Blood Flow Remodels Growing Vasculature During Vascular Endothelial Growth Factor Gene Therapy and Determines Between Capillary Arterialization and Sprouting Angiogenesis

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Background—For clinically relevant proangiogenic therapy, it would be essential that the growth of the whole vascular tree is promoted. Vascular endothelial growth factor (VEGF) is well known to induce angiogenesis, but its capability to promote growth of larger vessels is controversial. We hypothesized that blood flow remodels vascular growth during VEGF gene therapy and may contribute to the growth of large vessels.

Methods and Results—Adenoviral (Ad) VEGF or LacZ control gene transfer was performed in rabbit hindlimb semimembranosus muscles with or without ligation of the profound femoral artery (PFA). Contrast-enhanced ultrasound and dynamic susceptibility contrast MRI demonstrated dramatic 23- to 27-fold increases in perfusion index and a strong decrease in peripheral resistance 6 days after AdVEGF gene transfer in normal muscles. Enlargement by 20-fold, increased pericyte coverage, and decreased alkaline phosphatase and dipeptidyl peptidase IV activities suggested the transformation of capillaries toward an arterial phenotype. Increase in muscle perfusion was attenuated, and blood vessel growth was more variable, showing more sprouting angiogenesis and formation of blood lacunae after AdVEGF gene transfer in muscles with ligated PFA than in normal muscles. Three-dimensional ultrasound reconstructions and histology showed that the whole vascular tree, including large arteries and veins, was enlarged manifold by AdVEGF. Blood flow was normalized and enlarged collaterals persisted in operated limbs 14 days after AdVEGF treatment.

Conclusions—This study shows that (1) blood flow modulates vessel growth during VEGF gene therapy and (2) VEGF overexpression promotes growth of arteries and veins and induces capillary arterialization leading to supraphysiological blood flow in target muscles. (Circulation. 2005;112:3937-3946.)

Key Words: angiogenesis ■ gene therapy ■ ischemia ■ perfusion ■ peripheral vascular disease

Vascular endothelial growth factor (VEGF) is an absolute requirement for embryonic blood vessel formation, and its overexpression results in strong angiogenesis in various tissues in adults, suggesting that it could be used for the treatment of myocardial and peripheral ischemia.1-4 Recent studies from our laboratory and elsewhere have shown that the overexpressions of VEGF and the mature form of VEGF-D, another VEGFR-2 ligand, induce efficient proliferation of both vascular endothelial cells and pericytes via VEGFR-2 and NO-mediated mechanisms, leading to strong capillary enlargement in skeletal muscle and myocardium.1-3,5-7 This kind of angiogenesis is clearly different from the traditional concept that VEGF mainly induces sprouting angiogenesis in adults, resulting in new daughter vessels. There is also substantial controversy regarding whether VEGF or other VEGFR-2 ligands can, directly or indirectly, promote the growth of larger blood vessels than capillaries. Previously, VEGF has not been recognized as a molecule that promotes arteriogenesis, ie, collateral artery growth. However, for clinically relevant therapeutic blood vessel growth, it would be essential that the candidate growth factor is able to promote arteriogenesis in addition to angiogenesis.

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We hypothesized that local blood flow influences vessel growth during VEGF overexpression. To test this hypothesis, we injected adenoviral (Ad) VEGF (10^{11} viral particles/mL) intramuscularly into semimembranous muscles of rabbits with or without the ligation of the major arterial branch supplying blood to this muscle, the profound femoral artery (PFA). It was found that blood inflow remodels growing capillaries and that AdVEGF can promote the growth of large arteries and veins, leading to a 23- to 27-fold increase in muscle perfusion, a value that has not been described with any treatment before.

Methods

Please see the online-only Data Supplement for Methods. The video files and legends can also be found in the online-only Data Supplement.

Results

Ligation of PFA Leads to a Selective Vascular Defect in the Semimembranous Muscle

Digital subtraction angiograms of rabbit hindlimbs 6 days after GT in AdLacZ-transduced normal limb (a) and AdLacZ-transduced limb with PFA ligation (oper.) (b) are shown. Arrowheads indicate PFA; bars, the litigation site. c, AdVEGF GT in normal limb. d, AdVEGF GT with the ligation of PFA. X-ray contrast agent extravasates after AdVEGF treatment from distal PFA in normal limbs and from collaterals in limbs with ligated PFA (arrows in c and d).

AdVEGF overexpression increases vascular permeability in the transduced muscle, resulting in the leakage of the contrast agent (Figure 1c and 1d). After PFA ligation, collaterals grow from the distal arterial branches in the knee area (Figure 1d). Quantitatively, AdVEGF increased plasma protein extravasation 42- and 54-fold (AdLacZ 2.0-fold) in semimembranous muscles of normal and operated limbs, respectively, as assessed by modified Miles assay (P=NS, AdVEGF normal versus operated; P=0.05 versus AdLacZ).

VEGF-Induced Blood Vessel Growth Is Modulated by Blood Flow

In normal and operated limbs, native power Doppler signal is very low or absent 6 days after AdLacZ GT, respectively (Figure 2a and 2b; for Videos 1 and 2, see the online-only Data Supplement). AdVEGF treatment increases perfusion strongly in normal muscle, whereas in operated limbs the response is attenuated (Figure 2c and 2d; Videos 3 and 4).

