VEGF alleviates lower limb ischemia in diabetic mice by altering muscle fiber types

LIJING JIA1,2*, PEILIN ZHENG2*, HONGBO WANG3*, LIN KANG2, HAN WU2 and XIAOBING FU1

1Laboratory of Wound Healing and Cell Biology, Institute of Basic Medical Science, Chinese People's Liberation Army General Hospital, Beijing 100048; 2Department of Endocrinology, Shenzhen People's Hospital, The Second Clinical Medical College of Jinan University, The First Affiliated Hospital of Southern University of Science and Technology, Shenzhen, Guangdong 518020; 3Department of Respiratory Medicine, Tianjin Children's Hospital, Tianjin 300134, P.R. China

Received July 26, 2020; Accepted January 8, 2021

DOI: 10.3892/etm.2022.11176

Abstract. Lower limb ischemia caused by diabetic foot (DF) is one of the most serious complications of diabetes. The therapeutic role of VEGF in DF is well documented. However, the mechanism for action of VEGF is still not clear. The present study aimed to explore the effects of VEGF-mediated skeletal muscle fiber type switch in angiogenesis and the treatment of DF. C57BL/6 mice housed in cages equipped with a voluntary running wheel were used to access VEGF protein level and citrate synthase activity (by ELISA) as well as muscle fiber type changes (by immunofluorescence) in the gastrocnemius muscle. C57BL/6 mice were fed on a high-fat diet for 6 weeks and then injected with streptozocin to induce diabetic lower limb ischemia model. Control adenovirus (Ad-GFP) or Ad-VEGF-GFP were then injected into the left gastrocnemius of the ischemic diabetic mice. Blood flow perfusion was examined by laser Doppler imaging at 1, 3, 7 and 14 days after adenovirus transduction. On day 14, all mice were anesthetized and sacrificed. VEGF expression levels, citrate synthase activity and muscle fiber type changes in the gastrocnemius muscle were assayed by ELISA and immunofluorescence analysis of myosin heavy chain IIa (MHCIIa) expression, respectively. Transwell assays were performed to determine whether VEGF-treated C2C12 myotubes played a role on tubule formation and migration of HUVECs. It was found that VEGF levels and citrate synthase activity were upregulated after voluntary exercise, along with the increased frequency of oxidized muscle fibers. Notably, adenovirus-mediated VEGF overexpression in the muscle also increased the frequency of oxidized (MHCIIa-positive) muscle fibers, enhanced citrate synthase activity and ameliorated lower limb ischemia in diabetic mice. VEGF treatment enhanced the phosphorylation of PI3K, Akt and AMPK (assayed by western blotting), as well glucose consumption and metabolism (assayed by western blotting and glucose uptake assay), in the C2C12 myotubes. Interestingly, VEGF-treated C2C12 myotubes promoted the migration and tubule formation of HUVEC cells. The present findings suggest that skeletal muscle fiber conversion might be a potential approach for VEGF-mediated angiogenesis and disease treatment, which may provide new options for the prevention and treatment of DF.

Introduction

Diabetes and the complications arising from it have become a global public health problem. The rate of lower limb amputation is between 0.2-4.8% in diabetic patients worldwide, which is 25 times higher compared with that among non-diabetic individuals (1,2). A total of 85% of amputations occur after foot ulcers among diabetic patients. Peripheral artery disease (PAD) is not only the most important risk factor for diabetic foot (DF) ulcers, but also an independent factor to predict the outcome and recovery in patients with DF ulcers (3,4).

To date, the treatment of diabetic peripheral vascular lesions is limited to drug treatment; however, it is usually long-term, with high-cost and limited effectiveness (5). Vascular surgery or interventional therapy are ideal for non-extensive vascular lesions but unable to treat diffused vascular stenosis in lower extremity diabetic complications (6). Exercise can improve
metabolism and ameliorate vascular lesions (7). However, the ability to exercise is impaired in most patients with DF. These limitations have prompted further studies on the mechanism of DF to aid the development of novel and effective treatments.

