Adenoviral Gene Transfer With Soluble Vascular Endothelial Growth Factor Receptors Impairs Angiogenesis and Perfusion in a Murine Model of Hindlimb Ischemia

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**Background**—The purpose of the current study was to examine the contribution of endogenous vascular endothelial growth factor (VEGF) to ischemia-induced angiogenesis and perfusion.

**Methods and Results**—C57BL/6J mice (n=28) were subjected to unilateral hindlimb ischemia after intravenous injection of recombinant adenoviruses (10^9 plaque-forming units) encoding the ligand-binding ectodomain of VEGF receptor 1 (VEGFR1/Ad Flt1), VEGF receptor 2 (VEGFR2/Ad Flk1-Fc), a control murine IgG2α Fc fragment (Ad Fc), or vehicle (phosphate-buffered saline). Hindlimb perfusion was assessed by both laser Doppler and fluorescent microsphere injection 10 days after surgery. The role of endogenous VEGF in ischemia-induced angiogenesis and arteriogenesis was measured by capillary density and microangiography, respectively. Adenoviral gene transfer with soluble VEGFRs significantly attenuated hindlimb perfusion as assessed by laser Doppler and microsphere analysis (P<0.05). Furthermore, soluble VEGFRs significantly reduced ischemia-induced angiogenesis and collateral growth and inhibited histological recovery of muscle tissue. Adverse events consistent with ongoing vascular insufficiency such as limb necrosis or gangrene were observed only in animals expressing soluble VEGFRs and not in control animals.

**Conclusions**—Systemic, soluble receptor–mediated VEGF inhibition indicates an essential role for endogenous VEGF during postischemic angiogenesis and hindlimb perfusion. (Circulation. 2004;110:2424-2429.)

**Key Words:** collateral circulation ▪ genetics ▪ ischemia ▪ angiogenesis ▪ perfusion

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**Vascular endothelial growth factor (VEGF)** exerts pleiotropic effects on endothelial cell biology, including potent regulation of embryonic vasculogenesis, angiogenesis, proliferation, survival, and permeability. This dominant role in endothelial regulation has prompted numerous attempts to use VEGF to promote in vivo blood vessel growth in disorders associated with inadequate tissue perfusion. However, in contrast to encouraging results of exogenous delivery of VEGF in various animal models, prospective placebo-controlled trials in humans with recombinant or adenoviral vector–expressed VEGF have been unsuccessful in ameliorating coronary or peripheral artery disease.2,3

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- VEGF plays an important role in ischemia-induced angiogenesis, consistent with the known regulation of VEGF by hypoxia and the hypoxia-inducible factor family of transcription factors.4 In contrast, the role of VEGF in arteriogenesis, defined as the remodeling and growth of collateral arteries from a preexisting arteriolar network, remains controversial.5 Whereas some studies have failed to demonstrate a significant role for exogenously applied VEGF in arteriogenesis in animal hindlimb ischemia models, other investigators have reported stimulation of arteriogenesis by either exogenous VEGF or placental growth factor (PIGF).6–11

- We have shown that intravenous administration of adenoviruses encoding soluble ligand-binding ectodomains of VEGF receptor 1 (VEGFR1/Flt1) or VEGF receptor 2 (VEGFR2/Flk1) leads to liver transduction, followed by high-level circulating transgene expression in plasma lasting >3 weeks. Under these conditions, adenoviral expression of either Flt1 or Flk1 ectodomains elicits broad-spectrum and systemic antitumor efficacy through VEGF sequestration and inhibition of tumor angiogenesis in numerous animal models.12–14 Furthermore, these circulating soluble VEGFRs significantly attenuated VEGF-induced neovascularization in rodent corneal micropocket assays, indicative of potent and systemic VEGF neutralization.13,15 This adenoviral system thus represents a stringent system for the conditional and systemic VEGF inactivation in adult organisms, allowing convenient exploration of its function in physiology.