Transversal CD31-immunostained histological sections of the whole semimembranous muscles reveal highly increased vascularity 6 days after AdVEGF treatment in comparison to AdLacZ controls (Figure 2e to 2h). Higher magnifications demonstrate a more variable microvascular growth response to AdVEGF in muscles with ligated PFA than in normal muscles (Figure 2i to 2l). Importantly, no significant infiltrations of leukocytes or necrotic/regenerating myofibers were observed outside the needle track region despite rapid vascular growth and edema (Figure 2i to 2q).

In CD31/α-smooth muscle actin (α-SMA) double immunostainings at high magnification, capillaries are small and pericytes can hardly be observed in AdLacZ control (Figure 2m). Round, enlarged capillaries with thickened but loose pericyte coverage predominate in normal muscles after AdVEGF treatment (Figure 2n). In contrast, sprouting angiogenesis and formation of blood lacunae were observed after AdVEGF injections in muscles with ligated PFA (Figure 2o to 2q). Figure 2s to 2v demonstrates that normal and enlarged capillaries as well as blood lacunae are perfused, as shown by intra-arterial lectin injection resulting in red fluorescence on endothelium and intravascular red blood cells. The intramuscular injections of adenoviruses led to widespread transduction of skeletal muscle (Figure 2w), leading to abundant amounts of transduced VEGF165 in muscles (Figure 2y).

Videos 5 to 8 in the online-only Data Supplement show both low-power overviews and high magnifications of the whole CD31-immunostained semimembranous muscles of all groups, demonstrating distinct forms of blood vessel growth with AdVEGF.

Capillary Arterialization and Growth of Arteries and Veins by AdVEGF

Figure 3a to 3c shows normal appearance of capillaries, arteries, and veins in AdLacZ control muscle. Strong remodeling and enlargement of capillaries, arteries, and veins occur with AdVEGF (Figure 3d to 3f). To study the phenotype of enlarged microvessels, we used a histochemical staining for alkaline phosphatase (AP), which is known to be expressed in arterial capillaries but not in larger arteries or veins. AP was expressed in normal capillaries of AdLacZ-transduced mus-
Figure 2. Blood flow modulates vascular growth during VEGF overexpression and determines between capillary arterialization and sprouting angiogenesis. All images were taken 6 days after GT in semimembranosus muscles (brackets in a to d) either with or without the ligation of PFA (oper.). a to d, Transversal plane power Doppler ultrasound images show markedly increased perfusion with AdVEGF in comparison with AdLacZ controls. Gray scale signal is higher because of edema in AdVEGF-treated muscles. The gracilis muscle lies on top (asterisks in a to d). The distal branch of PFA is indicated by arrowheads in a to h. e to h, Transversal sections of the whole CD31-immunostained semimembranosus muscles show increased vasculature with AdVEGF. Arrows in e to h indicate needle tracks, and asterisks indicate sites where the images i to l were taken. Bar=1 cm. i to l, Higher magnifications show different microvesSEL growth patterns with AdVEGF. In normal muscles AdVEGF induces primarily uniform capillary vessel enlargement (k), whereas in the operated limbs the response is more variable, resulting in sprouting angiogenesis (arrow) and formation of blood lacunae (arrowhead) (l). m to r, α-SMA (brown) plus CD31 (blue) double immunostainings show the effects of AdVEGF on capillaries in detail. m, Normal capillaries in AdLacZ (arrowhead). n, Greatly enlarged capillaries (arrowhead) with a strengthened pericyte coverage in normal muscle after AdVEGF. o, Sprouting angiogenesis in operated limbs after AdVEGF (arrowheads). p to q, Large blood lacunae (asterisks). Endothelial cells and pericytes line the structures from surrounding skeletal myocytes, which in some cases are totally separated from one another to form “islands” (arrowhead). Bunches of endothelial cells and smooth muscle cells form septa between blood lacunae (thick arrows). Thin arrows show the extravascular space in between myocytes. r, Control immunostaining in which the primary monoclonal antibodies were omitted (the same site as in p). s to v, Intra-arterial injection of rhodamine-labeled Ricinus communis lectin with DAPI nuclear staining demonstrates that enlarged capillaries and blood lacunae are perfused. Inset in u, Control without lectin injection. v, Erythrocytes (negative for DAPI nuclear stain) inside lacunae (asterisks) in an operated and AdVEGF-treated limb without perfusion fixation. w and x, β-Galactosidase immunostaining (blue) shows that transduced cells (arrowheads) are found at a distance from the needle track (asterisks, unspecific brown staining) after nuclear-targeted AdLacZ GT. y and z, VEGF immunostainings. y, Transduced VEGF is primarily bound to interstitial extracellular matrix (arrowheads), but myocytes also stain faintly positively throughout the section. z, In AdLacZ muscle, only regenerating myofibers (arrowheads) near the needle track (asterisk) are positive for VEGF. Bar=50 μm in i to z except in v (25 μm).
cles, as shown by CD31 + AP double stainings (Figure 3g to 3i). Interestingly, AP staining was completely negative in enlarged capillaries by AdVEGF (Figure 3j to 3l). To exclude the possibility that the enlarged microvessels are venules, a histochemical staining for venous capillaries and venules, dipeptidyl peptidase IV (DPP), was used. As shown by triple AP + DPP + CD31 stainings, enlarged microvessels were also negative for DPP (Figure 3m and 3n).

