Adeno-Associated Virus-Mediated Vascular Endothelial Growth Factor Gene Therapy in Skeletal Muscle before Transplantation Promotes Revascularization of Regenerating Muscle

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ABSTRACT

Successful clinical transplantation of whole skeletal muscles can be limited by impaired muscle revascularization and regeneration. The aim of this study was to enhance the revascularization (and hence speed of regeneration) of transplanted whole muscles by transducing muscles with the vascular endothelial growth factor (VEGF) gene before transplantation, using a recombinant adeno-associated virus (rAAV). The rAAV encoding VEGF and green fluorescent protein (GFP) (rAAV.VEGF.GFP) was injected into the tibialis anterior muscles of adult BALB/c mice. One month after injection whole muscle autotransplantation was performed. Muscles were sampled 7 days after autografting. GFP expression was examined as an indicator of persistent transgene expression after grafting, and immunohistochemistry was used to identify VEGF, blood vessels, and newly formed myotubes. After grafting, GFP expression persisted only in a few surviving myofibers in the periphery of rAAV.VEGF.GFP-pretreated muscles, although abundant VEGF expression was seen in myogenic cells in all grafted muscles. Quantitative analysis demonstrated that, although only small numbers of rAAV.VEGF.GFP-transduced myofibers were present, whole muscle grafts preinjected with rAAV.VEGF.GFP were significantly more vascular than saline-injected and uninjected control muscle grafts. Furthermore, rAAV.VEGF.GFP-injected whole muscle transplants were further advanced in terms of regeneration (myotube formation) compared with the uninjected control muscle transplants. This study clearly shows that rAAV-mediated VEGF expression persists only in myofibers that survive the necrosis induced by muscle transplantation; however, this amount of VEGF results in significantly increased revascularization and regeneration of whole muscle transplants.

INTRODUCTION

Clinical whole muscle transplantation is used most commonly to correct facial palsy; however, the speed of regeneration of the transplanted muscles can limit the success of this technique (reviewed in Guelinckx and Sinsel1). It is well established that successful skeletal muscle regeneration is dependent on the ingrowth of new blood vessels, or revascularization (reviewed in Roberts and McGeachie2). Whereas rodent muscles regenerate extremely well after a major injury or whole muscle grafting,3-7 human muscles that are transplanted for therapeutic reasons or are severely injured are much larger than in rodents and therefore more prone to isch-

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emis. Nutrients cannot diffuse effectively throughout the large muscle mass to support surviving muscle precursor cells, and the speed of revascularization and consequent new muscle formation is too slow to prevent extensive fibrosis in the central region of the injured or transplanted muscle.\textsuperscript{5,8} It has been proposed that administration of agents that enhance regeneration to injured muscles will enable larger muscles to undergo more rapid and efficient repair.\textsuperscript{9} In the present study we investigate preloading of muscles to be transplanted with the angiogenic agent vascular endothelial growth factor (VEGF), delivered via an adenov-associated viral vector. It is proposed that the VEGF will promote more rapid revascularization and consequently enhance the speed and efficiency of regeneration in transplanted skeletal muscles.

VEGF is an extremely potent angiogenic growth factor associated with vasculogenesis during embryonic development, and several postnatal processes in both normal and abnormal tissues (reviewed in Refs. 10–12). It has been suggested that manipulation of angiogenic growth factor expression can be used clinically to promote revascularization in ischemic tissues, or to inhibit blood vessel growth in "angiogenic" diseases (i.e., tumor growth and diabetic retinopathy) (reviewed in Ferrara and Alitalo\textsuperscript{13}). Several studies have demonstrated that VEGF gene therapy promotes collateral blood vessel formation in ischemic skeletal muscles,\textsuperscript{14–18} and one study demonstrated revascularization of ischemic heart muscle after adenov-associated virus-mediated VEGF injection.\textsuperscript{19} However, previous studies have not examined the possibility of pretreating normal muscles with angiogenic growth factors, in preparation for enhanced revascularization, to accelerate regeneration after transplantation. In the present study we first transduced normal skeletal muscles with a recombinant aden-associated virus (rAAV) encoding the green fluorescent protein (GFP) reporter gene\textsuperscript{20,21} before whole muscle transplantation, to determine whether rAAV-mediated gene transfer persists after the transplanted ischemic muscle has undergone massive necrosis. Second, we used the same viral vector encoding the murine VEGF\textsubscript{165} gene, an isoform of VEGF (reviewed in Refs. 10–12), and encoding GFP (rAAV.VEGF.GFP) to transduce muscles 1 month before transplantation. At 7 days after transplantation grafted muscles were sampled and examined for expression of (1) GFP, (2) VEGF,\textsuperscript{22} (3) platelet endothelial cell adhesion molecule 1 (PECAM-1) on newly formed blood vessels,\textsuperscript{23,24} and (4) desmin in activated myoblasts and newly formed myotubes.\textsuperscript{5,7,25–27} The observed significantly enhanced revascularization and regeneration in rAAV.VEGF.GFP-injected muscles strongly supports the principle of using preloading with VEGF to prepare muscles for clinical transplantation.

