ARTICLE

doi:10.1016/j.ymthe.2004.08.007

Vascular Endothelial Growth Factor Stimulates Skeletal Muscle Regeneration *in Vivo*

Nikola Arsic,[1] Serena Zacchigna,[1] Lorena Zentilin,[1] Genaro Ramirez-Correa,[1] Lucia Pattarini,[1] Alessandro Salvi,[2] Gianfranco Sinagra,[2] and Mauro Giacca[1,3,*]

[1]*Molecular Medicine Laboratory, International Center for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy*
[2]*Cardiology Unit, Ospedale di Cattinara, Trieste, Italy*
[3]*Scuola Normale Superiore and Istituto di Fisiologia Clinica of CNR, Area della Ricerca, Via Moruzzi 1, 56100 Pisa, Italy*

**To whom correspondence and reprint requests should be addressed at the Molecular Medicine Laboratory, ICGEB, Padriciano 99, 34012 Trieste, Italy. Fax: +39 040 226555. E-mail: giacca@icgeb.org.*

Available online 17 September 2004

Vascular endothelial growth factor (VEGF) is a major regulator of blood vessel formation during development and in the adult organism. Recent evidence indicates that this factor also plays an important role in sustaining the proliferation and differentiation of different cell types, including progenitor cells of different tissues, including bone marrow, bone, and the central nervous system. Here we show that the delivery of the 165-aa isoform of VEGF-A cDNA using an adeno-associated virus (AAV) vector exerts a powerful effect on skeletal muscle regeneration *in vivo.* Following ischemia-, glycerol-, or cardiotoxin-induced damage in mouse skeletal muscle, the delivery of AAV-VEGF markedly improved muscle fiber reconstitution with a dose-dependent effect. The expression of both VEGF receptor-1 (VEGFR-1) and VEGFR-2 was upregulated both in the satellite cells of the damaged muscles and during myotube formation *in vitro;* the VEGF effect was mediated by the VEGFR-2, since the transfer of PlGF, a VEGF family member interacting with the VEGFR-1, was ineffective. These results are consistent with the observation that VEGF promotes the growth of myogenic fibers and protects the myogenic cells from apoptosis *in vitro* and prompt a therapeutic use for VEGF gene transfer in a variety of muscular disorders.

Key Words: muscle, gene therapy, adeno-associated virus, vascular endothelial growth factor, vascular endothelial growth factor receptor-2

INTRODUCTION

Vascular endothelial growth factor-A (VEGF-A) is a main regulator of blood vessel formation during embryogenesis and a potent inducer of neovascularization during adult life. The biological effects of the VEGF family members are transduced by three main receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR, Flk-1), and VEGFR-3 (Flt-4). The VEGFR-2 is the main receptor that mediates the angiogenic and permeabilizing effects of VEGF-A, through its powerful activity on endothelial cell proliferation and migration. The precise role of the other receptors in the angiogenic process still remains poorly understood; VEGFR-1 has been proposed as a decoy receptor, limiting the access of free VEGF to VEGFR-2, while VEGFR-3 seems to have a fundamental role on the lymphatic endothelium mainly through interaction with VEGF-C and VEGF-D (for reviews, see [1–3]).

Originally described as an endothelial-specific growth factor, recent evidence suggests that the effects of VEGF-A might extend to a variety of other cell types. In particular, the factor appears to have a direct neuroprotective potential, preventing neuronal cell death from ischemia and promoting neurogenesis *in vitro* and *in vivo* [4–6]. The demonstration that VEGF receptors are actually expressed by Schwann cells as well as by neurons is consistent with the direct trophic effect of VEGF-A on these cells [7]. Recently, the ability of VEGF-A to promote hepatocyte proliferation by the selective activation of VEGFR-1 has been exploited to reduce liver damage in mice exposed to a hepatotoxin [8]. Furthermore, VEGF-A and its receptors have been shown to play an important role during cartilage and bone formation, by promoting skeletogenesis; this effect might be secondary to the induction of neoangiogenesis into the perichondrium and the primary ossification center or might result from the direct promotion of osteoblast migration and differentiation [9–12]. Smooth muscle cells have also been shown to express both VEGFR-1 and VEGFR-2 and to respond to VEGF-A chemoattraction in culture [13,14].

1525-0016/$30.00

Finally, the effect of VEGF-A on hematopoietic cells has been described by several groups, showing that this factor is able to mediate monocyte chemotaxis [15], hematopoietic stem cell survival [16], and the recruitment of endothelial progenitor cells [17], through its interaction with VEGFR-1.

We have recently exploited the potential of vectors based on the adeno-associated virus (AAV)—which offer a unique opportunity to study the effects of gene expression for prolonged periods of time *in vivo,* in the absence of an inflammatory or immune response [18–20]—to assess the powerful angiogenic effect of the 165-aa isoform of VEGF-A (hereafter, VEGF) *in vivo* [21]. In the course of our studies, we unexpectedly observed that the expression of VEGF in the normal mouse skeletal muscle also resulted in the appearance of a notable subset of muscle fibers displaying a central nucleus, a widely recognized hallmark of muscle regeneration. This finding raises the important possibility that VEGF might also exert a direct effect on myogenesis *in vivo,* consistent with the observations that hypoxic muscle fibers express both VEGF and its receptors [22] and that the factor plays an important role in myoblast migration and survival [23].

Here we specifically demonstrate that AAV-VEGF administration exerts a powerful effect on muscle survival and regeneration following different types of muscle damage. This effect is mediated by its interaction with VEGFR-2 and involves the protection of myogenic cells from apoptosis and the stimulation of myogenic fiber growth. This observation prompts a therapeutic use of AAV-VEGF in a variety of muscular disorders.

RESULTS

Long-Term VEGF Expression in Skeletal Muscle Induces Muscle Fiber Regeneration

To investigate directly whether VEGF might affect the growth of skeletal muscle, we injected a purified AAV-VEGF preparation into the normoperfused right tibialis anterior muscles of six mice. Real-time PCR quantification of transgene expression in a parallel set of animals indicated that, under these conditions, the hVEGF mRNA is already detectable at 3 days after transduction and that its levels progressively increase over time (Supplementary Fig. 1), a kinetics that is consistent with previous observations [24]. One month after injection, a notable subset of muscle fibers (>5%) evidently displayed a central nucleus, a widely recognized hallmark of muscle regeneration (Fig. 1A). This effect was not evident after injection of an AAV vector expressing LacZ in a matched group of control animals (Fig. 1A) or of a variety of other AAV vectors used in the laboratory (not shown). In addition, this effect persisted for at least 3 months after transduction.

To quantify this effect better, we measured the areas of the muscle fiber cross sections in the AAV-VEGF- and AAV-LacZ-injected animals. The distribution of these values was relatively narrow and symmetric in the AAV-LacZ-injected muscles, with >75% of the fibers in the range 1–2 $\times$ 10^3 μm^2, and was indistinguishable from the uninjected normal muscle (not shown; Fig. 1B). In contrast, the fiber area distribution of the AAV-VEGF-expressing muscles skewed toward the left and included almost 20% of the fibers with a size of <1 $\times$ 10^3 μm^2 (as opposed to 10.7% in the normal muscle). In addition, this distribution was also much broader toward the right side, with almost 20% of the fibers being hypertrophic, with an area of >2.5 $\times$ 10^3 μm^2 (1% in the control muscle). The two distributions were different with a high statistical significance ($P < 1 \times 10^{-4}$).

