Capillary regression in vascular endothelial growth factor-deficient skeletal muscle

Kechun Tang, Ellen C. Breen, Hans-Peter Gerber, Napoleone M. A. Ferrara, and Peter D. Wagner. Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. Physiol Genomics 18: 63–69, 2004. First published April 13, 2004; 10.1152/physiolgenomics.00023.2004.—Skeletal muscle angiogenesis is an important physiological adaptation to increased metabolic demand, possibly dependent on vascular endothelial growth factor (VEGF), the increased expression of which is a known early response to exercise. To test the hypothesis that VEGF is essential to muscle capillary maintenance, we evaluated the consequences of targeted skeletal muscle inhibition of VEGF expression in postnatal, cage-confined VEGFloxP(+/−) mice. To delete VEGF, cre recombinase expression was accomplished using direct intramuscular injection of a recombinant adeno-associated cre recombinase expressing viral vector. Four weeks postsinfection, VEGF-inactivated regions revealed 64% decreases in capillary density and capillary-to-fiber ratio. Substantial apoptosis was also observed in VEGF-depleted regions. There was no evidence of rescue at 8 wk, with a persistent 67% reduction in capillary density and a 69% decrease in capillary density. These data implicate VEGF as an essential survival factor for muscle capillarity and also demonstrate insufficient VEGF-dependent signaling leads to apoptosis in mouse skeletal muscle.

SKELETAL MUSCLE DYNAMICALLY adapts to activity level or environmental limitations by altering structural and functional determinants of oxidative metabolism (21, 30). A key component of this adaptive response is the number of capillaries perfusing each muscle fiber, which increases with repeated activity. Increased capillarization allows for greater O2 availability and exchange of metabolites between the vasculature and myocytes. While skeletal muscle angiogenesis is an important physiological response to exercise in healthy individuals, impaired exercise capacity in patients with chronic renal failure, heart disease, or chronic obstructive pulmonary disease may in part be due to a reduction in the number of capillaries delivering oxygen and nutrients to the skeletal muscle (12, 22, 36).

Multiple signals generated during exercise, including mechanical events associated with muscle contraction and reduced muscle intracellular oxygen tension, have the potential to regulate vascular endothelial growth factor (VEGF) gene expression (6, 28, 29). VEGF is a well-known, potent angiogenic factor, which also functions as a vascular permeability and endothelial cell survival factor (13). We have recently demonstrated that a 1-h exercise session in untrained rats or humans leads to elevated gastrocnemius VEGF mRNA levels (6, 28). Furthermore, exercise-induced angiogenesis may be attenuated by neutralizing antibodies, which block soluble VEGF function (2). Thus VEGF has been proposed to be a critical factor for regulating the dynamic and temporal microvessel remodeling which occurs in skeletal muscle. However, an attempt to use traditional knockout of all three VEGF isoforms, VEGF120, VEGF165, VEGF188, or its receptors (Flk-1 and Flt-1) results in embryonic lethality (14, 15, 33). Conditional knockout of VEGF165 and VEGF188 isoforms using a Cre-loxP-mediated strategy to remove VEGF exon 6, allowing only expression of mouse VEGF120, has been reported to result in severe ischemic cardiomyopathy and a progressive reduction in skeletal muscle capillarity in the small percentage of mice that survived through postnatal day six (9). However, studies in other organ systems have suggested that lowering VEGF levels by either transcriptional or receptor blockade mechanisms leads to apoptosis of the capillary endothelial cells and eventual capillary attrition (1, 3, 23). In the present study, all three VEGF isoforms were specifically inactivated in skeletal muscle through the viral delivery of cre recombinase to muscle fiber regions of mice containing a floxed VEGF gene resulting in deletion of exon 3. The effect of lowering skeletal muscle VEGF was determined by correlating local VEGF levels with changes in capillary density, capillary-to-fiber ratio, and presence of apoptotic cells.

