Vascular Endothelial Growth Factor Overexpression in Ischemic Skeletal Muscle Enhances Myoglobin Expression In Vivo

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Abstract—Therapeutic angiogenesis using vascular endothelial growth factor (VEGF) is considered a promising new therapy for patients with arterial obstructive disease. Clinical improvements observed consist of improved muscle function and regression of rest pain or angina. However, direct evidence for improved vascularization, as evaluated by angiography, is weak. In this study, we report an angiogenesis-independent effect of VEGF on ischemic skeletal muscle, ie, upregulation of myoglobin after VEGF treatment. Mice received intramuscular injection with adenoviral VEGF-A or either adenoviral LacZ or PBS as control, followed by surgical induction of acute hindlimb ischemia at day 3. At day 6, capillary density was increased in calf muscle of Ad.VEGF-treated versus control mice (P<0.01). However, angiographic score of collateral arteries was unchanged between Ad.VEGF-treated and control mice. More interestingly, an increase in myoglobin was observed in Ad.VEGF-treated mice. Active myoglobin was 1.5-fold increased in calf muscle of Ad.VEGF-treated mice (P<0.01). In addition, the number of myoglobin-stained myofibers was 2.6-fold increased in Ad.VEGF-treated mice (P=0.001). Furthermore, in ischemic muscle of 15 limb amputation patients, VEGF and myoglobin were coexpressed. Finally, in cultured C2C12 myotubes treated with rhVEGF, myoglobin mRNA was 2.8-fold raised as compared with PBS-treated cells (P=0.02). This effect could be blocked with the VEGF receptor tyrosine kinase inhibitor SU5416. In conclusion, we show that VEGF upregulates myoglobin in ischemic muscle both in vitro and in vivo. Increased myoglobin expression in VEGF-treated muscle implies an improved muscle oxygenation, which may, at least partly, explain observed clinical improvements in VEGF-treated patients, in the absence of improved vascularization. (Circ Res. 2004;95:58-66.)

Key Words: vascular endothelial growth factor – myoglobin – angiogenesis – peripheral vascular disease – ischemia

Vascular endothelial growth factor-A (VEGF) is a major angiogenic growth factor.1 Several preclinical and clinical studies using VEGF (gene)therapy have shown promising results for the treatment of arterial obstructive disease.2–7 End-points in these clinical studies mainly consist of improved muscle function, as evaluated by exercise performance or angina classification, or regression of rest pain. It is assumed that these clinical improvements are induced by the induction of neovascularization. However, evidence for improved vascularization is scarcely reported. Moreover, intermittent claudication, implying a decreased function of limb musculature, does not correlate well with ankle brachial pressure index, a parameter of vascularization.8 In this study, we demonstrate an unanticipated effect after VEGF overexpression, namely an increase of myoglobin expression in skeletal muscle. Myoglobin is a cytoplasmic heme-containing protein, which is expressed in cardiomyocytes and skeletal myocytes. It functions as an intracellular oxygen reservoir in muscle cells and it facilitates oxygen supply, thereby maintaining cellular respiration during periods of high physiological demand, eg, during ischemia or exercise.9 In skeletal muscle, myoglobin is selectively expressed in oxidative muscle fibers, which mainly coexpress myosin heavy chain type I or IIA. Correspondingly, myoglobin is differentially upregulated in “red muscle,” which predominantly consists of oxidative fibers, as compared with “white muscle,” mainly consisting of glycolytic fibers.10 Studies of muscle composition in patients with limb ischemia showed a fiber-type switch in ischemic skeletal muscle from type IIb to IIA and subsequently to I fibers, thus toward myoglobin-containing, oxidative muscle fibers.11 Furthermore, myoglobin12−14 mice demonstrate compensatory mechanisms, eg, increased ex-
expression of hypoxia-inducible transcription factors and VEGF.\textsuperscript{12} The necessity for compensatory mechanisms in myoglobin-\textsuperscript{-/-} mice underscores the important role of myoglobin in maintaining a physiological oxygenation state of skeletal muscle. VEGF-mediated enhancement of myoglobin expression may thus result in an angiogenesis-independent improvement of muscle oxygenation state and, thereby, muscle function. Indeed, recent data suggest that VEGF may induce angiogenesis-independent effects resulting in the recovery of damaged muscle. This was corroborated by the finding that administration of recombinant VEGF increases the number of regenerating cells in a skeletal muscle transplantation model.\textsuperscript{13} Furthermore, both VEGF and VEGF-receptor 2 are restrictedly expressed in atrophic and regenerating muscle cells of human ischemic skeletal muscle, suggesting a direct effect of VEGF on these specific cells.\textsuperscript{14} In addition, VEGF modulates skeletal myoblast function via VEGF receptor pathways in culture.\textsuperscript{15}

In this study, we provide evidence that VEGF-A gene therapy acts on ischemic skeletal muscle by upregulating myoglobin expression in a mouse model of acute hindlimb ischemia. In addition, VEGF and myoglobin were coexpressed in ischemic muscle tissue of 15 limb amputation patients. Finally, an in vitro experiment in murine myotubes shows a direct stimulatory effect of VEGF on myoglobin mRNA expression in muscle cells. We propose that VEGF-mediated myoglobin expression might be beneficial for muscle function, explaining the observed clinical improvements in eg, exercise tests of VEGF-treated patients.