MATERIALS AND METHODS

Construction and in vitro analysis of recombinant AAV.VEGF.GFP

The construction of recombinant plasmid SSV.GFP and rAAV.GFP has previously been described.\textsuperscript{28} A cassette containing the cytomegalovirus (CMV) promoter and murine VEGF\textsubscript{165} was subcloned into recombinant plasmid SSV.GFP in place of the neo cassette. The resultant recombinant plasmid, SSV.VEGF.GFP, with the VEGF and GFP genes controlled by separate CMV promoters, was used for the generation of rAAV.VEGF.GFP according to the method previously described.\textsuperscript{28} The titer of rAAV.VEGF.GFP was determined by counting fluorescent cells among transduced 293 cells at 24 h post-transduction and the titer was expressed as transducing units (TU). Recombinant adenovirus (rAd) for cotransduction was also used in some experiments to enhance rAAV transduction and second-strand DNA\textsuperscript{29} synthesis. The rAd was prepared by transfection of 293 cells with adenovirus plasmid pFG140 (Microbix, Ontario, ON, Canada).\textsuperscript{28}

rAAV-mediated expression of VEGF was evaluated by Western blot analysis, using a commercial antibody, SC507 (Santa Cruz Biotechnology, Santa Cruz, CA). 293 cells were transduced with rAAV.VEGF.GFP and at 48 h post-transduction the supernatant from transduced 293 cells was harvested. A total of 40 μL of heparin-Sepharose beads (Sigma, St. Louis, MO), resuspended at 20 mg/mL in phosphate-buffered saline (PBS), was added to 1.5 mL of the supernatant. After a 4-h incubation at 4°C in a rotator, the beads were collected by centrifugation, washed with PBS, and then resuspended in 40 μL of loading buffer. A volume of the resuspended beads (20 μL) was electrophoresed in a 12% denaturing polyacrylamide gel and immunodetection of VEGF was performed as previously described.\textsuperscript{30}

Animals

Twenty-two female BALB/c mice (age, 6–8 weeks) were used. All mice were obtained from the Animal Resource Centre (Murdoch University, Western Australia, Australia) from certified specific pathogen-free breeding stock. Mice were housed in standard animal housing facilities at the University of Western Australia (Perth, Western Australia, Australia), and food and water were freely available at all times. All procedures were carried out under the guidelines of the National Health and Medical Research Council of Australia, and with the prior approval of the Animal Ethics and Experimentation Committee at the University of Western Australia.

In this study there were five treatment groups (A–E), and these are summarized in Table 1. Group A was used to identify the presence of the viral construct in myofibers
Table 1. Summary of Injection, Whole Muscle Graft, and Sampling Schedule\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Grafting times after pretreatment</th>
<th>Sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>rAAV.GFP injected + grafted</td>
<td>3 days (2)</td>
<td>1 month (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 month (2)</td>
<td>2 months (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 months (2)</td>
<td>+ 7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 month (4)</td>
<td>+ 7 days</td>
</tr>
<tr>
<td>B</td>
<td>rAAV.VEGF.GFP injected + grafted</td>
<td>1 month (4)</td>
<td>+ 7 days</td>
</tr>
<tr>
<td>C</td>
<td>rAAV.VEGF.GFP + rAd injected + grafted</td>
<td>1 month (4)</td>
<td>+ 7 days</td>
</tr>
<tr>
<td>D</td>
<td>Vehicle (PBS) injected + grafted</td>
<td>1 month (4)</td>
<td>+ 7 days</td>
</tr>
<tr>
<td>E</td>
<td>Uninjected + grafted</td>
<td>1 month (4)</td>
<td>+ 7 days</td>
</tr>
</tbody>
</table>

\(^a\)Numbers in parentheses represent number of mice used per time point (one TA muscle used per mouse), and the viral construct abbreviations used are detailed in Materials and Methods. In all cases injections were administered on day 0 and all grafted TA muscles were sampled 7 days after grafting.