MATERIALS AND METHODS

Animals. This study was approved by the University of California, San Diego, Animal Subjects Committee. Eight control (C57BL/6) and eight transgenic VEGFloxP mice, all aged 2–3 mo, were used for this study. VEGFloxP transgenic mice were provided by Dr. Napoleone Ferrara (Genentech, South San Francisco, CA), and details of the genetic engineering have previously been published (16). Recombinant AAVCre construction and delivery. AAV Helper-Free system (Stratagene, La Jolla, CA) was used for AAV/Cre production (31, 32, 37). The cre recombinase gene along with CMV promoter and SV40 poly-A sequence were inserted into the NotI site of pAAV-LacZ plasmid (Stratagene) to form a pAAV-Cre plasmid (19). This new, pAAV-Cre plasmid carries the gene cassette between two AAV2 LTRs in order to allow recombinant AAV to be assembled in strain 293 packaging cells. Recombinant AAV/Cre virus was produced by cotransfection of pAAV-Cre, pAAV-RC (expressing AAV2 rep/cap) and pHelper (expressing ADV E2A/EB), into strain 293 cells by calcium phosphate transfection (CaPhos Mammalian Transfection Kit; BD Sciences Clontech, Palo Alto, CA). Cells were collected and lysed using a freeze-thaw method. AAV/Cre recombinant virus titer was determined by hybridization of [α-32P]dCTP oligolabeled pAAV-Cre plasmid (Prime-It II random primer labeling kit, Stratagene) to serial dilutions of slot blotted viral supernatant and pAAV-Cre plasmid. To deliver AAV/Cre, mice were anesthetized...
with pentobarbital (40–60 mg/kg), and the midbelly of the gastrocnemius was injected with 5–10 μl (5 × 10^7 total AAV/Cre particles) of viral supernatant. Four animals in each group were killed at 4 wk postinfection and another four at 8 wk postinfection for histological studies.

**Immunohistochemistry.** At collection time, mice were euthanized with pentobarbital (65 mg/kg ip). The gastrocnemius muscle was frozen in isopentane and 10-μm sections were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), rinsed with PBS, and blocked with 10% goat serum/0.4% Triton X-100 in PBS. Cre recombinase and VEGF were detected by incubation with cre recombinase-specific (1:1,000) (Novagen, Madison, WI) or VEGF-specific (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in PBS/18% goat serum at 4°C overnight. Slides were subsequently incubated with biotinylated secondary antibody followed by Alexa Fluor 488-conjugated streptavidin (Molecular Probes, Eugene, OR) and viewed by fluorescent microscopy.

**Apoptosis.** TUNEL-positive apoptotic nuclei were detected in serial frozen sections using an in situ cell death detection kit (POD; Roche Diagnostic, Indianapolis, IN) and viewed by light microscopy.

**Capillary measurements.** Frozen 10-μm serial cross sections were prepared and stained using the sensitive alkaline phosphatase detection method, which stains muscle fibers yellow and capillary endothelial cells blue (18). Sections were first presoaked in precooled (−20°C) 1:1 mixture of acetone and chloroform and allowed to dry at room temperature. Slides were transferred to an incubation mixture containing 0.08% gly-pro-β-methoxy-β-naphthylamide and 0.034% fast blue in 0.1 M phosphate buffer, pH 7.5, for 60 min at 37°C, air dried overnight, and mounted. Sections were viewed by light microscopy and digitally imaged (each rectangular image being 1.118 mm × 0.86 mm). MATLAB 18.3 computer software was used to perform morphometric measurements. The total number of capillaries around each fiber (NCAF) was determined throughout entire cross-sectional regions of contiguous cre recombinase-expressing fibers and a surrounding region of noninfected fibers of an equal cross-sectional area. This circumference of noninfected fibers, surrounding the contiguous region of infected fibers, was chosen to avoid bias and minimize selection of a heterogeneous region. We analyzed 30–70 individual fibers from either AAV infected or noninfected regions. Fiber area was measured as the total sum of the area of all the fibers within a definable region divided by the number of fibers contained within the defined cross-sectional area. Detection of capillary number by alkaline phosphatase staining was also confirmed by immunohistochemical detection with antibodies specific for von Willebrand factor (Santa Cruz Biotechnology).

**RESULTS**

AAV/Cre-infected fibers in both control C57BL and VEGFloxP transgenic mice were confined to a contiguous region of 30–70 individual fibers as detected by immunohistochemical staining with an antibody specific for Cre recombinase (Figs. 1A–4A). Cre-positive fibers revealed a clear absence of VEGF expression in VEGFlloxP(+/+) mice (Figs. 1B and 2B) but not in wild-type mice (Fig. 3B and 4B). AAV/Cre-infected regions were also assessed for changes in capillarity by staining for alkaline phosphatase-positive vascular structures (Fig. 1C–4C) (18). In VEGFlloxP(+/+) mice, AAV/Cre-infected regions revealed substantial capillary regression, which was accompanied by the widespread appearance of TUNEL-positive apoptotic myocytes and capillary endothelial cells (Figs. 1D and 2D). In AAV/Cre-infected fiber regions, 13.4- and 12.3-fold increases in the number of apoptotic nuclei per square millimeter were measured compared with noninfected regions at 4 and 8 wk, respectively (Table 1). In wild-type mice infected with the AAV/Cre, changes in localized VEGF expression, capillarity, or apoptotic cells were not observed (Figs. 3, B–D, and 4, B–D, and Table 1). No inflammatory cells were identified in transfected fibers of control or VEGFloxP(+/+) mice at either 4 or 8 wk.