The dysfunction of vascular endothelial cells is the major cause of PAD, which has been documented in numerous studies in the past decades (8,9). Therefore, angiogenic factors, such as VEGF, are beneficial for ulcer healing (10). VEGF is known to act directly on numerous types of cells, including skeletal muscle cells (11). The skeletal muscle is the main organ for sugar metabolism in humans, also acting as the exoskeleton environment for vascular endothelial cell growth. Based on the source of ATP production, skeletal muscle fibers are divided into two types: Glycolytic and oxidative, and both can be converted to each other. In patients with long-term hypertension and diabetes, the proportion of oxidative muscle fibers is gradually decreased, with increased proportions of glycolytic muscle fibers (12,13). Moreover, the levels of sugar and energy metabolism in oxidative muscle fibers are higher compared with those in glycolytic muscle fibers (14,15). These observations led to the present study which hypothesized that VEGF might play a role in the transformation of skeletal fiber types and help improve vascular ischemia in DF. Therefore, the aim of the present study was to investigate the mechanism and effects of VEGF-induced muscle fiber conversion in angiogenesis and the treatment of DF.

Materials and methods

Animals. All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals published by the NIH (eighth edition, updated 2011) and ARRIVE guidelines (16). In total, 22 male C57BL/6 mice (age, 8 weeks, weight, 23.14±0.65 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were maintained at typical temperatures (20-22˚C) and humidity (40-60%) in the Chinese People's Liberation Army (PLA) General Hospital experimental Animal Center under specific pathogen free conditions with a 12-h light/dark cycle and access to food and water. Animal health and behavior were monitored every 2 or 3 days. All experiments were approved by the Ethics Committee of Chinese PLA General Hospital (approval no. 8157021269; Beijing, China).

Voluntary exercise training. In total, 12 C57BL/6 mice (age, 10 weeks at the start of the experiment) were divided into sedentary (sed, n=3) and voluntary exercise groups (n=9) in cages equipped with a voluntary running wheel (diameter, 11 cm). The wheel was connected to a counter for recording (17,18). After 0, 1, 2 and 4 weeks of exercise, three mice/time point were anesthetized with 3-4% isoflurane in air and sacrificed to induce myotube formation, C2C12 myoblasts with 60-80% confluence were seeded in DMEM differentiation medium containing 2% horse serum (Cytiva), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C, 5% CO₂. To induce myotube formation, C2C12 myoblasts with 60-80% confluence were seeded in DMEM differentiation medium containing 2% horse serum (Cytiva), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 5 days (19). The success of differentiation was validated by Giemsa staining of myotubes (cat. no. G4507; Sigma-Aldrich, Merck KGaA) and western blotting using anti-myosin heavy chain antibody (cat. no. sc-53095, 1:1,000, Santa Cruz Biotechnology).

For Giemsa staining, cells were fixed in 100% methanol at room temperature (RT) for 10 min. After air dry, Giemsa stain was added and stained for 30 min at RT. After rinsing with deionized water, cells were examined at x20 magnification under a light microscope (DP73; Olympus Corporation).

Recombinant proteins and antibodies. Recombinant murine VEGF (rmVEGF) was purchased from PeproTech, Inc. The anti-cytochrome c oxidase (COX) IV (cat. no. ab16056, 1:2,000), anti-peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α; cat. no. ab54481, 1:1,000), anti-glucose transporter member (GLUT) 4 (cat. no. ab654, 1:1,000) antibodies were purchased from Abcam. The anti-PI3K (cat. no. 4292, 1:1,000), phosphorylated (p)-PI3K antibody (Tyr458/Tyr199; cat. no. 4228; 1:1,000), AMPKα antibody (cat. no. 2532; 1:1,000), p-AMPKα antibody (Thr172; cat. no. 2535; 1:500), Akt antibody (cat. no. 4685S; 1:250), p-Akt antibody (Ser473) (cat. no. 4060S; 1:250) antibodies were purchased from Cell Signaling Technology, Inc. The anti-β-actin (cat. no. 854-s, 1:2,000) was purchased from HuaBio.

