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Superior neovascularization and muscle regeneration in ischemic skeletal muscles following VEGF gene transfer by rAAV1 pseudotyped vectors

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Abstract

Recombinant adeno-associated virus serotype 2 (rAAV2) vector has been widely employed for gene therapy. Recent progress suggests that the new serotypes of AAV showed a better performance than did AAV2 in normal tissues. Here, we evaluate the potential role of human vascular endothelial growth factor (VEGF) gene transfer using rAAV vector pseudotyped with serotype 1 capsid proteins (rAAV1) in the treatment of muscle ischemia. In ischemic skeletal muscles, the rAAV1-LacZ vector allowed higher level, broader distribution, and long-lasting gene expression compared with the rAAV2-LacZ vector. Muscle VEGF165 production following the rAAV1-VEGF165 vector injection was 5–10 times higher than that following the rAAV2-VEGF165 vector injection. VEGF165 production mediated by the rAAV1-VEGF165 vector stimulated a large set of neovascularization with relatively mature vascular structures and enhanced muscle regeneration in the ischemic skeletal muscles. Thus, the rAAV1-VEGF165 vector mediated gene transfer may be a therapeutic approach to peripheral vascular diseases.

Keywords: Adeno-associated virus; Muscle; Serotype 1; Gene delivery; Vascular endothelial growth factor; Ischemia

AAVs are members of the *Parvovirus* family and require helper viruses for efficient replication and expression. There are at least 11 known AAV serotypes, AAV1-6, avian AAV, bovine AAV, canine AAV, equine AAV, and ovine AAV [1]. Of these, AAV serotypes 2, 3, and 5 are believed to be of human origin, while AAV1 appears to be of nonhuman primate origin [2]. rAAV vectors are frequently utilized as gene delivery vehicles due to their attractive features. AAVs are not associated with any known human diseases, rAAV vectors can infect both dividing and nondividing cells, and long-term gene expression following rAAV vector mediated gene delivery has been demonstrated in a number of tissues [3–5]. Currently, AAV serotype 2 based rAAV vectors (rAAV2 vectors) are mostly employed in animal experiments and clinical trials [6–10]. rAAV2 vector mediated gene delivery into skeletal muscles has been explored for treating diseases such as hemophilia B due to factor IX deficiency and anemia due to erythropoietin deficiency [10–13]. However, most of these initial successes in rAAV2 vector mediated muscle gene transfer were performed in small animal models, which were not successfully translated into nonhuman primate models or clinical trials [10,14]. Further studies have shown that rAAV2 vectors are restricted in tropism, so ideal gene transfer efficiency cannot be achieved with rAAV2 vectors in a range of cell types [15,16]. Cells initially transduced by rAAV2 vectors may

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also be eliminated by the host immune response, as high prevalence of neutralizing antibodies to AAV2 capsid proteins was observed in normal human population [17–22].

Driven by these limitations, new generations of rAAV vectors have been developed to make it possible to recapture the best features of the native virus. Hence, recent studies have focused on comparing the AAV serotype-specific virion shells on vector transduction in normal tissues and found that new serotypes of AAVs may exhibit different tropism, likely due to the utility of different sets of primary- and co-receptors, although the receptors for most AAVs are currently unknown [23,24]. It has been demonstrated that rAAV2 based vectors pseudotyped with serotype 1 capsid proteins (rAAV1 vectors) allow superior gene transfer into normal skeletal muscles [25,26]. This finding implies that it is possible to achieve a more superior effect in the treatment of muscle diseases by using new rAAV1 pseudotyped vectors as gene delivery vehicles.

VEGF is a major regulator of blood vessel formation during development and in the adult organism. It has been widely used for vascular therapeutic purposes. Clinical trials of the therapeutic angiogenesis using VEGF as functional gene have entered into a phase II/III study. However, the results to date from the current clinical trials are not ideal as expected or did not prove to be an efficient therapeutic strategy [27–29]. Because the naked plasmid DNA or adenovirus vectors were used to deliver VEGF gene in these human angiogenic experiments, the single-delivery and the short-term expression of VEGF gene were considered as important reasons of non-ideal results [30,31]. In the process of stimulating neovascularization, premature cessation of VEGF gene expression may lead to the regression of new vessels [30]. Repeated administration of vectors is limited by the increased number of times of operation, risk of operation or suffering of patients. As a result, developing superior vectors for gene therapy to achieve a longer, stable expression of angiogenic cytokines is also an area of active research.

In this report, we have studied whether rAAV2 based vectors pseudotyped with the AAV1 capsid proteins (referred to as rAAV1 vectors) could be used to treat the skeletal muscle ischemia. We showed that the rAAV1 vectors allowed higher level, broader distribution, and long-lasting gene expression in the ischemic skeletal muscles compared with the rAAV2 vectors. Importantly, a large set of new vessel formation and superior muscle regeneration were observed in the ischemic skeletal muscles following the rAAV1-VEGF165 vector gene transfer.

Materials and methods

rAAV vector construction and production. The VEGF165 cDNA was obtained by PCR from a human heart cDNA library. To generate the pSNAV1/VEGF165, the VEGF165 cDNA was inserted into the AAV2 vector plasmid pSNAV1 under control of the CMV promoter and the SV40 polyadenylation signal [32]. Large-scale rAAV production and purification were described previously [33]. In brief, the pSNAV1/VEGF165 was transfected into BHK-21 cells and cultured in RPMI 1640 medium supplemented with 10% FBS. The rAAV vector producing cells were selected with 400 μg/ml G418 for 15 days. For production of the rAAV2-VEGF165 vector, the resulting G418 resistant cells at about 90% confluence were infected with helper recombinant rHSV/rep2cap2 viruses containing rep and cap genes from the AAV2 genome. For production of the rAAV1-VEGF165 vector, the resulting G418 resistant cells were infected with recombinant rHSV/rep2cap1 viruses containing rep gene from AAV2 and cap gene from AAV1. Forty-eight hours later, cells and media were harvested, chloroform was added at 10% (v/v), and the resulting mixture was shaken vigorously to release rAAV particles and to inactivate helper rHSV/rep2cap2 or rHSV/rep2cap1 viruses. The rAAV containing supernatant was harvested following centrifugation at 12,000*g* for 10 min at 4 °C. Subsequently, polyethylene glycol and NaCl were added at 10% (w/v) and 1.0 M, respectively. Following overnight incubation at 4 °C, the rAAV particles were precipitated by centrifugation at 12,000*g* for 10 min at 4 °C. The precipitated rAAV particles were resuspended in PBS solution and extracted with an equal volume of chloroform. The upper layer was harvested and further purified by ion-exchange column chromatography. The rAAV2-GFP, rAAV1-GFP, rAAV2-LacZ, and rAAV1-LacZ vectors were generated in the same manner. Viral titer was determined by dot blot DNA analysis by using purified plasmid DNA as standard. Titers are given as vector genomes (vg)/ml. Vectors were stored at 4 °C in PBS. The vector preparations used in this study showed titers between 10^{12} and 10^{13} vg/ml.

rAAV transduction of myotubes in vitro. Murine myogenic C2C12 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in six-well plates at 5×10^4 cells per well, allowed to reach confluence, and then differentiated to myotubes by culturing in DMEM supplemented with 2% horse serum (differentiation medium). Cells were incubated for 5 days in differentiation medium prior to transduction with the AAV vectors. Vector transduction was performed in serum-free DMEM, and vector preparations were added at 1×10^5 vg per cell. The serum-free DMEM was replaced by differentiation medium after 2 h. Supernatants were collected daily following vector transduction, and myotubes were subsequently harvested for RNA isolation.

