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AAV-mediated VEGF gene transfer into skeletal muscle stimulates angiogenesis and improves blood flow in a rat hindlimb ischemia model

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Abstract

Objectives: Clinical trials on therapeutic angiogenesis using vascular endothelial growth factor (VEGF) are ongoing, however the benefits of these therapies are still controversial. To establish a more efficient gene transfer method for ischemic diseases, we investigated the therapeutic potential of adeno-associated virus (AAV)-mediated VEGF gene transfer. **Methods:** We produced $VEGF_{165}$-expressing AAV vectors (AAV-VEGF). HEK-293 cells were transduced with AAV-VEGF in vitro and VEGF expression and secretion were examined. We used a rat ischemic hindlimb model and AAV-VEGF was administered intramuscularly into the ischemic limb. Gene expression was evaluated by RT-PCR and ELISA. Six weeks after gene transfer, we measured the blood flow of limb vessels and the skin temperature of limbs. Histochemical examination was performed to illustrate capillary growth. **Results:** Western blotting and ELISA revealed VEGF protein expression and secretion from AAV-VEGF-transduced HEK-293 cells. VEGF mRNA and protein expression was consistently observed in the injected muscle at least 10 weeks after the injection, while no VEGF mRNA could be detected at remote organs. The mean blood flow in AAV-VEGF-transduced ischemic limbs was significantly higher than in AAV-LacZ-transduced limbs. Capillary density was significantly higher in AAV-VEGF-injected tissues than in AAV-LacZ-injected tissues. **Conclusions:** This study demonstrates that (1) AAV-mediated VEGF gene transfer into rat skeletal muscles is efficient and stable without ectopic expression, and (2) AAV-mediated VEGF gene transfer stimulates angiogenesis and thereby improves blood flow in a rat hindlimb ischemia model. These findings suggest that AAV-mediated VEGF gene transfer may be useful for treatment of ischemic diseases.

Keywords: Angiogenesis; Atherosclerosis; Cytokines; Gene therapy; Growth factors; Ischemia

1. Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a heparin-binding dimeric glycoprotein and a principal angiogenic factor stimulating migration, proliferation, and expression of various genes in endothelial cells [1,2]. VEGF is synthesized by cells around the vasculature and affects endothelial cells as a paracrine factor. Its expression is upregulated by hypoxia and various cytokines. We previously reported increased circulating VEGF levels in patients with acute myocardial infarction [3]. VEGF has been shown to stimulate the development of collateral vessels in animal models of peripheral and myocardial ischemia [4–

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6]. There are many patients with ischemic diseases for whom pharmacological intervention is ineffective and therapy is limited to surgical revascularization or endovascular interventional therapy. Promoting the formation of new collateral vessels in the ischemic myocardium and leg muscles is an important role for the treatment of such disorders. Recently, clinical trials of therapeutic angiogenesis using the VEGF gene have been initiated. Several uncontrolled clinical trials showed that VEGF gene transfer resulted in clinical improvement in patients with ischemic diseases [7,8]; however, the benefits of these therapies are still controversial [9].

Beneficial gene transfer vehicles are necessary for the clinical application of gene therapy. Recombinant viral vectors based on a non-pathogenic human parvovirus, adeno-associated virus (AAV), have a number of attractive features, including lack of cytotoxicity, ability to transduce both dividing and non-dividing cells [10], and long-term transgene expression [11–13]. AAV vectors can transduce efficiently to the skeletal muscles [13], cardiac myocytes [14,15], neurons [12,16,17], lungs [11], hepatocytes [18], renal cells [19], and endothelial cells [20], and have been evaluated in clinical trials for hemophilia B [21] and cystic fibrosis [22].

In the present study, in order to establish a more efficient and stable gene transfer method for ischemic diseases, we produced VEGF-expressing AAV vectors and assessed the efficiency and stability of AAV-mediated gene transfer into rat hindlimbs by intramuscular injection. Furthermore, we investigated whether AAV-mediated VEGF gene transfer could stimulate angiogenesis and thereby improve the hemodynamic deficit in the ischemic limb of a rat model.

2. Methods

2.1. Plasmid construction and vector production

The parent AAV vector plasmid pAAV-LacZ contains the LacZ reporter gene with the human cytomegalovirus (CMV) immediate early promoter and simian virus 40 polyadenylation signal sequence between the inverted terminal repeats (ITRs) of the AAV-2 genome [16]. The pAAV-VEGF vector plasmid was constructed by excising the LacZ reporter gene from the pAAV-LacZ and replacing it with the full-length human $VEGF_{165}$ cDNA isolated from the pUC18 vector [23].