**Contrast-Enhanced Ultrasound Imaging and 3D Reconstructions of the Vasculature After Angiogenic Therapy**

Figure 4a to 4d and Videos 9 to 12 in the online-only Data Supplement illustrate the difference in blood flow between AdLacZ- and AdVEGF-treated normal limbs and limbs with ligated PFA as assessed by power Doppler imaging and intravenous bolus administration of the second-generation contrast agent (SonoVue). In limbs with PFA ligation, AdVEGF increases blood flow primarily in the distal region, while a perfusion deficit is still present in the proximal part of the muscle because of PFA ligation (Figure 4d). 3D reconstructions of the vasculature were performed to visualize the effect of AdVEGF on perfusion and collateral growth within the whole muscles (Figure 4e to 4h and Videos 13 to 16).

**Relative Blood Volume Measurement With Contrast-Enhanced MRI**

To confirm the strong perfusion increase by AdVEGF measured with contrast-enhanced ultrasound (CEU) imaging, contrast-enhanced and dynamic susceptibility contrast (DSC)
MRI protocols were performed for relative blood volume and perfusion measurements, respectively, exploiting intravenous contrast agent SHU-555A (carboxydextran-coated superparamagnetic iron oxide particles [Resovist]). As shown by contrast-enhanced MRI (Figure 5), the relative blood volume was found to be strongly increased by AdVEGF in normal muscle. In postcontrast images taken at the steady state of contrast agent distribution (Figure 5b and 5e), regions with high blood volume appear black in the AdVEGF-treated limb because superparamagnetic contrast agents decrease the MRI signal by enhancing T2* relaxation. The effect of AdVEGF versus AdLacZ on regional blood volume is best visualized by R2* maps that show the difference between T2* relaxation rates before and after contrast administration, ie, the contrast agent concentration and thus blood volume in muscles (Figure 5c and 5f).

CEU Perfusion Measurement Correlates Significantly With DSC-MRI and Provides Information on the Structure and Function of the Vascular Bed

The CEU signal intensity–time curves illustrate the kinetics of intravenous bolus–injected SonoVue contrast agent in semimembranous muscles 6 days after AdVEGF or AdLacZ GT (Figure 6a). CEU perfusion index between the transduced and contralateral intact muscle was calculated by using the ratio of the peak intensity values. Furthermore, the time to the arrival of the contrast agent was derived from the curves, which gives information on peripheral vascular resistance. The shape of the curve reflects different vessel types in the vascular bed: The presence of large arteries and shunting without a normal capillary bed produces a steep curve with a high peak intensity (such as AdVEGF in normal muscle), whereas a normal vascular network with normal-sized capillaries yields a flat curve (AdLacZ). Furthermore, after AdVEGF GT in limbs with ligated PFA, the peak intensity is lower and the shape of the curve is flatter, reflecting the absence of this large conducting artery (Figure 6a).

DSC-MRI data were acquired and quantified analogously to the CEU data with the use of intravenous bolus injection of Resovist and a FLASH pulse sequence. The calculated R2* is directly proportional to concentration of the contrast agent, and therefore the resulting R2*-time curve (Figure 6b) was similar to that obtained by CEU imaging. The slight differences in the curve shape between CEU and MRI probably indicate some differences in the contrast agent behavior or detection between these 2 different imaging modalities.

As shown in Figure 6c, a comparison of 4 different methods to calculate a perfusion index in normal muscles transduced with AdVEGF or AdLacZ was performed. In all methods, the peak tracer signal intensities between the transduced and contralateral limbs were used. Importantly, the microsphere method likely underestimates perfusion in cases in which potent angiogenic growth factors such as VEGF significantly enlarge capillary size (diameter >15 μm) so that microspheres (15 μm) cannot be retained in them. The perfusion indices calculated with the use of native power Doppler ultrasound, CEU, or DSC-MRI were very similar after AdVEGF GT (23- to 27-fold). Furthermore, it was found that the CEU perfusion index correlated significantly with the corresponding index measured with DSC-MRI (r=0.89, P<0.01), showing that muscle perfusion assessment with the novel CEU technique is comparable to the previously established method.
Growth of the Whole Vascular Tree by AdVEGF
Results in Supraphysiological Perfusion and a Decrease in Peripheral Resistance

The CEU perfusion index was significantly enhanced (8.3-fold) 3 days after AdVEGF injections in normal muscles (Figure 7a). After reaching the maximal 27-fold increase at 6 days, perfusion decreased rapidly, reaching baseline at 2 weeks (1.4-fold). After PFA ligation, the maximal perfusion with AdVEGF was clearly attenuated but still significantly elevated 2 weeks after GT (2.7-fold, 0.4-fold in AdLacZ operated).

Total areas (percentage) of arteries, veins, and microvessels were measured quantitatively in the transduced muscles (Figure 7b). AdVEGF induced manifold increase in the total arterial, venous, and capillary areas in both groups. However, vascular growth was even more pronounced in muscles with ligated PFA because of the effect of blood lacunae and enlarged collateral arteries. Collaterals persisted in operated AdVEGF animals, but abundantly enlarged capillaries and large blood lacunae disappeared completely only 2 weeks after GT (Figure 7b and 7c).

In CEU imaging, the contrast agent arrived ~2-fold faster in normal muscles treated with AdVEGF than AdLacZ, reflecting a significant reduction in peripheral vascular resistance as the result of microvascular enlargement (Figure 7d). Contrast agent arrival was also quicker in operated AdVEGF animals at 6 days than in normal AdLacZ controls, probably because of shunting via large blood lacunae.