Materials and Methods

Gene Transfer in a Mouse Model of Acute Hindlimb Ischemia

Vectors
An adenoviral vector carrying the VEGF gene (Ad.VEGF) was constructed using cDNA encoding the human VEGF\textsubscript{165} isoform under control of the cytomegalovirus (CMV) promoter with SV40 polyadenylation signal. VEGF\textsubscript{165} cDNA was cloned into plasmid CMV\textsubscript{10}, as previously described.\textsuperscript{16} An adenoviral vector carrying the Escherichia coli LacZ reporter gene (Ad.LacZ), under control of the CMV promoter was used as a control. Production of adenovirus stocks was performed as previously described.\textsuperscript{17}

Surgical Procedure
All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO, Leiden, The Netherlands). Male C57BL/6 mice (TNO), aged 10 weeks, were randomly allocated into two groups (n=6).

The effect of VEGF gene transfer on collateral vessel growth in the upper hindlimb was analyzed in one group (group A). For this, muscles of the left upper hindlimb surrounding the femoral artery were injected at T=0 with 40 μL of either Ad.VEGF or Ad.LacZ (1.5×10\textsuperscript{10} pfu/mL).

In the second group, the effect of VEGF gene transfer on VEGF and myoglobin expression and capillary growth was studied in the calf muscle (group B). For this, the gastrocnemius muscle of both limbs was injected at T=0 with either 40 μL of PBS, Ad.VEGF, or Ad.LacZ (1.5×10\textsuperscript{10} pfu/mL).

At day 3, ischemia of the left hindlimb was induced in animals of both groups by coagulation of the left femoral artery proximal to the bifurcation of deep and superficial femoral artery. At day 6, collateral vessel growth was studied by performing postmortem angiography in mice of group A. In mice of group B, the gastrocnemius muscles of both hindlimbs were dissected for further analysis.

Angiography
To study collateral vessel growth, angiography of both hindlimbs was performed using polyacrylamide-bismuth contrast (Sigma), as previously described (for limited modifications, see the expanded Materials and Methods available in the online data supplement at http://circres.ahajournals.org).\textsuperscript{18} Grading of collateral filling was performed in a single blinded fashion and was based on the Rentrop classification.\textsuperscript{19} Grading was as follows: 0 = no filling of collaterals, 1 = filling of collaterals only, 2 = partial filling of distal femoral artery, and 3 = complete filling of distal femoral artery.

Perfusion Imaging
Blood flow was determined in the paws of Ad.VEGF- or Ad.LacZ-treated mice (n=6) using laser doppler perfusion imaging (LDPI) (Moor Instruments), as previously described.\textsuperscript{20}

C2C12 Cells in Culture
C2C12 myoblasts, obtained from the American Type Culture Collection, Rockville, Md, were used to induce differentiation to myotubes by supplementing the growth medium with 2% horse serum, 1 μmol/L insulin, and 2.5 μmol/L dexamethasone. After 7 days of culture mature myotubes were incubated for 3, 6, 9, and 12 hours with 5 ng/mL human VEGF recombinant protein (RELIA Tech.) in differentiation medium (n=4). In a separate experiment (n=6), myotubes were incubated for 6 hours with 5 ng/mL human VEGF recombinant protein combined with the VEGF receptor tyrosine kinase inhibitor SU5416 (100 nmol/L) (kindly provided by Dr K Hoekman, Amsterdam). Subsequently, cells were washed with PBS and lysed in 1 mL GTC-βME buffer (4 mol/L Guanidinium-isothiocyanate, 25 mmol/L Na-citrate, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol), pH 7.0, and stored at −80°C for further RNA analysis.

Determination of Protein Expression

Immunohistochemistry
Immunohistochemistry was performed on paraffin-embedded muscle sections using antibodies for myoglobin (Santa Cruz Biotechnology), VEGF (Santa Cruz Biotechnology), CD31 (Pharmingen), and on cryosections for VEGFR-1 and VEGFR-2 (antibodies kindly provided by Dr HA Weich, Germany). In addition, cryosections were histochemically stained for nicotinamide dehydrogenase tetrazolium reductase (NADH-TR), as previously described.\textsuperscript{21} Stainings were quantified from randomly photographed sections (3 to 6 images per section) using image analysis (Qwin, Leica). Myoglobin expression in mice was expressed as follows: (area of muscle fibers stained for myoglobin/total area of muscle fibers)×100%. Intensity of VEGF and myoglobin staining of human skeletal muscle sections was studied at the different levels of each amputated limb in a single blinded fashion.

Analysis of Active Myoglobin
Concentration of active myoglobin in samples of murine gastrocnemius muscle was determined by peroxidase-based activity assay, as previously reported.\textsuperscript{22} Myoglobin was calibrated against a standard of horse myoglobin (Sigma).

VEGF ELISA
Human VEGF\textsubscript{165} levels in murine blood samples were assayed by ELISA (R&D systems) according to the manufacturer’s protocol.