Real-time RT-PCR analysis. Total RNA was isolated from the transduced myotubes using TRIzol reagent (Invitrogen). cDNA syntheses from 2 μg of total RNA were performed by using M-MLV reverse transcriptase and oligo dT-primers (Promega). The VEGF165 mRNA level in transduced myotubes was evaluated by real-time PCR analyses using SYBR Green dye and CFD 3200 system (MJ Research, USA) according to the standard procedure. One microliter of RT products was used for PCR amplification with primers specific for human VEGF165 cDNA and for mouse β-actin. Mouse β-actin served as an internal control for RT-PCR efficiency. The primer sequences used are as follows: VEGF165 forward, 5′-ATG AAC TTT CTG CTG TCT TGG GTG-3′; VEGF165 reverse, 5′-TCA CCG CCT CGG CTT GTC ACA T-3′; β-actin forward, 5′-ATC TGG CAC CAC ACC TTC-3′; β-actin reverse, 5′-AGC CAG GTC CAG ACG CA-3′. Expression of VEGF165 mRNA relative to β-actin mRNA was calculated based on the threshold cycle (C_T) as $2^{-\Delta\Delta C_T}$, where ΔC_T is the difference between the mean C_T value of VEGF165 and that of β-actin and $\Delta\Delta C_T$ is the difference between the ΔC_T values of the experimental group samples and the mean ΔC_T value of the control samples.

Mouse hind limb ischemia model and gene transfer into ischemic skeletal muscles. Six- to eight-week-old male BALB/c mice were purchased from the animal department at Peking University Health Science Center (Beijing, China). Mice were kept under specific pathogen free conditions supplied with sterile food and acidified water. The care of mice complied with the Guide for the Care and Use of Laboratory Animals. All experimental manipulation of animals was conducted according to protocols approved by the Care and Use of Laboratory Animals published by the Commission of Science Technology of People's Republic of China.

Hind limb ischemia was performed as described before [34]. In brief, mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Under a surgical microscope, a vertical longitudinal incision was made in the right hind limb, the right external iliac and femoral arteries were dissected free from the origin of the external iliac

artery, ligated, and completely excised. Subsequently, the skin above was sutured.

Ten days after induction of ischemia, the thigh muscles of the ischemic right hind limb were injected with 100 μl of the purified vector preparations containing 3×10^{11} vg of either the rAAV1-VEGF165 or the rAAV2-VEGF165 vector in five injections: two injections in the medial thigh, one in the lateral thigh, one in the distal thigh close to knee, and one in the proximal thigh close to the ligated site. Each group contained 25–28 mice and the control group mice were injected with 100 μl PBS. Mice were sacrificed at various time points post-injection for characterization of gene transfer efficiency, the effects on vessel genesis and muscle regeneration.

Another set of experiments was performed to evaluate the dosage effect of the rAAV1-VEGF165 vector. Following the induction of ischemia, the ischemic thigh muscles of each mouse were injected with 100 μl of the purified rAAV1-VEGF165 vector preparations containing 3×10^{9}, 3×10^{10}, or 3×10^{11} vg. Each group contained 10–15 mice and PBS injections were used as the control.

Muscle extract preparation and assessment of VEGF expression. Frozen muscles were pulverized in liquid N_2, homogenized in 3 ml ice-cold RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology) per gram muscle weight, and incubated on ice for 30 min. Homogenate was centrifuged for 15 min at 14,000*g* and frozen at −80 °C. The muscle extracts (100 μg) were separated by 12% SDS–PAGE and electrotransferred to pure nitrocellulose blotting membranes (Pall Corporation). Immunoblotting was performed with a primary mouse monoclonal anti-VEGF antibody (1:1000 dilution) (sc-7269, Santa Cruz Biotechnology) or mouse monoclonal anti-α-tubulin (1:3000 dilution) (T5168, Sigma) for overnight at 4 °C, followed by secondary horseradish peroxidase-conjugated goat anti-mouse antibodies (1:3000 dilution) (sc-2005, Santa Cruz Biotechnology) for 1 h at room temperature, the specific binding of the antibody was visualized with an ECL detection system (Cell signaling).

Culture media and muscle extracts were measured for VEGF content by using the Quantikine human VEGF immunoassay (R&D Systems, Minneapolis, MN). Human VEGF concentrations were also measured in murine plasma at 4 weeks after vector injection using the same kit.

X-gal staining and immunohistochemical analysis. To examine gene transfer efficiency, the relevant muscles were collected at various time points after vector injection. The fresh tissue was mounted in OCT, was snap-frozen in liquid nitrogen, and then stored at −80 °C. Frozen sections were cut at 7 μm thick, and fixed in 4% paraformaldehyde for 10 min, washed three times for 5 min with PBS. Sections were then incubated in a solution of pH 7.3, 10 mM Tris–HCl containing 0.005% Na-desoxycholate, 0.01% Nonidet P40, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml X-gal staining solution for 24 h at 37 °C. Subsequently, muscle sections were counterstained with hematoxylin. Random cross-sections from the middle portion of the muscle were examined under light microscopy. The estimation of the percentage of muscle fibers expressing β-galactosidase was determined by a blinded observer.

For immunohistochemical evaluation, the relevant muscles were fixed in 4% paraformaldehyde solution, embedded in paraffin, and sectioned. For defining the capillary density, sections were stained against a mouse monoclonal antibody against von Willebrand factor (vWF, sc-14014, Santa Cruz Biotechnology, Santa Cruz, CA). Staining against the proliferating cell nuclear antigen (PCNA) by using the mouse mAb sc-56 (Santa Cruz Biotechnology) was used to define proliferating cells. Similarly, staining against smooth muscle α-actin (α-SMA) by using mouse mAb clone 1A4 (A2547, Sigma) was used to define vessel smooth muscle cells. The ABC kit (sc-2017, Santa Cruz Biotechnology) or BCIP/NBT kit (sc-24981, Santa Cruz Biotechnology) was used for immunohistochemical staining. We routinely counterstained the section with hematoxylin or eosin as stated in figure legends. Quantification of capillaries, arterioles, and the fiber loss area was performed on histological sections from the upper, middle, and lower regions of the thigh muscle in a blinded fashion by three different examiners [35,36]. Three sections of each region were observed and each group contained six animals. Image ProPlus software (Media Cybernetics, Silver Spring, MA) was used to quantify the analysis, and values were presented as means ± SEM or percentages ± SEM.

Angiography. Angiography was performed at 6 weeks post-vector injection. Anesthetized mice were first injected with heparin at 1000 U/kg, subsequently, about 1 ml Pb_3O_4 turpentine suspension (1 g Pb_3O_4 in 1.5 ml turpentine) was manually injected from the left ventricle cavity, and filming was performed using an X-ray system (output 37 kV at 420 mA · s) (DGx-3 type, Beijing Wandong, China). The angiographic scores were classified as negative (−), mild (+), moderate (++), or marked (+++). Each angiographic degree was defined as follows: negative (score 0): no appearance or almost no appearance of femoral artery, collateral artery, and arterioles; mild (score 1): appearance of proximal femoral artery close to ligation site that did not exceed the knee, few collateral artery, and few arterioles; moderate (score 2): appearance of longer femoral artery that exceeded the knee, more collateral arteries, and more arterioles; marked (score 3): obvious femoral artery including arteria profunda, the arteria saphena, arteria poplitea, or appearance of tibial arteries and obvious collateral arteries, arterioles. All evaluations were performed in a double-blind fashion.

