

Unilateral hindlimb ischemia was created in NOD or C57BL/6 mice as previously described.[20,21] The animals were anesthetized with pentobarbital (160 mg/kg intraperitoneally) following which an incision was performed in the skin overlying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated and the artery, as well as all side-branches, were dissected free and excised. The skin was closed using a surgical stapler.

Monitoring of Hindlimb Blood Flow

After anesthesia, hair was removed from both legs using a depilatory cream, following which the mice were placed on a heating plate at 37°C for 10 minutes to minimize temperature variations.

Hindlimb perfusion was measured using a laser Doppler perfusion imager (LDPI) system (Lisca Inc., North Brunswick, NJ). The LDPI uses a beam from a 2-mW helium-neon laser that sequentially scans a 12 × 12 cm tissue surface to a depth of a few hundred microns. During the scanning procedure, the moving blood cells shift the frequency of incident light according to the Doppler principle. A photodiode collects the back-scattered light, and the original light intensity variations are transformed into voltage variations in the range of 0 to 10 V. A perfusion output value of 0 V was set to 0% perfusion, whereas 10 V was set to 100%. When the scanning procedure is terminated and the back-scattered light collected from all of the measured sites, a color-coded image representing the microvascular blood flow distribution appears on a monitor. The perfusion signal is split into six different intervals, each displayed in a separate color. Low or no perfusion is displayed in dark blue, whereas the highest perfusion interval is displayed in red. The stored perfusion values behind the color-coded pixels are then available for analysis.

Consecutive measurements were obtained after scanning the same region of interest (leg and foot) with LDPI. Color photographs were recorded and analysis performed by calculating the average perfusion of the ischemic and nonischemic foot. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) *versus* right (normal) limb. Serial changes in perfusion have been previously shown to correlate with changes in capillary density and endothelial incorporation of bromodeoxyuridine (BrDU).[20]

Tissue Preparation

The mice were sacrificed at predetermined arbitrary time points after surgery with an overdose of sodium pentobarbital. For immunohistochemistry, whole ischemic and nonischemic limbs were immediately fixed in methanol overnight. After bones had been carefully removed, 3-μm thick tissue sections were cut and paraffin embedded. For total protein extraction, isolated tissue samples were rinsed in phosphate-buffered saline (PBS) to remove excess blood, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Immunohistochemistry

Histological sections, 5 μm thick, prepared from paraffin-embedded tissue samples of the lower limbs were used for immunohistochemical analysis. Identification of endothelial cells was performed by immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) using a rat monoclonal antibody (MAb) directed against mouse CD31 (Pharmingen, San Diego, CA). Immunohistochemical localization of VEGF was performed using a rabbit polyclonal antibody directed against human VEGF amino terminal peptides 1–20 (Santa Cruz Biotechnology, Santa Cruz, CA) that cross-reacts with murine VEGF.

Immunoperoxidase staining was performed as previously described.[20,22] In brief, sections were incubated in 3% hydrogen peroxide to block endogenous peroxide activity. To prevent nonspecific antibody binding, sections were preincubated for 20 minutes in PBS containing 10% horse serum. Next, sections were incubated with the primary antibodies directed against either CD31 or VEGF at appropriate dilutions overnight at 4°C. Sections were then rinsed for 15 minutes with PBS, followed by incubation with biotinylated secondary antibody for 30 minutes at room temperature. After a 15-minute wash, sections were treated with streptavidin-horseradish peroxidase (HRP) complex (Biogenex, San Ramon, CA) at room tem-

Figure 1. Left: Representative results of laser Doppler perfusion imaging recorded at serial time points after surgery in NOD and C57 mice. A color scale illustrates blood flow variations from minimal (dark blue) to maximal (red) values. As shown in upper left frame: NI, nonischemic (right) limb; T, tail; and I, ischemic (left) limb of mice. Right: Laser Doppler perfusion ratio over time after surgery in NOD and C57 mice. At day 14 after surgery, the LDPI flow ratio was significantly reduced in the NOD mice, and this difference persisted through day 35 postoperatively.

perature for 30 minutes. Sections were rinsed with PBS and incubated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride dihydrate (for VEGF staining) or 3-amino-9-ethylcarbazole (for CD31 staining). Sections were finally counterstained with 20% Gill's hematoxylin and subsequently covered. Negative control slides were prepared by substituting preimmune rat serum for CD31 and preimmune rabbit serum for VEGF antibody staining.