and animals were injected with an rAAV encoding the GFP reporter gene and driven by the CMV promoter (rAAV.CMV.GFP, from this point on abbreviated as rAAV.GFP). Some injected muscles were subjected to whole muscle grafting, to determine the extent and efficiency of adult skeletal muscle transduction with the rAAV vector after the induction of necrosis and regeneration that results from transplantation. Groups B–E were used to examine the effects of transfer of the murine VEGF\(_{165}\) gene (using an rAAV encoding the GFP reporter gene) on whole muscle graft revascularization and regeneration in vivo. Group B mice were injected with rAAV.CMV.VEGF.GFP (from this point on abbreviated as rAAV.VEGF.GFP) before grafting. Group C mice were injected with rAAV.VEGF.GFP combined with a helper recombinant adenovirus (rAd) to determine whether rAd enhanced rAAV-mediated transgene transfer, because there is evidence that the presence of rAd may enhance the crucial step of rAAV DNA second-strand synthesis.\(^{31}\) Groups D and E were vehicle (PBS, pH 7.6) and uninjected controls, respectively.

Injections and whole muscle transplantation

All mice in groups A–D received a single injection into the midregion of the left tibialis anterior (TA) muscle. Mice were injected with either rAAV.GFP (Table 1, group A), rAAV.VEGF.GFP (groups B and C), or PBS (group D). Mice were anesthetized (1.5% halothane) and a small incision was made in the skin overlying the lower TA muscle on the anterior surface of the left hindlimb. A total injection volume of 25–50 μL, containing 2.5–4.5 × 10\(^{10}\) TU, was administered by inserting a 0.3-

mL insulin syringe with a 29-gauge needle in longitudinal orientation to the muscle. The plunger was depressed gently as the needle was withdrawn to minimize leakage of the solution from the muscle along the needle track. A single suture was used to close the incision.

At 3 days (group A), 1 month (groups A–E), or 2 months (group A) after injection, whole muscle transplants were performed. The whole TA muscle transplantation technique is based on a standard whole muscle grafting technique that is used extensively in muscle regeneration studies.\(^{5,7,25–27}\) The standard technique requires relocation of the extensor digitorum longus (EDL) muscle over the TA muscle. However, for the purpose of the present study it was necessary to modify this technique and graft the larger TA muscle because the EDL muscle was not easily accessible to administer a viral injection before transplantation. Briefly, mice were anesthetized (1.5% halothane) and an incision was made from the knee to the ankle in the skin overlying the TA muscle. The distal tendon of the TA muscle was located, dissected from its surrounding fascia, and severed completely. A pair of forceps was used to grasp the distal TA tendon and to carefully lift the TA muscle from its bed, dissecting away the surrounding tissue with fine tissue scissors. When the TA muscle was completely isolated to the knee, the proximal tendon was severed so that the entire muscle was free. The muscle was then replaced within its cavity and the skin sutured closed. All grafted TA muscles were sampled 7 days after autografting. At sampling, all injected and/or grafted TA muscles were bisected transversely and embedded on a cork block in gum tragacanth (Sigma) with the cut surfaces at the block face so that sections were taken through the midregion
of the TA muscle. Muscles were snap-frozen in liquid nitrogen with isopentane quenching. All muscles injected with rAAV.GFP (Table 1, group A) were serially sectioned throughout the entire muscle and examined by fluorescence microscopy for the presence of GFP-positive myofibers. Tissue sections from all other groups were used for fluorescence microscopy or immunohistochemistry.

**Immunohistochemistry**

Fresh frozen tissue sections (5 µm) of grafted muscles from groups B–E were taken and placed onto Silane (Sigma)-coated glass slides for immunohistochemistry. All sections were fixed in cold acetone, air dried, and washed in PBS. Sections were then treated with hydrogen peroxide (3% [v/v] in doubly distilled water) to block endogenous peroxidase activity, and with horse serum (10% [v/v] in PBS) to block nonspecific primary antibody binding.

Patterns of VEGF expression in the mouse muscles were examined with a rabbit anti-VEGF antibody, SC507 (Santa Cruz Biotechnology), diluted 1:100 in PBS–1% bovine serum antigen (BSA)–0.1% sodium azide, and with a biotinylated anti-rabbit secondary antibody (Jackson Laboratories, Bar Harbor, ME) diluted 1:200 in PBS–1% BSA–0.1% sodium azide. New blood vessels in revascularized autografted TA muscles were assessed by immunohistochemical localization of PECAM-1. The rat anti-PECAM-1 antibody was kindly supplied by L. Sorokin (Erlangen, Germany), and was applied at a dilution of 1:200 in PBS–1% BSA–0.1% sodium azide. Primary antibody detection was carried out with a biotinylated anti-rat secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:200 in PBS–1% BSA–0.1% sodium azide. Whole muscle graft regeneration was examined with a rabbit anti-desmin antibody (Dako, Carpinteria, CA) applied at 1:500 in PBS–1% BSA–0.1% sodium azide, followed by detection with biotinylated anti-rabbit immunoglobulins (Jackson Laboratories) diluted 1:200 in PBS–1% BSA–0.1% sodium azide.