RNA Analysis

Total RNA Isolation
Total RNA was extracted from frozen murine gastrocnemius muscle using the RNEasy isolation kit (QIAGen) according to the manufacturer’s protocol. RNA isolation from C2C12 cells was performed according to Chomczynski et al, as previously described.\textsuperscript{23}
Real-Time RT-PCR
To prevent contamination of genomic DNA in PCR, RNA samples were treated with DNase before cDNA synthesis using RQ1 RNase-free DNase (Promega) according to the manufacturer’s protocol.
Total RNA was reversed transcribed into cDNA as described in the expanded Materials and Methods available in the online data supplement at http://circres.ahajournals.org.
Primers pairs and probes for studying expression of human VEGF-A, mouse myoglobin, and mouse HPRT by real-time RT-PCR were designed using the specific criteria as described in the primer express software (Perkin Elmer). Mouse PCR primer sets for Troponin I (slow), cytochrome c oxidase IVa (Cox IVa), and the peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1 (PGC-1α) were purchased (Applied Biosystems). Samples were normalized to housekeeping gene expression [GAPDH (Perkin Elmer) or HPRT for muscle or C2C12 samples, respectively]. PCR was performed as described in online data supplement. Myoglobin expression was calibrated against a construct containing myoglobin cDNA cloned into a pMOSBlue plasmid vector. Primers for mouse VEGFR1/FLT1 and VEGFR2/FLK1 were used for semiquantitative PCR as previously reported.24

Statistical Analysis
Results are expressed as mean±SEM. Tests to compare means were performed as appropriate. A value of P<0.05 was considered statistically significant.

Results

Effects of Ad.VEGF Gene Transfer in Mouse Model

Effect of Ad.VEGF Treatment on Collateral Vessel Growth
Three days after Ad.VEGF or Ad.LacZ gene transfer by intramuscular injection in one upper hindlimb, ischemia was induced by femoral artery occlusion in mice. All mice showed abnormal mobility of the occluded hindlimb until 3 days after occlusion. Spontaneous collateral vessel growth was already detectable within the first 3 days after occlusion, and a collateral vessel network was completely developed after 7 days (Figure 1A and 1B). No significant difference in collateral vessel growth between Ad.VEGF- and Ad.LacZ-treated mice 3 days after occlusion was observed (angiographic degrees 1.9±0.4 and 1.9±0.3, respectively; P=0.50, n=7) (Figure 1C through 1E). Correspondingly, there was no significant difference in blood flow recovery between ischemic limbs of Ad.VEGF- versus Ad.LacZ-treated mice, as measured with LDPI at various time points (n=6) (Figure 1F).

Effect of Ad.VEGF Treatment on Muscle Color and Capillary Formation
One day after Ad.VEGF injection in the gastrocnemius muscle of both hindlimbs, human VEGF (4.1±0.8 ng/mL) was detectable in the plasma. At day 6, VEGF concentration was below the detection limit of the assay. In addition, real-time PCR for human VEGF mRNA showed VEGF expression in the gastrocnemius muscle of Ad.VEGF-treated mice 6 days after injection, whereas the Ad.LacZ- and PBS-treated mice were negative (data not shown). This indicates that VEGF gene transfer resulted in a transient expression of VEGF protein.

Although no difference in collateral formation was observed, a striking red color of the treated muscle in the occluded limb was observed in all VEGF-treated mice, but not in Ad.LacZ-treated mice (Figure 2).

Moreover, the number and area of capillaries in gastrocnemius muscle of both occluded and nonoccluded limb was increased after Ad.VEGF treatment as compared with Ad.LacZ, as visualized by CD31 staining (Figure 3). Capillary density in Ad.VEGF-treated mice was 409±74 versus 185±24 capillaries/mm² in the Ad.LacZ-treated mice for

Figure 1. Angiograms of mouse hindlimb after femoral artery occlusion. A, One day after occlusion, no distal filling of arteries was observed. B, After 7 days, substantial collaterals were visualized originating from the arteria femoralis profunda (open arrow) and arteria circumflexa femoris lateralis (solid arrow) with distal filling of the arteria saphena and poplitea. C and D, Representative angiograms of Ad.VEGF- or Ad.LacZ-treated mouse, respectively. E, Rentrop score of angiographic images demonstrated no significant difference in collateral vessel growth between Ad.VEGF- and Ad.LacZ-treated mice 3 days after femoral artery occlusion (n=7). F, Quantitative evaluation of blood flow expressed as a ratio of ischemic to nonischemic limb. There was no significant difference in blood flow recovery between ischemic limbs of Ad.VEGF- vs Ad.LacZ-treated mice (n=6).
occluded limb \((P=0.008, n=6)\), and 129±31 versus 39±11 capillaries/mm² for nonoccluded limb, respectively \((P=0.01)\). Moreover, a marked increase of area/capillary in both occluded and nonoccluded limb was noted in the Ad.VEGF versus Ad.LacZ group (149±11 versus 97±7 \(\mu\)m² for occluded limb; \(P=0.001\); and 147±11 versus 106±11 for nonoccluded limb, respectively; \(P=0.02\)). In addition, histological examination showed local extravasation of erythrocytes in some areas of muscle damage after femoral artery occlusion. The number of extravascular erythrocytes was not different between the two groups of mice (data not shown).