Immunofluorescence on frozen sections. The center part of gastrocnemius muscle from mice trained with voluntary exercise was obtained and immediately immersed in liquid nitrogen (-196˚C). Tissues were embedded in optimal cutting temperature compound (cat. no. 4583; Sakura Finetek USA, Inc.) at −196˚C). Tissues were quickly immersed in liquid nitrogen (−196˚C). Tissues were embedded in optimal cutting compound (cat. no. 4583; Sakura Finetek USA, Inc.) and cut into 6-µm-thick sections. Sections were then fixed in 4% polyformaldehyde solution at RT for 10 min, permeabilized in 0.3% Triton X solution, blocked with goat serum (cat. no. ZLI-9022; ZSBG-Bio; OriGene Technologies, Inc.) at RT for 30 min, and stained with anti-CD31 (1:50, cat. no. MCA2388, Bio-Rad Laboratories) and myosin heavy chain IIa (MHCIIa) antibodies (1:200, cat. no. sc-53095, Santa Cruz Biotechnology) at 4˚C overnight followed by incubation with FITC- (cat. no. ZF-0315, 1:300; ZSBG-Bio; OriGene Technologies, Inc.) or Alexa Fluor 594- (cat. no. ZF-0513, 1:300; ZSBG-Bio; OriGene Technologies, Inc.) conjugated secondary antibodies at RT for 30 min. The images were captured at x40 magnification using a confocal microscope (TCS SP8; Leica Microsystems GmbH). The fluorescence intensity was quantified using ImageJ (version 1.52a; National Institutes of Health) (20).

ELISA. Gastrocnemius muscle samples from mice trained with voluntary exercise were cut into small pieces (~5-mm), and quickly immersed in liquid nitrogen (-196˚C). Tissues were then homogenized and centrifuged at 8,000 x g for 15 min at 4˚C. The supernatant was used to assay the concentration...
of VEGF (cat. no. EK0541; Boster Biological Technology) and enzyme activity of citric acid synthase (cat. no. MAK057; Sigma-Aldrich, Merck KGaA) according to manufacturer's protocols.