In the current studies, we administered adenoviruses encoding soluble Flt1 and Flk1 ectodomains in the setting of a

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murine hindlimb ischemia model to examine the contribution of endogenous, as opposed to exogenous, VEGF activity to ischemic angiogenesis and arteriogenesis.

Methods

Mouse Model of Hindlimb Ischemia

Unilateral hindlimb ischemia was introduced in 5- to 6-month-old male C57BL/6J mice (n = 28, Jackson Laboratories, Bar Harbor, Me.). In brief, mice were anesthetized by injection with etomidate (35 mg/kg IP). Under sterile conditions, the superficial and deep femoral arteries were ligated with 7-0 silk sutures (Ethicon). In addition, the superficial femoral artery was excised down to the reentry of collateral branches arising from the lateral circumflex artery. A sham procedure (preparation of arteries without ligations) was performed on the contralateral leg. As appropriate, 3 days before surgery, animals received a single intravenous (tail) injection (10⁶ plaque-forming units [PFU]) of Ad Fc, Ad Flt1, Ad Flk1-Fc (each n = 8), or vehicle solution (phosphate-buffered saline [PBS], n = 4), as described later.

Animals were euthanized 10 days after surgery by exsanguination (rupture of the right atrium) under deep anesthesia (etomidate 50 mg/kg) after injection of microspheres. The adductor and calf muscles of both legs were harvested and snap-frozen in isopentane solution supercooled by immersion in LN₂. The time point of sacrifice was based on the time course of ischemia-induced angiogenesis in pilot studies and previously described systemic expression kinetics of the adenoviral constructs. Investigators involved in the surgical procedure and subsequent analyses were blinded to the treatment groups. All animals were housed in a temperature-controlled animal facility with a 12-hour light/dark cycle and had free access to tap water and rodent chow. The study protocol was approved by the Administrative Panel on Laboratory Animal Care of Stanford University.

Adenovirus encoding the murine Flk1 ectodomain fused to murine IgG2a Fc (Ad Flk1-Fc) and a control adenovirus encoding murine IgG2a Fc alone (Ad Fc) have been previously described. The Ad Flt1 construct is identical to the one previously described, with the addition of an N-terminal hemaglutinin (HA) tag. Large-scale adenoviral preparations were produced by infection of 293 cells, banded over CsCl gradients, and dialyzed into 4% sucrose. Circulating plasma expression of Flt1 and Flk1 was confirmed by ELISA of plasma obtained by retro-orbital phlebotomy on the day of surgery and 1 day before sacrifice. In addition, hematocrit levels were measured at these time points.

Laser Doppler Perfusion

In vivo muscle perfusion was measured with a calibrated laser Doppler probe (Perimed PFD3) placed on the adductor muscles by using a 3-dimensional micromanipulator stage. Measurements (perfusion units) were recorded for 2 minutes on the middle and distal portion of the adductor muscles of both legs before ligation of the arteries, immediately after induction of hindlimb ischemia, and on the day of sacrifice. Average perfusion was expressed as the ratio of the ischemic versus nonischemic leg. The device was primarily used to document the initial reduction in muscle perfusion after hindlimb surgery.

Microsphere Analysis

On the day of sacrifice, a left-sided hemithoracotomy was performed and ultrasonicated red fluorescent microspheres (15 μm, Molecular Probes) were injected into the beating left ventricle under microscopic visualization (150 000 particles). Injection time was 1 minute to avoid streaming of spheres that occurs with bolus injection. The particle amount was based on recommendations from the Fluorescent Microsphere Resource Center and tested in a pilot project aimed to detect at least 400 spheres in the adductor muscles of nonischemic legs. With this amount of spheres, there is 95% confidence that the observed flow in the region is within 10% of true flow. Microspheres were visually counted in 70-μm frozen cross sections from the adductor muscles of both legs, and the amount of spheres per milligram of muscle tissue was calculated. At fixed intervals, 10-μm sections were cut for histomorphometry and immunohistochemistry. Kidneys served as reference organs proximal to the occlusion site.