Finally, we also observed that when AAV-VEGF was injected into the gastrocnemius or the tibialis anterior, prior to the induction of acute ischemia by the resection of the femoral artery, the tissue viability of the ischemic area was remarkably preserved at day 20 after surgery, with the damaged muscle showing large areas containing small regenerating fibers with a central nucleus (Fig. 1C). A more extensive survey of the protective and proregenerative effect of AAV-VEGF injection after acute ischemia is shown in Supplementary Fig. 2.

VEGF Promotes the Growth of Myogenic Fibers and Protects Myogenic Cells from Apoptosis

The above-reported observations suggest that, in addition to its well-documented angiogenic properties, VEGF might also exert a direct effect on muscle fibers. We reasoned that these effects would imply that the muscle cells should express at least one of the VEGF receptors and therefore looked for the presence of VEGFR-1 and VEGFR-2 in C2C12 myogenic cells and in primary mouse myoblasts. We detected expression of these receptors in both cell types through RT-PCR amplification of the total RNAs from these cells and by Western blotting on total cell lysates (not shown). Of note, we found that the expression of both receptors was strikingly increased upon switching the cultures to a differentiation medium. As shown by immunocytochemistry, myotubes formed by the differentiation of C2C12 cells (Fig. 2A) expressed a myogenic differentiation marker—myosin heavy chain (MHC)—as well as high levels of VEGFR-1 and VEGFR-2. The expression of both receptors was already detectable as early as 2 days after switching the cultures to the differentiation medium and subsequently remained very high. The same effects were observed upon differentiation of the primary myoblasts from mouse skeletal muscle (Supplementary Fig. 3).

To assess the activity of VEGF along the differentiation process, we switched C2C12 cells at 85% confluence to a differentiating medium and either supplemented them or not with recombinant VEGF at a concentration of 100

doi:10.1016/j.ymthe.2004.08.007

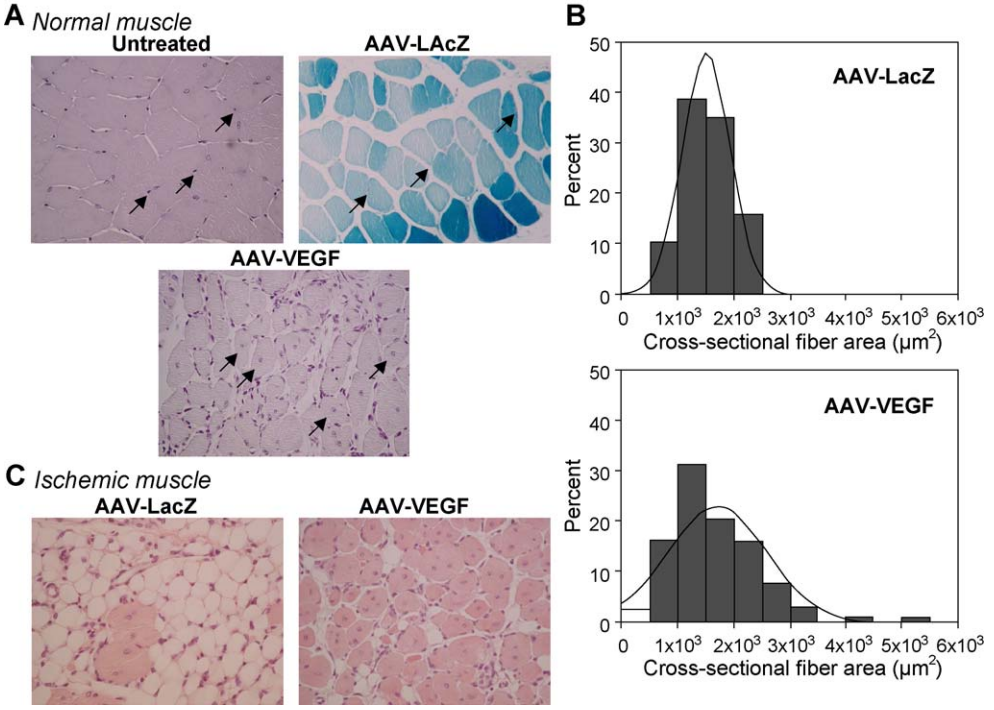

FIG. 1. Sustained expression of VEGF in normoperfused and ischemic skeletal muscle induces muscle fiber regeneration. (A) Hematoxylin-stained sections of normoperfused muscle tibialis anterior from untreated and AAV-LacZ- or AAV-VEGF-injected mice, at 1 month after transduction. Muscles injected with AAV-LacZ were also stained for β-galactosidase expression. Long-term expression of VEGF in normoperfused muscles induced the appearance of small fibers with a central nucleus (arrows), a hallmark of an ongoing regeneration process. (B) Fiber size analysis in normal muscles injected with AAV-LacZ or AAV-VEGF. The histograms show the distribution of the fiber cross-sectional areas (μm^2) with a normal distribution curve superimposed. Data were obtained from the analysis of 20 cross sections from six different animals per group. The fiber area distribution of the AAV-VEGF-expressing muscles skewed toward the left compared to the control and also included a significant number of fibers with large cross-sectional areas. (C) Hematoxylin and eosin-stained histological sections of AAV-LacZ- and AAV-VEGF-treated muscles at 20 days after induction of acute ischemia. A massive fiber loss with adipose substitution was evident in control ischemic muscles; in contrast, the VEGF-treated samples showed substantial recovery, with the presence of several regenerating fibers (×400 original magnification).

ng/ml. As shown in Fig. 2B, the number of myotubes expressing MHC, detected by immunofluorescence, at day 3 from induction was clearly increased in the cultures supplemented with VEGF. In particular, in the cultures without VEGF, >80% of MHC-positive cells were mononucleated (myocytes) and <2.5% of the multinucleated myofibers contained three or more nuclei. In contrast, the distribution of the number of nuclei was far more dispersed in the cultures treated with VEGF, with ~50% of the MHC-positive cells being mononucleated and >20% of the myofibers containing three or more nuclei per fiber ($P < 1 \times 10^{-4}$). Consistent with the profusogenic role of VEGF, the length of the mono- or polynucleated MHC-positive cells was significantly increased in the cultures exposed to VEGF ($P < 0.05$; Fig. 2C).

The increase in the number, length, and nuclear content of the differentiated C2C12 cells upon VEGF treatment clearly indicated that this growth factor has an important effect in promoting muscle fiber growth. This notion was further reinforced by the analysis of the rate of C2C12 cell proliferation after treatment with VEGF, as measured by the 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay scoring for cell metabolic activity. Under both proliferating and differentiating conditions, the addition of VEGF determined a decrease in the proliferation rate of the cells, with a dose-dependent response (Fig. 2D). The same effect was also observed by exposing primary mouse myoblasts to differentiating conditions in the presence of VEGF (Supplementary Fig. 4A).