Discussion
VEGF has been generally considered as an angiogenic but not very arteriogenic growth factor. This study demonstrates that VEGF, likely via the effects of increased blood flow, is a strong modulator of the whole vascular tree, including large arteries and veins, in both normal and hypoperfused muscles. In normal muscles, capillaries are enlarged and transformed toward an arterial phenotype by VEGF in a process that we call capillary arterialization. Unlike in arteriogenesis (collateral artery growth from preexisting small arterial anastomoses), in capillary arterialization it is the capillary vessels that start to enlarge and strengthen their wall. This kind of

Figure 5. Relative blood volume measurement with contrast-enhanced T2*-weighted MRI with the use of intravenous carboxydextran-coated superparamagnetic iron oxide particles (Resovist) 6 days after AdLacZ or AdVEGF GT in normal limbs. a, d, Transversal plane precontrast MRI at the midthigh level showing both limbs. Semimembranosus muscles are outlined with dashed lines. Note muscle edema as well as free fluid (asterisks) surrounding AdVEGF-transduced semimembranous muscle and in the subcutaneous space of the contralateral intact limb. b, e, Postcontrast MRI 6 minutes (steady state) after intravenous bolus administration of Resovist. Note intensive signal loss (enhanced T2* relaxation) in AdVEGF-treated muscle due to strongly increased blood volume and thereby high concentration of contrast agent, whereas there is no difference in signal intensity between AdLacZ-transduced and intact muscle. Contrast effect is also clear in large vessels (arrowheads, superficial femoral artery; arrows, PFA). c, f, ΔR2* maps calculated from the signal intensity drop are directly proportional to the concentration of the contrast agent, and therefore ΔR2* maps can be interpreted as relative blood volume maps so that bright areas have high blood volume (semimembranous after AdVEGF GT) and dark areas low blood volume.
capillary arterialization by AdVEGF results in a significant reduction in peripheral resistance and a very high perfusion increase (23- to 27-fold). On the other hand, in muscles with compromised perfusion, AdVEGF generates variable blood vessel growth also composed of sprouting angiogenesis and formation of large blood lacunae. The excess vasculature regresses in normal limbs, but collateral arteries persist in ischemic limbs after VEGF withdrawal.

Imaging of growing vasculature and quantitative measurement of perfusion are very important in the assessment of clinical efficacy of angiogenic therapies. Strong vascular growth by AdVEGF involving manifold capillary enlargement and increased vascular permeability interferes with many standard vascular imaging and perfusion measurement techniques such as x-ray angiography, the microsphere method, and, as shown previously, contrast-enhanced MRI with the use of low-molecular-weight extracellular contrast agents such as gadolinium. In these circumstances, the contrast agent must not extravasate and the method must not assume physiological capillary size. CEU imaging is very attractive for quantitative perfusion measurement after angiogenic therapies because it meets these requirements, is noninvasive, is approved for humans, provides quantitative data on blood flow kinetics, and enables 3D reconstruction of vasculature without nephrotoxic contrast agents or ionizing radiation. DSC-MRI with the use of relatively large contrast agent particles is feasible as well but is more time consuming and technically challenging. CEU is increasingly used for perfusion measurement in various tissues including human skeletal muscle. Here, the perfusion indices measured with the 2 independent methods, CEU and DSC-MRI, were in good agreement.

AdVEGF at $10^{11}$ viral particles/mL increased perfusion 23- to 27-fold in normal rabbit skeletal muscle 6 days after intramuscular injections. To the best of our knowledge, a perfusion increase of this magnitude has not been described previously with any medical treatment. In fact, the perfusion achieved with AdVEGF in resting muscles is about the same order of magnitude as the increase observed during maximal skeletal muscle exercise. Thus, VEGF overexpression seems to bypass the physiological control of muscle perfusion, which is normally tightly adapted to metabolic needs, leading to supraphysiological perfusion. The enormous blood flow enhancement is easier to understand when the theoretical effect of Poiseuille’s law (blood flow in cylindrical vessel is proportional to the fourth power of the vessel radius) is combined with the fact that the capillary mean size was increased 20- to 28-fold by AdVEGF.

AP, an enzyme capable of hydrolyzing organic phosphate esters, is expressed on the arterial side of the capillaries and arterioles but not in large arteries, and thus it has been widely used to demonstrate increases in capillary density after various angiogenic therapies. However, we found that both AP and DPP, a venous capillary and venule marker, activities were absent in AdVEGF-induced enlarged capillaries. Similar AP downregulation has been found to occur in tumor vasculature and in hypoxic skeletal muscle, both of which involve increased vascular growth and VEGF. The downregulation of AP and DPP activity in enlarged microvessels together with the enhanced coverage with α-SMA-positive pericytes suggests that capillaries were transformed toward an arterial phenotype in muscles treated with AdVEGF. Furthermore, the downregulation of AP by VEGF overexpression corroborates the previously known fact that AP is
not an optimal staining method for capillaries after angiogenic therapies.

The short arrival times (decreased peripheral resistance) and the steep shape of CEU and DSC-MRI signal intensity–time curves indicate the presence of large arteries and enlarged capillaries and the lack of normal small-sized capillary bed after AdVEGF treatment. The enlargement of arteries (collaterals) persists at 2 weeks in operated AdVEGF limbs. Our findings are similar to those in some tumors that have high blood velocity likely because of shunting. Although excess blood flow may not be very useful to the local environment, decreased peripheral resistance increases blood flow in upstream collaterals, which probably contributes to arteriogenesis via enhanced shear stress and wall strain. This blood flow, however, may be detrimental for the overall function of the grafts.