Lower limb ischemia model in diabetic mice and adenovirus transduction. A total of 10 8-week-old male C57BL/6 mice were fed with high-fat diet (Research Diets, Inc.) for 6 weeks and then injected with 100 mg/kg streptozocin (STZ, Sigma-Aldrich; Merck KGaA) on day 0. Diabetic mice were screened for random blood glucose levels and confirmed by two consecutive tests of tail vein blood glucose of >11 mmol/l on days 2 and 7 after STZ injection. On day 8, diabetic mice were intraperitoneally injected with pentobarbital (50 mg/kg, Sigma-Aldrich; Merck KGaA) and then received surgery to ligate the femoral artery on the left lower limb, using the right lower limb as the control (skin not cut open) (21). On day 9, laser Doppler imaging apparatus (PeriCam PSI System; PIMSoft version 1.5.4.8078; Perimed, AB) was used to detect the blood flow perfusion in lower limbs to ensure the ischemia was successful in seven mice (monitoring distance, 15 cm; area height; 4.5 cm; width, 5.0 cm; area, 110 cm²; modeling failed in three mice, which were sacrificed immediately) (22). Adenovirus (Ad)-VEGF-green fluorescent protein (GFP) and Ad-GFP were produced by Hanbio Biotechnology Co., Ltd. based on the sequence of VEGF transcript (pAdEasy-EF1-MCS-3flag-CMV-EGFP; accession no. of VEGF, NM_001025366.2; 1x10⁹ pfu/ml each). On day 10, a total of 200 µl Ad-VEGF-GFP or Ad-GFP was injected at three sites of the left gastrocnemius of ischemic diabetic mice (three mice/group, one mouse was sacrificed). A pilot experiment showed that ischemia could be released 2 weeks after surgery. Therefore, the blood flow perfusion in those mice was examined by laser Doppler imaging at 1, 3, 7 and 14 days after adenovirus transduction. The speed of blood flow under the skin was indicated as perfusion unit in different colors: Red indicated the most rapid blood flow, dark blue indicated the slowest blood flow, and yellow indicated moderate speed of blood flow (21,22). To eliminate the influence of environmental factors and individual variations, the ratio of blood flow in the ischemic limb and control limb in each mouse was used to analyze the data. On day 14, all mice were anesthetized by 3-4% isoflurane in air and sacrificed by cervical dislocation with x40 magnification under a fluorescence microscope by incubating at 95°C for 2 sec, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec for 2 min. The following primers were used: Mouse VEGFR1 (forward, 5’-AAAGCGGACGCTCCTAACCC-3’) and reverse, 5’-AGGAGCCAAAGAGGGTCG-3’), mouse VEGFR2 (forward, 5’-GGTGGCCTTCGCGCAAGTGAT-3’) and reverse, 5’-CATGTCCTCGTGTGGTGTC-3’) and mouse β-actin (forward, 5’-CCACGCAATGAAAAAGATCAAGATCAT-3’ and reverse, 5’-ATCTGCTGGAGGTGGGACCG-3’). Subsequently, 1% agarose gel with ethidium bromide was used for electrophoresis.

After the formation of myotubes in C2C12 cells, 5, 10, 20 ng/ml rmVEGF was added into cells incubated at 37°C for various durations (6, 12 and 24 h). Control cells were treated with 20 ng/ml BSA. qPCR was performed with TB Green Premix Ex Taq (Tli RNaseH Plus; Takara Biotechnology Co., Ltd.) using the following thermocycling conditions: 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The gene expression levels were quantified as a fold change against GAPDH using the 2^ΔΔCq method (24).

The following primers were used: mouse MHCIIa (forward, 5’-CGAGATCCTCGGAAAGTGA-3’) and reverse, 5’-ATA TCTCTGCCCCTGACCA-3’). mouse GAPDH (forward, 5’-CGTGTTCCTATCCCCAAATGT-3’ and reverse, 5’-TGT CATCATTGTGGAGGTTT-3’).

HUVEC migration assay. A total of 200 µl HUVECs (2x10⁵ cells/ml in the aforementioned DMEM) were seeded into the upper Transwell chambers in triplicate (24-well plate with 8.0-µm pore insert, cat. no. 3422, Corning, Inc.). The lower chamber was added 800 µl DMEM differentiation medium containing either 3x10⁵ cells/ml untreated C2C12 myotubes, or 3x10⁵ cells/ml VEGF-treated C2C12 myotubes (20 ng/ml rmVEGF-treated for 12 h at 37°C, but no VEGF in Transwell afterwards), or 20 ng/ml rmVEGF for 24 h at 37°C. The cells in the lower chamber were fixed with 100% methanol at RT for 20 min and stained with 0.1% crystal violet solution at room temperature for 20 min (Beijing Solarbio Science & Technology Co., Ltd.) (25). A total of eight fields of view in each chamber were captured at x100 magnification using a confocal microscope (TCS SP8; Leica Microsystems GmbH).

Tubule formation of HUVECs. A total of 800 µl HUVECs (2x10⁵ cells/ml in the aforementioned DMEM) were seeded into the lower Transwell chamber in triplicate (24-well plate with 8.0-µm pore insert; cat. no. 3422; Corning, Inc.). The upper chamber was added 200 µl DMEM differentiation medium containing either 3x10⁵ cells/ml untreated C2C12 myotubes, or 3x10⁵ cells/ml VEGF-treated C2C12 myotubes (20 ng/ml rmVEGF-treated for 12 h at 37°C, but no VEGF in Transwell afterwards), or 20 ng/ml rmVEGF. After 24 h culture at 37°C, nine fields of view in each chamber were captured at x100
magnification under an inverted phase contrast light microscope (TH4-200; Olympus Corporation). HUVEC tube length was analyzed using ImageJ software (version 1.52a) (26).