We also explored the possibility that the increased differentiation promoted by VEGF might be paralleled by the protection of these cells from apoptotic cell death. To address this issue, we treated C2C12 cells with camptothecin (50 nM), a well-known apoptosis-triggering agent, for 5 h and analyzed the percentage of cells reactive to annexin V on the surface by flow cytometry (Fig. 2E). The percentage of cells expressing this marker—but still excluding propidium iodide, an indicator of necrosis—decreased from 29.0 to 7.5% after treatment with hrVEGF (50 ng/ml). In a consistent manner, the protective effect of VEGF was highly decreased (21.2% of annexin V-positive cells) when the cells were also treated with SU1498 (5 μM), an inhibitor of the VEGFR-2 tyrosine

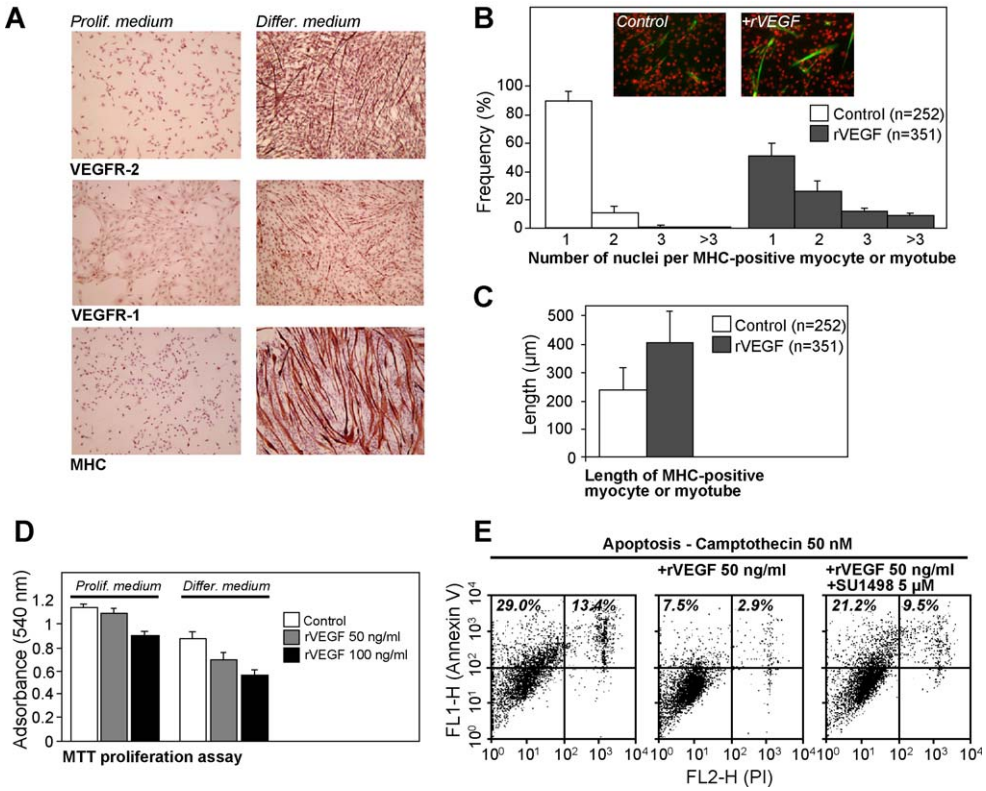

FIG. 2. Effects of VEGF on myogenic cells *in vitro.* (A) Immunocytochemistry performed on C2C12 cells using specific anti-mouse VEGFR-2, VEGFR-1, and slow myosin heavy chain (MHC) antibodies. Cells under proliferating conditions showed very low levels of positivity for all three antibodies (images on the left). In contrast, both VEGF receptors were markedly upregulated in cells cultured for 4 days in differentiation medium (images on the right), showing massive myotube formation and positive staining for the differentiation marker MHC. (B) Frequency distribution of the number of nuclei present in differentiated C2C12 cells with or without treatment with VEGF (100 ng/ml). Cells were cultured under differentiating conditions for 3 days followed by immunofluorescence with an antibody against MHC. The histograms show the frequency distribution of the number of nuclei present in the MHC-positive myocytes (one nucleus) and myofibers (two or more nuclei). After VEGF treatment, the total number of MHC-positive cells was significantly increased (351 vs 252 in 10 microscopic fields from four independent experiments; $P < 0.01$), with a higher content of multinucleated myofibers. Representative immunofluorescence images are shown at the top. (C) Length of MHC-positive myocytes and myotubes. The length of the MHC-positive, differentiating C2C12 cells of the experiment described in B was significantly increased after treatment with VEGF. (D) MTT proliferation assay. In C2C12 cells, the addition of VEGF decreased the cell proliferation rate, with a dose-dependent response, during proliferation as well as in the early phase of differentiation. (E) Flow cytometry analysis of C2C12 cells stained for annexin V reactivity and propidium iodide (PI) incorporation after treatment with camptothecin. The percentage of annexin-positive and PI-negative apoptotic cells (upper left quadrants) induced by the camptothecin treatment was significantly decreased in the presence of recombinant VEGF 50 ng/ml. The protective effect of VEGF was highly diminished when the cells were also treated with SU1498, an inhibitor of the VEGFR-2 tyrosine kinase activity.

kinase activity. This observation also suggests the specific involvement of the VEGFR-2 in mediating the antiapoptotic effect of VEGF. We noticed similar findings in primary myoblasts treated with camptothecin in the presence or absence of hrVEGF (Supplementary Fig. 4B).

The Delivery of AAV-VEGF Promotes Dose-Dependent Recovery after Muscle Damage

To explore the therapeutic potential of AAV-VEGF administration after skeletal muscle damage independent of hypoxia, we studied the effects of AAV-VEGF transduction in CD1 mice after glycerol (50% v/v) injection into the tibialis anterior muscle. This treatment resulted in the destabilization of the cytoplasmic membrane followed by cell death. In the absence of treatment, muscle regeneration was brought to a complete recovery in ~35 days. We administered doses of 3×10^8 AAV-VEGF vector particles 5 days before glycerol injection, immediately after glycerol, or 5 days after glycerol, to assess the effects of timing of vector administration. We sacrificed the treated animals at day 20 after injury and three independent investigators who were blinded to the experimental procedures evaluated the extent of the damaged area in transverse muscle sections. At this time point, the control muscles still showed a large area of degeneration (>15% of the transversal muscle section area), with a massive substitution of muscle fibers with adipose tissue and only a few regenerating muscle fibers (Fig. 4A). In contrast, all the AAV-VEGF-treated muscles showed a remarkable reduction in the damaged area (Fig.