Analysis of Capillary Density

Capillaries, identified by positive staining for CD31 and appropriate morphology, were counted by a single observer blinded to the treatment regimen under a 20× objective and a 5× lens to determine the capillary density (mean number of capillaries per square millimeter).[20] A total of 20 different fields from the two muscles were randomly selected, and the number of capillaries were counted for each field.

Northern Blot Analysis of VEGF mRNA Expression

Total tissue RNA was isolated from ischemic hindlimb muscles of mice by phenol/chloroform extraction.[23] Twenty μg of RNA/lane were separated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham) by blotting. The membrane was hybridized with ^{32}P-labeled probe specific for VEGF, a 675-bp *Eco*RI/*Bgl*II fragment of plasmid pSVI.VEGF.21.[24] Hybridization was carried out as previously described.[23]

Western Blot Analysis of VEGF Protein Expression

Whole-cell protein extracts were obtained after homogenization of ischemic and control muscles of both young and old animals. A total of 200 μg of protein per sample

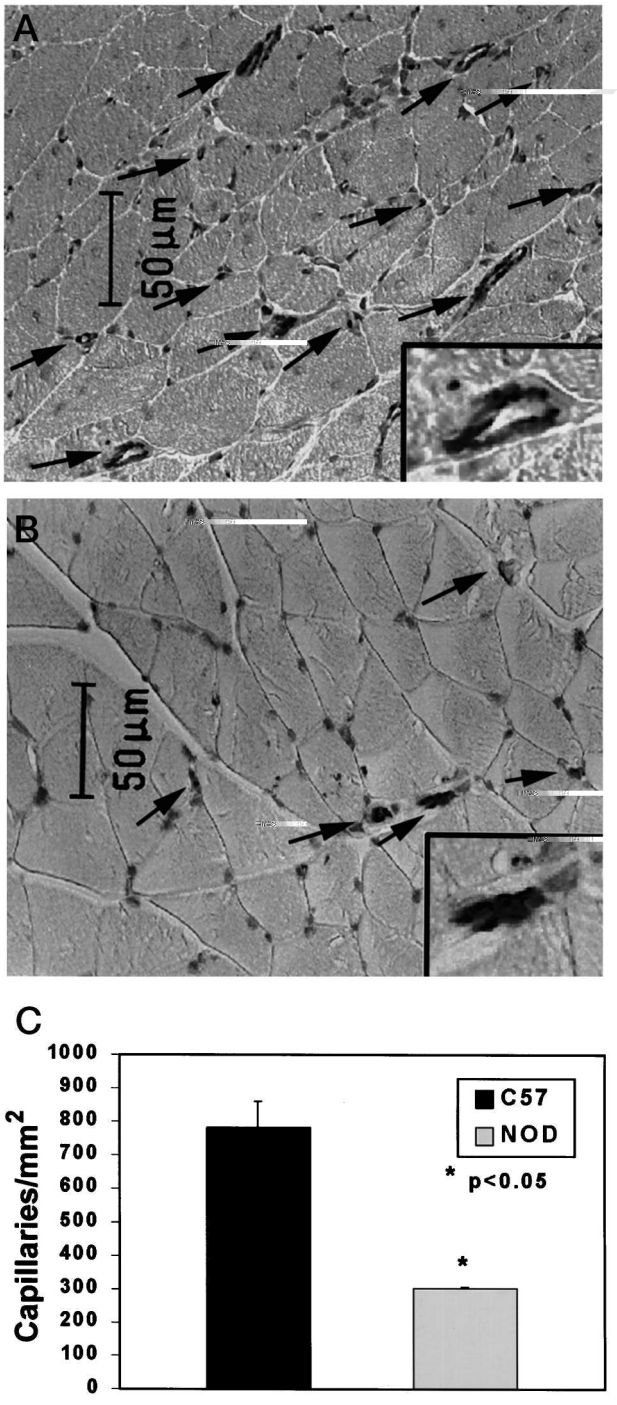

Figure 2. CD31 staining of ischemic muscles from C57 and NOD mice at time of sacrifice (A and B) showed a significant reduction in the capillary density in diabetic mice (C).