Glucose uptake assay. In total, 2×10^5 cells/ml C2C12 cells were induced to differentiate into myotubes in a six-well plate in DMEM differentiation medium and then starved in serum-free DMEM for 6 h at 37°C. Cells were then treated with either 20 ng/ml BSA (control), or 100 nmol/l insulin (Sigma-Aldrich; Merck KGaA) or 20 ng/ml rmVEGF at 37°C for 12 h. The level of glucose in the supernatant was assayed according to manufacturer's instructions (cat. no. 09000240660; Shanghai Mingdian Biotechnology Co., Ltd.).

Statistical analysis. Statistical analyses were performed with GraphPad Prism software (version 8.3.0; GraphPad Software, Inc.). Data are presented as the mean ± standard deviation and were analyzed with unpaired Student's t-test (two-tailed) or one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Voluntary exercise induces VEGF expression and skeletal fiber type switch. Mouse housing cages were first set up equipped with a voluntary running wheel (Fig. 1A). After 1, 2 and 4 weeks of exercise, mouse gastrocnemius muscle tissues were collected to investigate whether there were any changes in the muscle fiber types in those mice. Immunofluorescence staining found that the frequency of oxidized muscle fibers (red, MHCIIa-positive) was significantly increased after 1-week (46.050±0.919 vs. 36.400±0.990; P<0.05), 2-week (50.600±2.970 vs. 36.400±0.990; P<0.01), 4-week (60.050±1.626 vs. 36.400±0.990; P<0.001) exercise compared with that in sedentary mice (Fig. 1B). Significantly increased oxidized muscle fibers were also observed between 2- and 4-week exercise (50.600±2.970 vs. 60.050±1.626; P<0.05; Fig. 1B). The present study proceeded to measure VEGF levels in gastrocnemius muscle by ELISA. It was found that VEGF expression in skeletal muscle samples was significantly increased compared with the sed group even after 1 week of exercise (205.962±9.712 vs. 449.164±15.280 pg/ml; P<0.0001) and further increased with longer exercise duration (556.818±22.659 and 549.366±14.410 pg/ml after 2 or 4 weeks exercise, respectively; both P<0.0001 vs. sed group; Fig. 1A). Moreover, the enzyme activity of citric acid synthase in skeletal muscle was also enhanced compared with that in sedentary mice (Fig. 1B). Significantly increased VEGF levels in gastrocnemius muscle were also observed between 1 and 2 weeks exercise (0.029±0.002 vs. 0.036±0.001; P<0.01; Fig. 1D). These data suggested that skeletal fiber type switch might be involved in VEGF-induced angiogenesis.

VEGF-induced oxidized muscle fibers promote the migration and tubule formation of HUVECs. The present study has showed that VEGF improved blood perfusion and induced muscle fiber type switch. Subsequently, whether the changes of muscle fiber types could influence angiogenesis was assessed using C2C12 myoblasts and HUVECs. C2C12 myoblasts were cultured and differentiated into myotubes, which was validated by the changes in morphology, which changed from round shape to elongated tubule shape (Fig. S2A-D) and the expression of MHC (Fig. S2E). The expression of VEGFR1 in C2C12 myotubes was confirmed by semi-quantitative PCR (Fig. S3A). Different concentrations (5, 10 and 20 ng/ml) of the recombinant murine VEGF proteins (rmVEGF) were added into C2C12 myotubes for various durations to screen for the optimal treatment protocol. RT-qPCR showed treatment of 20 ng/ml VEGF for 12 h had the greatest effect on MHCIIa expression (Fig. S3B), which was used in subsequent experiments. The expression of oxidized muscle fibers (MHCIIa, red) in C2C12 myotubes also increased after treating with 20 ng/ml VEGF for 12 h, but not with 20 ng/ml BSA (Fig. S3C).