doi:10.1016/j.ymthe.2004.08.007

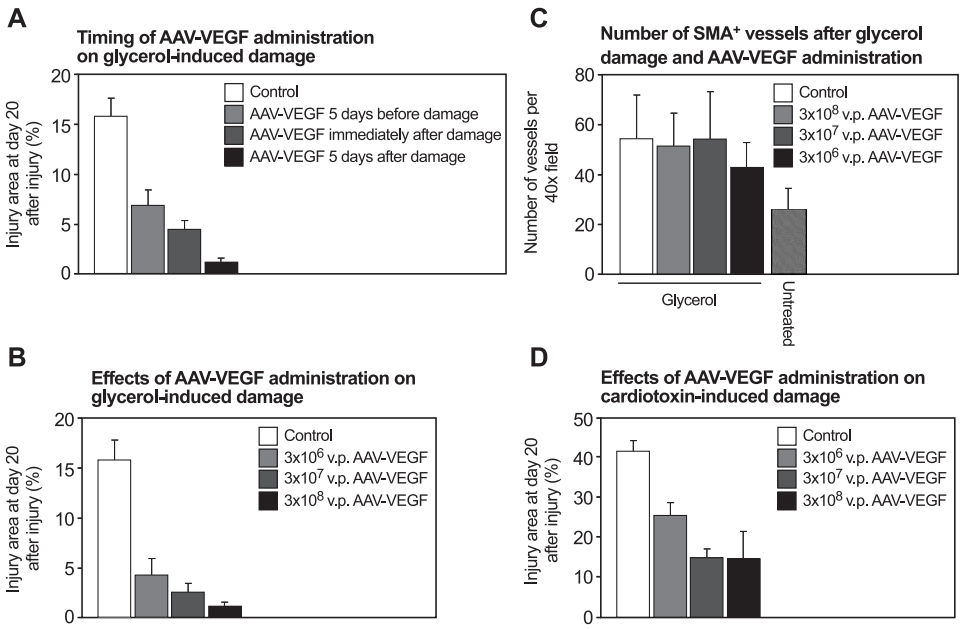

FIG. 3. Quantification of the VEGF protective effects on glycerol- and cardiotoxin-induced muscle damage. (A) Quantification of injured areas in digital images of muscle sections treated with AAV-VEGF at different time points after glycerol damage. Doses of 3×10^8 viral particles were administered 5 days before, immediately after, or 5 days after injury; analysis of the injured area was performed 20 days after injury. The results of these experiments clearly show that the maximum efficacy was obtained by injecting the vector 5 days after injury ($P < 0.01$ for the comparison between the most effective treatment with any of the other two). (B) Quantification of injured areas in regenerating muscles at 20 days after glycerol injury. The injection of increasing doses of AAV-VEGF at day 5 after injury (from 3×10^6 to 3×10^8 viral particles) dramatically improved the regeneration process in a dose–response manner ($P < 0.001$ for all doses). (C) Quantification of α-SMA-positive blood vessels in glycerol-injured muscles. Animals (5 per group) were injured with glycerol and 5 days later injected with PBS or AAV-LacZ (control groups) or with increasing doses of AAV-VEGF165. Animals were then sacrificed 20 days after muscle injury and muscle sections were immunostained for α-SMA. The histograms show the means and SD of the numbers of vessels per microscopic field. The column on the right (Untreated) shows the number of vessels in normal, untreated muscles for comparison. (D) Quantification of injured areas in control and AAV-VEGF-treated muscle sections at 20 days after cardiotoxin-induced damage, showing dose–response reduction of the damaged areas after injection of AAV-VEGF ($P < 0.001$ for all vector doses).

3A). Maximum efficacy was obtained by injecting the vector 5 days after injury (damage in <1% of the transversal muscle section area, compared with ~4 and ~7% for the simultaneous injection and the injection before injury, respectively ($P < 0.01$ for the comparison between the most effective treatment with any of the other two; Fig. 3A)). The observation that the highest efficacy of AAV-VEGF administration is obtained when the vector is injected after the damage is consistent with the notion that VEGF might affect myogenesis directly in addition to its well-established proangiogenic role.

We proceeded to investigate the effects on muscle regeneration of different doses of AAV-VEGF (from 3×10^6 to 3×10^8 viral particles) injected 5 days after glycerol-induced damage. A remarkable dose-dependent response was observed. The degenerated area was ~4% of the muscle section with the lowest AAV-VEGF amount, ~3% with the intermediate amount, and less than 1% with the highest amount ($P < 0.001$ for all doses; Fig. 3B shows quantification and Fig. 4 shows histology). In the last group of animals, the entire damaged region was replaced by regenerating muscle fibers showing a central nucleus (Fig. 4D). In the same group of animals, we also counted the number of blood vessels after immunostaining with an antibody against smooth muscle α-actin. We found that, in all the injured animals, the number of arteriolae was slightly increased (~2.5 times) compared to normal muscles, irrespective of the dose of injected vectors; this small increase is most likely due to the inflammatory response after muscle injury (Fig. 3C). This result is again consistent with the notion that the effect on VEGF on muscle regeneration is not mediated by its angiogenic activity.

Finally, we injected the same scalar doses of AAV-VEGF into the tibialis anterior muscles of mice that were treated, 5 days in advance, with 1 mM cardiotoxin, a powerful inducer of muscle fiber degeneration. The damage induced by this treatment is more severe than that obtained with glycerol, with broader fiber degeneration and extensive infiltration of inflammatory cells (shown at day 20 after damage in Fig. 5A); in the absence of treatment, muscle fiber recovery is complete after ~40

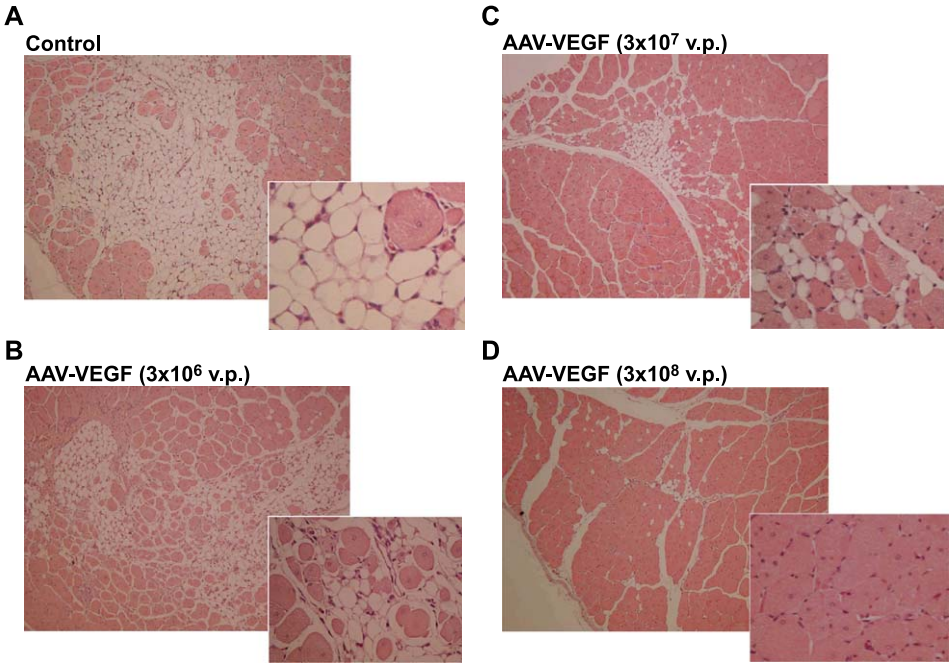

FIG. 4. Histological sections of glycerol-damaged muscles after AAV-VEGF treatment. (A) Hematoxylin and eosin-stained muscle section at day 20 after glycerol injury. The muscle was injected with PBS 5 days after damage. The inset shows a 400× original magnification of the injured area, showing massive fiber loss. (B–D) Glycerol-injured muscles were injected with different doses of AAV-VEGF (from 3×10^6 to 3×10^8 viral particles) at day 5 after injury; animals were sacrificed at day 20 after injury. The marked improvement in muscle regeneration correlates with the number of administered vector particles.

days (not shown). Treatment with AAV-VEGF resulted in a remarkable improvement in the regeneration process. At day 20 after injury, with the lowest dose (3 × 106 viral particles), the damaged area encompassed ~25% of the transversal muscle section area, compared with >40% of the control ($P < 0.01$). The size of the injured area was lower still with the two higher AAV doses (~13% for both doses; Fig. 3D shows quantification and Figs. 5A–5D show histology). Recovery was almost complete at day 20 after injury with the use of even higher doses of AAV-VEGF (9×10^{11} viral particles; data not shown).