**Ad.VEGF-Induced Myoglobin Expression in Ischemic Skeletal Muscle In Vivo**

Activation of the myoglobin gene in transgenic mice results in a distinct red color of most muscles. Therefore, it was analyzed whether the VEGF-induced red color of muscle could be attributable to the upregulation of myoglobin after VEGF treatment. In Ad.VEGF-treated mice, indeed, an increase in the number of muscle fibers that positively stained for myoglobin was observed in sections of gastrocnemius muscle after femoral artery occlusion (Figure 4A and 4B).

**Figure 2.** Gross appearance of gastrocnemius muscle samples after Ad.VEGF and Ad.LacZ treatment, 3 days after femoral artery occlusion. Note the red color of Ad.VEGF-treated muscle (A through D), whereas Ad.LacZ-treated muscle was clearly paler (E through H).

**Figure 3.** A and B, CD31 staining of ischemic gastrocnemius muscle after treatment with Ad.VEGF or Ad.LacZ, respectively, \((\times 100)\). C, Quantification of both capillary density and area per capillary in gastrocnemius muscle, depicted as capillaries/mm² and \(\mu\)m², respectively \((^*P<0.05, ^{**}P<0.01, ^{†}Red colored muscle, n=6)\).
The observed difference in myoglobin staining was quantified by image analysis (Figure 4C). The percentage of myoglobin-positive muscle fibers in gastrocnemius muscle was significantly higher in the Ad.VEGF group as compared with Ad.LacZ group (18±4.3 versus 7.0±4.1%; P=0.001, n=6). Moreover, Ad.VEGF treatment increased the number of mice positive for myoglobin as compared with Ad.LacZ (100% versus 50%). To determine the effect of Ad.VEGF treatment on functional myoglobin, active myoglobin content of gastrocnemius muscle was determined by a peroxidase-based activity assay (Figure 4D). Treatment with Ad.VEGF resulted in a significant increase of active myoglobin in gastrocnemius muscle after femoral artery occlusion as compared with Ad.LacZ or PBS treatment (64±6.8, 43±3.9, or 21±2.4 μg myoglobin/mg dry-weight, respectively; P≤0.01, n=5). In addition, Ad.LacZ treatment increased active myoglobin levels as compared with PBS (P<0.001). No significant difference in active myoglobin induction between Ad.VEGF and Ad.LacZ group was observed in the nonischemic limb.

Finally, to assess whether the increase of myoglobin was accompanied by an increase of mitochondrial content of the muscles, NADH-TR reactivity was determined in gastrocnemius muscle cryosections. Ad.VEGF treatment was accompanied by a significant increase in oxidative fibers in ischemic muscle as compared with Ad.LacZ (P=0.03, n=5) (Figure 4E).
Limb Amputation Patients: Coexpression of VEGF and Myoglobin in Ischemic Muscle of Limb Amputation Patients

To determine whether a similar correlation between VEGF and myoglobin also exists in patients with peripheral arterial obstructive disease, skeletal muscle samples were collected from amputated limbs of 15 patients. Patients consisted of 7 males and 8 females with a mean age of 67 years (range 52 to 88 years). In general, VEGF staining was absent in nonischemic gastrocnemius muscle near the amputation level (Figure 5A), whereas, more distally, cytoplasmic VEGF staining was evident in some muscle fibers of soleus muscle near the Achilles tendon. This was accompanied by histological signs of chronic ischemia, e.g., disorganized muscle composition and adipose cells (Figure 5B). More distally, VEGF was present in a large number of muscle fibers in the severely ischemic interosseus muscle and was especially abundant in regenerating and atrophic muscle fibers with abundant VEGF expression. Moreover, both VEGF and myoglobin expression in muscle were significantly elevated with an increased level of ischemia ($P=0.018$ and 0.047, respectively). Furthermore, an increased expression of VEGFR-1 and -2 was observed in cryosections of more distally located.

Figure 5. Representative photomicrographs of muscle from patients that underwent limb amputation. ($\times 200$). A, In nonischemic muscle, VEGF was absent. B, In more distally located soleus muscle, VEGF was expressed in cytoplasm of some muscle fibers. C, Interosseus muscle between the toes showed most signs of chronic ischemia, such as many regenerating and atrophic muscle fibers with abundant VEGF expression. D, Staining for myoglobin demonstrated an almost identical expression pattern of individual muscle fibers as VEGF in a sequential cross-section of ischemic interosseus muscle (compare C and D). E, VEGF receptor 1 was strongly expressed around vessels and muscle fibers in ischemic muscle. F, VEGF receptor 2 was also expressed around muscle fibers in ischemic muscle. G, CD31 staining of a sequential section showed localization of vascular cells adjacent to muscle fibers.
ischemic muscle. Importantly, VEGFR-1 was not only expressed around vessels, but also around muscle fibers, suggesting muscle cell membrane-specific expression (Figure 5E). Staining for VEGFR-2 showed a similar expression pattern around muscle fibers of ischemic interosseous muscle (Figure 5F). Comparison of staining for VEGF receptors with anti-CD31 endothelial staining (Figure 5G) confirmed that VEGF receptors were additionally expressed on muscle fibers, thus not restrictedly expressed on the closely adjacent vascular cells. In both gastrocnemius and soleus muscle, however, VEGFR-2 was only expressed on vascular endothelial cells (data not shown).