was separated on a 12% polyacrylamide gel and electroblotted on nitrocellulose membranes.[25] The membrane was blocked with 10% nonfat dry milk in 0.2% Tween phosphate buffered saline (T-PBS) and then probed with 1:250 of rabbit polyclonal anti-human VEGF antibody (Sigma) for 3 hours at room temperature. After incubation with primary antibody, the blot was washed three times in T-PBS, followed by incubation for 1 hour

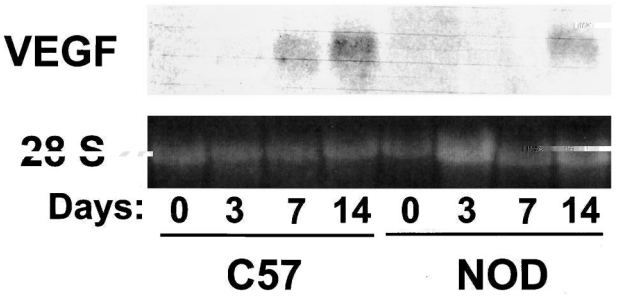

Figure 3. Expression of VEGF mRNA. At baseline (nonischemic hindlimbs, day 0), the level of VEGF mRNA expression was almost undetectable both in C57 and NOD mice. However, in ischemic hindlimbs, VEGF mRNA expression was significantly reduced in NOD *versus* C57 mice from day 3 to day 14 after surgery.

with 1:4000 of anti-rabbit horseradish peroxidase IgG (Santa Cruz Biotechnology). The blot was then washed in T-PBS, and antigen-antibody complexes were visualized after incubation for 1 minute with enhanced luminescence reagent (Amersham) at room temperature, followed by exposure to Kodak XAR-5 film.

Intramuscular Adenoviral Transfection

Mice were transfected with E1-deleted recombinant adenovirus (1×10^9 pfu) expressing either *LacZ* containing a nuclear localization sequence (*nls-LacZ*) or murine VEGF cDNA. Transfection was performed by four direct intramuscular injections into the thigh muscles of the ischemic hindlimb using a 27-gauge needle at the time of surgery.

Results

Analysis of Native Angiogenesis

Hindlimb perfusion was evaluated postoperatively by serial LDPI studies (Figure 1). Restoration of perfusion was significantly slower in NOD mice *versus* controls. At day 14 after surgery, the ratio of blood flow between the ischemic and the normal limb was 0.49 ± 0.04 compared with 0.73 ± 0.06 for the C57 mice ($P \leq 0.005$). This impairment in blood flow recovery was consistent throughout the duration of the study, so that perfusion remained impaired up to the time of sacrifice (day 35) with values of 0.50 ± 0.05 *versus* 0.90 ± 0.07 in the NOD and C57 mice, respectively ($P \leq 0.001$).

Tissue sections from the ischemic hindlimb muscles were examined histologically as described above. As shown in Figure 2, there was a significant reduction in capillary density in the NOD mice at 35 days postoperatively with values of 302 ± 4 capillaries/mm^2 *versus* 782 ± 78 for NOD mice *versus* C57 controls ($P \leq 0.005$).

VEGF mRNA Expression

At baseline (nonischemic hindlimbs, day 0), the level of VEGF mRNA expression was almost undetectable both in C57 and NOD mice. However, in ischemic hindlimbs,