For all tissue sections the secondary antibody was labeled with peroxidase-conjugated avidin D (Vector Laboratories), applied at a 1:200 dilution in PBS–1% BSA. The color reaction was then carried out with diamino-benzidine (Sigma). Sections were washed thoroughly, counterstained with hematoxylin, mounted with DPX (BDH Chemicals, Poole, UK), and coverslipped.

**Analysis**

For all muscle grafts in groups B–E, one section was stained for each of VEGF, PECAM-1, and desmin, whereas two nonserial sections from each sample were stained (one each for PECAM-1 and desmin). PECAM-1 and desmin analysis was also carried out for group A to determine whether the injection of the adenovirus-associated virus encoding GFP alone (in the absence of VEGF) had an effect on graft revascularization and/or regeneration. All tissue sections were placed on a standard light microscope connected to a personal computer and mounted with a video camera. Images of fields of view were taken with ImagePro Plus (Microsoft, Redmond, WA) software and an automated microscope stage movement mechanism. All fields were nonoverlapping and the entire graft area was sampled. An M168 Weibel grid22 copied onto an overhead transparency was placed over the computer screen, and the total number of points falling on vascular space (i.e., within a blood vessel lumen or on a blood vessel wall) was counted. This process was repeated for all fields and the overall percentage vascularity for each sample was calculated as

\[
\text{Percent vascularity (sample)} = \left(\frac{P_C(F \times P_T)}{P_T}\right) \times 100
\]

where \(P_C\) is the total number of points falling on vascular space (combined for all field counts in each sample), \(F\) is the total number of field counts for each sample, and \(P_T\) is the total number of points on the grid (i.e., 168).

The overall mean percentage vascularity and standard deviation were calculated for each treatment group by combining the data from all four samples in each group.

Whole muscle graft regeneration was assessed by counting the total number of myotubes (large desmin-positive cells26) in each tissue section. Nonoverlapping images were obtained as described above, using the automated stage movement apparatus. To account for differences in tissue section area between samples the total number of myotubes for each section was calculated per unit area (mm²) as

\[
\text{Myotubes/mm}^2 = \frac{M_T}{M_V(F \times A)}
\]

where \(M_T\) is the total number of myotubes counted for each section, \(F\) is the total number of field counts, and \(A\) is the area of the field of view (calculated at ×20 magnification as 0.0763402 mm²).

The number of myotubes per millimeter squared was determined for each sample and all four samples in each treatment group were combined to calculate the mean and standard deviation.

Mean blood vessel and myoblast/myotube counts were determined for each treatment group and differences between groups were tested for statistical significance by nonparametric (Kruskal–Wallis) one-way analysis of variance (ANOVA), using SigmaPlot 2.0 software. To determine whether there was a direct relationship between revascularization and regeneration for each treatment group, correlation coefficients were calculated (Microsoft Excel 97).
RESULTS

rAAV-mediated VEGF expression in vitro

The transduction of rAAV.VEGF.GFP into 293 cells resulted in high levels of GFP expression 24 h after transduction (Fig. 1a). Western blot analysis demonstrated the presence of a pair of bands, approximately 22 kDa in size, indicating the expression of VEGF (both glycosylated and nonglycosylated forms) in the supernatant of rAAV.VEGF.GFP-transduced 293 cells 48 h after transduction (Fig. 1b, lane 1). VEGF was not detected in supernatants collected from nontransduced (Fig. 1b, lane 3) and rAAV.GFP-transduced 293 cells (Fig. 1b, lane 4).

rAAV.GFP injections

All muscles that were injected with rAAV.GFP and sampled before or after transplantation were completely serial sectioned to determine the extent of rAAV-mediated transduction of skeletal muscle in vivo. There was no reporter gene expression (at 10 days) observed in the single muscle that was transplanted 3 days after injection and sampled 7 days later.

In the single muscle that was sampled 1 month after rAAV.GFP injection, occasional muscle fibers expressed the reporter gene (Fig. 2a). In the two muscles that were transplanted 1 month after injection and sampled on day 7 after transplantation many myofibers expressed GFP, but these myofibers were located only at the periphery of the graft (Fig. 2b). By 2 months after injection there were many myofibers expressing the reporter gene and these were widely distributed throughout the muscle (Fig. 2c). Muscles that were transplanted 2 months after injection and sampled 7 days later contained GFP-positive myofibers in the periphery (Fig. 2d), with a pattern of distribution similar to that of the 1-month grafted samples (Fig. 2b).