The decreased angiogenic response in VEGF-inactivated fibers was quantitated by morphometry (Fig. 5), and the total

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*Fig. 1. Four weeks postinfection of VEGFlloxP(+/+) mice with AAV/Cre reveal capillary regression. Gastrocnemii from VEGFlloxP(+/+) transgenic mice were infected with 5 × 10^7 aden-associated virus-expressing Cre recombinase (AAV/Cre) particles, intramuscularly. Four weeks later, infected fibers from VEGFlloxP(+/+) transgenic mice were identified by nuclear immunostaining of serial cross sections with a Cre recombinase antibody (green) (A) and revealed lower levels of VEGF at the myocyte periphery (green) (B), a loss of capillaries detected by alkaline phosphatase staining (purple) (C), and TUNEL-positive apoptotic cells (brown) (D). VEGF: vascular endothelial growth factor.*
number of capillaries, individual fibers, and fiber surface areas were measured. These data were used to calculate the ratio of capillaries to muscle fibers (Fig. 5A) and capillary density (Fig. 5B) within AAV/Cre-infected regions compared with adjacent noninfected regions of an equivalent cross-sectional surface area within the same muscle section. These measurements revealed that AAV/Cre-infected regions of VEGFloxP(+/+) gastrocnemius demonstrated 64% and 67% (P < 0.01 for both) decreases in capillary/fiber ratio at 4 and 8 wk postinfection, respectively, compared with noninfected regions of the same gastrocnemius. These results are reflected in similar 64% (4 wk) and 69% (8 wk) decreases in the capillary density in AAV/Cre-infected gastrocnemius regions (P < 0.01 for both). Neither at 4 or 8 wk was there an effect of infection on fiber density.

Fig. 2. Eight-week AAV/Cre-infected VEGFloxP(+/+) mice show evidence of long-term effects on skeletal muscle capillarity. Gastrocnemii from VEGFloxP(+/+) transgenic mice were infected with 5 × 10⁹ AAV/Cre particles, intramuscularly. Eight weeks later, infected fibers from VEGFloxP(+/+) transgenic mice were identified by nuclear immunostaining of serial cross sections with a Cre recombinase antibody (green) (A) and revealed lower levels of VEGF at the myocyte periphery (green) (B), a loss of capillaries detected by alkaline phosphatase staining (purple) (C), and TUNEL-positive apoptotic cells (brown) (D).

Fig. 3. Four-week AAV/Cre-infected C57BL mice infected do not display altered VEGF levels or capillarity. Gastrocnemii from control, C57BL mice were also infected with 5 × 10⁹ AAV/Cre particles, intramuscularly. Four weeks later, infected fibers were identified by nuclear immunostaining of serial cross sections with a Cre recombinase antibody (green) (A) and did not reveal lower levels of VEGF at the myocyte periphery (green) (B), loss of capillaries detected by alkaline phosphatase staining (purple) (C), or increased TUNEL-positive apoptotic cells (brown) (D). Bar equals 100 μm.
Table 1. Number of apoptotic-positive nuclei

<table>
<thead>
<tr>
<th>Group</th>
<th>Week</th>
<th>Infected Region</th>
<th>Noninfected Region</th>
</tr>
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<tbody>
<tr>
<td>AAV/Cre</td>
<td>4</td>
<td>248.3 ± 38.12*</td>
<td>18.5 ± 2.67</td>
</tr>
<tr>
<td>AAV/LacZ</td>
<td>4</td>
<td>20 ± 3.13</td>
<td>22 ± 2.05</td>
</tr>
<tr>
<td>AAV/Cre</td>
<td>8</td>
<td>196 ± 46.74*</td>
<td>16 ± 3.29</td>
</tr>
<tr>
<td>AAV/LacZ</td>
<td>8</td>
<td>19 ± 4.13</td>
<td>17 ± 3.87</td>
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Values are means ± SE (n = 4) of no. of apoptotic-positive nuclei per mm². Gastrocnemius of VEGFfloxP mice were infected with AAV/Cre or AAV/LacZ (control), *Significant (P < 0.01) compared with the surrounding noninfected fiber regions or fiber regions from AAV/LacZ infected mice.

area in infected vs. noninfected regions in either VEGFloxP or wild-type mice. Loss of capillarity thus persisted through 8 wk without evidence of initiated compensatory mechanisms to restore oxygen conductance to affected skeletal muscle fibers. In wild-type mice, the capillary-to-fiber ratio and capillary density were also not different between AAV/Cre noninfected and infected regions at either the 4- or 8-wk time points, further suggesting no deleterious effect of the viral vector itself (Fig. 5, A and B).