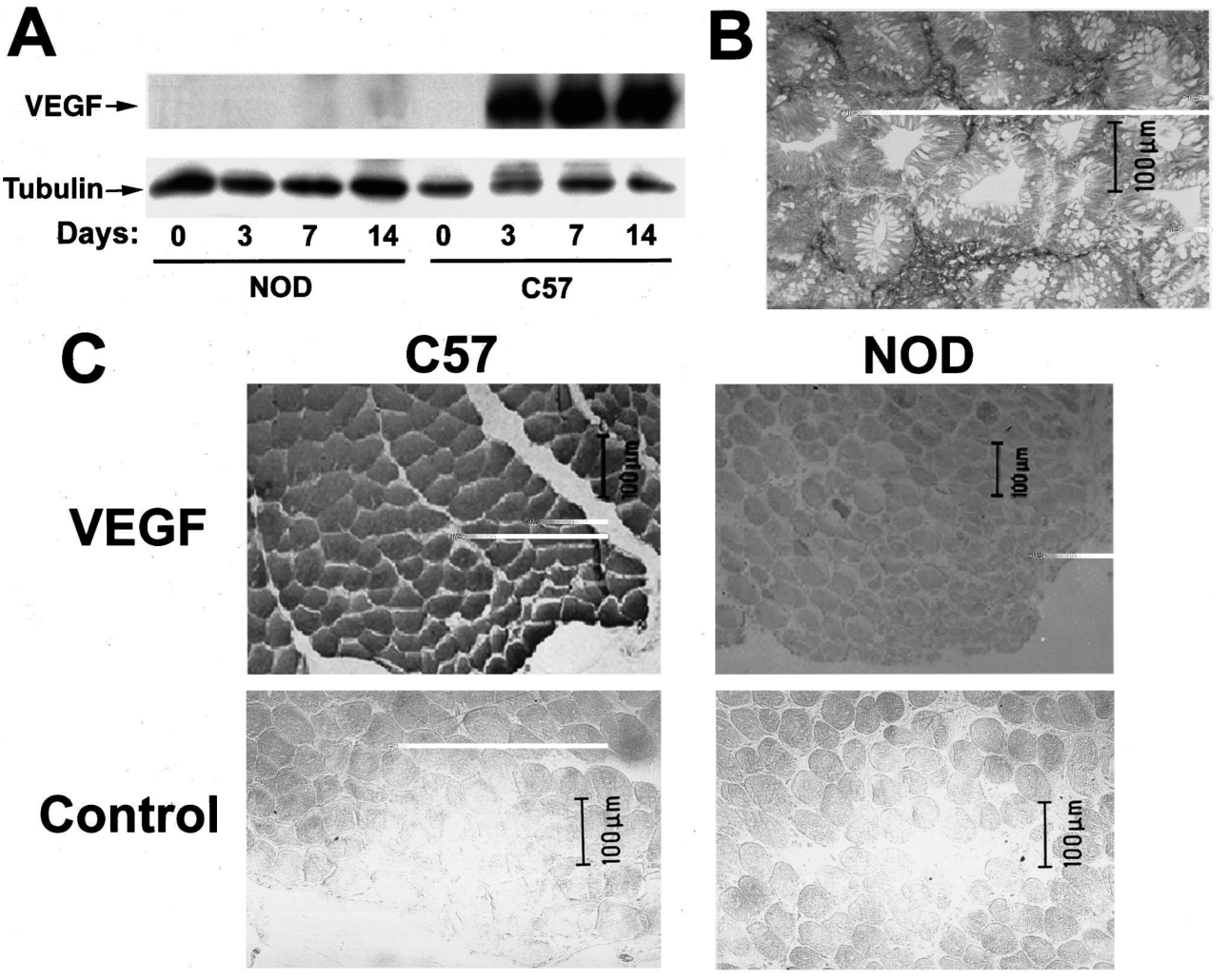

Figure 4. Expression of VEGF protein. A: Western blot analysis of VEGF protein expression in ischemic muscles harvested at different time points after hindlimb surgery in diabetic (NOD) and normal (C57) mice. The level of VEGF protein expression was significantly reduced in NOD *versus* C57 mice from day 3 to day 14 after surgery. B: Positive control (human colon carcinoma, ×50 hematoxylin counter stain) for VEGF immunostaining. C: Immunostaining for VEGF in ischemic tissues of C57 and NOD mice, 7 days after surgery. Immunostaining confirmed the results of the Western blot analysis, showing a lower level of VEGF expression in the ischemic muscles of NOD *versus* C57 mice. Negative control slides (control) were prepared in C57 and NOD mice by substituting preimmune rabbit serum for VEGF antibody staining.