Perfusion study with China ink microsphere suspension. For labeling the vessel structure and assessing the blood flow capacity in the ischemic muscles following the rAAV1-VEGF vector gene transfer, perfusion study with China ink microsphere suspension was performed at 6 weeks post-vector injection. Mice were anesthetized as described above and perfused from the left ventricle cavity with a China ink microsphere suspension consisting of 3 g of isinglass in 100 ml China ink. The relevant muscles were fixed in 4% paraformaldehyde and embedded in paraffin. Thick cross-sections of 150 μm were prepared and assessed for vessel continuation under a light microscope.

Measurement of capillary permeability using Evans blue dye extravasation. Mice were injected with 100 μl of 1% Evans blue solution via tail vein. The dye was allowed to circulate for 30 min and thereafter the mice were killed. The relevant muscles were harvested and weighed. Dye was extracted from the muscles for 24 h in 2 ml formamide at 55 °C. Extracted dye concentration was determined by measuring the absorbance at 620 nm in comparison with a standard curve of 0.75–25 μg/ml Evans blue in formamide. After extraction, the relevant muscles were dried at 60 °C for 48 h and re-weighed [37]. Extravasation is expressed as nanograms of Evans blue per milligram of dry muscle weight (WT) to correct for tissue edema.

Statistical analysis. ANOVA with Bonferroni's correction for multiple comparisons was used in this study. A P value of <0.05 was considered statistically significant.

Results

Comparison of rAAV1 and rAAV2 vector mediated gene transfer efficiency in myotubes in vitro

Our preliminary data showed that the rAAV1-GFP vector allowed superior gene transfer efficiency into in vitro differentiated myotubes in comparison with the rAAV2-GFP vector (data not shown). To investigate whether the data achieved with the GFP encoding vectors could be translated into the VEGF165 encoding vectors, C2C12 cell derived myotubes were infected with the rAAV1-VEGF165 and the rAAV2-VEGF165 vector, and the secretion of VEGF into supernatant was determined daily for 6 days after vector transduction with an ELISA kit. Substantial levels of VEGF secretion were measured at day 2 after vector infection and the VEGF secretion reached a plateau at day 3 for both vectors. In all measurements, the rAAV1-VEGF165 vector infected myotubes secreted 3- to 4-fold more VEGF compared with that from the rAAV2-VEGF165 vector infected myotubes. No measurable

production of VEGF was found in supernatants from rAAV1- or rAAV2-GFP vector infected myotubes. (Fig. 1A). The myotubes infected by the VEGF165 encoding vectors were also assessed for VEGF mRNA expression at day 6 post-infection by real-time RT-PCR. A 3-fold increase in the VEGF mRNA expression was observed in the rAAV1-VEGF165 vector infected myotubes compared with that from the rAAV2-VEGF165 vector infected myotubes ($P < 0.001$, Fig. 1B).

Superior gene transfer into ischemic skeletal muscles by the rAAV1 vectors in vivo

We next compared in vivo gene transfer into ischemic mouse skeletal muscles between the rAAV1 and the rAAV2 vectors. Ten days following initiation of muscle ischemia in mouse hind limb, 3×10^{11} vg of the rAAV1-LacZ or the rAAV2-LacZ vector was injected into the thigh muscles and β-galactosidase (β-gal) expression was assessed after 2 weeks, 1, 6, or 9 months by immunohistochemistry. Markedly different kinetics and distribution of β-gal expression in ischemic skeletal muscles were observed between the rAAV1-LacZ and the rAAV2-LacZ vector (Fig. 2). At 2 weeks after vector injection, few weakly β-gal expressing skeletal muscle cells were observed in both rAAV1-LacZ and rAAV2-LacZ vector injected ischemic muscles (Fig. 2A). By 1 month, the β-gal expression in two groups reached the maximum, 100% of the muscle fibers in the rAAV1-LacZ injected ischemic muscles were expressing β-gal, whereas fewer than 20% of muscle fibers were expressing β-gal following the rAAV2-LacZ vector injection. Of particular note, the β-gal expressing muscle fibers following the rAAV2-LacZ vector injection were limited to areas surrounding the injection sites. In contrast, a broader distribution of β-gal expression including muscle fibers and vessels was observed in the rAAV1-LacZ vector injected muscles (Fig. 2B). No β-gal staining was detected in muscles injected with PBS or the rAAV1-VEGF165 and the rAAV2-VEGF165 vector (data not shown). At 6 months and 9 months post-rAAV vector injection, substantial fraction of the muscle fibers were still expressing β-gal in rAAV1-LacZ vector injected ischemic muscles. β-gal expression was barely detectable in rAAV2-LacZ vector injected ischemic muscles (Figs. 2C and D).

Superior production of VEGF in ischemic skeletal muscles following injection of the rAAV1-VEGF165 vector

The rAAV1-VEGF165 and the rAAV2-VEGF165 vector were injected into the ischemic thigh muscles under the same conditions as for the LacZ encoding rAAV vector. The production of human VEGF was quantified in relevant muscle extracts and blood samples at 1 month after gene transfer. The average VEGF concentration was 205 ± 88 pg/mg total protein in the rAAV1-VEGF165 vector injected muscles, compared with 38 ± 23 pg/mg total protein in the rAAV2-VEGF165 injected muscles ($P < 0.001$, $n = 5$, Fig. 3A). Western blot analysis on representative muscle extracts showed that strong staining with the expected molecular weight of 21–28 kDa was detected in the rAAV1-VEGF165 vector injected muscles, weak staining at the same range of molecular weight was observed in the rAAV2-VEGF165 vector injected muscles, and no such band was observed in extracts derived from muscles injected

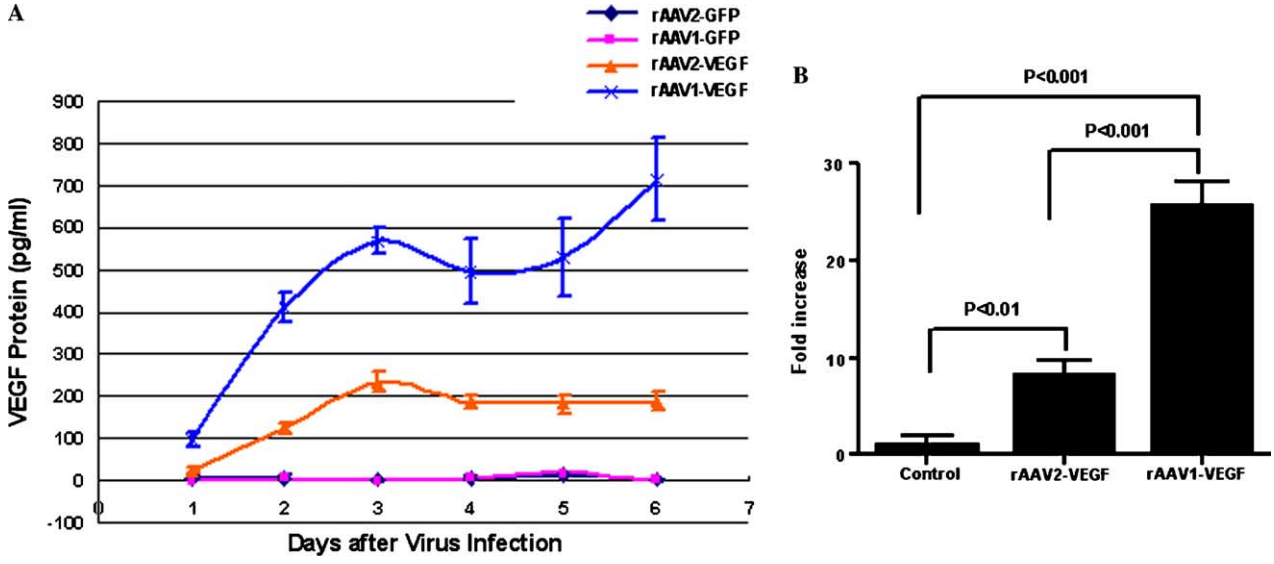

Fig. 1. Improved VEGF production in differentiated myotubes following the rAAV1-VEGF165 vector infection in vitro. (A) Human VEGF165 secretion from C2C12 derived myotubes following infection with the rAAV1-VEGF165 or the rAAV2-VEGF165 or the control rAAV (1 or 2)-GFP vector was quantified in supernatants daily by using an ELISA kit. The means and their standard deviations of the VEGF protein concentrations from three independent experiments are shown. (B) Real-time RT-PCR quantification of VEGF mRNA levels in C2C12 derived myotubes at day 6 following rAAV vector infection as in (A). The mean and SEM of the increase of the VEGF mRNA levels between the vector and mock infected groups from three independent experiments are shown.