DISCUSSION

The results presented demonstrate that withdrawal of VEGF leads to the sustained regression or loss of capillaries perfusing muscle fibers in adult, cage-confined mice with nonrestricted activity levels. Insufficient VEGF levels initiate apoptotic pathways in cellular elements of the skeletal muscle. Altered myocyte VEGF levels may potentially downregulate the expression of bcl2- or Akt-dependent signaling mechanisms thought to be important in protection from apoptosis (17). This is supported by the recent observation that regional adenovirus delivery of Akt to ischemic rabbit myofibrils is associated with enhanced VEGF levels and the recruitment of capillaries to the periphery of Akt overexpressing skeletal muscle fibers (35).

Choice of targeted Cre-loxP method to ablate muscle VEGF.

To experimentally determine VEGF function in skeletal muscle, a Cre-loxP-mediated strategy of gene inactivation was used to deplete available VEGF throughout skeletal muscle fiber regions. This was accomplished by local injection of a recombinant cre recombinase adeno-associated viral vector directly into mouse gastrocnemii of VEGFloxP(+/+) transgenic mice (31). Subsequent cre recombinase expression resulted in conditional deletion of mouse VEGF exon 3, which is flanked by loxP sites in this transgenic mouse strain (16), and prevented expression of all three VEGF isoforms, VEGF120, VEGF164, and VEGF188. This experimental approach allows a degree of temporal and spatial control over inactivation of any gene surrounded by bacteriophage P1 loxP sequences (32), and makes functional analysis of skeletal muscle VEGF in postnatal mice possible. This is an important consideration since knockout of embryonic VEGF is lethal. Furthermore, complicating developmental effects and potential long-term compensatory pathways were avoided by choosing to study regional cre recombinase delivery to mature, postnatal mice as opposed to using a muscle-specific promoter (i.e., muscle creatine kinase promoter) to drive cre recombinase expression in skeletal muscle throughout embryonic development. Another advantage of this experimental approach was that AAV/Cre-infected fibers were confined to a definable contiguous region of muscle fibers. Infected fibers could be localized by immunohistochemical detection of cre expression, and the identical fibers could be tested for changes in VEGF level, capillarity, and apoptotic progression. Areas of diminished capillarization and reduced VEGF expression closely overlaid that of cre expression, suggesting that there was little or no availability of

Fig. 4. Eight-week AAV/Cre-infected C57BL mice display normal capillary parameters and apoptosis. Gastrocnemii from C57BL mice were infected with 5 × 10⁷ AAV/Cre particles, intramuscularly. Eight weeks later, infected fibers were identified by nuclear immunostaining of serial cross sections with a Cre recombinase antibody (green) (A) and revealed unchanged levels of VEGF at the myocyte periphery (green) (B), capillarity (purple) (C), and TUNEL-positive apoptotic cells (brown) (D).
**VEGF in skeletal muscle**

Overall, this is a powerful tool for studying the local effects of VEGF from surrounding normal regions or via the blood flow/gastrocnemius muscles. Morphometric confirmation of decreased capillarity in VEGF-loxP(+/+) gastrocnemius muscles infected with AAV/Cre. Four (4W) and eight weeks (8W) postinfection with AAV/Cre, capillary number, number of fibers, and fiber surface area were measured in VEGF-loxP(+/+) and C57BL/6J (wild type) in a contiguous area of infected fibers (+) and surrounding, noninfected (−) fiber regions of an equivalent area. From these determinants, the capillary/fiber ratio (number of capillaries/fiber) (A) and the capillary density (capillary number/fiber cross-sectional area) (B) were calculated. Error bars are SE; n = 4–6 mice. Student’s t-test was used to estimate significant differences between noninfected (−) and infected (+) regions; *P < 0.01.

VEGF from surrounding normal regions or via the blood supply to maintain muscle structure in the infected fibers. Overall, this is a powerful tool for studying the local effects of VEGF in skeletal muscle fibers within their native extracellular and cellular environment.