VEGF mRNA expression was significantly reduced in NOD *versus* C57 mice, especially at day 7 and 14 after surgery (Figure 3).

VEGF Protein Expression

Endogenous expression of VEGF protein was determined for NOD and C57 mice by Western blot analysis of protein extracts obtained from hindlimb muscles harvested at different time points postoperatively. Figure 4A shows the time course of VEGF expression in NOD and C57 mice after operative induction of hindlimb ischemia. In both C57 and NOD mice, VEGF levels were undetectable at baseline (nonischemic hindlimbs, day 0). However, in ischemic hindlimbs, the level of VEGF protein expression was significantly reduced in NOD *versus* C57 mice from day 3 to day 14 after surgery. Immunostaining confirmed the results of the Western blots by showing a lower level of VEGF expression in the tissues retrieved from NOD *versus* C57 mice at day 7 after surgery (Figure 4, B and C) and identifying the skeletal myocytes as the cell source of VEGF expression. The specificity of the immunostaining was confirmed using negative controls (Figure 4C, control) prepared in C57 and NOD mice by substituting preimmune rabbit serum for VEGF antibody staining.

VEGF Supplementation in Diabetic Mice

We used an adenoviral vector encoding for the murine VEGF sequence to provide replacement therapy for deficient VEGF expression in the NOD mice. Adeno-VEGF was injected intramuscularly at a dose of 1×10^9 pfu immediately after the surgery ($n = 7$). NOD mice injected with the same dose of an adenoviral construct coding for nuclear-specific β-galactosidase (adeno-*nls-LacZ*) ($n = 5$) were used as controls. Figure 5, A and B illustrates the macroscopic and histological sections of ischemic muscles stained with X-Gal solution 3 days after intramuscu-