The *in Vivo* Activity of VEGF on Muscle Fiber Regeneration is Mediated by VEGFR-2

The finding that VEGF exerts a powerful role on muscle fiber regeneration *in vivo* implies that the skeletal myoblasts and the regenerating fibers express one or more VEGF receptors, as observed on the differentiating

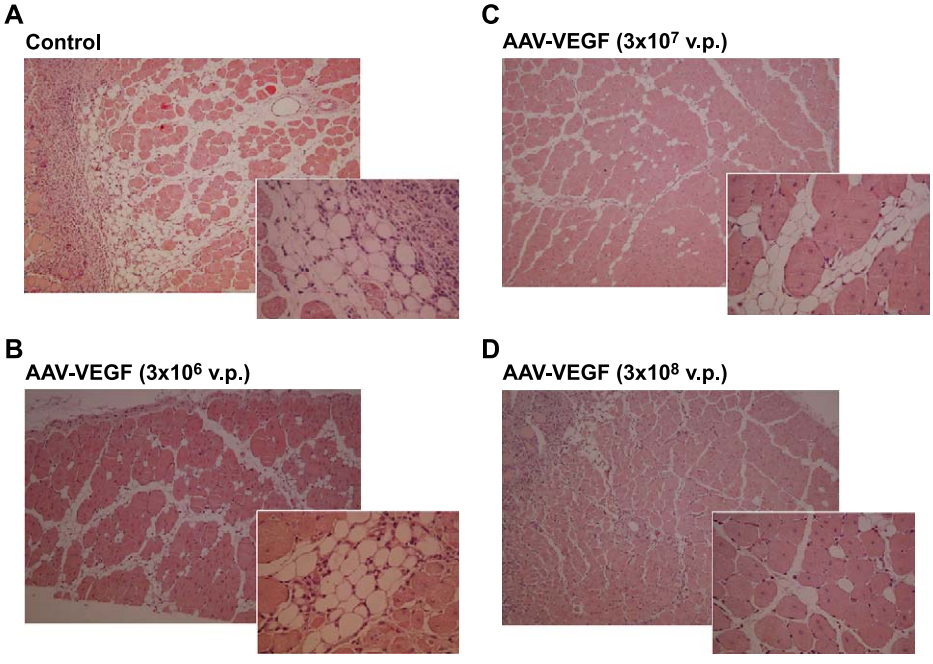

FIG. 5. AAV-VEGF promotes recovery of muscle injury after cardiotoxin injection. (A–D) The tibialis anterior muscles of mice were injected with 25 μl of 1 mM cardiotoxin. Escalating doses of AAV-VEGF (3×10^6, 3×10^7, 3×10^8 viral particles) were injected into damaged muscles at 5 days after cardiotoxin injection. Hematoxylin and eosin-stained muscle sections were examined 20 days after injury. A remarkably faster, dose-dependent regeneration was observed in the AAV- VEGF-injected animals (B to D) compared to controls (A).

doi:10.1016/j.ymthe.2004.08.007

C2C12 and primary myoblast cultures *in vitro*. We therefore investigated the presence of VEGFR-1 and VEGFR-2 in normal mouse skeletal muscle as well as at different time points after glycerol-induced damage.

Normal muscle fibers did not express levels of VEGFR-1 or VEGFR-2 detectable by immunohistochemistry; in contrast, injury resulted in a marked increase in the presence of these receptors (Fig. 6). In particular, both receptors were highly expressed by elongated cells surrounding the newly formed fibers—identifiable by the presence of a central nucleus—with a half-moon appearance resembling that of activated satellite cells at the edge of the regenerating fibers. Expression was detectable early after injury and persisted until the late stages of the regenerative process. In addition, we also found highly expressed VEGFR-2 on the surface of mature muscle fibers at early time points after injury (shown at day 7 after injury in Fig. 6).

Which receptor mediates the muscle regenerative effect of VEGF? To address this question, we constructed an AAV vector expressing the mouse placental growth factor (PlGF), a member of the VEGF family that specifically targets VEGFR-1 [25,26]. We assessed expression of PlGF from this vector by Western blotting following the transduction of CHO cells in culture (not shown). We injected different doses of AAV-PlGF into the tibialis anterior muscles of mice 5 days after glycerol-induced damage, similar to the experiments performed with AAV-VEGF. However, in contrast with AAV-VEGF, we could detect no effect on the extent of the damaged area. Even one dose of 1.2×10^{11} AAV-PlGF viral particles, which is 400 times higher than the dose of AAV-VEGF that reduced muscle damage to <1% (Fig. 3B), only marginally affected the area of muscle degeneration (Fig. 7). This result strongly suggests that the effect of VEGF on

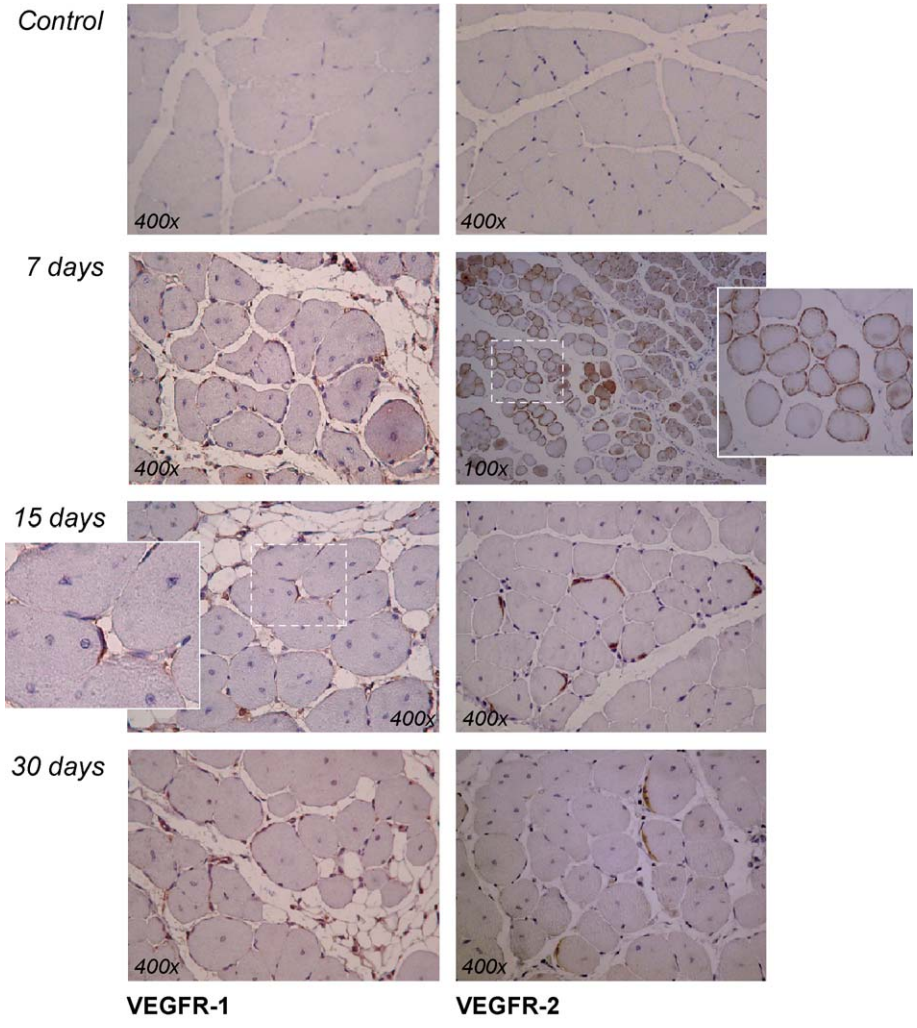

FIG. 6. VEGFR-1 and VEGFR-2 are expressed in regenerating muscles. Immunohistochemistry for VEGFR-1 and VEGFR-2 on untreated and glycerol-damaged muscle sections. Regenerating muscle showed robust expression of both receptors in elongated cells surrounding muscle fibers and resembling satellite cells. Expression of the receptors was detectable early after injury (7 days) and persisted until the late stages of the regenerative process (30 days). In addition, VEGFR-2 was also highly expressed on the surface of mature muscle fibers at the earlier days after injury (shown at day 7 after injury). In contrast, neither VEGFR-1 nor VEGFR-2 was detectable in normal muscle sections (control).