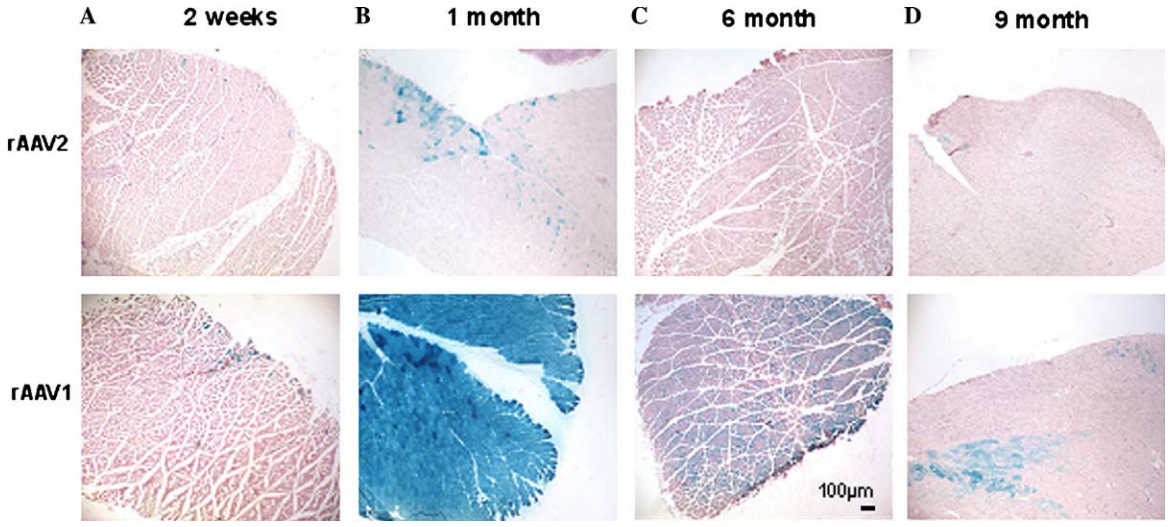

Fig. 2. Superior gene transfer of the rAAV1-LacZ vector into ischemic skeletal muscles. Mouse right thigh muscles were injected with 3×10^{11} vg of the rAAV1-LacZ or the rAAV2-LacZ vector, mice were killed at the indicated time point after vector injection, and the relevant muscles were stained for β-gal expression. Representative muscle sections from mice in each group examined are shown. Scale bar: 100 μm.

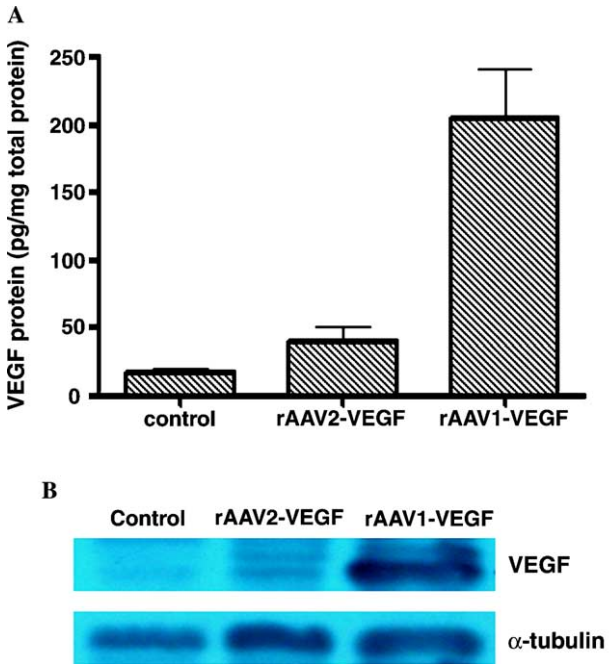

Fig. 3. Improved VEGF production in ischemic muscles following the rAAV1-VEGF165 vector infection. Ten days following hind limb ischemia induction, the ischemic thigh muscles were injected with 3×10^{11} vg of the rAAV1-VEGF165 or the rAAV2-VEGF165 vector, and the VEGF production in the relevant muscles was assessed at 1 month after vector injection. (A) Measurements of the human VEGF165 protein concentrations in the relevant muscle extracts by ELISA, the VEGF concentrations (mean ± SEM) from five animals in each group are shown. (B) Western blot analysis on VEGF protein production in the relevant muscle extracts. Muscle extracts (100 μg protein) were separated by 12% SDS–PAGE, blotted onto a nitrocellulose membrane, stained against VEGF specific antibodies, and visualized with an ECL kit. As a control for equal protein loading and transfer efficiency, the same membrane was stripped and stained against α-tubulin specific antibodies. Data shown are representative of three independent experiments.

with PBS or the LacZ encoding rAAV vectors (Fig. 3B). Simultaneously, we assessed whether the VEGF production could be detected in circulation. Human VEGF was not detected in the blood samples from the mice injected with the rAAV1-VEGF165 or the rAAV2-VEGF165 vector by ELISA, suggesting that the VEGF secretion from the injected muscles to circulation was below the levels for ELISA detection. It is also likely that the half-life of VEGF in circulation is rather short [38], thus the VEGF concentrations in the circulation were below the ELISA detection limit, irrespective of the high production levels in the rAAV1-VEGF165 vector infected muscles.

rAAV1-VEGF165 vector mediated gene transfer in ischemic skeletal muscles improved angiogenesis, arteriogenesis, and muscle regeneration

To evaluate the biological consequences of the elevated VEGF production following the rAAV1-VEGF165 vector gene transfer into ischemic skeletal muscles, we first evaluated the consequence on the formation of new vessels following injection of the rAAV1-VEGF165 or the rAAV2-VEGF165 vector into ischemic skeletal muscles at 6 weeks post-vector injection. The number of capillaries in the relevant muscle cross-sections was defined by staining for vWF, a marker of vessel endothelial cells (Figs. 4A and C) [39]. The rAAV1-VEGF165 vector infected ischemic muscles contained significantly more capillaries compared with that following the rAAV2-VEGF165 vector infection, the mean densities of the newly formed capillaries being 147 ± 13 per mm^2 and 70 ± 6 per mm^2 for the rAAV1-VEGF165 and the rAAV2-VEGF165 vector injected ischemic muscles, respectively ($P < 0.001$, $n = 6$, Fig. 4C). The rAAV2-VEGF165 vector injected ischemic muscles contained about 2-fold more capillaries compared