HUVECs were cultured alone, with C2C12 myotubes, VEGF (positive control) or VEGF-treated C2C12 myotubes in Transwell chambers. The migrated HUVECs were then quantified and normalized (HUVECs, 100±20.84%; HUVECs + C2C12, 103.1±7.07%; HUVECs + rmVEGF, 373.5±9.184%; HUVECs + rmVEGF-treated C2C12, 262.2±12.37%). Treatment of VEGF significantly promoted HUVEC migration (P<0.0001, HUVECs + rmVEGF vs. HUVECs or HUVECs + C2C12; Fig. 4A-E). Treatment of VEGF-treated C2C12 myotubes also significantly enhanced HUVEC migration (P<0.0001; HUVECs + rmVEGF-treated C2C12 vs. HUVECs or HUVECs + C2C12; Fig. 4A-E).

Similarly, the tubule formation of HUVEVs were quantified and normalized (HUVEVs, 100±16.67%; HUVEVs + C2C12, 122.2±20.97%; HUVEVs + rmVEGF, 844.4±91.41%; HUVEVs + rmVEGF-treated C2C12, 500.0±52.04%).
Both VEGF treatment (P<0.0001, HUVECs + rmVEGF vs. HUVECs or HUVECs + C2C12) and co-culture with VEGF-treated C2C12 myotubes (P<0.0001 for HUVECs + rmVEGF-treated C2C12 vs. HUVECs or HUVECs + C2C12 group) significantly enhanced HUVEC tubule formation (Fig. 4F-J).

Glucose consumption and PI3K/Akt/AMPK signaling pathways are involved in the VEGF-induced oxidization of C2C12 myotubes. The molecular mechanism underlying VEGF-induced oxidization of C2C12 myotubes was subsequently investigated. Since skeletal muscle is one of the most important organs involved in glucose consumption, it was first
Figure 2. VEGF overexpression in gastrocnemius induces muscle fiber type switch in ischemic mice. (A) GFP marker levels 14 days after Ad-VEGF-GFP and Ad-GFP injection in skeletal muscle tissues were assessed by fluorescence microscopy. Scale bar=200 µm. (B) VEGF levels at 14 days as determined using ELISA. (C) Immunofluorescence staining of MHCIIa fibers (red) of ischemic gastrocnemius following treatment with Ad-VEGF-GFP or Ad-GFP. Scale Bars, 100 µm. (D) Citrate synthase activity in gastrocnemius. Data are presented as the mean ± standard deviation and are representative of 2 independent experiments. n=3 mice/group in each independent experiment. *P<0.01 and ***P<0.001. GFP, green fluorescent protein; Ad, adenovirus; VEGF, vascular endothelial growth factor.

Figure 3. VEGF overexpression in gastrocnemius improves blood perfusion in ischemic limbs. (A) Laser Doppler monitoring of blood flow at the indicated time points after injecting Ad-GFP alone or Ad-VEGF-GFP. The color scale of all images was analyzed by setting the lowest perfusion value to 0 and the highest perfusion value to 200. (B) Blood flow imaging data were calculated by normalizing the ischemic limb data to the non-ischemic limb values. Data are presented as the mean ± standard deviation and are representative of two independent experiments. n=3 mice/group in each independent experiment. *P<0.05 vs. Ad-GFP. GFP, green fluorescent protein; Ad, adenovirus; Isc, ischemic; VEGF, vascular endothelial growth factor.
evaluated whether VEGF treatment altered glucose consumption in C2C12 tubule. Compared with the control group (BSA treated), glucose consumption in the VEGF group was significantly increased (0.829±0.150 vs. 2.126±0.220 mmol/l; P<0.001; Fig. 5A), which was significantly lower compared with the positive control insulin group (2.918±0.182 mmol/l). The expression of molecules involved in glucose consumption, including PGC-1α, GLUT4 and COX IV, was markedly increased following VEGF treatment (Fig. 5B). Furthermore, several pathways, including PI3K, Akt and AMPK signaling, were activated in VEGF-treated cells, but not in BSA-treated cells (Fig. 5C).