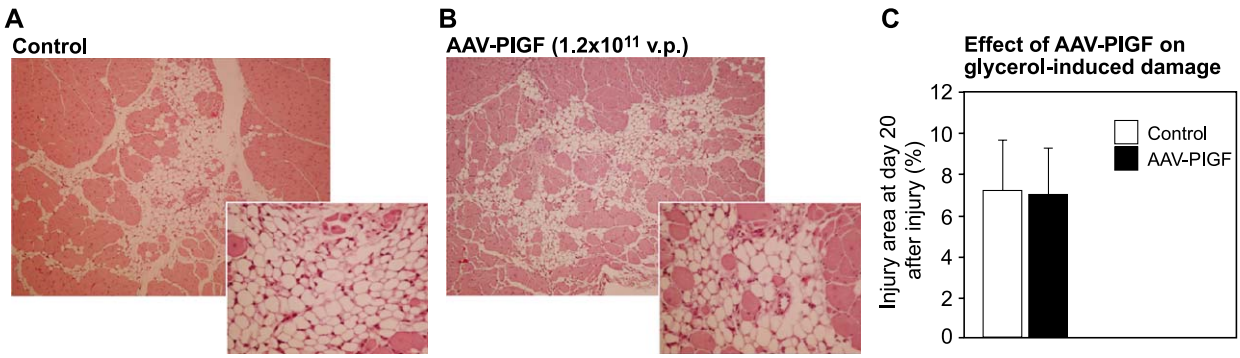

FIG. 7. Effects of AAV-PlGF on muscle regeneration after glycerol-induced damage. (A, B) Hematoxylin and eosin-stained histological sections of muscles injected with either PBS (control) or 1.2×10^{11} AAV-PlGF particles 5 days after glycerol-induced muscle damage. Samples were harvested 20 days after injury. (C) Quantification of the injured muscle areas in digital images of muscle sections from control and AAV-PlGF-treated muscles. AAV-mediated overexpression of mouse PlGF, which interacts only with VEGFR-1, did not exert any protective or proregenerative effect on the damaged muscle tissue.

muscle regeneration is mediated by its interaction with VEGFR-2.

Discussion

The results presented in this paper indicate that VEGF possesses a novel biological role in stimulating skeletal muscle fiber regeneration *in vivo*. The direct effect of VEGF on myogenic cells is supported by a number of observations. *In vitro,* hrVEGF promotes the fusion of myogenic cells to form myotubes and protects these cells from apoptotic cell death; differentiating myocytes and myotubes express high levels of both VEGFR-1 and VEGFR-2, as detected by both immunocytochemistry and immunofluorescence. *In vivo,* the long-term expression of VEGF using AAV vectors under normal conditions promotes the appearance of a regenerating muscle phenotype in the injected areas, with several muscle fibers containing a central nucleus and a much broader muscle fiber size distribution, including small (regenerating) and enlarged (hypertrophic) fibers. Under conditions of ischemic or chemical damage (the latter obtained by glycerol or cardiotoxin), the muscle satellite cells express high levels of both VEGFR-1 and VEGFR-2. Finally, and perhaps most strikingly, the delivery of VEGF determines a dramatic decrease in the size of the damaged area and in the time required for complete regeneration.

Other studies in the past have addressed the effects of VEGF overexpression in normoperfused skeletal muscle, merely indicating a strong angiogenic effect of the factor. In particular, the implantation of genetically modified myoblasts into nonischemic muscle caused an accumulation of endothelial cells and macrophages, followed by networks of vascular channels and hemangiomas [27,28]. The pattern of VEGF expression using AAV vectors is clearly different. The production of the factor progressively increases over time, while its expression persists for longer periods at levels that are probably lower than those obtained by genetically modified myoblasts. These properties might favor the direct effect of the factor on the muscle fibers.

The effects of VEGF on the promotion of muscle recovery after damage are likely to be exerted by different mechanisms. The factor has a very well known proangiogenic activity; in addition, our results show that it also prevents apoptosis and promotes muscle fiber growth. These three effects most likely cooperate in improving recovery after muscle damage. An additional possibility is that VEGF, by mobilizing bone marrow progenitor cells [29,30], might favor muscle regeneration through transdifferentiation or fusion of these cells [31,32], although recent evidence argues against this event [33,34].

Interestingly, the regenerative effects of AAV-VEGF injection are greater when the vector is injected 5 days after damage, an observation that indicates that the major therapeutic role of VEGF has to be attributed to its direct activity on the myofiber regeneration. This result is consistent with the high levels of expression of the VEGF receptors on muscle satellite cells and muscle fibers at this time point after damage ([22] and Fig. 5 in this work). In this respect, it is also worth noticing that the efficiency of AAV vector transduction is highly increased after muscle damage, with a decreased time lapse before the onset of transgene expression [35].

Muscle regeneration is a complex biological process. After damage, satellite cells, which are normally in the G0 phase of the cell cycle, reenter the cell cycle and proliferate, thus providing a sufficient number of cells necessary for repair. This proliferative phase ends with the appearance of the first small regenerating myotubes at approximately 3 days after injury [36,37]. At this time point, part of the proliferated cells become quiescent again, while the remaining ones start to fuse to form multinucleated myotubes (terminal differentiation), an event that is followed by the maturation of these myotubes into muscle fibers (biochemical differentiation) [38–40]. Concomitant with these events is the process of macrophage accumulation and the disman-

doi:10.1016/j.ymthe.2004.08.007

tling of the damaged fibers. The results obtained by studying the effects of hrVEGF on myotube formation *in vitro* indicate that this most likely stimulates the terminal differentiation phase, by increasing maturation of the multinucleated myofibers. This effect is most possibly achieved by promoting the fusion of the differentiated, MHC-positive myocytes to form myofibers that are longer and contain more nuclei.

To identify the receptor that mediates these effects of VEGF on myogenic cells one must consider that both VEGFR-1 and VEGFR-2 are upregulated in the differentiating myogenic cells, in myotubes *in vitro,* and in satellite cells *in vivo* after muscle damage. In cultured myoblasts and C2C12 cells, treatment with SU1498, an inhibitor of the VEGFR-2 protein kinase, abolishes the protective effect of VEGF on camptothecin-induced cell apoptosis, suggesting the participation of VEGFR-2 in mediating the VEGF signal. Most strikingly, *in vivo* overexpression of PlGF, an agonist of the VEGFR-1 but not of the VEGFR-2, was not able to promote muscle regeneration after damage, not even at very high doses of vector. These results clearly point to the VEGFR-2 as the main mediator of the effect of VEGF on myogenic cells. Elucidation of the biochemical pathway triggered after VEGFR-2 activation in these cells clearly requires further investigation. In this respect, however, it is worth noticing that at least two signaling pathways that are important for muscle survival and regeneration are known to be set in motion by the activation of the VEGFR-2 in endothelial cells, namely the PI3K/Akt and the MAP kinase pathways. The activation of Akt signaling in the muscle cells is important to inhibit apoptosis during differentiation [41,42] and to control the myofiber size [43,44]. In endothelial cells, Akt signaling after activation of the VEGFR-2 by VEGF is crucial for endothelial cell survival [16]. Intriguingly, insulin growth factor-1, a powerful promoter of muscle regeneration that stimulates muscle differentiation through Akt [45], also increases VEGF synthesis in C2C12 cells [46], indirectly suggesting the involvement of VEGF in the regeneration process. Accordingly, muscle fibers transduced by a constitutively active Akt formed *in vivo* also produce increased levels of VEGF and show signs of muscle hypertrophy [46]. Another important biochemical pathway in muscle cell differentiation involves MAP kinase signaling, which leads to the increased expression and activity of the MyoD protein [47]. The same pathway is also known to be activated in endothelial cells by the interaction of VEGF with VEGFR-2.