Immunohistochemistry

Capillary densities were determined by immunofluorescence in 10 randomly chosen, nonoverlapping fields of 10-μm cross sections made through the midbelly of the adductor muscles with monoclonal anti-CD31 (BD Pharmingen) and fluorescent anti-rat IgG secondary (Molecular Probes) antibodies. To avoid overestimation of capillary density secondary to myocyte atrophy or underestimation caused by interstitial edema, the capillary–myocyte fiber ratio was determined by counting the number of capillaries and myocytes per field (×20 objective). Capillaries were automatically counted with Image Pro Plus 4.5.1 software (Media Cybernetics, Inc). Myocytes were outlined with an antibody against laminin (Chemicon) and fluorescent anti-rabbit IgG secondary antibody (Molecular Probes) and counted visually.

Microangiography

Postmortem microangiograms under maximum vasodilation with sodium nitroprusside (10⁻⁴ mol/L) were performed with barium sulfate contrast medium (1 g/mL, E-Z-EM) injected into the thoracic aorta. Images were acquired with a Faxitron x-ray imager (Hewlett-Packard), and films were exposed for 30 seconds at 40 kV and subsequently developed. Additional angiographies were performed in male Sprague-Dawley rats (Jackson Laboratories) injected with Ad Flt1, Ad Flk1-Fc, or Ad Fc (5×10⁹ PFU) under conditions identical to those of the mouse studies (see earlier sections). The angiographic score was determined by pixel analysis (Image Pro Plus 4.5.1) and expressed as the ratio of the ischemic to nonischemic limb. The area of interest was delineated by the position of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg.

Statistical Analysis

Statistical analysis was performed with an SPSS software package (version 12.0). For comparisons between treatment groups, the Kruskal-Wallis test was applied; comparisons within groups were performed with the Wilcoxon signed-rank test. All data are given as mean±SEM. Statistical significance was accepted at P<0.05.

Results

Soluble VEGFRs Produce Sequelae of Chronic Vascular Insufficiency After Hindlimb Ischemia

Male C57BL/6J mice (5 to 6 months old) received a single intravenous (tail) injection of 10⁶ PFU of Ad Flt1 (sVEGFR1), Ad Flk1-Fc (sVEGFR2), Ad Fc (encoding an antibody IgG2a Fc fragment) (n = 8 each), or PBS (n = 4), followed 3 days later by introduction of hindlimb ischemia by ligation of the superficial and deep femoral arteries. By ELISA measurement, plasma expression levels of soluble VEGFRs at the time of surgery were 0.56±0.10 μg/mL and 9.0±1.7 mg/mL for Flt1 and Flk1-Fc, respectively, consistent with previous values sufficient to produce systemic VEGF inhibition. Interestingly, VEGF blockade was associated with sequelae of chronic vascular insufficiency after hindlimb ischemia, because foot or toe gangrene confined to the operated leg was observed only in animals that received either Ad Flt1 (2/8) or Ad Flk1-Fc (3/8) but not PBS (0/4) or Ad Fc (0/8), suggestive of a functional role for endogenous VEGF in postischemic revascularization.
Soluble VEGFRs Inhibit Histological Recovery After Hindlimb Ischemia

Histological analysis of Ad Flt1– and Ad Flk1-Fc–treated mice with hindlimb ischemia was performed to correlate the gross anatomic findings of digit necrosis and gangrene with cellular changes. Centralization of myocyte nuclei indicating regenerative muscle tissue was absent in nonischemic hindlimbs (Figure 1a) but was prominent by day 10 after surgery in ischemic muscle (Figure 1b). At this time point, ischemic calf sections from Ad Fc– (Figure 1c) or PBS– (data not shown) treated mice showed no gross tissue necrosis, whereas the ischemic calf muscles of mice expressing soluble VEGFRs such as Ad Flk1-Fc exhibited considerable myocyte necrosis (Figure 1d), consistent with participation of endogenous VEGF in the preservation of myocyte function after an acute ischemic insult. Within thigh muscles, myocyte necrosis was not present in any of the treatment groups, likely owing to proximal collateral circulation upstream of the surgical vascular interruption.