VEGF likely promotes capillary arterialization and arteriogenesis indirectly because pericyte and smooth muscle cell proliferation has not been demonstrated to occur directly on VEGF stimulation in vitro. Previously, blood flow has been shown to be a critical determinant of vessel maintenance and growth. Elevated circumferential wall strain may force vessels exposed to increased blood pressure to strengthen their wall by hypertrophy of their pericyte and smooth muscle cell coverage. Furthermore, a very high degree of arteriogenesis was recently achieved in a model with the use of an arteriovenous fistula, which strongly increased blood flow in collaterals after the ligation of the main artery. Thus, mechanical factors derived from increased blood flow and pressure, occurring subsequently to microvascular effects by VEGF, appear necessary for capillary arterialization and growth of arteries and veins (Figure in the online-only Data Supplement).

Our results showing that VEGF may be arteriogenic agree with previous studies in which mice deficient in VEGF120 and VEGF164 show impaired development of retinal arteries but not veins, and transgenic cardiac overexpression of VEGF164 leads to increased arteriolar but decreased venular capillary formation. In 2003, 3 independent research groups reported transformation of capillaries toward an arterial phenotype by VEGF or VEGF-D. Furthermore, a decade ago VEGF administration...
was reported to enhance collateral artery growth, and more recently VEGFR-2 receptor antagonism was shown to completely inhibit collateral growth. These results suggest an important role for VEGF in the growth of muscular vessels. Although VEGF may also directly contribute to the arteriализation of blood vessels because VEGF appears to promote the arterial fate even before the onset of circulation, the results of the present study with the use of the arterial ligation model suggest that blood flow is crucial for capillary arteriализation and collateral growth by VEGF in adults.

Capillary arteriализation played the predominant role in normal muscles after AdVEGF injections, whereas in operated limbs with a perfusion deficit a different response was observed, with other forms of blood vessel growth being more prevalent. Classic sprouting angiogenesis, which is not the most common form of capillary growth in normal muscles with AdVEGF, took place in muscles with the ligation of PFA. Furthermore, massive blood lacunae were formed from preexisting capillaries in other areas of the same muscles. The lacunae were perfused as shown by lectin injections and intravascular erythrocytes and formed in between the myofi bers, in contrast to a study using AAV-VEGF overexpression in which the lacunae grew inside myocytes. In addition to the microenvironmental VEGF concentration, local blood flow appears crucial in molding the growing capillaries into ones that best serve the needs of current circumstances: Into daughter vessels via sprouting angiogenesis if newly formed vessels are needed or into large shunts to direct excess blood flow elsewhere. In future studies, it is necessary that the significance of the different forms of VEGF-induced neovascularization on local muscle metabolism and exercise tolerance will be carefully addressed. In addition, the intramuscular AdVEGF165 dose and injection volumes must be optimal in clinical trials to obtain a clinically relevant effect on muscle perfusion but avoid excess tissue edema and formation of blood lacunae.

In nonischemic skeletal muscle, all the effects by AdVEGF were transient, lasting no longer than adenoviral gene expression (<2 weeks). Thus, in normal tissues increased blood flow and immature pericycle coverage alone are insufficient to protect the enlarged vessels from regression without the presence of VEGF. Mathematical models also suggest that shear stress alone does not lead to stabilization of vessels, but additional factor(s) must be present. However, it appears that long-term VEGF overexpression may stabilize the vessels and produce persistent increases in perfusion in most normal tissues. Collateral arteries in the operated limbs resisted regression, probably because they provide crucial blood flow to muscles with compromised perfusion and they are formed from structurally mature preexisting arteriolar anastomoses. The persistence of collateral blood flow resulted in normal or even slightly elevated (2.7-fold) perfusion values in the operated AdVEGF-treated limbs at 2 weeks.

In conclusion, the results of this study indicate that adenoviral VEGF overexpression alone can orchestrate the growth of the whole vascular tree and induce supraphysiological perfusion increases in the target muscle. Thus, VEGF gene therapy is very promising for the induction of clinically relevant therapeutic vascular growth in patients with ischemic disease, but the correct dosage and duration of expression are the key issues for clinical success.

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Disclosure

None.

References


Vascular endothelial growth factor (VEGF) is the master regulator of blood vessel growth that has been previously thought to induce effects exclusively at the capillary level. However, the growth of collaterals bypassing the stenosis or occlusion in the conducting artery would be highly desirable in patients with myocardial or peripheral ischemia. This study shows that adenoviral VEGF overexpression promotes the growth of the whole vasculature in both normal and ischemic rabbit hindlimbs. This occurs secondarily to the great enlargement of capillaries that results in very high capillary perfusion and reduced peripheral resistance, leading to compensatory growth of upstream arteries and downstream veins. In normal muscles, excess blood flow resulting from VEGF overexpression transformed capillaries toward arterioles that functioned as shuntlike vessels. Muscle perfusion increased 23- to 27-fold under these conditions. In ischemic muscles, VEGF induced capillary sprouting in addition to the formation of large vessel structures. These results show the enormous potential of VEGF to reshape the existing vasculature. Clinical success in future trials will require optimization of the indications of therapy as well as the dose and duration of VEGF overexpression. VEGF gene therapy could potentially be used in combination with conventional revascularization procedures as adjuvant therapy to enhance capillary perfusion, reduce peripheral resistance, and improve patency of the grafts via better blood flow.