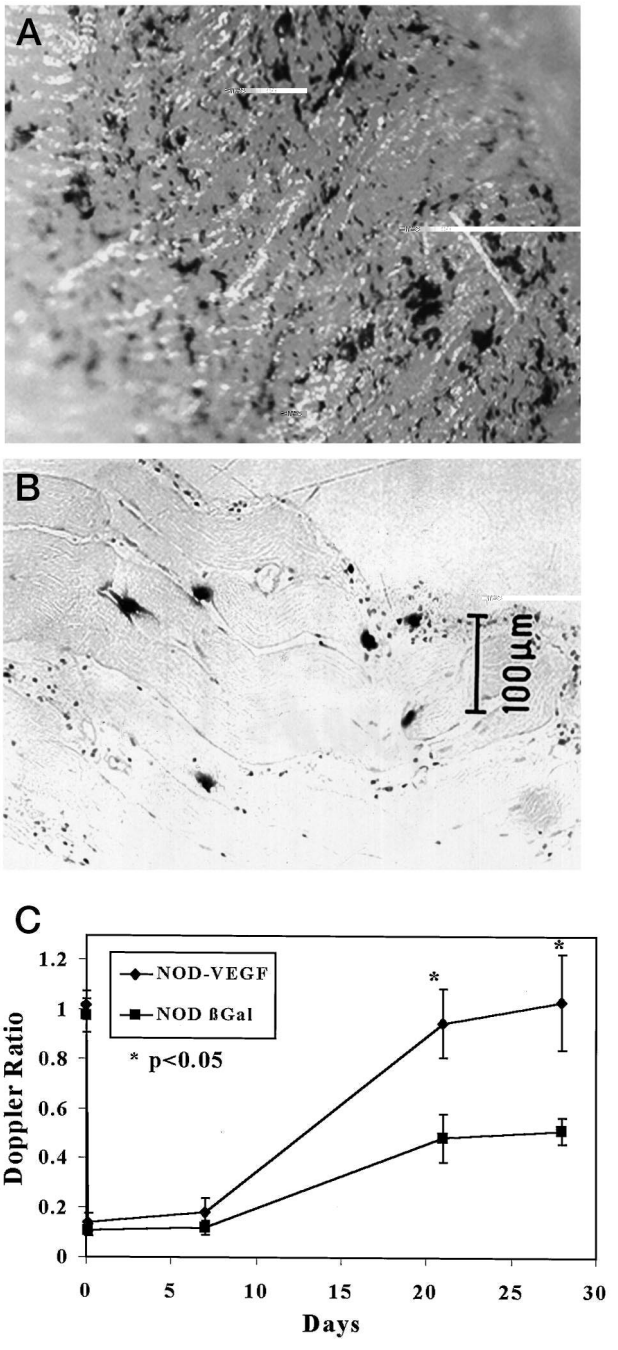

Figure 5. VEGF supplementation in diabetic mice. (A and B) Macroscopic and histological sections of ischemic muscles stained with X-Gal solution 3 days after intramuscular injection of the adeno-*nls-LacZ* construct. The efficiency of transfection is indicated by the positive blue staining of the myocyte nuclei. NOD mice receiving adeno-VEGF gene transfer showed significant improvement in hindlimb perfusion as assessed by LDPI analysis of hindlimb blood flow. This improvement in hindlimb perfusion was consistent throughout the duration of the study (C).

lar injection of the adeno-*nls-LacZ* construct. The efficiency of the transfection was confirmed by the positive blue staining of the myocyte nuclei. As shown on Figure 5C, NOD mice receiving VEGF replacement therapy showed significant improvement in hindlimb perfusion as assessed by LDPI. At day 21 after surgery, the ratio of ischemic to normal hindlimb blood flow was 0.9 ± 0.2 for NOD mice transduced with adeno-VEGF *versus* 0.5 ± 0.1 for NOD mice receiving adeno-*nls-LacZ* ($P \leq 0.05$). This improvement in hindlimb perfusion was consistent for the duration of the study with final values of 1.0 ± 0.2 *versus* 0.5 ± 0.2 in the VEGF and adeno-*nls-LacZ* mice, respectively ($P \leq 0.05$). These results were confirmed at the microvascular level. As seen on Figure 6, there was a significant increase in capillary density in NOD mice injected with adeno-VEGF (Figure 6A) compared with mice receiving adeno-*nls-LacZ* (Figure 6B). Necropsy examination disclosed the capillary density in adeno-VEGF-treated mice to be 903 ± 224 capillaries/mm^2 *versus* 326 ± 57 for adeno-*nls-LacZ*-transduced mice (Figure 6C, $P \leq 0.05$).

Discussion

The present study is, to our knowledge, the first one to document the unfavorable impact that diabetes may have on neovascularization of ischemic tissue. Although diabetes has been widely recognized has a major risk factor for cardiovascular diseases,[1] the extent to which it may modulate angiogenesis in the setting of tissue ischemia has been limited to the study of certain types of ocular pathology;[26–28] these studies established that hypoxia-induced up-regulation of VEGF leads to pathological angiogenesis. In contrast, we investigated the potential for diabetes to modulate nutrient angiogenesis that constitutes a compensatory response to nonocular tissue ischemia. We used a model of hindlimb ischemia in the NOD mouse, which has been shown to develop a form of diabetes with clinical features similar those of the human type-I, insulin-dependent diabetes.[18,19] Impairment of angiogenesis following hindlimb ischemia in these mice was documented by two different techniques. First, LDPI showed that blood flow was significantly reduced in the ischemic hindlimb of NOD mice when compared with the C57 mice. This difference was seen at 14 days after surgery and persisted without evidence of resolution to the end of the follow-up period (35 days). Second, impairment of angiogenesis in the diabetic mice was confirmed at the microvascular level by documenting a statistically significant reduction in the capillary density at 35 days after surgery. This result is consistent with necropsy examination of human hearts, which showed that capillary density was reduced in tissue sections from diabetics *versus* nondiabetic patients with myocardial infarction.[29]

The mechanisms by which diabetes may affect angiogenesis are potentially diverse. Angiogenesis is a complex process that involves activation, migration, and proliferation of endothelial cells.[30] Recent studies have documented that endothelium-dependent relaxation is impaired in both the peripheral and coronary arteries of insulin-dependent diabetic patients.[31,32] *In vitro* studies of endothelial cells have also shown that a high glucose concentration is associated with decreased endothelial cell proliferation, cell cycle prologation, and alterations in endothelial cell cytoarchitecture.[33–35] Taken together,