Figure 1. Effects of VEGF blockade on histological appearance of ischemic limbs at day 10 after surgery. a, Nonischemic thigh from Ad Fc–treated animal. Myocytes have angular shape with peripheral nuclei (×20). b, Ischemic thigh from Ad Fc–treated animal. Myocytes have lost their polygonal shape and have centralized nuclei, indicating ischemic tissue is undergoing regeneration (×20). c, Ischemic calf muscle from Ad Fc–treated animal. Portions of calf show myocytes with centralized nuclei and moderate inflammatory infiltrates (×10). d, Ischemic calf muscle from Ad Flk1-Fc–treated animal. Almost entire cross section shows centralized myocyte nuclei and extensive infiltration of inflammatory cells. Part of muscle tissue has undergone necrosis (×10). Abbreviations are as defined in text.

Soluble VEGFRs Inhibit Ischemia-Induced Angiogenesis

To correlate VEGF blockade–induced histological changes in the ischemic limb with changes in angiogenesis, neovascularization was assessed by CD31 immunofluorescence. Ten days after surgical ischemia, capillary densities (capillaries per myocyte) were markedly lower in the ischemic adductor muscle of Ad Flt1– and Ad Flk1-Fc–treated mice compared with PBS– or Ad Fc–treated animals (PBS, 1.95±0.11; Fc, 1.95±0.07; Flt1, 1.44±0.03; and Flk1, 1.45±0.05 capillaries/myocyte; P<0.01 for Flt1 and Flk1 versus PBS or Fc; Figure 2).

Soluble VEGFRs Inhibit Hindlimb Reperfusion

To assess the effect of VEGF blockade on blood flow to the ischemic limb, laser Doppler and fluorescent microsphere analyses were performed. Preoperative adductor muscle perfusion as assessed by laser Doppler measurements was similar in all experimental groups, with an average perfusion ratio (ischemic leg/nonischemic leg) of 1.00±0.03 (n=28, Figure 3). Immediately after surgery, the perfusion ratio in all experimental groups decreased to 0.31±0.02 after ligation of arteries (P<0.001, n=28, Figure 3). In animals treated with PBS or Ad Fc, a significant rise in the perfusion ratio was observed after 10 days, consistent with physiological restoration of muscle perfusion, whereas this increase was severely inhibited in Ad Flt1 and Ad Flk1-Fc mice (Figure 3). As an alternative and more precise method to assess tissue perfusion, fluorescent microsphere analysis was performed. In PBS– or Ad Fc–treated mice, relative perfusion of ischemic versus nonischemic limbs 10 days after surgery was lower than in sham-operated controls (PBS, 61.7±9.7%; Fc, 58.2±5.7% normalized to sham as 100%, Figure 4), consistent with an overall decreased perfusion after surgery. However, both Ad Flt1– and Ad Flk1-Fc–treated mice exhibited significantly less perfusion (Flt1, 29.0±7.0%; Flk1, 30.1±6.6%, P<0.05 versus PBS or Fc, Figure 4), suggestive of a causative effect of VEGF blockade.