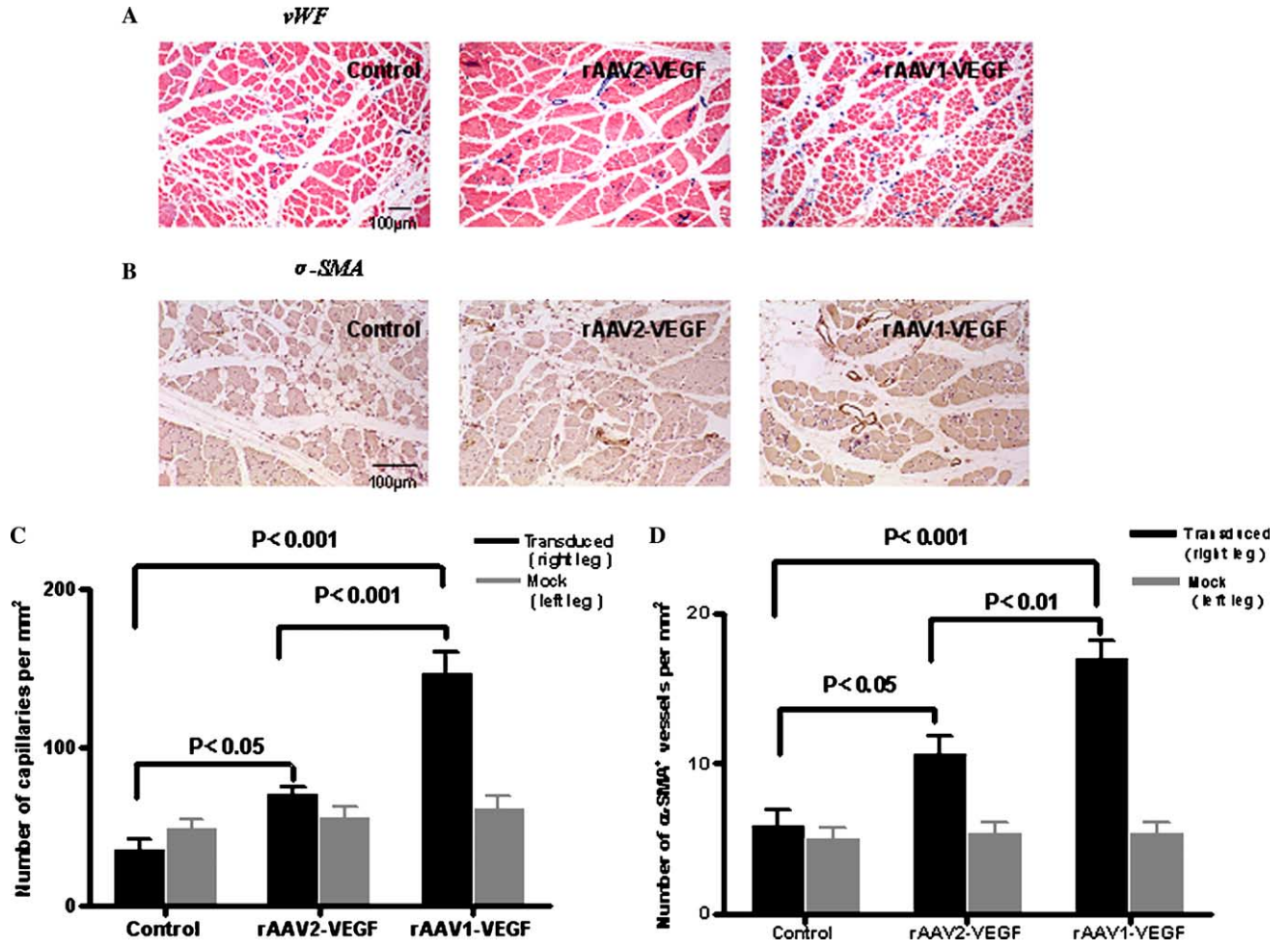

Fig. 4. Improved formation of capillaries and arterioles in ischemic muscles following injection with the rAAV1-VEGF165 vector in comparison with the rAAV2-VEGF165 vector. Representative staining for vWF defining vessel endothelial cells (A) and α-SMA defining vessel smooth muscle cells (B) at 6 weeks post-vector infection are shown, scale bars are indicated. The data of the vWF and the α-SMA staining from six animals are summarized in (C) and (D), respectively.

with the control ischemic muscles injected with PBS (36 ± 7 per mm^2, $P < 0.05$, $n = 6$, Fig. 4C). No angioma-like structure was observed. To investigate whether the formation of arterioles in the ischemic muscles was similarly improved following a rAAV1-VEGF165 vector injection, the relevant muscle cross-sections were stained with α-SMA, a marker of vessel smooth muscle cells. As shown in Fig. 4B, a thick layer of smooth muscle cells positively stained by anti-α-SMA antibodies represented arterioles. The ischemic muscles injected with the rAAV1-VEGF165 vector contained about 3.5-fold more arterioles compared with that in the control group, while the rAAV2-VEGF165 vector injected ischemic muscles contained about 2-fold more arterioles compared with that in the control group (Fig. 4D). Furthermore, we compared the anatomy of collateral arteries and arterioles of three groups using angiography at 6 weeks post-vector injection (Fig. 5A). The arrangement of collateral arteries and arterioles was similar in the rAAV2-VEGF165 and the rAAV1-VEGF165 vector injected ischemic muscles. In the rAAV1-VEGF165 vector injected ischemic muscles, more tortuous collateral arteries and arterioles connect the arteria profunda with the arteria saphena, arteria poplitea, or tibial arteries. Angiographic analysis showed that the rAAV1-VEGF165 vector treatment significantly enhanced blood supply compared with the rAAV2-VEGF165 vector treatment or the control group ($P < 0.01$, Fig. 5B).

Next, the relevant muscles were morphologically examined at 6 weeks post-vector injection (Fig. 6A). The control ischemic muscles without injection of VEGF165 encoding rAAV vectors showed a massive fiber loss accompanied by adipose substitution. The rAAV2-VEGF165 vector injected ischemic muscles indeed exhibited muscle fiber regeneration, yet with substantial adipose substitution. However, a markedly improved muscle fiber regeneration with a less degree of adipose substitution was observed in the rAAV1-VEGF165 vector injected ischemic muscles. To quantify the regenerated muscle fibers in the necrotic areas and their surrounding areas, the percentages of the fiber loss area were calculated from the sections derived from upper, middle, and lower parts of the ischemic muscles. Histological analysis showed that the percentages of

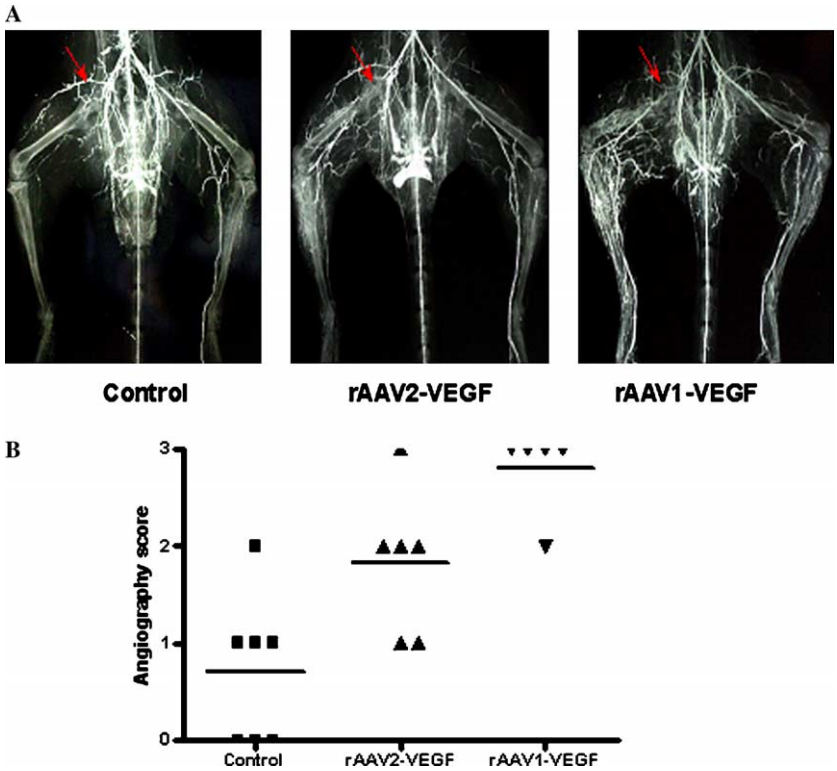

Fig. 5. Angiogram of mouse thigh vasculature at 6 weeks post-injection with PBS, the rAAV2-VEGF165 or the rAAV1-VEGF165 vector at 3×10^{11} vg per mouse. The ligation sites (indicated by arrows) can be seen in the right hind limb. Denser network of collateral vessels can be observed in the ligated limb following the rAAV1-VEGF165 vector treatment compared with that following the rAAV2-VEGF165 vector treatment (A). The angiography scores from 5 to 7 mice are summarized in (B), the average of angiography scores is indicated.