CLINICAL PERSPECTIVE

Vascular endothelial growth factor (VEGF) is the master regulator of blood vessel growth that has been previously thought to induce effects exclusively at the capillary level. However, the growth of collaterals bypassing the stenosis or occlusion in the conducting artery would be highly desirable in patients with myocardial or peripheral ischemia. This study shows that adenoviral VEGF overexpression promotes the growth of the whole vasculature in both normal and ischemic rabbit hindlimbs. This occurs secondarily to the great enlargement of capillaries that results in very high capillary perfusion and reduced peripheral resistance, leading to compensatory growth of upstream arteries and downstream veins. In normal muscles, excess blood flow resulting from VEGF overexpression transformed capillaries toward arterioles that functioned as shuntlike vessels. Muscle perfusion increased 23- to 27-fold under these conditions. In ischemic muscles, VEGF induced capillary sprouting in addition to the formation of large vessel structures. These results show the enormous potential of VEGF to reshape the existing vasculature. Clinical success in future trials will require optimization of the indications of therapy as well as the dose and duration of VEGF overexpression. VEGF gene therapy could potentially be used in combination with conventional revascularization procedures as adjuvant therapy to enhance capillary perfusion, reduce peripheral resistance, and improve patency of the grafts via better blood flow.
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Online Data Supplement

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Methods

**Gene transfer**

New Zealand white rabbits (2.0-2.5kg, n=35) received i.m. injections of adenoviruses (Ad) encoding either human VEGF-A$_{165}$ or β-galactosidase (LacZ) driven by the CMV-promoter with or without the simultaneous ligation of the profound femoral artery (PFA). Rabbits were anesthetized with fentanyl-fluanisone (Hypnorm, Janssen, 0.2 ml/kg) and midazolame (Dormicum, Roche, 1.5 mg/kg). Five 0.1 ml injections of the viral solution were performed with a 25G needle into the right semimembranosus muscle at the titer of $10^{11}$vp/ml.$^1$ Human clinical grade first generation serotype 5 replication deficient (E1, partially E3 deleted) adenoviruses produced under GMP conditions in 293 cells and analyzed to be free from endotoxin and microbiological contaminants were used.$^2$ Rabbits were sacrificed either six or 14 days after gene transfer (GT). Blood flow in the semimembranosus muscles was analyzed with contrast-enhanced ultrasound (CEU) imaging before and just after the ligation of PFA, and three, six, nine and 14 days after GT. As independent methods of blood flow measurement, contrast-enhanced MRI and fluorescent microspheres were used at sacrifice as described below. After sacrifice, muscle samples were collected for immunocytochemistry and histochemistry. All animal experiments were approved by the Experimental Animal Committee, University of Kuopio.
Perfusion measurement with ultrasound imaging

Perfusion in transduced and contralateral intact rabbit semimembranosus muscles was analyzed with an Acuson Sequoia 512 system and 15L8 transducer (Siemens) at 14 MHz using the power Doppler mode with or without the administration of ultrasound contrast agent. Transversal plane video clip (2 s, 12.5 frames/s) without contrast enhancement was first obtained (power Doppler at 14 MHz, dynamic range 10 dB, power 0 dB, mechanical index 0.63, gain 50 and depth 20 mm). Then, two consecutive longitudinal plane video clips of 10 s (power Doppler at 8.5 MHz, dynamic range 10 dB, power -18 dB, mechanical index 0.60, gain 40 and depth 20 mm) were captured starting at the same time of 0.3 ml bolus injection of the second generation contrast agent (sulphur hexafluoride in a phospholipid shell, approx. 2x10^8 bubbles/ml, mean diameter 2.5 µm, SonoVue, Bracco) via the ear vein. No blooming of the CEU signal was observed with the mechanical index used.

It has been previously shown that the backscatter signal from microbubbles correlates in a linear manner with the concentration of bubbles in silico and in vitro as well with blood flow in vivo according to the classical dye dilution theory.3-6 Recently, power Doppler CEU was also validated in skeletal muscle perfusion measurement in humans.7

The power Doppler signal intensity (dB) of the video clips was quantified with Datapro 2.13 program (Noesis) and signal intensity-time curves8 after i.v. bolus administration of the contrast agent are presented for each group. A perfusion index was calculated from the native power Doppler ultrasound data as the ratio of the average signal intensities during the 2 s video clips between the transduced and contralateral intact semimembranosus muscles. For the CEU perfusion index, the ratio between the transduced and contralateral semimembranosus muscles using the peak signal intensities
after i.v. bolus contrast agent administration was calculated. These perfusion indices as well as those measured with MRI and microspheres are not absolute measures of tissue perfusion (blood flow / tissue volume i.e. blood volume x blood velocity / tissue volume) but reflect the difference in maximal circulating tracer concentration between the transduced and contralateral intact muscle and therefore can be used as measures of perfusion. It should be also noted that the risk of overestimating perfusion with power Doppler CEU is alleviated by the fact that attenuation of the CEU signal occurs at high concentrations of the contrast agent.\(^9\) In addition to the perfusion index, the time from i.v. injection to the arrival of contrast agent in the transduced muscle was calculated which is dependent on peripheral vascular resistance.

For the 3D reconstruction of vasculature, the semimembranosus muscle was scanned using the 15L8 transducer from its proximal origin to the distal end at a constant pace during Sonovue infusion (power Doppler at 14 MHz, gain 40, gray scale imaging off). The video files were decompiled into individual image files and 3D volume rendering (resolution 1/1, maximum density) was performed with 3D Doctor after automatic object segmentation according to manufacturer’s instructions (Able Software).