VEGF expression

Four groups (two treatment and two control groups) were used to study the effects of rAAV-mediated VEGF delivery to skeletal muscle (Table 1, groups B–E). Group

![Fig. 1](image-url)  
**FIG. 1.** (a) Fluorescence micrograph showing high levels of GFP expression in cultured 293 cells at 24 h posttransduction. (b) Western blot analysis of VEGF expression in supernatants from 293 cells transduced with rAAV.VEGF.GFP (lane 1), 293 cells transduced with SSV.VEGF.GFP DNA (lane 2), 293 cells transduced with rAAV.GFP (lane 3), and nontransduced 293 cells (lane 4). Lane 5 contains the molecular weight marker (KaleidoScope; Bio-Rad). Note the presence of two bands of approximately 22 kDa in size in lane 1, demonstrating expression of both glycosylated and nonglycosylated forms of VEGF, and the absence of VEGF expression in the controls (lane 3 and 4).
FIG. 2. Fluorescence microscopy showing GFP-positive myofibers (arrows). Muscles in (a)–(d) were injected with rAAV.GFP (group A) and sampled before (a and c) or after (b and d) grafting. (a) Muscle sampled 1 month after injection; (b) muscle grafted 1 month after injection and sampled 7 days later; (c) muscle sampled 2 months after injection; and (d) muscle grafted 2 months after injection and sampled 7 days later. GFP-positive myofibers (arrows) were also observed after grafting TA muscles that had been injected with (e) rAAV.VEGF.GFP (group B) and (f) rAAV.VEGF. GFP plus rAd (group C). Note that in all muscles that were grafted (b, d, e, and f) GFP-positive myofibers were observed only at the periphery, near the edge of the transplant, at 7 days. In all grafted muscles the periphery (p) and center (c) are indicated. Bars: 100 μm.

B was treated with rAAV.VEGF.GFP only, and group C was injected with rAAV.VEGF.GFP in conjunction with a helper adenovirus. Groups D and E were saline-injected and uninjected controls, respectively. In groups B and C the rAAV.VEGF.GFP was tagged with GFP. Fluorescence microscopy demonstrated that occasional GFP-expressing myofibers (indicating the presence of rAAV.VEGF.GFP) were present at 7 days in the periphery of whole muscle grafts that had been preinjected with either rAAV.VEGF.GFP (Fig. 2e) or rAAV.VEGF.GFP plus rAd (Fig. 2f). GFP-positive myofibers were not observed in any of the PBS-injected or uninjected control muscles. Immunohistochemical localization of VEGF was also used to examine the distribution of total VEGF expression (endogenous and virally mediated) throughout rAAV.VEGF.GFP-treated and control grafted muscles. There were no obvious differences in VEGF staining intensity between rAAV.VEGF.GFP-treated (groups B and C) and control whole muscle grafts (groups D and E). In all cases, many myotubes at the periphery of muscle grafts expressed VEGF strongly, and there was uniform pale positive staining in the central necrotic zone of the TA autograft at 7 days. Most VEGF expression was localized to myogenic cells, while few inflammatory cells were positively stained (data not shown).

Whole muscle graft revascularization

Immunohistochemical localization of PECAM-1 was used to identify blood vessels within the grafted muscles 7 days after transplantation. This antibody was localized to large and small blood vessels, with extremely high specificity. In all grafts the general pattern of blood ves-
AAV-MEDIATED VEGF GENE THERAPY

Vessel staining was similar (Fig. 3b), with many blood vessels in the peripheral regenerating zone (Fig. 3c and d), and only occasional small blood vessels within the central necrotic zone at this time (Fig. 2d–f). There were no obvious differences in the general staining pattern of blood vessels between any of the rAAV-injected whole muscle grafts (groups A–C; Fig. 3c and e) and the PBS-injected or un.injected control muscle grafts (groups D and E; Fig. 3d and f).

However, careful quantitation of blood vessels revealed that the percentage vascularity of whole muscle grafts that had been preinjected with VEGF as either rAAV.VEGF.GFP, or as rAAV.VEGF.GFP plus rAd, was significantly higher than that of the rAAV.GFP-injected, PBS-injected, and un.injected controls (Fig. 4A). The whole muscle transplants in the rAAV.VEGF.GFP plus rAd-treated group (group C) were slightly more vascular than those injected with rAAV.VEGF.GFP alone (group B); however, this difference was not statistically significant. In group B, the percentage vascularity (5.34 ± 0.44) was 2-fold that of the un.injected control grafts (2.80 ± 0.32).

The muscles injected with rAAV.GFP alone (i.e., no VEGF) or PBS and then grafted 1 month after injection were not significantly different in their percentage vascularity compared with the un.injected control muscle grafts (Fig. 4A).