the fiber loss areas were $45.4 \pm 4.7\%$, $32.1 \pm 2.0\%$, and $20.2 \pm 5.3\%$ in the control, the rAAV2-VEGF165 vector injected, and the rAAV1-VEGF165 vector injected group, respectively. Compared with the control group, the rAAV1-VEGF165 vector injection significantly improved the muscle regeneration ($P < 0.01$, $n = 6$). However, such a trend did not reach statistic significance between the rAAV1-VEGF165 and the rAAV2-VEGF165 injected group (Fig. 6B). The increased muscle fiber regeneration following the rAAV1-VEGF165 vector infection coincided with enhanced cell proliferation in the damaged muscle areas, as demonstrated by higher frequencies of cells expressing PCNA in the rAAV1-VEGF165 vector infected muscles compared with the rAAV2-VEGF165 vector infected muscles (Fig. 6C).

To further verify whether the rAAV1-VEGF165 vector injection was paralleled by increased blood flow in ischemic muscles, perfusion studies using China ink were performed to display newly formed capillaries and arterioles. At 6 weeks post-vector injection, the density of newly formed capillaries and arterioles was increased in the rAAV2-VEGF165 vector injected ischemic muscles in comparison with that in the PBS injected ischemic muscles. A marked increase in the capillary and arteriole density was observed in the rAAV1-VEGF165 vector injected ischemic muscles in comparison to that following the rAAV2-VEGF165 vector injection (representative cross-sections shown in Fig. 7). Thus, the newly formed vessels indeed allow blood flow in the previously ischemic muscles.

As VEGF is also a vascular permeability factor, we further analyzed vascular leakiness by Evans blue dye extravasation. Evans blue dye was injected into mouse circulation via tail vein. The extravasation of Evans blue dye in the relevant skeletal muscles was assessed after a circulation period of 3 h. A massive dye infiltration was macroscopically evident in the rAAV1-VEGF165 vector injected muscles in comparison with that in the rAAV2-VEGF165 vector injected muscles, which is confirmed by spectrophotometric quantification of the Evans blue dye in the relevant muscle samples following formamide extraction (Fig. 8).

To explore the possibility that an appropriate rAAV1-VEGF165 vector dosage may produce VEGF at levels sufficient for vessel genesis but insufficient for vessel permeabilization, ischemic muscles were injected with the rAAV1-VEGF165 vector preparations at 3×10^{11}, 3×10^{10}, or 3×10^{9} vg. At 6 weeks post-vector injection, similar extent of arteriole genesis was observed in the previous ischemic muscles following the injection with 3×10^{10} vg of the rAAV1-VEGF165 vector or 3×10^{11} vg of the rAAV2-VEGF165 vector (Fig. 9A). Interestingly, the newly formed vessels in muscles injected with 3×10^{10} vg of the rAAV1-VEGF165 vector were significantly less leaky compared to those in muscles injected with 3×10^{11} vg of the rAAV1-VEGF165 vector (Fig. 9B).

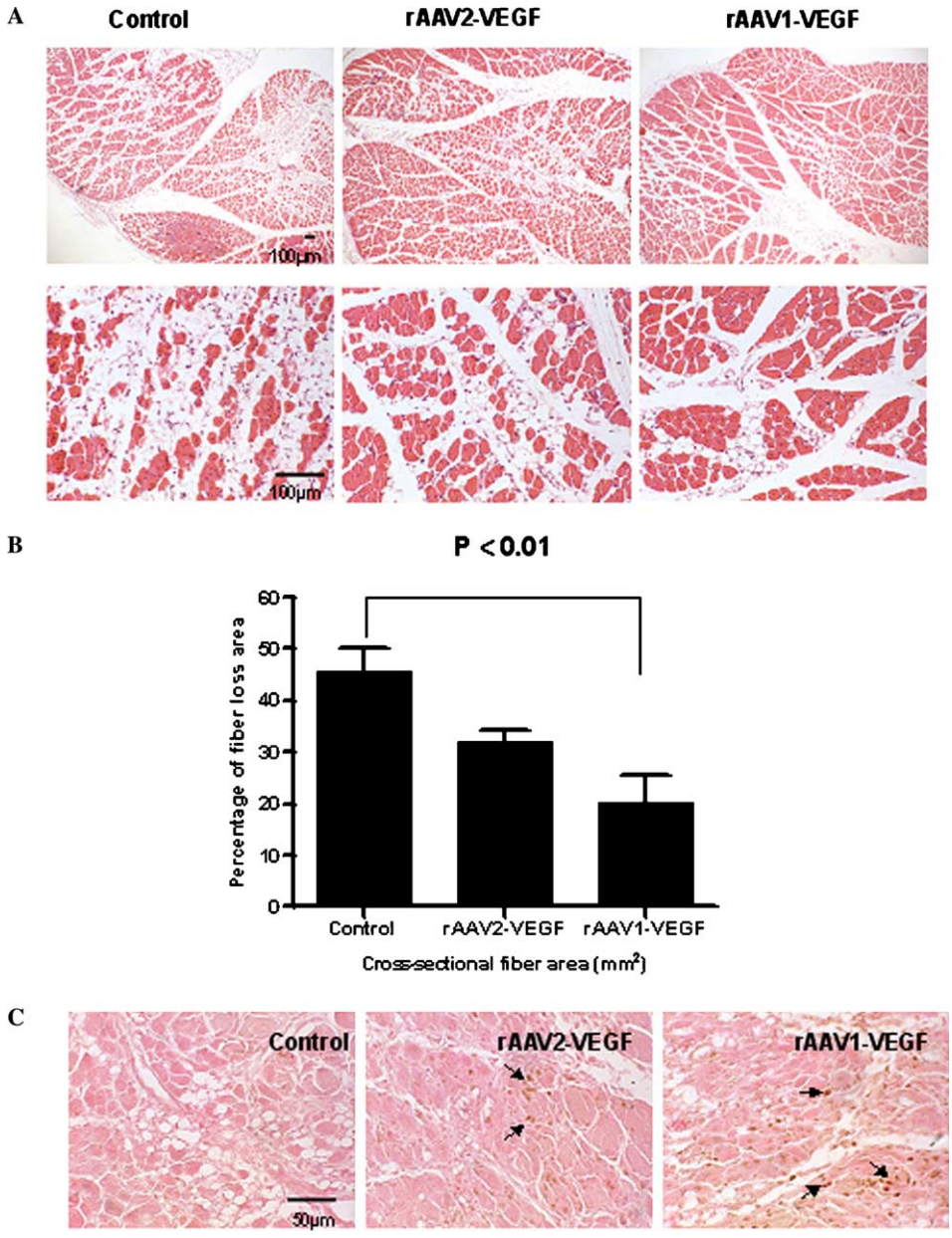

Fig. 6. Improved muscle fiber regeneration and cell proliferation in ischemic skeletal muscles following the rAAV1-VEGF165 vector infection. The ischemic thigh muscles were injected with 3×10^{11} vg of the rAAV1-VEGF165 or the rAAV2-VEGF165 vector, and morphological analysis with hematoxylin and eosin staining (A), assessment of fiber loss areas (B), and immunohistochemical staining for PCNA (arrows) (C) were performed at 6 weeks after vector injection. The percentages of fiber loss were calculated from the sections of necrotic areas and their surrounding areas derived from upper, middle, and lower parts of the ischemic muscles. Nine different fields were observed for each mouse and each group contained six mice. The nuclei positively stained for PCNA were mainly around or in the damaged muscle areas in both groups. Scale bars are indicated.