**Cultured Myotubes: Increased Myoglobin mRNA Levels by VEGF in Murine Myotubes in Culture**

To more critically assess whether VEGF can directly act on muscle cells, the effect of VEGF was studied in vitro in differentiated murine myotubes (C2C12 cells). First, expression of VEGF receptor 1 and 2 mRNA in myotubes was confirmed by semiquantitative RT-PCR (Figure 6A). Secondly, the effect of human VEGF recombinant protein (rhVEGF) on myoglobin mRNA expression was studied at various time points. PBS-treated cells served as control. Myoglobin mRNA expression gradually increased, being 2.8-fold elevated at 9 hours \((P=0.02, n=4)\), and decreased thereafter (Figure 6B). However, in PBS-treated cells, no increase in myoglobin mRNA expression was observed. Real-time RT-PCR measurements of myoglobin mRNA were within the linear range of a calibration curve using a construct containing myoglobin cDNA, indicating that the observed differences in PCR signal for myoglobin represent a true difference in mRNA levels (see online data supplement). To provide more evidence that the observed effects of VEGF are mediated through VEGF receptor pathways, we studied whether VEGF-induced myoglobin expression could be inhibited using the VEGF receptor tyrosine kinase inhibitor SU5416 in C2C12 cells. Induction of myoglobin mRNA expression with rhVEGF was completely inhibited when rhVEGF was combined with SU5416 (fold-induction versus PBS 2.91 ±0.94 or 0.71 ±0.13, respectively; \(P=0.05, n=6\)) (Figure 6C). To study whether VEGF induces muscle cell specification leading to more oxidative type I myotubes, expression of a set of genes characteristic of type I fibers, including Troponin I, Cox IVα, and PGC-1α, was studied. There was a significant 2-fold increased expression of mRNA for Troponin I (slow) 6 hours after VEGF treatment \((P=0.04, n=3)\). In addition, Cox IVα and PGC-1α mRNA expressions were increased by 1.8- and 2.8-fold respectively, although this increase did not reach statistical significance for both genes \((P=0.07 and 0.21, \text{respectively}, n=3)\) (Figure 6D).

**Discussion**

Our data indicate that VEGF gene therapy results in enhanced myoglobin expression in ischemic skeletal muscle in mice. Furthermore, we show coexpression of VEGF and myoglobin in muscle biopsies from patients after limb amputation caused by peripheral arterial obstructive disease, which correlates with the degree of ischemia. In addition, we provide evidence for a direct regulation of myoglobin by VEGF in murine myotubes in culture. Importantly, these data may explain the puzzling inconsistencies shown in previous clinical trials with VEGF. VEGF-mediated increase of muscle myoglobin may clarify, at least partly, the observed clinical improvements of VEGF-treated patients, in particular the regression of intermittent claudication, rest pain, or angina, in the absence of improved vascular status.

Although VEGF is a well-known inducer of angiogenesis, its role in the induction and development of larger conductance collaterals via arteriogenesis is still a matter of debate.26–28 In this study, it was shown that collateral vessel
growth was not enhanced by Ad.VEGF treatment in a mouse model. Nevertheless, a marked red color of gastrocnemius muscle in the ischemic limb was apparent in Ad.VEGF-treated mice. Indeed, capillary formation, a possible reason for this red color, was significantly increased in Ad.VEGF-treated muscle. Despite an increased capillary density, we also observed a marked enlargement of capillaries in Ad.VEGF-treated muscle, as previously described.29 Nevertheless, we were puzzled by the fact that an increase in capillary density was only correlated with a distinct redness of muscle in ischemic VEGF-treated limbs and not in the other groups.

A second possible explanation for the red color of muscle was that VEGF treatment induced extravasation of erythrocytes, because VEGF may increase vascular permeability.30 However, there was no evidence for an increased number of extravascular erythrocytes in histological sections of Ad.VEGF-treated muscle. Thus, we hypothesized that other factors affecting the color of muscle might play a role. Interestingly, activation of the myoglobin gene in transgenic Ad.VEGF-treated muscle. Thus, we hypothesized that other factors affecting the color of muscle might play a role. Interestingly, activation of the myoglobin gene in transgenic mice results in a distinct red color of most muscles.25 In physiological conditions, the gastrocnemius muscle contains a relatively low percentage of myoglobin-containing oxidative fibers, ie, around 1% to 8% of type-I and 30% to 32% of type-IIa fibers in mice, and can be readily distinguished from dark red, type-I–enriched soleus muscle.31 However, color of gastrocnemius and soleus muscle was similar in limbs of Ad.VEGF-treated mice after femoral artery occlusion. Therefore, we analyzed the myoglobin content in the treated gastrocnemius muscles. Indeed, the number of myoglobin-stained muscle fibers and active myoglobin content were increased in calf muscle of Ad.VEGF-treated mice. Thus, VEGF-mediated myoglobin expression may, at least partly, explain the red color of gastrocnemius muscle in these mice.