Figure 4. rmVEGF-treated oxidative fibers enhance the migration and tubule formation of HUVECs after a 24-h culture in Transwell chambers. Crystal violet staining of migrated HUVECs. (A) HUVECs alone. (B) HUVECs with C2C12 myotubes. (C) rmVEGF-treated HUVECs. (D) HUVECs with rmVEGF-treated C2C12 cells. Scale bars, 50 µm. (E) The number of migrated cells in each condition was normalized to the HUVECs group and analyzed. An inverted microscope was used to assess the tubule formation of HUVECs. (F) HUVECs alone. (G) HUVECs with C2C12 myotubes. (H) rmVEGF-treated HUVECs. (I) HUVECs with rmVEGF-treated C2C12 cells. Scale bars, 50 µm. (J) The length of the formed tubes in each condition was normalized to the HUVECs group and analyzed. Data are presented as the mean ± standard deviation and are combined from three independent experiments. ****P<0.0001. HUVEC, human umbilical vein endothelial cells; rmVEGF, recombinant murine VEGF.
Discussion

The present study indicated that VEGF could induce the switch of muscle fiber types by promoting the formation of oxidative fibers in vivo and in vitro. Furthermore, the present study showed that VEGF-induced oxidation of muscle fibers improved migration and tubule formation of HUVECs. In addition, the present study provided the molecular mechanism for VEGF-induced oxidation of muscle fibers. These data may provide a novel insight into VEGF-mediated therapy in DF.

Voluntary exercise in the running wheel for 1 week was sufficient to induce the increase in the proportion of oxidative skeletal muscle fibers and the activity of citric acid synthase, which is consistent with a previous study showing that exercise could promote skeletal muscle type transformation and vascular angiogenesis (27). However, multiple myokines, including VEGF, are also influenced by exercise (28-30). Therefore, in subsequent experiments, adenoviruses were injected into the skeletal muscle to locally upregulate VEGF expression. Ameliorated ischemia in mice with DF was observed, suggesting that VEGF in the muscle alone is sufficient to induce angiogenesis.

Skeletal muscles consist of a variety of fiber types with different functions. Based on the expression of the main myosin heavy chain subtypes, rodent fibers present with I, Ila, IId/x and IIb subtypes, while humans present with I, Ila, and IId/x subtypes (4). According to the oxidation capacity, rodent skeletal muscles are divided into oxidized fibers (I and Ila) and glycolytic fibers (IIb and IId/x). Under normal conditions, the number of oxidized and glycolytic skeletal muscle fibers is balanced but these fiber types may be readily transformed into each other in a number of settings. For example, aerobic exercise can induce the transformation of glycolytic muscle fibers into oxidized muscle fibers, characterized by a higher mitochondrial content, which...
was also observed in the present study (31-33). VEGF deficiency in mouse skeletal muscle abrogated exercise-induced angiogenesis and the increase in the proportion of oxidative fibers, indicating the importance of VEGF originating from muscles (32).

It was further determined whether the fiber type of skeletal muscle had an effect on vascular endothelial cell activity. Due to the lack of VEGF knockout mice, the present study was unable to fully analyze the mechanism of VEGF-mediated muscle fiber type switch and vascular angiogenesis in vivo. Instead, the commonly used myoblast (C2C12 cells) and epithelial (PUMC-HUVEC-T1 cells) cell lines were used to investigate the role of oxidized muscle fibers on vascular angiogenesis. It was found that VEGF-oxidized C2C12 myoblasts promoted the migration and tubule formation of HUVECs, confirming the close association between oxidative muscle fibers and angiogenesis (34).