Discussion

Recent studies have reported that reporter gene transfer into muscle by vector based on AAV1 showed a much more superior transduction efficiency than did rAAV2 vector. In this study, we have demonstrated for the first time the potential role of the pseudotyped rAAV1 vector mediated functional VEGF gene transfer in the treatment of skeletal muscle ischemia. By using the rAAV1-LacZ vectors, we demonstrated that the rAAV1 vector mediated gene transfer into ischemic skeletal muscles could result in higher level, broader distribution, and long-lasting gene expression than the rAAV2 vector. Importantly, extensive vessel genesis with the formation of relatively mature vascular structures and superior muscle regeneration were achieved in ischemic skeletal muscles following injection of the rAAV1-VEGF165 vector preparations. Finally, injection of a relatively lower dose of the rAAV1-VEGF165 vectors was found sufficient for vessel genesis but apparently insufficient for vessel permeabilization.

Ischemic cardiovascular diseases such as peripheral arterial disease (PAD), ischemic heart disease, and vascular by-

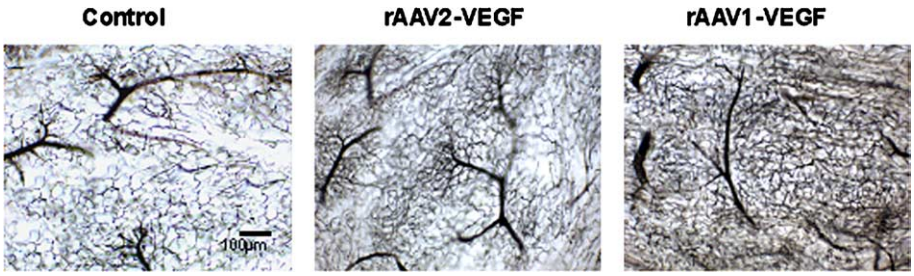

Fig. 7. Improved blood flow in the ischemic muscles following injection with the rAAV1-VEGF165 vectors. Six weeks post-vector injection, mice were perfused with China ink microsphere suspension. Thick cross-sections of 150 μm of the relevant muscles were prepared and examined under light microscope. Representative sections from relevant muscles derived from five animals in each group are shown.

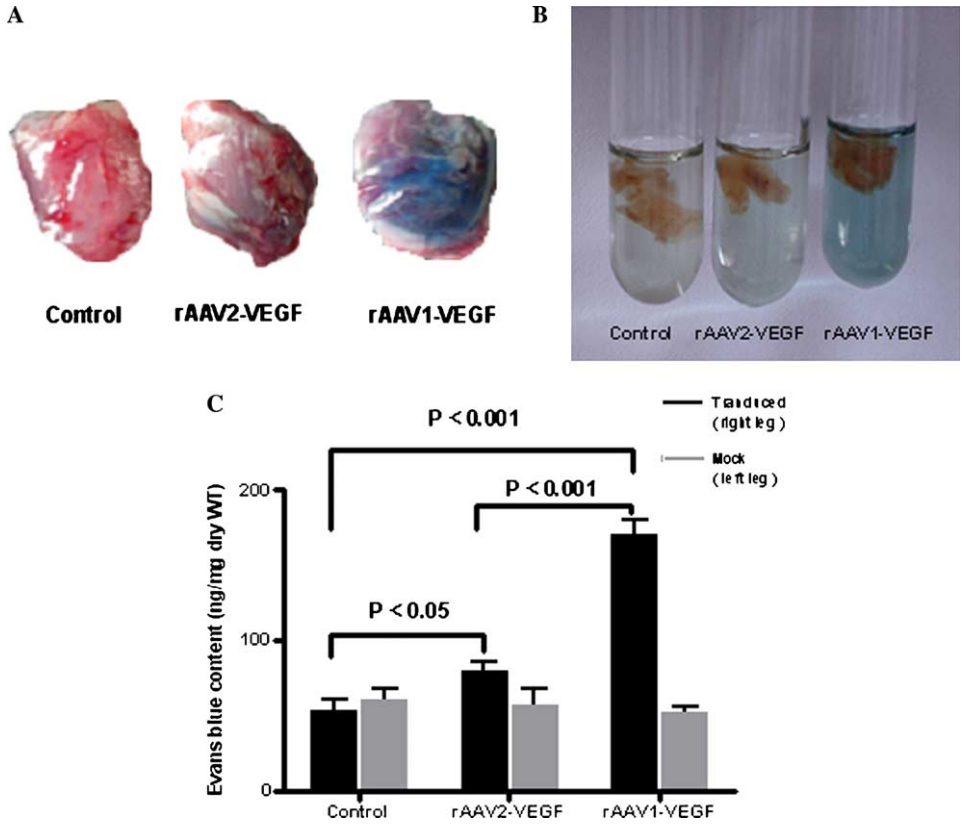

Fig. 8. The newly formed vessels are leaky in ischemic muscles following the rAAV2-VEGF165 or rAAV1-VEGF165 vector injection at 3×10^{11} vg per mouse. At 6 weeks following vector injection, Evans blue dye extravasation study was performed via tail vein injection. The relevant muscles were harvested following a circulation period of 30 min. Both macroscopic examination (A and B) and spectrophotometric quantification (C) showed a massive Evans blue dye infiltration in the rAAV1-VEGF165 injected muscles in comparison with that in the rAAV2-VEGF165 injected muscles. Data shown in (C) are means ± SEM of nanograms of Evans blue per milligram of dry muscle weight (WT) from four animals in each group.

pass graft occlusion are significant public health problems. Therapeutic angiogenesis that seeks to improve ischemic tissue perfusion by the formation of new vessels has emerged as a promising therapeutic strategy for the treatment of the ischemic cardiovascular diseases. However, recent results of clinical angiogenic trials are not ideal as expected or not proved to be an efficient therapeutic strategy [27–29]. Data from these trails suggest that insufficient duration expression of single-delivery of angiogenic growth factors, limited gene transfer efficiency, and gene expression localized to the site of delivery all are of key importance for clinically unsuccessful vascular growth factor therapy [30,31]. rAAV2 vector has been a favorable tool for gene transfer into muscles. Promising long-term muscle gene expression following rAAV2 vector gene transfer has been achieved in model studies performed in small animals [3–5], but expected biological consequences were not achieved in nonhuman primate models or patients following rAAV2 vector mediated muscle gene transfer because of neutralizing antibodies [10,14]. Gene expression following rAAV2 vector gene transfer in muscles appeared to be localized at the vector injection sites due to restricted tropism [39,40]. Broader