VEGF-mediated myoglobin expression in vivo may be regulated by several molecular mechanisms. Firstly, VEGF may act directly on muscle fibers via membrane-bound VEGF receptors, resulting in stimulation of myoglobin expression. VEGF has previously been thought to be an endothelial cell specific mitogen. However, recent data demonstrate that it also has an effect on a large variety of cell types, eg, vascular smooth muscle cells, neural cells, endothelial precursor cells, and monocytes.32–35 In this study, we found abundant expression of VEGF receptor 1 and 2 at the margins of muscle fibers in ischemic muscle of the amputated limbs, suggesting an effect of VEGF on these specific cells via binding to its receptor. Upregulation of both VEGF-receptors in ischemic muscle may explain the observation that myoglobin expression was only increased in the ischemic limb of Ad.VEGF-treated mice and not in the nonischemic control limb.

To show that VEGF can directly act on skeletal muscle cells through classic VEGF receptor pathways, we cultured murine myoblasts (C2C12) that were differentiated into mature myotubes. First, we observed expression of VEGF receptor 1 and 2 mRNA in C2C12 cells, as previously described.15 Second, treatment with VEGF resulted in a significant 2.8-fold induction of myoglobin mRNA as compared with control. Third, the VEGF-induced myoglobin mRNA expression was completely blocked when VEGF was combined with the VEGF receptor tyrosine kinase inhibitor SU5416. Finally, VEGF-induced myoglobin expression was accompanied by an increased expression of genes characteristic of type I oxidative fibers, indicating activation of myofiber specialization.

We cannot exclude that VEGF may also act indirectly on myoglobin expression by stimulating the chemotaxis of leukocytes (eg, monocytes), which on their turn secrete factors that stimulate myoglobin expression. This hypothesis is supported by the observation that active myoglobin content was partially increased in the control virus-treated mice. Another explanation for an indirect effect of VEGF on myoglobin expression may lay in the protective properties of VEGF toward motor neurons against ischemic death.36 It has been shown that decreased neuromuscular activity elicits transitions from oxidative toward glycolytic muscle fiber phenotypes.37 VEGF may therefore, indirectly, induce a fiber type transition toward more oxidative, myoglobin-containing muscle fiber types.

Finally, studies of pathways downstream of neural activity have implicated NFAT and Mef2 transcription factors under the regulation of calcium signaling through calcineurin, a calcium/calmodulin-dependent protein phosphatase, in the control of myoglobin regulation.38,39 Interestingly, VEGF has been shown to activate the calcium-calcinurin pathway in endothelial cells.40 It remains to be determined whether VEGF is also able to activate the calcium-calcinurin pathway in skeletal muscle cells, and whether this can ultimately lead to activation of myoglobin transcription.

In conclusion, VEGF gene therapy offers a dual therapeutic effect for the improvement of ischemic muscle condition, first, via the induction of new capillaries, and second, via improved muscle oxygenation by increased myoglobin.

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Expanded Materials and Methods

Gene transfer in a mouse model of acute hindlimb ischemia

Vectors
An adenoviral vector carrying the VEGF gene (Ad.VEGF) was constructed using cDNA encoding the human VEGF\textsubscript{165} isoform under control of the cytomegalovirus (CMV) promoter with SV40 polyadenylation signal. VEGF\textsubscript{165} cDNA was isolated from human umbilical vein endothelial cells by Reverse Transcriptase PCR, using the following primers: 5’-GGATCCCCCTCGGGCCTCCGAAACCATGAAC-3’ and 5’-TCTAGAGAGAGATCTGGTTCCCGAAACGCTG-3’. Subsequently, VEGF cDNA was cloned into plasmid CMV10, as previously described.\textsuperscript{1} An adenoviral vector carrying the \textit{Escherichia coli} LacZ reporter gene (Ad.LacZ), encoding β-galactosidase under control of the cytomegalovirus promoter was used as a control. Production of adenovirus stocks using the Ad5 E1 transformed human embryonic retina cell line 911 and other virus techniques were performed as previously described.\textsuperscript{2}

Surgical procedure
All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO). Male C57BL/6 mice, aged 10 weeks, were anesthetized with Hypnorm (25mg/kg, Janssen Pharmaceutica) and Dormicum (25mg/kg, Roche) intraperitoneally before surgery. Animals were randomly allocated into two groups (n=6).
The effect of VEGF gene transfer on collateral vessel growth in the upper hindlimb was analyzed in one group (group A). For this, muscles of the left upper hindlimb surrounding the femoral artery were injected at T=0 with 40µl of either Ad.VEGF or Ad.LacZ (1.5x10^{10} pfu/ml).

In the second group, the effect of VEGF gene transfer on VEGF and myoglobin expression and capillary growth was studied in the calf muscle (group B). For this, the gastrocnemius muscle of both limbs was injected at T=0 with either 40µl of PBS, Ad.VEGF or Ad.LacZ (1.5x10^{10} pfu/ml).