FIG. 3. (a) Desmin and (b–f) PECAM-1 staining of rAAV.VEGF.GFP-injected (groups B and C) and uninjected control (group E) muscle grafts examined 7 days after transplantation. (a) Desmin-stained section showing many desmin-positive myotubes in the peripheral regenerating zone (arrowheads) and activated myoblasts “cuffing” the necrotic myofibers toward the central zone (arrows). This was a typical staining pattern and there were no qualitative differences between treatment groups. (b) Low-power image showing peripheral regenerating zone p and central necrotic zone c, with many blood vessels (arrows) stained by the PECAM-1 antibody in the periphery while the center contained few blood vessels at 7 days. There were no obvious differences in PECAM-1 staining between rAAV.VEGF.GFP-treated (c and e) and control (d and f) muscle grafts. High-power images of the peripheral zones (c and d) showed many darkly stained blood vessels (arrows) interspersed among newly formed myotubes whereas in the central necrotic zones of the grafts (e and f) only occasional small blood vessels (arrowheads) were found between necrotic myofibers (arrows). Bars: 100 μm.
FIG. 4. (Top) Percentage vascularity and (bottom) number of myotubes per millimeter squared in whole muscle grafts. Muscles had been preinjected with rAAV.VEGF.GFP (AAV.VEGF) (Table 1, group B), rAAV.VEGF.GFP with helper adenovirus (AAV.VEGF+Ad) (Table 1, group C), rAAV.GFP (AAV.GFP) (Table 1, group A), and sterile phosphate-buffered saline (PBS) (Table 1, group D). Uninjected controls (uninj) (Table 1, group E) were also examined. All values shown represent means ± standard deviation (n = 4 mice for all groups except for rAAV.GFP, where n = 2). Differences between the injected groups and the uninjected controls were tested for significance by one-way ANOVA (*p < 0.05, **p < 0.005).

Whole muscle graft regeneration

All grafts at 7 days after transplantation were composed of a large central necrotic zone containing many necrotic myofibers and inflammatory cells, and a peripheral regenerating zone characterized by many newly formed multinucleated myotubes. Desmin immunohistochemistry was used to localize myoblasts and myotubes in regenerating whole muscle grafts. Desmin staining was predominantly localized to the periphery of all whole muscle grafts, where many myoblasts and myotubes were present (Fig. 3a), whereas the central necrotic zones were completely devoid of regenerating cells. An intermediate zone was present between the peripheral regenerating and central necrotic zones, in which activated myoblasts were seen within and “cuffing” necrotic sarcoplasm of myofibers (Fig. 3a).

Quantitative analysis of myotube numbers for all treatment groups demonstrated that there were significantly more myotubes per millimeter squared in whole muscle grafts preinjected with rAAV.VEGF.GFP alone (group B), or in combination with a helper adenovirus (group C), compared with the uninjected control grafts (Fig. 4B). In both cases there were almost 3-fold more
myotubes per unit area compared with the uninjected controls. In contrast to the data for whole muscle graft revascularization, there were no significant differences in myotube numbers between the muscles that had been injected with VEGF before grafting (Table 1, groups B and C), and the rAAV.GFP- and PBS-injected control grafts (Table 1, groups A and D, respectively), and in these latter two control groups there were also significantly more myotubes compared with the uninjected controls.

To determine whether there is a direct relationship between revascularization and regeneration (as measured by myotube formation), correlation coefficients ($r_{xy}$) for percentage vascularity and muscle regeneration (myotubes/mm²) were calculated for all VEGF and control treatment groups (groups A–E). There was a high positive correlation between revascularization and regeneration in all treatment groups, ranging from $r_{xy} = 0.598$ in the uninjected controls to $r_{xy} = 0.991$ in the rAAV.VEGF.GFP plus rAd-injected muscles. This confirms that skeletal muscle regeneration is dependent on revascularization even in untreated control muscle.

**DISCUSSION**

This study demonstrates that adeno-associated virus-mediated transfer of the VEGF gene before whole skeletal muscle transplantation results in significantly enhanced revascularization of muscles posttransplantation. This occurs despite the persistence of only occasional myofibers surviving in the periphery of the grafted muscles that express the reporter gene after transplantation. Furthermore, we have clearly demonstrated that skeletal muscle regeneration (myotube formation) is strongly correlated with revascularization. These results support our hypothesis that gene therapy with angiogenic growth factors before clinical muscle transplantation should accelerate the efficiency of muscle regeneration after grafting.