*Perfusion and blood volume measurement with MRI*

MRI was carried out in a 4.7T magnet (Magnex, Abington, UK) interfaced to Varian UNITYINOVA (Varian inc., Palo Alto, USA) console, with actively shielded gradients (diameter 23 cm, max. gradient strength 7 G/cm, Magnex). An in-house-built surface coil (diameter 38 mm) was used for MRI which consisted of dynamic susceptibility contrast (DSC) imaging of perfusion and $T_2^*$-weighted gradient echo pre- and post-contrast steady state multi-slice images to get high quality representative images of relative blood volume.
For quantitative perfusion index measurement a DSC-MRI protocol was performed in which transversal MR images from a single slice in the center of the thigh were non-interruptedly acquired using a FLASH pulse sequence as follows: time-to-repetition (TR)=9ms, time-to-echo (TE)=5ms, field of view (FOV)=6x6cm², the number of readout points (NP)=64, the number of points in phase encoding direction (NV)=64, slice thickness (SLT)=5mm and the number of averages (NA)=1, acquisition time 0.58s/image. Ten images were acquired before and 65 after an i.v. bolus injection of 0.7ml of SHU-555A, carboxydyextran coated superparamagnetic iron oxide (SPIO) particles (Resovist, 0.5mmol Fe/ml, Schering).

To obtain relative blood volume maps T₂*-weighted gradient echo images were acquired before contrast agent injection and again 6 min after the bolus injection to determine the steady-state distribution of the i.v. contrast agent using the parameters as follows: TR=2 s, TE=18 ms, FOV = 6 x 6 cm², NP=256, NV=128, SLT=2.5 mm and NA=2. Ten transversal slices perpendicular to the femur bone were acquired covering the mid thigh region of both limbs.

SPIO particles enhance T₂⁺ relaxation leading to reduced signal intensity in T₂*-weighted MRI.¹⁰ SPIO particles are more suitable for perfusion measurement than extracellular contrast media such as small gadolinium complexes which leak rapidly from hyperpermeable vessels to extravascular space in tumors and after VEGF gene transfer in skeletal muscle.¹⁻¹² SHU-555A is indicated for the detection of focal liver lesions and can also be used for perfusion measurement.¹⁰¹³ As a relatively large SPIO particle (mean particle size 62nm), SHU-555A is expected to be superior to smaller SPIO particles in perfusion assessment of very hyperpermeable vessels such those induced by VEGF overexpression.
MRI Analysis

$\Delta R_2^*$ is the difference between $T_2^*$ relaxation rates pre- and post-contrast administration. $R_2^*$ is approximated to be directly proportional to contrast agent concentration in perfused tissue. Assuming that the contrast agent remains in intravascular space and is evenly distributed in blood volume under steady state conditions, $\Delta R_2^*$ can be used to visualize relative blood volume. $\Delta R_2^*$ maps were reconstructed from the same slice as in the DSC-MRI protocol using equation

$$\Delta R_2^* = \frac{\ln(I_{\text{pre}}) - \ln(I_{\text{post}})}{TE}, \quad [1]$$

where $I_{\text{pre}}$ and $I_{\text{post}}$ are the measured pre- and post-contrast images, respectively. For DSC-MRI, the signal intensity from the semimembranosus muscle was determined and normalized using the baseline level defined from images 10-15 (to exclude the saturation effect in the first 1-9 images). To get the contrast agent concentration-time curves, $\Delta R_2^*,\text{bolus}$ values were calculated using equation

$$\Delta R_2^*,\text{bolus} (i) = \frac{-\ln(I_{\text{bolus}}(i))}{TE}, \quad [2]$$

where $I_{\text{bolus}}$ is the scaled intensity from the semimembranosus muscle and $i$ refers to the image number. The MRI perfusion index was calculated as the ratio of peak $\Delta R_2^*,\text{bolus}$ (maximal contrast agent concentration) between the transduced and contralateral intact semimembranosus muscles. More detailed analysis of perfusion using the gamma variate function was found impractical due to very low perfusion in intact control muscle.
Perfusion measurement with microspheres

Microsphere perfusion index between transduced semimembranosus muscles was measured at rest with red fluorescent microspheres (2x10^6, 15µm in diameter, FluoSpheres, Molecular Probes) injected into the left ventricle just before sacrifice. After sacrifice, microspheres were extracted from muscle samples with the sedimentation method according to the manufacturer's instructions. The microsphere perfusion index was calculated as the ratio of red fluorescence between the transduced and contralateral intact semimembranosus muscles. Yellow-green microspheres were used as internal controls for pipeting errors.

Digital subtraction angiography

To demonstrate the effects of PFA ligation and the extravasation of X-ray contrast medium from growing VEGF-induced vessels, selective digital subtraction angiography (DSA) of the hindlimbs was performed six days after GT with a 4F right coronary artery catheter (Cordis) introduced into the common carotid artery using a power-injection of 6 ml contrast medium (2 ml/s, 320 mgI/ml, Omnipaque, Amersham). Serial DSA images were recorded at the rate of two images/s for 5 s (Siremobil 2000, Siemens), and the image representing the best arterial filling was chosen for analysis.
Modified Miles assay for measurement of plasma protein extravasation

Evans Blue dye (30 mg/kg) was injected i.v. 30 min before sacrifice as described previously.\textsuperscript{1} After sacrifice the animals were perfusion-fixed with 1.5L of 1% paraformaldehyde (PFA) in 0.05M citrate buffer (pH 3.5) via the left ventricle. Extravasated Evans Blue dye bound to plasma proteins (mostly albumin) was extracted from transduced and contralateral intact semimembranosus muscle samples by incubation in formamide overnight at 60°C. The amount of Evans Blue dye was determined on the basis of absorbance at 610 nm and the ratio between transduced and intact muscle samples was calculated normalized to the tissue weight.