The observation that VEGF promotes muscle fiber regeneration when delivered a few days after muscle damage opens the way to possible, important therapeutic applications in the treatment of acute and chronic muscular diseases of different origins, including traumatic injury (in which the regenerative process might be accelerated) or inherited muscular dystrophies (in which a sustained stimulus for muscle regeneration might prove beneficial). In this respect, we wish to point out that the use of vectors based on AAV offers an important possibility to maintain sustained expression of the VEGF gene over prolonged periods of time in the absence of inflammation or vector-induced immune response, a property that might prove advantageous in several clinical applications.

On a final note, for a long time VEGF has been considered an endothelial-specific growth factor that promotes a powerful angiogenic response. The observation that VEGF also induces myofiber regeneration in the skeletal muscle now extends the recent evidence that challenges this notion. Beyond angiogenesis, the interaction of VEGF with its receptors is important in maintaining survival and promoting differentiation of cells with a progenitor phenotype in a broad array of different tissues, including bone marrow, bone, the central nervous system, and skeletal muscle. These observations pave the way to the possible exploitation of VEGF gene transfer under varying conditions of damage in adult tissues to provide cellular protection and tissue regeneration.

MATERIALS AND METHODS

Cell Culture and Reagents

Primary myoblast cultures were prepared from newborn CD1 mice (2–7 days of age) according to [48]. Myoblasts were plated on gelatin-coated flasks and cultured in proliferation medium (DMEM, 10% fetal bovine serum, 10% horse serum, 0.5% chick embryo extract, 1% penicillin–streptomycin and 1‰ amphotericin B, 4.5 g glucose/liter). Differentiation was induced by switching the myoblast cultures to a low-serum differentiation medium (DMEM, 0.4% UltraserG (BioSepra Sa, France), 4.5 g glucose/liter).

C2C12 myogenic cells were maintained in high glucose DMEM plus 10% fetal bovine serum and induced to form myotubes by 3–4 days culture in differentiation medium. When indicated, hrVEGF165 (R&D Systems) was added twice a day to a final concentration of 100 ng/ml.

Production, Purification, and Characterization of rAAV Vectors

The rAAV vectors used in this study were prepared as already described [21,49].

Animals and Experimental Protocols

Animal care and treatments were conducted in conformity with institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, December 12, 1987). All experiments were performed in male CD1 mice, 4–6 weeks of age.

Animal model of hind-limb ischemia and muscle injury. Unilateral hind-limb ischemia was induced by resecting a 2.5-cm segment of the left femoral artery. The lower leg muscles were harvested 20 days after the induction of ischemia and their viability was tested by staining with tetrazolium red (2,3,5-triphenyltetrazolium chloride; Sigma), an indicator of enzymatic redox reactions. Injury in the tibialis anterior muscle was induced by injecting 25 μl of 1 mM cardiotoxin (Sigma) or 25 μl of 50% v/v glycerol in two injection sites.

Intramuscular administration of rAAV vectors. In the hind-limb ischemia mouse model, the recombinant vector solution (100 μl; 3 × 1011 AAV-VEGF vector particles) was injected into the tibialis anterior,

adductor, and gastrocnemius muscles (one, two, and one injection per muscle, respectively).

The normoperfused muscles or the muscles treated with glycerol and cardiotoxin were injected with different doses of AAV-VEGF or AAV-PlGF in a total volume of 25 μl and in two distinct injection sites. Muscles were harvested 20 days after damage, fixed in 2% formaldehyde, and embedded in paraffin. Control animals were injected with either PBS or 3×10^{11} AAV-LacZ. All the experiments were performed in groups including four to six animals.

Immunostaining and Histological Evaluation

Staining of 2-μm muscle histological sections was performed as described [21]. Myogenic cells (C2C12, myoblasts) cultured on multichamber slides (Nalge Nunc International) were fixed in 2% paraformaldehyde followed by permeabilization in 0.1% Triton X-100 for immunofluorescence or fixed in cold (−20°C) methanol for immunocytochemistry. Monoclonal anti-mouse Flk-1 (Santa Cruz Biotechnology, sc-6251) and rabbit polyclonal anti-Flt-1 (Santa Cruz Biotechnology, sc-316) antibodies were used to detect VEGF receptors on cultured cells and muscle tissue samples. A monoclonal anti-mouse skeletal slow MHC antibody (Sigma, clone NOQ7.5.4D) was used to visualize differentiating myocytes and myofibers. A secondary antibody conjugated with a green fluorochrome (Alexa 449, Jackson ImmunoResearch Laboratories) was used for immunofluorescence. The procedures for immunohistochemistry were undertaken according to the Vectastain Universal ABC kit and MOM Kit (Vector Laboratories). Signals were developed using 3,3′-diaminobenzidine as the substrate for the peroxidase chromogenic reaction (Lab Vision Corp., Fremont, CA, USA).

Apoptosis and MTT Assays

To study apoptosis, subconfluent myogenic cells were serum-starved and then exposed for 4 days at 12-h intervals to hrVEGF 165 (50 ng/ml). The VEGFR-2 inhibitor SU1498 (5 μM; Calbiochem) was added every 12 h from day 2 to day 4; camptothecin (50 nM for C2C12 cells and 50 μM for primary myoblasts; Calbiochem) was added on day 4, 5 h before the cells were harvested. Both reagents were diluted in DMSO. Detection of apoptotic cells was performed by detecting annexin V expression on a flow cytometer (FACSCalibur; BD) using the Annexin- V-FLUOS kit (Roche), using propidium iodide to distinguish apoptosis from necrosis.

The effect of VEGF on myogenic cell proliferation was assessed by plating cells in 96-well plates (5000 cells/well). After overnight incubation, the medium was replaced by fresh proliferation or differentiation medium containing different concentrations of hrVEGF. After 3 days incubation, proliferation was assessed using the MTT conversion kit (Boehringer) according to the manufacturer's instructions.

Quantification of Myotube Length and Ploidy

Myotube differentiation was induced in C2C12 cells in the presence or absence of hrVEGF165. After staining of slow MHC by immunofluorescence, digital images of the cell cultures were acquired with an Axiovert 100M confocal laser-scanning microscope (Zeiss LSM 510). Ten fields from four independent experiments (10× objective) were randomly taken for each treatment. The length of all positive slow MHC fibers was analyzed using the measurement tools of the LSM510 2.02 software. The quantification of the number of nuclei per MHC-positive fiber was performed by three independent investigators blinded to experimental procedures.

Quantification of Glycerol and Cardiotoxin Muscle Injury areas

To quantify glycerol-induced injury, microphotographs of histological samples of transversal sections of the tibialis anterior muscle were examined in a blinded fashion by three different examiners. Quantification was performed on histological sections from the upper, middle, and lower regions of the tibialis anterior muscle (three sections per region; n = 4 animals per group). The areas of injury, identified by the absence of myofibers, were quantified using the ImageJ software (NIH Software) and expressed as the percentage ± SD of the total cross-sectional area of the tissue section.

Real-Time PCR

The expression of hVEGF in the transduced muscles was assessed by real-time quantification using the TaqMan technology. Briefly, total RNA was extracted from the AAV-VEGF-injected tibialis anterior muscles of 16 animals at 3, 7, 14, and 28 days after injection (four animals per group). RNA (4 μg) was reverse-transcribed and subjected to quantification using an Applied Biosystems Assay-on-Demand for human VEGF. All values are expressed as number of molecules using an external calibration curve. The same procedure was also used for the quantification of hVEGF in the tibialis anterior of the uninjected, contralateral leg; in this case, the levels of hVEGF mRNA were below the levels of detection after at least 40 PCR cycles. Amplifications were carried out in an ABI Prism 7000 Sequence Detection System.