It is well documented that excellent muscle regeneration occurs after initial muscle degeneration in such grafted muscles and that new muscle formation is well advanced by 1 week after grafting (reviewed in Roberts and McGeachie). It should be noted that the mouse TA muscle is about 4-fold greater in mass than the EDL muscles that are used in many studies of mice, and this means that progression of regeneration to the center of the necrotic graft is still incomplete by 1 week in the TA autografts. Persistence of some myofibers at the graft edge occurs because of the diffusion of nutrients from the surrounding environment, and in the present study it was these surviving myofibers only that expressed the viral construct after grafting.

**rAAV-mediated gene therapy in skeletal muscle**

Preliminary studies assessed the transduction efficiency of rAAV.VEGF.GFP in cultured 293 cells. The majority of cells were transduced by this vector at 24 h as indicated by the presence of GFP expression. Furthermore, Western blot analysis confirmed excellent VEGF expression by the transduced cells at 48 h. These findings were used as the basis to determine the efficiency of skeletal muscle transduction in vivo, using a recombinant adeno-associated virus encoding the green fluorescent protein reporter gene (GFP), and to examine whether reporter gene expression persisted after the muscle necrosis and subsequent regeneration. Reporter gene expression was present in normal (nongrafted) skeletal muscles 1 and 2 months after injection. In muscles that were grafted 3 days after injection there was no reporter gene expression, although this was not surprising as previous in vitro studies have shown that the conversion of single-stranded to double-stranded rAAV DNA occurs in an ongoing fashion, so that reporter gene expression increases progressively over time. In accordance with this, we observed that muscles that were grafted 2 months after rAAV injection contained many more myofibers that expressed the reporter gene compared with muscles transplanted at 1 month. In all cases positive myofibers were located only in the graft periphery, strongly indicating that rAAV-mediated gene expression persists only in cells that survive the massive necrosis that occurs after whole muscle grafting.

Several studies have demonstrated highly efficient and stable long-term rAAV-mediated gene transfer in skeletal muscle. Similar to these previous studies, we observed excellent rAAV-mediated reporter gene expression throughout nongrafted muscles for up to 2 months. The present study is the first (that we are aware of) to examine whether rAAV-mediated reporter gene expression persists in muscles that have subsequently undergone extensive necrosis and regeneration. Reporter gene expression was observed only in peripheral mature myofibers that survived whole muscle transplantation, and there was little or no persistent rAAV-mediated reporter gene expression in myofibers that undergo necrosis, or in the activated myoblasts that arise from satellite cells associated with necrotic myofibers (e.g., "cuffing" myoblasts) that subsequently proliferate and fuse to form new muscle fibers (myotubes).

Several viral families have been examined as potential gene therapy vectors. Recombinant retroviruses and lentiviruses are highly efficient vectors that can induce long-term transgene expression, and studies have reported the use of "improved" retroviral vectors that induce easily regulated transgene expression. Recombinant adenoviral (rAd) vectors are advantageous in that they can transduce both dividing and nondividing cells,
although transgene expression can be limited to the short term and this vector family can be highly immunogenic in humans. Both of these problems can be somewhat ameliorated by removing many of the active viral genes ("attenuation").\[40,42-44\] One study demonstrated that a hybrid viral vector, which combined adenoviral components with specific components of other viruses, resulted in improved transfection of dividing cells in vitro.\[43\] However, recombinant adeno-associated viruses (rAAVs) are currently favored as one of the most promising viral vectors in gene therapy, as they can induce long-term transgene expression in both dividing and nondividing cells with low immunogenicity.\[44\] In the present study an rAAV vector was chosen for its ability to transfet nondividing (muscle) cells, and because of its low immunogenicity, as immune cells play a crucial role in skeletal muscle regeneration. We inserted a 661-base pair coding region of the murine VEGF165 gene into an rAAV that also encoded the GFP reporter gene to allow in vivo detection of transduced cells.

**Effects of VEGF gene therapy on skeletal muscle revascularization and regeneration**

Having established that some rAAV-mediated transgene expression persists after whole muscle transplantation, we applied this vector to VEGF gene therapy. Muscles were injected with rAAV.VEGF.GFP alone, or in combination with a helper adenovirus, and then grafted at 1 month. The results showed that although only occasional myofibers continued to express the GFP reporter gene that was used to tag the viral vector, there was a significant increase in the revascularization and regeneration (myotube formation) of the whole muscle transplants. However, because VEGF is an extremely potent angiogenic growth factor it is likely that even small increases in the amounts of VEGF protein within a tissue can have profound effects on local blood vessel growth.\[10\] Furthermore, previous studies have demonstrated that the expression of VEGF receptors, which are localized to endothelial cells with extremely high specificity (reviewed in Refs. 10–12), is upregulated in hypoxic tissues.\[46\] Therefore, the significant effects of only small amounts of VEGF gene expression observed in the present study may be augmented by hypoxia-induced upregulation of VEGF receptors on surviving endothelial cells in the muscle periphery.