Figure 6. Capillary density after VEGF supplementation. NOD mice transduced with the adeno-VEGF construct (A) developed more vessels in response to ischemia than mice receiving adeno-*LacZ* (B). Thirty-five days after surgery, there was a significant increase in the capillary density in diabetic mice supplemented with VEGF (C).

these observations support the notion that endothelial dysfunction associated with diabetes could contribute to the impaired angiogenesis in the setting of tissue ischemia.

Growth factors, particularly endothelial cell mitogens, represent a second essential element in the promotion and regulation of angiogenensis. VEGF, an endothelial cell-specific mitogen has been shown to be a critical growth factor in therapeutic [7,8,11,12,16,17] and pathological[26,36,37] angiogenesis. The absence of a single VEGF allele in the developing embryo is sufficient to prohibit vascular development, and VEGF appears to lie downstream of several, if not all, other angiogenic cytokines.[24,38–40] In the present study, we showed that the magnitude of VEGF expression in ischemic hindlimbs of diabetic mice was significantly reduced in comparison with that observed in normal C57 mice. The lower levels of VEGF expression in NOD mice was documented by Northern blot, Western blot, and immunohistochemical studies of tissues isolated from the ischemic hindlimbs. Interestingly, a similar defect in VEGF regulation has also been reported in diabetic mice in the context of wound healing.[41] These observations contrast with studies on angiogenesis in the context of diabetic retinopathy. In this situation, high levels of VEGF have been identified in ocular fluids of diabetic patients.[26] The mechanisms by which VEGF expression could be different from one tissue to the other are not known. It is possible that the transcriptional or posttranscriptional regulation of VEGF could vary depending on the cell type (skeletal myocytes *versus* retinal cells). Alternatively, differences in the degree and type of ischemia (chronic *versus* acute) could account for the differences in VEGF expression in different tissues.

The pivotal role of VEGF in diabetes-related impairment of angiogenesis was confirmed by replacement of this growth factor in the NOD mice. Using direct intramuscular injection of a replication-deficient adenovirus coding for murine VEGF, we demonstrated a statistically significant improvement in both hindlimb blood flow and capillary density in diabetic mice, achieving values similar to those of normal C57 mice. Supplementation of angiogenic growth factors as recombinant protein therapy[7–13] or gene transfer[14–17] has been used in animal models of myocardial and limb ischemia to augment the development of new vessels. More recently, this strategy has been used to amplify neovascularization in selected patients with peripheral[17,42] and coronary artery disease.[43] The extent to which the use of such therapy may be affected by specific risk factors in patients with vascular diseases is not known. We have recently shown that endogenous hypercholesterolemia impairs angiogenesis, but does not preclude a favorable response to therapeutic angiogenesis.[44,45] Similarly, whereas aging is associated with impaired angiogenesis, it does not preclude augmented neovascularization in response to exogenous VEGF administration.[46] Diabetics represent an additional large subgroup of patients with a high incidence of cardiovascular diseases. The present series of experiments suggests that such patients, in whom the development of collateral blood vessels in response to tissue ischemia may be impaired because of insufficient VEGF expression, may benefit from a strategy of VEGF replacement. It is appropriate, however, to underscore the fact that the present study involves a total time frame of 35 days; whether such success can be duplicated indefinitely in humans with a multiyear history of insulin-requiring diabetes remains to be determined.

Finally, the results of these experiments may have implications for the high incidence of restenosis[47] and poor long-term outcomes,[48] which have been reported for diabetics treated by percutaneous revascularization. Experimental work from our laboratory[25] and others[49,50] has suggested a role for VEGF in the maintenance of endothelial integrity and consequently primary or secondary neointimal thickening. If VEGF expression by the arterial smooth muscle cells of diabetic patients is compromised to the extent observed in the skeletal myocytes of the diabetic mouse hindlimb, the consequent disruption of endothelial integrity may promote recurrent luminal narrowing.

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