Immunohistochemistry and histochemical stainings

After sacrifice, the animals were perfusion fixed with 1.5l of 1% paraformaldehyde in 0.05M citrate buffer (pH 3.5) via the left ventricle. Skeletal muscle samples were then immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin.\textsuperscript{1,14} Muscle samples for histochemical alkaline phosphatase (AP) and dipeptidyl peptidase IV (DPP) stainings were collected from non-fixed animals and the samples were frozen in isopentane cooled down with liquid nitrogen.

Avidin-biotin-HRP and alkaline-phosphatase systems (Vector Laboratories) with 3’-5’-diaminobenzidine (DAB, Zymed) and Vector Blue (Vector) color substrates were used, respectively, for immunohistochemistry on 7 \( \mu \)m thick 4% paraformaldehyde-fixed paraffin-embedded sections.\textsuperscript{1,14} The endothelium was immunostained using a mouse mAb
against CD31 (DAKO, dilution 1:50) and pericytes and SMCs with a mAb against α-smooth muscle actin (α-SMA, clone 1A4, Sigma, 1:250). Transduced β-galactosidase and VEGF were immunostained using mAbs (VEGF: clone sc-7269, Santa Cruz, 1:500 and β-galactosidase: Cat.# Z3783, Promega, 1:500) and Vector Blue and DAB as chromogens, respectively. Proliferation marker BrdU (20mg/kg, Sigma) given 3h before sacrifice was stained with a mAb (Clone Bu20a, DAKO, 1:100) followed by CD31 staining on the same sections. Controls for immunostainings included incubations with irrelevant class- and species-matched immunoglobulins and incubations in which the primary antibody was omitted.

AP and DPP activity on blood vessels was demonstrated by the azo-coupling method on frozen sections. Briefly, for AP+CD31 double staining the sections were incubated in medium containing 10 mg of Naphthol AS-BI phosphate (disodium salt, Sigma) dissolved in 0.5 ml of N,N-dimethylformamide (Aldrich) and 10 mg of Fast Blue BB Salt (Fluka) dissolved in 10 ml of veronal-acetate buffer (pH 9.2) for 90 min. After stopping the reaction in distilled H2O, the sections were immunostained for CD31 according to the standard protocol. For triple AP-DPP-CD31 stainings, the sections were first stained for AP as described above. Thereafter, DPP staining was performed using Gly-Pro-4-β-naphthylamide (Cat.# G-9262, Sigma) as the substrate. The incubation was performed at room temperature under microscopic control for 90 minutes. After stopping the reaction in distilled H2O, the sections were immunostained for CD31.

To visualize vessels that are perfused, Rhodamine-labeled Ricinus Communis lectin (1mg in 10ml of saline, Cat.# RL-1082, Vector) was injected into the common femoral artery using a 4F catheter just before sacrifice in a subset of AdVEGF and AdLacZ treated animals. Standard paraffin-embedded sections were prepared and counterstained with Dapi (Molecular Probes, Cat.# D-1306) for fluorescent microscopy. Red fluorescence
on endothelium indicates vessels that are perfused and therefore connected to systemic circulation.

**Preparation of images and video files**

Photographs of histological sections were taken using Olympus AX70 microscope (Olympus Optical) with analySIS imaging software (Soft Imaging System) and were further processed for publication with Adobe Photoshop (Adobe). The video files of histology were made with ImageMatics StillMotion Creator 1.7 (StageTools LLC). Ulead Media Studio Pro 7 (Ulead Systems Inc.) was used for the processing of video files. The video files were encoded by the ISO Mpeg-4 v1 codec (Microsoft). In case the video files cannot be played, this codec can be downloaded at http://www.microsoft.com/windows/windowsmedia/format/codecdownload.aspx. Mac users may have to download the latest Microsoft (or equivalent) Media Player at http://www.microsoft.com/windows/windowsmedia/software/Macintosh/osx/default.aspx.

**Histological blood vessel measurements**

The capillary mean area (µm²) and total capillary vessel area of muscle area (%) were measured from ten fields of CD31 immunostained sections of semimembranosus muscles at 200X magnification. The total area of arteries and veins of muscle area (%) was measured from α-SMA-immunostained sections at 40X magnification covering the muscle. All measurements were performed in a blinded manner.
Statistical analyses

Results are expressed as means + SEM if not otherwise noted. Statistical significance was evaluated using ANOVA followed by independent samples t-test or the Kruskal-Wallis test followed by Mann-Whitney U-test where appropriate. Correlation analyses were performed by the Pearson test. \( P<0.05 \) was considered statistically significant.
References


Intact muscle

AdVEGF in normal muscle

AdVEGF in ischemic muscle

VEGF overexpression

VEGFR-2

PI3K/Akt and MAPK

NO

EC proliferation and chronic pericyte relaxation

Capillary enlargement

Increased capillary blood pressure and flow in normal muscle

Increased shear stress and wall strain

Rich ECM via plasma protein extravasation

Pericyte/SMC proliferation

Capillary arterialization

Growth of normal arteries and veins

Attenuated perfusion increase and uneven flow in ischemic muscle

Sprouting angiogenesis and formation of blood lacunae

Decreased peripheral resistance

Collateral artery growth