Patterns of VEGF expression were examined by immunohistochemistry, and no differences in the distribution or types of VEGF-expressing cells were observed between the VEGF-treated whole muscle transplants and the controls. Although it was not possible for us to distinguish between endogenously produced VEGF and that associated with the viral vector, we also observed GFP expression in myofibers in the periphery of these whole muscle grafts. Although the presence of GFP cannot conclusively be used to indicate VEGF expression, our preliminary in vitro and Western blot analyses strongly suggest that these two reporter genes are usually coexpressed. Further studies are required with VEGF isoforms from different species in combination with quantitative protein analyses to determine the actual difference in VEGF production that was induced by rAAV-mediated VEGF gene transfer.

VEGF was predominantly expressed in myogenic cells in the periphery of all grafted muscles. VEGF expression has been detected in most normal tissues and in tumors (reviewed in Klagsbrun and D'Amore\[47\]), and basal VEGF mRNA levels were demonstrated in resting human muscle although its localization to specific cell types was not examined.\[48\] Because we have also demonstrated that muscle regeneration correlates extremely highly with revascularization, the expression of VEGF by activated myoblasts and newly formed myotubes was not surprising. However, on the basis of our observation of GFP expression only in mature myofibers in the periphery that survived transplantation, upregulated expression of VEGF induced by rAAV-mediated gene transfer is likely to originate only in surviving myofibers, whereas VEGF expression observed in activated myoblasts and newly formed myotubes is likely to be endogenous.

The mechanisms by which muscle pretreatment with VEGF causes enhanced whole muscle transplant revascularization are presently unclear. It is most likely that increased amounts of VEGF protein produced by the rAAV-transduced myofibers directly act on surviving endothelial cells in the muscle periphery to stimulate vascular sprouting and new vessel formation. However, it must also be taken into consideration that the pretreatment of muscles with VEGF probably stimulates vascular growth before transplantation, and thus more rapid revascularization of the muscle transplants resulted from the presence of a larger number of surviving peripheral vessels. There is also the possibility that VEGF expressed by myogenic cells exerted autocrine/paracrine effects on these cells to augment regeneration directly, although this is extremely unlikely as VEGF receptors have not previously been detected on any nonendothelial cell types.\[12,49\] Application of this VEGF treatment is expected to have a far more dramatic effect in the clinical situation, because it is well documented that the speed of revascularization is a limiting factor that can lead to fibrosis and a lack of new muscle formation in the center of large grafts. Ideally, the next stage is to further test rAAV VEGF gene therapy in a larger model of muscle grafting (e.g., in dogs).

One of the major problems in VEGF gene therapy is the possibility of deleterious effects of elevated VEGF
protein in the bloodstream on distant tissues. There are not only implications for compounds angiogenic disease such as tumor growth and diabetic retinopathy, but VEGF also acts as a vascular permeability factor and a blood clotting/coagulation promoter, and these properties may also be extremely destructive. Therefore, it is absolutely essential that a mechanism be introduced by which the newly introduced VEGF gene can be inactivated once the therapeutic benefits have been accomplished.

Although there were significantly more myotubes (indicating more advanced skeletal muscle regeneration) in the whole muscle grafts that had been injected with rAAV.VEGF.GFP, or with rAAV.VEGF.GFP plus rAd, compared with the un.injected controls, this was also the case for myotube numbers in the injected control muscle grafts (rAAV.GFP and PBS). This indicates that the injection per se has influenced the subsequent myotube formation in grafts and this may be the result of the injection trauma activating the satellite cells before transplantation. In addition, the myotube counts within each treatment group were highly variable. Previous studies in our laboratory have demonstrated large biological variation in myotube counts between different whole EDL muscle grafts. However, in light of the highly conclusive revascularization data presented in this study, and previous studies that have unequivocally demonstrated the dependence of skeletal muscle regeneration on revascularization (reviewed in Roberts and McGeeachie), it seems likely that further investigation would also reveal a significant effect of rAAV.VEGF.GFP pretreatment on the efficiency of new myotube formation, especially in large muscle grafts.

In summary, this study has demonstrated that rAAV-mediated transfer of the VEGF gene into skeletal muscles in preparation for whole muscle transplantation results in significantly enhanced revascularization of the muscles posttransplantation. Although further studies are required to identify methods of "switching off" VEGF expression once the clinical benefits of this gene have been realized, the results of the present study strongly support the use of VEGF gene therapy in pretreating whole muscles to enhance muscle regeneration for clinical transplantation.

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