Endothelium-Dependent Relaxation of Collateral Microvessels After Intramuscular Gene Transfer of Vascular Endothelial Growth Factor in a Rat Model of Hindlimb Ischemia

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Background—Recent investigations have demonstrated the ability of vascular endothelial growth factor (VEGF) to augment the development of collateral arteries in vivo. In vitro studies have suggested that the use of VEGF also improves the endothelium-dependent relaxation of collaterals at the microvascular level. The purpose of this study was to determine in vivo the extent to which vasomotor responses of collateral microvessels are altered after VEGF treatment.

Methods and Results—Ischemia was induced in the hindlimb of 35 rats by excision of the femoral artery. Immediately thereafter, 400 µg of a plasmid encoding VEGF or β-galactosidase (control) was transfected into limb muscles. Four weeks later, synchrotron radiation microangiography, with a spatial resolution of 30 µm, was performed to document the reactivity of collateral microvessels. Administration of the endothelium-dependent vasodilator acetylcholine failed to induce dilation of collateral microvessels in control animals. By contrast, profound dilation of collaterals was observed after acetylcholine in VEGF-treated animals. This response was evident in vessels with a linear appearance but not in those with an undulating appearance. The resulting blood flow in the ischemic limb after administration of acetylcholine in the control animals was only 64.6 ± 17.0% of that of the contralateral normal limb, whereas blood flow was augmented to 106.1 ± 8.4% in VEGF-treated animals (P<0.05).

Conclusions—These results demonstrate in vivo that the use of VEGF restores impaired vasomotor responses in some types of collateral microvessels, which may help to provide a basis for understanding the microcirculation after therapeutic angiogenesis with VEGF. (Circulation. 1998;98:1261-1263.)

Key Words: angiogenesis ■ collateral circulation ■ endothelium ■ microcirculation

Collateral vessels respond to numerous vasoactive agents and play an active role in regulation of blood flow to ischemic tissues. However, the vasomotor responses of collaterals, whether these collaterals are large conduit vessels or small arterioles, differ markedly from those of innate vessels. Specifically, vasomotor responses of relatively large collateral vessels to endothelium-dependent agents such as acetylcholine (ACh) have been shown angiographically to be impaired in ischemic limbs in vivo.1 In vitro studies also have shown that endothelium-dependent relaxation of coronary collaterals is depressed markedly at the microvascular level.2

Recent investigations have established that exogenously administered angiogenic growth factors can induce the formation of new blood vessels and enhance collateral blood flow to ischemic tissues.3 A number of naturally occurring growth factors could potentially induce or accelerate angiogenesis by stimulating endothelial cell proliferation and migration. Among these, the most potent endothelial mitogen may be vascular endothelial growth factor (VEGF).4

Previous in vivo studies have suggested that VEGF not only augments the formation of collateral vessels but also modifies their vasomotor responses. Bauters et al1 demonstrated angiographically that administration of VEGF results in improved endothelium-dependent responses of relatively large collateral vessels. A more recent study demonstrated in vitro that VEGF improves preservation of endothelium-dependent relaxation of collaterals at the microvascular level.5 Whether impaired responses of collateral microvessels can be improved by the use of VEGF remains to be confirmed in vivo.

The purpose of this study was to document in vivo the altered endothelium-dependent responses of naturally occurring collateral vessels at the microvascular level and to investigate the extent to which the function of these microvessels is modified after VEGF administration.

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Methods

Plasmids
The plasmid phVEGF<sub>165</sub> used in this study contains the cDNA for recombinant human VEGF<sub>165</sub> under the transcriptional control of the cytomegalovirus promoter/enhancer (a gift from Genentech Inc). The biological activity of VEGF<sub>165</sub> secreted from the cells transfected with phVEGF<sub>165</sub> was confirmed previously. A promoter-matched reporter plasmid pCMVβ (Clontech) encoding β-galactosidase was used in control transfection experiments.

Direct Transfection of Limb Muscles
The endothelium-dependent microvessel reactivity of collaterals was investigated in a model of rat limb ischemia. Male Wistar rats weighing 300 to 350 g (Charles River Japan) were anesthetized with an injection of sodium pentobarbital 40 mg/kg IP, after which the left femoral artery was surgically excised to induce limb ischemia. Rats were then transfected with either VEGF (n=17) or β-galactosidase (n=18) plasmid. The plasmid (400 μg) was dissolved in saline (in a final volume of 1.0 mL) and injected directly into the limb muscles with a syringe with a 27-gauge needle. For each animal, DNA was injected into 5 different sites in the 3 major thigh muscles: the adductor (2 sites), quadriceps (2 sites), and semimembranos (1 site). After 5 injections (80 μg · 0.2 mL⁻¹ · site⁻¹) were completed, the incision was closed in layers.

Microangiographic Assessment
Four weeks after transfection, 10 VEGF-treated and 12 control animals were reanesthetized, and angiographic assessment was performed with synchrotron radiation (SR) microangiography with a spatial resolution of 30 μm. After obtaining a baseline angiogram, we allowed a 10-minute interval to reestablish basal hindlimb blood flow. ACh (Sigma) prepared in saline (final volume of 0.4 mL) was then infused over 2 minutes (0.2 μg · kg⁻¹ · min⁻¹), after which a second angiogram was recorded. Preliminary studies have shown that the dose of ACh used here significantly dilates normal limb arteries (Figure 1A and 1B) and increases limb blood flow to 199.3±31.5% (n=5) without an effect on the systemic blood pressure. 