At day 3, ischemia of the left hindlimb was induced in animals of both groups by coagulation of the left femoral artery proximal to the bifurcation of deep and superficial femoral artery. At day 6, collateral vessel growth was studied by performing post-mortem angiography in mice of group A. In mice of group B, the gastrocnemius muscles of both hindlimbs were dissected for further analysis.

**Angiography**

To study collateral vessel growth, angiography of both hindlimbs was performed using polyacrylamide-bismuth contrast (Sigma) 3 days after femoral artery occlusion, as previously described.\(^3\) Briefly, papaverin (2 mg/ml, Centrafarm) was injected into the aorta to induce vasodilation, followed by contrast medium at constant pressure (100 mmHg). Post-mortem angiographic images were acquired by röntgenographic exposure using a Faxitron X-ray machine. Detailed photographs of the hindlimb region on the radiographs were taken with a Hitachi digital camera. Grading of collateral filling was performed in a single blinded fashion and was based on the Rentrop classification.\(^4\) Grading was as follows: 0=no filling of collaterals, 1=filling of
collaterals only, 2=partial filling of distal femoral artery, 3=complete filling of distal femoral artery.

**Perfusion Imaging**
Repeated blood flow measurements over the paws were obtained at baseline, immediately after femoral artery occlusion, and serially over 2 weeks in Ad.VEGF- or Ad.LacZ-treated mice (n=6) through the use of Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments), as previously described. Perfusion is expressed as a ratio of left (ischemic) to right (non-ischemic) limb.

**Sample collection from patients**
Samples of skeletal muscle were obtained after informed consent from 15 patients after lower limb amputation because of critical ischemia, according to the guidelines of the Institutional Review Board. Muscle biopsies were performed at the non-ischemic amputation level (m.gastrocnemius) and at ischemic levels near the Achilles tendon (m.soleus) and between the toes (m.interossius).

**C2C12 cells in culture**
C2C12 myoblasts, obtained from the American Type Culture Collection, Rockville, MD (kindly provided by Dr CWGM Löwik, Leiden), were maintained in a humidified atmosphere of 5% CO$_2$/95% air and passaged by standard trypsinization in a growth medium consisting of DMEM supplemented with 20% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 mg/ml) and grown until approximately 50% to 70% confluency. Differentiation to myotubes was initiated by replacing the medium with DMEM supplemented with 2% horse serum, penicillin (50 U/ml), streptomycin
(50 mg/ml), 1 µM Insulin and 2.5 µM Dexamethasone (differentiation medium). After 7 days of culture mature myotubes were formed. Cells were incubated for 3, 6, 9 and 12 hours with 5 ng/ml human VEGF recombinant protein (RELIA Tech.) in differentiation medium (n=4). In a separate experiment (n=6), myotubes were incubated for 6 hours with 5 ng/ml human VEGF recombinant protein combined with the VEGF receptor tyrosine kinase inhibitor SU5416 (100 nM) (kindly provided by Dr K Hoekman, Amsterdam). Subsequently, cells were washed with PBS and lysed in 1 ml GTC-βME buffer (4 M Guanidinium-isothiocyanate, 25 mM Na-citrate, 0.5% sarcosyl, 0.1 M 2-mercatoethanol), pH 7.0, and stored at -80 °C for further RNA analysis.

**Determination of protein expression**

**Immunohistochemistry**

Immunohistochemistry was performed on 4% formaldehyde-fixed, paraffin-embedded sections of murine and human skeletal muscle using antibodies for myoglobin (Santa Cruz Biotechnology), VEGF (Santa Cruz Biotechnology), CD31 (Pharmingen), and on frozen sections using VEGFR-1 and VEGFR-2 (kindly provided by Dr HA Weich, Germany). In addition, cryo-sections were histochemically stained for nicotinamide dehydrogenase tetrazolium reductase (NADH-TR), as previously described. Myoglobin expression, NADH-TR reactivity, capillary density and area per capillary were quantified from randomly photographed sections (3-6 images per section) using image analysis (Qwin, Leica). Myoglobin expression in mice was expressed as:

\[
\frac{\text{area of muscle fibers stained for myoglobin}}{\text{total area of muscle fibers}} \times 100\%.
\]

Intensity of VEGF and myoglobin staining of human skeletal muscle sections was studied at the different levels of each amputated limb in a single blinded fashion.
**Analysis of active myoglobin**

Concentration of active myoglobin in samples of murine gastrocnemius muscle was determined as previously described. Briefly, muscle samples were washed in ice-cold Tyrode (in mM: NaCl 146; KCl 6; NaHCO₃ 20; Glucose 5.6; KH₂PO₄ 0.3; CaCl₂·2H₂O 3; MgCl₂·6H₂O 0.5, human serum albumin (1 mg/ml)) gassed with 95% oxygen and 5% carbon dioxide, pH 7.4, at 10°C for 15 minutes. Subsequently, samples were frozen in liquid nitrogen, freeze-dried overnight, weighed, and homogenized in a solution containing KCl and Tris-HCl. The homogenate was centrifuged (6000 rpm, 10 minutes, 4°C) and the supernatant was added to a reaction medium, consisting of water, ethanol with ortho-tolidine (Sigma) and tertiary-butyl-hydro-peroxide (Sigma). Absorbance was determined at 436 nm after 60 minutes of incubation at room temperature. Myoglobin was calibrated against a standard of horse myoglobin (Sigma). Concentration of active myoglobin was expressed in µg myoglobin per mg dry weight.