Soluble VEGFRs Inhibit Postischemic Collateral Vessel Formation

The ability of exogenously applied VEGF to induce vascular remodeling and arteriogenesis in the setting of hindlimb ischemia has been somewhat controversial. However, given that surgically treated animals receiving Ad Flt1 and Ad Flk1-Fc manifested histopathologic features and reduced perfusion con-
sistent with severe limb ischemia (Figures 1, 3, and 4), we hypothesized that endogenous VEGF might contribute to post-
ischemic arteriogenesis. Angiographic scores (ratio of ischemic limb to nonischemic limb) of postmortem angiograms taken
after nitroprusside vasodilation were significantly greater in mice treated with PBS or Ad Fc compared with animals treated
with either Ad Flt1 or Ad Flk1-Fc (Figure 5a). In PBS- and Ad
Fc–treated animals, prominent collateral vessels developed in
the ischemic left leg relative to the nonischemic right leg. In
contrast, in Ad Flt1– and Ad Flk1-Fc–treated animals, angiog-
detectable collateral growth in ischemic hindlimbs was
markedly reduced relative to the ischemic extremity of PBS
or Ad Fc mice. Representative angiographic results for Ad Fc
and Ad Flt1 groups are presented (Figure 5b). Similar inhibition
of angiographic collateral development was observed in the
ischemic extremities of Ad Flt1– or Ad Flk1-Fc–treated rats
(data not shown).

Discussion
The present study describes findings consistent with an
essential role for endogenous VEGF in postischemic angi-
genesis and limb perfusion. We have previously shown that
adenoviral expression of soluble VEGFR ectodomains pro-
duces potent, systemic, and conditional VEGF neutralization
in adult animals.12–14 Here, adenovirus-expressed soluble Flt1
and Flk1-Fc significantly impaired ischemia-induced neovas-
cularization at the capillary level (Figure 2) and, more
importantly, markedly attenuated perfusion of ischemic limbs
(Figures 3 and 4). Both findings were independent of possible
confounding effects caused by adenoviral infection or intra-
venous injection, because neither neovascularization nor limb
perfusion was affected by vehicle solution (PBS) or Ad Fc.
Decreased limb perfusion was also associated with impair-
ment in collateral growth (arteriogenesis), as indicated by
postmortem microangiograms (Figure 5). Macroscopic and
microscopic signs of severe tissue damage and ongoing
ischemia, such as toe or foot gangrene or histological focal
myocyte necroses, were observed only with Ad Flt1 and Ad
Flk1-Fc and not in control groups in either these or other
studies. These gross anatomic and histological sequelae
specific for VEGF blockade are further consistent with a
crucial role for endogenous VEGF family members during
postischemic vascular remodeling.

VEGF exerts dominant functions during physiological and
pathological angiogenesis, with enhancement of tumor growth
by promoting tumor angiogenesis and stimulation of capillary
growth in animal models of acute or chronic tissue ischemia.1 In
animal models of limb ischemia, local injection of VEGF or
adenoviral VEGF gene therapy enhances ischemia-induced neo-
vascularization and limb perfusion,7,18–20 although clinical trials
have not reduced this effect to practice.

Although the importance of VEGF in angiogenesis is
undisputed, its role in arteriogenesis remains controversial,5
and the weak arteriogenic activity of exogenously delivered
VEGF and PIGF has been reported to be macrophage depend-
ent.9 Arteriogenesis occurs in a hypoxia-independent fash-
ion and is driven by inflammation and biomechanical forces
such as increased shear stress. A role for endogenous VEGF
in arteriogenesis has not been previously demonstrated.5
Because direct measurement of flow and pressure within
single collateral blood vessels is not technically feasible in
mice, we performed postmortem microangiograms to study
the impact of VEGF blockade on ischemia-induced collateral
growth. Mice treated with soluble VEGFRs exhibited angiog-
graphic evidence of decreased collateral formation (Figure 5),
concordant with ischemia-induced perfusion deficits ob-
served by laser Doppler and fluorescent microsphere studies
(Figures 3 and 4). These observations are consistent with but
not unequivocally demonstrative of a role for VEGF as an
endogenous regulator of arteriogenesis. Our study is limited,
because although we performed angiography under condi-
tions of nitroprusside-induced vasodilation, we cannot ex-
clude the possibility that soluble VEGFRs could impair such
vasodilation, leading to a decrease in the observed number of
collateral vessels independent of effects on arteriogenesis.