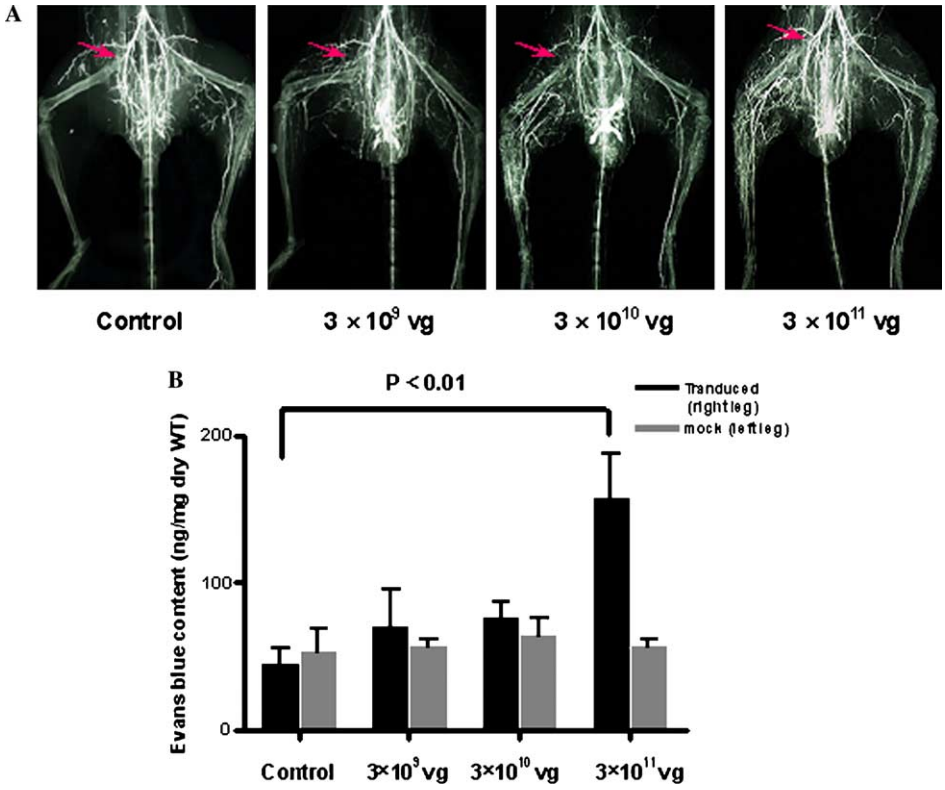

Fig. 9. Reduced rAAV1-VEGF165 vector dosage generated less leaky vessels. Ischemic muscles were injected with 3×10^{11}, 3×10^{10}, or 3×10^{9} vg of the rAAV1-VEGF vectors. At 6 weeks post-vector injection, vessel genesis was evaluated with angiography (A) and the vessel permeability was assessed with Evans blue extravasation (B). Representative angiograms are shown in (A), the means and SEM of extracted Evans blue in the ischemic muscles from each group of 5 to 7 mice are summarized in (B).

spreading of injected vectors into the injected muscles would be required for therapeutic levels of gene expression. Recent reports have suggested that different serotypes of AAVs exhibit different tropism, as their capsid proteins likely interact with distinct cellular receptor(s) for viral attachment and internalization [41,42]. Compared with the most commonly utilized rAAV2 vectors, rAAV1 vectors have been demonstrated to allow high levels of gene expression in normal skeletal muscles [24,25].

The rAAV1 vectors used in this study were generated by pseudotyping the corresponding rAAV2 vectors. Our preliminary data demonstrate that the rAAV1-GFP vector allowed superior gene transfer efficiency in comparison with the rAAV2-GFP vector in C2C12 derived myotubes. Analogous findings have been recently reported in cardiomyocytes [43]. In mouse ischemic skeletal muscles, distinct patterns of gene expression were achieved following injection of the rAAV1-LacZ or the rAAV2-LacZ vector. Based on the assessed time points, gene expression peaked at 1 month following vector injection. A significantly broader distribution of β-gal expression was achieved following the rAAV1-LacZ vector injection. In contrast, β-gal expression was restricted to the areas surrounding the rAAV2-LacZ vector injection sites. As the genomes of the rAAV1-LacZ and the rAAV2-LacZ vector are the same, the differences in the capsid proteins between these two vectors are very likely to be responsible for their strikingly different transduction efficiency in ischemic skeletal muscles. Examination of the β-gal expression at 6 months or 9 months post-vector injection suggested that adequate levels of long-term gene expression in ischemic skeletal muscles can be achieved with the rAAV1 vector, but rAAV2 vector may only allow low or undetectable levels of gene expression under the same conditions. These results can be explained by the following reasons. (1) the cell-surface attachment mechanisms of rAAV2 vectors can be dissected into two functionally uncoupled processes. rAAV2 vectors first bind to ubiquitously expressed cell-surface heparan sulfate proteoglycan (HSPG) as primary receptor for attachment [44]; subsequently, the entry of attached vectors is mediated by co-receptors such as human fibroblast growth factor receptor 1 (FGFR1) or αVβ5 integrin [45,46]. The relative abundance of the HSPG, FGFR1, and αVβ5 integrin predominantly determines whether a specific cell type is permissive or not to the rAAV2 vectors. However, the receptors for AAV1 are currently unknown. The differences in binding affinity to different receptors between the rAAV1 and the rAAV2 vector may account for the differences in LacZ gene transfer efficiency. (2) it is likely that a relatively strong immune response to rAAV2 capsid proteins was induced, as a result of which the β-gal expressing muscle cells were eliminated; (3) high levels

of β-gal expression at 1 month after vector injection were largely dependent on the vector genomes of episomal form, which were degraded at 6 months or 9 months after vector injection.

We have been particularly interested in the biological consequences following the rAAV1-VEGF165 vector gene transfer in ischemic skeletal muscles. Higher levels of VEGF expression were indeed detected in the rAAV1-VEGF165 vector injected ischemic skeletal muscles. Based on the time course assessment of β-gal expression following the rAAV1-LacZ vector injection, it is likely that a long-lasting and broad-distributing VEGF production was also achieved following the rAAV1-VEGF165 vector injection. In agreement with higher levels of VEGF production, significantly more capillaries (defined by vWF staining), arterioles (demonstrated by α-SMA staining and angiography), and superior muscle regeneration were generated in the rAAV1-VEGF165 vector injected ischemic skeletal muscles compared with that following the rAAV2-VEGF165 vector injection. These data, in turn, are supported by the high frequencies of the PCNA positive cells in the rAAV1-VEGF165 vector injected ischemic skeletal muscles. Functionally, the newly formed capillaries and arterioles indeed allowed blood flow as examined in angiography and China ink perfusion assay. Restored blood flow in ischemic skeletal muscles very likely contributed to muscle fiber regeneration, therefore, superior muscle fiber regeneration was achieved in the rAAV1-VEGF165 vector injected ischemic skeletal muscles. It is also likely that high levels of the VEGF production in the rAAV1-VEGF165 vector injected ischemic skeletal muscles directly contributed to muscle fiber regeneration [36]. However, the newly formed capillaries and arterioles were leaky at high vector dosage, likely due to persistent production of the VEGF, as VEGF is also a potent vascular permeability factor. Finally, we demonstrated that the biological consequences following the rAAV1-VEGF165 vector injection were vector dosage dependent. Injection of a relatively lower dosage of the rAAV1-VEGF165 vector was also effective in the new vessel formation but caused much less leakiness, suggesting that a way to overcome the leakage may be to try a lower dose in the future experiment.

In summary, our studies demonstrate that the rAAV1 vectors can allow long-term, broader distribution and high level gene expression in ischemic skeletal muscles in comparison with the rAAV2 vectors. rAAV1 vector mediated VEGF gene transfer improved more superior neovascularization and muscle fiber regeneration than rAAV2 vector did. Thus, rAAV1 vector mediated transfer of VEGF gene may facilitate the treatment of muscle ischemia.

Acknowledgments

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