**VEGF as an essential capillary survival factor.** The effects of VEGF inactivation were observed within 4 wk of a one-time AAV/Cre delivery and suggest that critical VEGF levels must be maintained to preserve muscle capillary-to-fiber ratios. The data collected from our mouse skeletal muscle targeted knock-out model of VEGF gene inactivation is from a mixed region of skeletal myocytes, rather than pericytes or smooth muscle cells. Angiopoietins are also present in white and red gastrocnemii of sedentary rats. The transcript ratio of angiopoietin 2 to angio-

**Potential mechanism of capillary regression.** One mechanism to destabilize capillary structure is to shift the balance of pro- and anti-angiogenic factors. In our analysis, inactivating the VEGF gene abolished approximately two-thirds of the capillaries in an AAV/Cre-infected region. Thus the remaining third of the capillaries appear to be refractory or VEGF-independent vessels. Studies in which VEGF expression was conditionally turned on and then off in the heart and liver suggest that the duration of elevated VEGF expression (greater than 2 wk) is a critical turning point for establishing VEGF-independent, refractory vessels. Furthermore, in tumor and retina model systems, capillaries are thought to remodel into VEGF-independent vessels as the endothelial cell layer is surrounded by pericytes or smooth muscle cells. However, mouse skeletal muscles exist as several diverse types based on their make up of varying proportions of glycolytic and oxidative fiber types (5, 8, 10). Signals stemming from these diverse skeletal myocytes, rather than pericytes or smooth muscles cells, are likely to regulate VEGF-dependent changes in the endothelial cell. Such a range of myocyte-endothelial interactions may allow an additional level of regulation over capillary formation or regression dependent on skeletal muscle fiber composition. For example, Lloyd and colleagues (24) have reported that ligated white gastrocnemius muscle responds to exercise training by increasing capillarity. In contrast, the same exercise stimulus results in a blunted angiogenic response in red gastrocnemius. Interestingly, VEGF as well as angiopoietin 1 and angiopoietin 2 were reported to be at a lower expression levels in white gastrocnemius (composed primarily of glycolytic fibers) compared with the red gastrocnemius (composed predominantly of oxidative fibers). Angiopoietins 1 and 2 antagonistically bind to the same endothelial receptor, Tie-2, and are reported to have opposing functions (26). Angiopoietin 1 functions to stabilize capillaries, while angiopoietin 2 has a dual role. In the presence of VEGF, angiopoietin 2 augments angiogenesis, whereas in the absence of VEGF it promotes capillary destabilization (20, 25).

Furthermore, differences in the ratio of angiopoietin 2 to angiopoietin 1 transcripts are also present in white and red gastrocnemii of sedentary rats. The transcript ratio of angiopoietin 2 to angiopoietin 1 in white gastrocnemius has been reported to be 0.87, whereas the ratio in red gastrocnemius is only 0.4 (24). Thus lowering the already limited amount of VEGF in white gastrocnemius could potentially shift the balance of angiopoietin 2 to VEGF proportionally more than it would in red gastrocnemius. Red gastrocnemius contains higher VEGF levels and a lower ratio of angiopoietin 2 to angiopoietin 1, which would favor capillary stabilization. The balance and overall levels of angiogenic regulators in glycolytic white gastrocnemii may contribute to a muscle type design that is more sensitive to changes in VEGF and able to dynamically regulate capillarity with modest changes in angiogenic-dependent stimuli. The data collected from our mouse skeletal muscle targeted knock-out model of VEGF gene inactivation is from a mixed region of gastrocnemius, composed of a heterogeneous population of glycolytic and oxidative fibers (mostly type IIA and IIB fibers and a small proportion of type I fibers) with presumably varying amounts VEGF transcript levels similar to the rat (5, 7). Thus it is reasonable to speculate that a portion of the capillary population may be refractory to depletion of VEGF.
Future VEGF-based skeletal muscle therapy. Currently, several animal studies and clinical trials are ongoing to test the possibility of delivering VEGF to ischemic limbs. Viral vectors expressing various VEGF isoforms have shown promise toward improving oxygen delivery and blood flow to impaired muscles (27, 34). The present study not only supports the rationale behind the use of VEGF to restore oxygen supply in capillary-depleted skeletal muscle of patients with renal failure, heart disease, or peripheral ischemia but also demonstrates the importance of critical VEGF levels for maintaining capillarity in otherwise healthy individuals. Understanding the mechanism of action of VEGF in normal and compromised skeletal muscle will allow greater insight into therapeutic strategies for patients with impaired exercise capacity secondary to many of the chronic illness occurring in older, sedentary populations.

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GRANTS

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REFERENCES