Theoretically, systemic VEGF blockade could be associ-
ated with neutralization of circulating and/or tissue VEGF.
Although plasma VEGF concentrations with and without
hindlimb ischemia in PBS-treated mice were not significantly
altered at day 10 (28 versus 34 pg/mL, \( P = 0.53 \)), we have
observed mild ischemic increases in muscle VEGF, consist-
tent with other reports,21 which are further superinduced 4–
to 5-fold by Ad Flt1 or Ad Flk1-Fc (B.Y.Y.T. and C.J.K.,
unpublished data), consistent with a local muscle response to
VEGF blockade. Although we are unable to distinguish
between free and soluble receptor-bound mouse VEGF,
soluble Flt1 and Flk1-Fc proteins are present at the micro-
gram per milliliter level in hindlimbs of adenovirus-treated
animals, suggesting a vast molar excess of soluble receptor
sufficient to neutralize the observed levels (pg/mL) of VEGF
(data not shown).

Prior data indicating a role for endogenous VEGF during
postischemic hindlimb perfusion have not been entirely consis-
tent. Couffinhal et al22 first demonstrated that intraperitoneal
administration of a neutralizing VEGF antibody impaired neo-
vascularization in a murine hindlimb ischemia model, although
a limited number of animals were studied and assessment of
limb perfusion was limited to laser Doppler analyses. In a
different study, neutralizing anti-VEGFR1 or anti-VEGFR2
antibodies did not inhibit perfusion, whereas inhibition was
obtained with the small-molecule VEGFR tyrosine kinase inhib-
itor ZK202650. Angiography to visualize collateral vessels was
not performed after ZK202650 treatment, and the lack of effect
of the neutralizing antibodies was thought to be possibly sec-
ondary to their poor pharmacokinetic properties.23 Such phar-
macokinetic issues, which could compromise antibody or small-
molecule approaches, are less prominent with adenoviral
delivery of either Flt1 or Flk1 ectodomains, which provide
continuous plasma transgene expression and robust plasma
levels sufficient to provide systemic inhibition of tumor growth
and angiogenesis.12–14 The current studies, which included 2
distinct soluble VEGFRs, strongly argue for a role for endoge-
 nous VEGF during postischemic limb perfusion.

We have observed that adenoviral VEGF blockade can be
associated with increases in hematocrit (B.Y.Y.T. and C.J.K.,
manuscript in preparation). In the current study, VEGF
blockade was associated with mild elevations in hematocrit in
the range of 52% to 58% from a baseline of 45% to 48%.
Although this could theoretically impair perfusion, adenoviral expression of erythropoietin sufficient to elevate hematocrit to >60% did not produce distal gangrene, inhibition of postischemic angiogenesis, or inhibition of perfusion measured by laser Doppler or fluorescent microspheres (data not shown), suggesting that alterations in viscosity are unlikely to underlie the present results.

Although powerful, the soluble-receptor approach is unable to precisely define the relative contributions of distinct endogenous VEGF family members, because Flt1 binds PIGF, VEGF-A, and VEGF-B, whereas Flk1/KDR interacts with VEGF-A, VEGF-C, VEGF-D, and VEGF-E. The common phenotypes of Ad Flt1 and Ad Flk1-Fc treatment, however, are consistent with a role for VEGF-A, which is bound by both ectodomains. Although the present data are consistent with a role for VEGF in arteriogenesis, our results do not exclude and may in fact be fully consistent with the involvement of monocyte recruitment and/or shear stress of effects on local or systemic hemodynamics. VEGF blockade has been associated with increases in blood pressure in both rodents and humans, presumably through inhibition of vascular permeability factor activity. Thus, changes in vascular tone and systemic hemodynamics could have an impact on vascular remodeling in this animal model through impaired vasodilation after VEGF inhibition. However, the current studies with systemic expression of 2 distinct soluble VEGFRs clearly demonstrate an important role for endogenous VEGF during postischemic vascular remodeling and perfusion. These studies further underscore the ability of using...
adenovirus to explore physiological functions of growth factor pathways in adult organisms by conditional inactivation strategies.

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