Quantitative angiographic analysis of collateral vessel development was performed by directly counting the number of vessels that crossed a line drawn diagonally across the mid thigh (perpendicular to the femur). This analysis was performed by a single observer who was blinded to the treatment regimen.

Measurements of Limb Blood Flow by Use of Colored Microspheres
Colored microspheres were used to determine the regional perfusion of the limb muscles at 4 weeks after transfection. Briefly, 7 VEGF-treated and 6 control animals were reanesthetized. A polyethylene catheter was then introduced through the carotid artery into the aortic arch. After ACh was infused intra-arterially, Dye-Trak microspheres (15 μm in diameter, Triton Technology) were injected. Animals were killed, and muscle samples were obtained from the medial thigh of both limbs. Samples were digested with potassium hydroxide, and microspheres were reclaimed with a vacuum filter. The dye from the microspheres was extracted with dimethyl formamide. These dye samples were then analyzed with a spectrophotometer. On the basis of the optical density (OD) measurements, the percent limb flow (flow in the ischemic limb expressed as a percentage of that in the contralateral normal limb) was calculated from the following equation: (OD of the ischemic limb/OD of the normal limb)×(tissue weight of the normal limb/tissue weight of the ischemic limb)×100 (%).

Statistical Analyses
Results are expressed as mean±SEM. Statistical significance was evaluated by unpaired Student’s t test. A value of P<0.05 was considered statistically significant.

Results

Angiographic Assessment of Collateral Microvessels
Quantitative analysis of collateral vessels showed that the number of collateral vessels in the VEGF-treated group was significantly higher than in the controls (18.1±1.3 versus 10.7±1.3, P<0.01).

Administration of ACh to the normal limb induces relaxation of microvessels (Figure 1A and 1B, arrows). This response was blunted in collaterals of any size in control animals (Figure 1C and 1D, arrows). In contrast, VEGF-treated animals showed improved vasodilatory responses regardless of the vessel size (Figure 1E and 1F). Importantly, this vasodilatory effect was observed only in the arteries with a relatively linear appearance (arrows) but not in those with a corkscrew appearance (open arrows).

Limb Blood Flow Measurement
At 4 weeks after transfection, blood flow to the ischemic limbs after ACh in the control animals was reduced to
64.6 ± 17.0% of that of the normally perfused limb. By contrast, ischemic limb blood flow in VEGF-transfected animals was completely restored to the level in their normal limbs (106.1 ± 8.4%, P < 0.05 versus controls) (Figure 2).

Discussion

SR microangiography demonstrated that endothelium-dependent relaxation of collateral microvessels improved markedly in response to VEGF. Although VEGF has been suggested to induce endothelium-derived relaxing factor–dependent vasorelaxation, the contribution of this effect on collateral dilation was not significant in this study because expression of the transfected VEGF gene was limited to < 3 weeks. The resultant limb blood flow measured by colored microspheres also increased significantly in the VEGF-treated animals. Preliminary studies performed in our laboratory also showed that 4 weeks after induction of ischemia, blood flow to the ischemic limb in VEGF-treated animals had already returned to the level of the normal limb before ACh administration, which was = 50% of that seen after ACh. Thus, = 50% of the limb flow after ACh administration in VEGF-treated animals seemed to be due to ACh-induced dilation of collateral vessels.

At least 2 different mechanisms could contribute to the improvement in the vasodilatory responses of collateral microvessels. First, VEGF significantly increases blood pressure in ischemic limbs; therefore, the characteristics of flow and perfusion pressure in arterioles distal to the site of vessel occlusion should change significantly. Development of an increase in perfusion pressure may lead to repair of dysfunctional endothelium in distal vasculatures. The second possibility is that VEGF leads to a direct improvement in endothelial function. Asahara et al demonstrated that VEGF accelerates recovery of endothelium-dependent reactivity of balloon-injured vessels. Thus, VEGF may modulate qualitative aspects of endothelial cell function by directly repairing endothelial cells damaged by protracted ischemia and thereby restore normal, endothelium-dependent blood flow.

Another major finding of this study is that VEGF improved the vasomotor response of linear but not of tortuous collateral vessels. We have documented recently, by SR microangiography, that tortuous collaterals exist in ischemic but not in normal limbs. It is possible that linear collaterals result from the functional opening and/or dilation of preexisting vessels, whereas tortuous microvessels result from remodeling of preexisting vessels or are newly formed and thus possess functionally different endothelial cells that have a smaller vasodilatory response to ACh.

We chose ACh to examine endothelium-dependent vasomotor reactivity because it can be used in human patients. However, ACh has vasoconstrictive actions in vascular smooth muscle cells, and in this regard, other agents, such as substance P, may also be useful for examining endothelium-dependent reactivity of collaterals in animals.

In summary, the use of VEGF restores the impaired vasomotor responses of a selected group of microvascular collaterals. These data may provide new insights regarding collateral microcirculation and therapeutic angiogenesis.

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