**VEGF ELISA**

Human VEGF$_{165}$ levels in murine blood samples were assayed by ELISA (R&D systems) according to the manufacturer’s protocol.

**RNA analysis**

**Total RNA isolation**

Total RNA was extracted from frozen murine gastrocnemius muscle using the RNEasy isolation kit (QIAGen) according to the manufacturer’s protocol. RNA isolation from C2C12 cells was performed according to Chomczynski et al, as
previously described. RNA samples were electrophoresed on a 1% agarose gel and quality was confirmed by staining with ethidium bromide.

**Real-time RT-PCR**

To prevent contamination of genomic DNA in PCR, RNA samples were treated with DNAse prior to cDNA synthesis using RQ1 RNase-free DNase (Promega) according to the manufacturer’s protocol.

One microgram of total RNA was reversed transcribed into cDNA in a final volume of 20 µl using 15 U AMV transcriptase (Promega) in transcription buffer (10 mM Tris-HCL, 50 mM KCL, 0.1% Triton X-100) supplemented with 1 mM deoxyribonucleotides (dNTPs), 1U RNAsin ribonuclease inhibitor and 0.5 mg oligo(dT)$_{15}$ primer. Samples were incubated at 42°C for 60 minutes. The reaction was stopped by incubating at 99°C. Samples were stored at –20°C until PCR analysis.

Primers pairs and probes for studying expression of human VEGF-A, mouse myoglobin and mouse HPRT by real-time RT-PCR were designed using the specific criteria as described in the primer express software (Perkin Elmer), as depicted in Table I. Mouse PCR primer sets for Troponin I (slow), cytochrome c oxidase IVa (Cox IVa) and the peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1 (PGC-1α) were purchased (Applied Biosystems). Samples were normalized to housekeeping gene expression (GAPDH (Perkin Elmer) or HPRT for muscle or C2C12 samples respectively). PCR was performed using Q-PCR mastermix (Eurogentec) in a 25µl reaction volume. After 2 minutes of incubation at 50°C the enzyme was activated by incubation at 95°C for 10 minutes followed by 40 PCR cycles consisting of 15 seconds denaturation at 95°C and hybridization at 60°C for 1 minute. Myoglobin expression was calibrated against a construct containing
myoglobin cDNA cloned into a pMOSBlue plasmid vector at concentrations ranging from 500 to 0.05 pg/ml. Primers for mouse VEGFR1/FLT1 and VEGFR2/FLK1 were used for semi-quantitative PCR as previously reported.\textsuperscript{9}

**Statistical Analysis**

Results are expressed as mean±SEM. Comparisons between means were performed using the Student T-test (2 groups), one-way ANOVA (>2 groups) or Mann-Whitney U test (non-parametric data). Comparisons of immunostaining intensity between different levels of human limbs were performed with the Signs-test. A p-value <0.05 was considered statistically significant.
<table>
<thead>
<tr>
<th>Primers pairs and probe for real-time quantitative RT-PCR</th>
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<tr>
<td>human VEGF-A S</td>
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<td>human VEGF-A AS</td>
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<td>human VEGF-A P</td>
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<tr>
<td>murine myoglobin S</td>
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<td>murine myoglobin AS</td>
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<td>murine myoglobin P</td>
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<td>murine HPRT S</td>
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<th>Primers pairs for semi-quantitative RT-PCR</th>
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<tr>
<td>murine VEGFR1 S</td>
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<tr>
<td>murine VEGFR1 AS</td>
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<tr>
<td>murine VEGFR2 S</td>
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<td>murine VEGFR2 AS</td>
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**Table I.** Primers pairs (and probes) for studying expression of human VEGF-A, murine myoglobin, murine HPRT, murine VEGFR1/FLT1 and murine VEGFR2/FLK1 by RT-PCR. S=sense, AS=antisense and P=probe.
Additional figure

Figure 1: Calibration of real-time PCR method for myoglobin

To more critically assess whether VEGF can directly act on muscle cells, the effect of VEGF was studied in vitro in differentiated murine myotubes (C2C12 cells). The effect of human VEGF recombinant protein on myoglobin mRNA expression was studied at various time points. PBS-treated cells served as control. Myoglobin mRNA expression gradually increased, being 2.8-fold elevated at 9 hours (p=0.02, n=4) and decreased thereafter. However, in PBS-treated cells no increase in myoglobin mRNA expression was observed. Real-time RT-PCR measurements of myoglobin mRNA were within the linear range of a calibration curve using a construct containing myoglobin cDNA, indicating that the observed differences in PCR signal for myoglobin represent a true difference in mRNA levels (Figure 1).
References


Figure 1. Calibration curve for myoglobin real-time PCR using a construct containing myoglobin cDNA cloned into a pMOSBlue plasmid vector at concentrations ranging from 500 to 0.05 pg/ml (open data points). All measurements either after VEGF or PBS treatment were within the linear range of this calibration curve (solid data points).