There are four muscle fiber types found in mice. Different subtypes of muscle fibers have distinct mitochondrial characteristics (35,36). MHCIIa-positive oxidative fibers exhibit high mitochondrial content and fusion rates (35). PGC-1α is an essential regulator in mitochondrial biogenesis (37). In addition, it was demonstrated that it plays an important role in exercise-induced adaptation of skeletal muscle (31,38). Systemic or muscle-specific knockout PGC-1α resulted in a decrease in the ratio of oxidized muscle fibers (39). In addition, specific expression of PGC-1α in skeletal muscle induced oxidized muscle fibers (40). To the best of our knowledge, a limited number of studies have examined the effects of VEGF on PGC1-α and mitochondrial biogenesis. In adipose and brain tissues, treatment with VEGF increased PGC1-α expression and promoted mitochondrial biogenesis (41,42). However, VEGF facilitated the cytoplasmic localization of PGC1-α in endothelial cells (43). The present study observed increased expression of PGC-1α in VEGF-treated C2C12 myoblasts, suggesting that the changes of skeletal muscle type mediated by VEGF were possibly mediated by PGC-1α. Meanwhile, an increased mitochondria content in oxidative muscle fibers was also suggested by the enhanced activity of citric acid synthase and increased COX IV expression.

Glucose is the major energy source for skeletal muscles and skeletal muscle is the organ which consumes the largest amount of glucose in the body (44). Several GLUTs are expressed in muscles to facilitate the transportation of glucose into cells (45). GLUT1 and GLUT3 are selectively expressed in fetal and regenerating fibers (46,47). GLUT4 is the main glucose transporter in skeletal muscle, and highly expressed in oxidative fibers (48). It was found that oxidized muscle fibers expressed higher levels of GLUT4, indicating the increased glucose uptake capacity of skeletal muscle to improve the hyperglycemic conditions. The present study observed an elevated expression level of GLUT4 protein in C2C12 myotubes following VEGF treatment, which might facilitate the consumption of glucose and mitochondrial biogenesis.

Although the present study found that an increase of VEGF in the skeletal muscle could promote angiogenesis in vitro and alleviate lower limb ischemia in the diabetic mice, several limitations exist. First, the present study did not use a tissue-specific promoter in the adenovirus, which means that rather than myofibers, other cells could have been transfected with the VEGF gene. Although the present study performed in vitro experiments to assess the effects of VEGF-treated myofibers on angiogenesis, considering the complexity in vivo, further studies, such as utilizing adenoviruses containing a skeletal muscle specific promoter or transgenic mice with skeletal muscle-specific VEGF overexpression, are required for further clarification. Second, the present study used femoral artery ligation to induce ischemia. Although it is widely used to mimic ischemia in the lower limb of patients with DF, this acute lesion may not fully mimic the chronic ischemia in patients. Further studies are required to utilize a novel mouse model to better mimic the process of PAD in diabetic patients.

In conclusion, the present study extended prior observations of the protective effects of VEGF in DF by inducing the conversion of skeletal muscle fiber types and supported the potential therapeutic utility of VEGF in the future.

Acknowledgements

Not applicable.

Funding

This work is supported by the Shenzhen Municipal Science and Technology Innovation Committee Project (grant no. KCXFZZ202002010100045, JCYJ20160422150209240), China Postdoctoral Science Foundation (grant no. 2014M562547), National Natural Science Foundation of China (grant no. 81550035) and National science foundation of Guangdong province (grant no. 2020A1515010085).

Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

Authors' contributions

LK and XF discussed and designed the study, and critically edited the manuscript. LJ and HBW conducted the experiments and analyzed the data. PZ and HW analyzed the data and wrote the manuscript. LJ and HBW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of Chinese PLA General Hospital (approval no. 8157021269; Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
References