Statistical Analysis

Statistical comparison between treated and control groups was performed by the two-tailed Student t test on paired samples; the non-parametric Kolmogorov–Smirnov test was used to compare continuous distributions. All analyses were performed using the StatView 4.5 statistical software package for the Macintosh (Abacus Concepts, Berkeley, CA, USA).

ACKNOWLEDGMENTS

This work was supported by grants from the Progetto Finalizzato "Genetica Molecolare" of the Consiglio Nazionale delle Ricerche, Italy; from the FIRB program of the Ministero dell'Istruzione, Universita' e Ricerca, Italy; and from the Fondazione Cassa di Risparmio of Trieste, Italy. We are very grateful to Barbara Boziglav and Mauro Sturnega for excellent technical support and to Suzanne Kerbavcic for editorial assistance.

RECEIVED FOR PUBLICATION MARCH 14, 2004; ACCEPTED AUGUST 9, 2004.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2004.08.007.

REFERENCES

1. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J., and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* **407:** 242–248.
2. Gale, N. W., and Yancopoulos, G. D. (1999). Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* **13:** 1055–1066.
3. Ferrara, N., Gerber, H. P., and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat. Med.* **9:** 669–676.
4. Lambrechts, D., *et al.* (2003). VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat. Genet.* **34:** 383–394.
5. Jin, K., Zhu, Y., Sun, Y., Mao, X. O., Xie, L., and Greenberg, D. A. (2002). Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **99:** 11946–11950.
6. Jin, K. L., Mao, X. O., and Greenberg, D. A. (2000). Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc. Natl. Acad. Sci. USA* **97:** 10242–10247.
7. Schratzberger, P., *et al.* (2000). Favorable effect of VEGF gene transfer on ischemic peripheral neuropathy. *Nat. Med.* **6:** 405–413.
8. LeCouter, J., *et al.* (2003). Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. *Science* **299:** 890–893.
9. Zelzer, E., *et al.* (2002). Skeletal defects in VEGF(120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* **129:** 1893–1904.
10. Deckers, M. M., Karperien, M., van der Bent, C., Yamashita, T., Papapoulos, S. E., and Lowik, C. W. (2000). Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology* **141:** 1667–1674.
11. Mayr-Wohlfart, U., *et al.* (2002). Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* **30:** 472–477.

doi:10.1016/j.ymthe.2004.08.007

12. Street, J., *et al.* (2002). Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc. Natl. Acad. Sci. USA* **99:** 9656–9661.
13. Ishida, A., *et al.* (2001). Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J. Cell. Physiol.* **188:** 359–368.
14. Wang, Z., Castresana, M. R., and Newman, W. H. (2001). Reactive oxygen and NF-kappaB in VEGF-induced migration of human vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **285:** 669–674.
15. Barleon, B., Sozzani, S., Zhou, D., Weich, H. A., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* **87:** 3336–33343.
16. Gerber, H. P., *et al.* (2002). VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature* **417:** 954–958.
17. Lyden, D., *et al.* (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Med.* **7:** 1194–1201.
18. Snyder, R. O., *et al.* (1997). Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum. Gene Ther.* **8:** 1891–1900.
19. Bouchard, S., *et al.* (2003). Long-term transgene expression in cardiac and skeletal muscle following fetal administration of adenoviral or adeno-associated viral vectors in mice. *J. Gene Med.* **5:** 941–950.
20. Chirmule, N., Propert, K., Magosin, S., Qian, Y., Qian, R., and Wilson, J. (1999). Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther.* **6:** 1574–1583.
21. Arsic, N., *et al.* (2003). Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. *Mol. Ther.* **7:** 450–459.
22. Rissanen, T. T., *et al.* (2002). Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in ischemic skeletal muscle and its regeneration. *Am. J. Pathol.* **160:** 1393–1403.
23. Germani, A., *et al.* (2003). Vascular endothelial growth factor modulates skeletal myoblast function. *Am. J. Pathol.* **163:** 1417–1428.
24. Fisher, K. J., *et al.* (1997). Recombinant adeno-associated virus for muscle directed gene therapy. *Nat. Med.* **3:** 306–312.
25. Autiero, M., *et al.* (2003). Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat. Med.* **9:** 936–943.
26. Carmeliet, P., *et al.* (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.* **7:** 575–583.
27. Springer, M. L., *et al.* (2003). Localized arteriole formation directly adjacent to the site of VEGF-induced angiogenesis in muscle. *Mol. Ther.* **7:** 441–449.
28. Springer, M. L., Chen, A. S., Kraft, P. E., Bednarski, M., and Blau, H. M. (1998). VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell* **2:** 549–558.
29. Hattori, K., *et al.* (2001). Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J. Exp. Med.* **193:** 1005–1014.
30. Asahara, T., *et al.* (1999). VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18:** 3964–3972.
31. Gussoni, E., *et al.* (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401:** 390–394.
32. LaBarge, M. A., and Blau, H. M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111:** 589–601.
33. Balsam, L. B., Wagers, A. J., Christensen, J. L., Kofidis, T., Weissman, I. L., and Robbins, R. C. (2004). Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* **428:** 668–673.
34. Murry, C. E., *et al.* (2004). Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* **428:** 664–668.
35. Abadie, J., Blouin, V., Guigand, L., Wyers, M., and Cherel, Y. (2002). Recombinant adeno-associated virus type 2 mediates highly efficient gene transfer in regenerating rat skeletal muscle. *Gene Ther.* **9:** 1037–1043.
36. Yan, Z., *et al.* (2003). Highly coordinated gene regulation in mouse skeletal muscle regeneration. *J. Biol. Chem.* **278:** 8826–8836.
37. Kawai, H., Nishino, H., Kusaka, K., Naruo, T., Tamaki, Y., and Iwasa, M. (1990). Experimental glycerol myopathy: a histological study. *Acta Neuropathol. (Berlin)* **80:** 192–197.
38. Nadal-Ginard, B. (1978). Commitment, fusion and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* **15:** 855–864.
39. Endo, T., and Nadal-Ginard, B. (1987). Three types of muscle-specific gene expression in fusion-blocked rat skeletal muscle cells: translational control in EGTA-treated cells. *Cell* **49:** 515–526.
40. Stockdale, F. E. (1992). Eclipse of the identifiable myoblast. *Symp. Soc. Exp. Biol.* **46:** 1–7.
41. Fujio, Y., Guo, K., Mano, T., Mitsuuchi, Y., Testa, J. R., and Walsh, K. (1999). Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Mol. Cell Biol.* **19:** 5073–5082.
42. Alessi, D. R., *et al.* (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15:** 6541–6551.
43. Bodine, S. C., *et al.* (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* **3:** 1014–1019.
44. Rommel, C., *et al.* (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* **3:** 1009–1013.
45. Tureckova, J., Wilson, E. M., Cappalonga, J. L., and Rotwein, P. (2001). Insulin-like growth factor-mediated muscle differentiation: collaboration between phosphatidylinositol 3-kinase-Akt-signaling pathways and myogenin. *J. Biol. Chem.* **276:** 39264–39270.
46. Takahashi, A., *et al.* (2002). Myogenic Akt signaling regulates blood vessel recruitment during myofiber growth. *Mol. Cell. Biol.* **22:** 4803–4814.
47. Gredinger, E., Gerber, A. N., Tamir, Y., Tapscott, S. J., and Bengal, E. (1998). Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. *J. Biol. Chem.* **273:** 10436–11044.
48. Rando, T. A., and Blau, H. M. (1994). Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* **125:** 1275–1287.
49. Deodato, B., *et al.* (2002). Recombinant AAV vector encoding human VEGF165 enhances wound healing. *Gene Ther.* **9:** 777–785.