We produced AAV vectors without the use of a helper adenovirus, as described previously [24]. The vector-production process involved cotransfection of human embryonic kidney cell line 293 (HEK-293) with the following plasmids: the AAV vector plasmid (pAAV-LacZ, pAAV-VEGF), the AAV helper plasmid, and the adenovirus helper plasmid, using the calcium phosphate method. Then, the AAV vectors were harvested and purified after two sequential continuous CsCl gradients as described [24]. The vector particle titer was determined by quantitative DNA dot-blot hybridization of DNase I-treated vector stocks.

2.2. In vitro transduction and Western blotting

HEK-293 cells were transduced with AAV vectors by adding the indicated amount of vector stock diluted in 10% fetal bovine serum (FBS) containing DMEM/F-12 medium for 24 h. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (1% Nonidit P-40, 50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 200 U/ml aprotinine, 1 mmol/l PMSF). The cell lysates (10 μg of protein) were separated by 12% polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Immunoblotting was performed with anti-human VEGF antibody (Sigma, St. Louis, MO, USA) and the specific binding of the antibody was visualized with an ECL detection system (Amersham, UK).

2.3. Assay for VEGF concentration

HEK-293 cells were incubated with AAV-VEGF-containing medium for 24 h. Then, cells were washed with PBS twice and incubated in serum-free medium. Twenty-four hours after replacement of the culture medium, VEGF concentrations of the culture supernatant were measured with an enzyme-linked immunosorbent assay (ELISA) method using the Biotrak human VEGF ELISA system (Amersham) according to the manufacturer's instructions. In brief, 50 μl of each sample was added to an anti-human VEGF precoated plate and incubated at room temperature for 2 h. The plate was washed three times, and then biotinylated antibody reagent was added to the plate and incubated for 1 h. After washing, streptavidin-HRP reagent was added and incubated for 1 h. Finally, substrate solution was added to the plate and the optical density at 450 nm was determined. The standard curve was linear from 15.6 to 1000 pg/ml of VEGF. VEGF levels of the serum were also determined using the same ELISA system.

2.4. Intramuscular administration of AAV vectors

All animal experiments were performed in accordance with the *Jichi Medical School Guide for Laboratory Animals*, 1993. Male Sprague–Dawley rats (200–250 g) were anesthetized with diethyl ether. A skin incision about 5 mm in length was made over the tibialis anterior muscle and the fascia identified. AAV vectors were diluted in 50 mM Hepes-buffered saline and carbon black was added (Pelikan ink, Günther Wagner) to allow tracing of the injection site. The vector suspension containing AAV-LacZ (1.0×10^{13} particles/200 μl) and AAV-VEGF (1.8×10^{13}

particles/200 μl) was injected with a 29-gauge needle into two different sites (100 μl/site) in the tibialis anterior muscle.

2.5. RT-PCR

Gene expression at the mRNA level was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA of muscle tissues and remote tissues (brain, heart, liver, spleen, kidney, testes) was isolated using RNA STAT-60 (TET-TEST, Friendswood, TX, USA). Extracted RNA was treated with DNase I (Takara Shuzo, Tokyo, Japan) to eliminate DNA contamination. The synthesis of first-strand cDNA was performed under the conditions recommended in the ProSTAR First Strand RT-PCR Kit (Stratagene, La Jolla, CA, USA). The PCR amplifications were performed using human VEGF specific primers (sense, 5′-GAGGGCAGAATCATCACGAAGT; antisense, 5′-TGAGAGATCTGGTTCCCGAAAC-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (sense, 5′-TATTGGGCGCCTGGTCACCA-3′; antisense, 5′-CCACCTTCTTGATGTCATCA-3′). GAPDH mRNA served as an internal standard. The PCR products were electrophoresed on ethidium bromide-stained 2.0% agarose gels.

2.6. Rat hindlimb ischemia model and gene transfer

Male Sprague–Dawley rats (200–250 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A longitudinal incision was made in the right thigh, after which the right femoral artery was surgically excised to induce limb ischemia. Rats were then transduced with AAV-VEGF (2.0×10^{13} particles; $n=8$) or AAV-LacZ (1.5×10^{13} particles; $n=8$) via an intramuscular injection. The vector suspension (100 μl/site) was injected into four different sites in the major thigh muscles (quadriceps and adductor). Three rats received sham operations for preliminary assessment of hemodynamic examination.

2.7. Blood flow measurement and thermography

Six weeks after gene transfer, each rat was re-anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and the lower body coats were shaved. The skin temperature of the rat hindlimb was measured with infrared thermography (TH3106ME, NEC San-ei Instruments, Tokyo, Japan).

Blood flow at the tibialis posterior artery was measured by a transit-time ultrasound flowmeter (T206, Transonic Systems, Ithaca, NY, USA) using a perivascular flowprobe. The tibialis posterior artery was dissected free and perivascular flowprobes placed according to the manufacturer's instructions. Blood flow of both the ischemic and contralateral limbs was recorded simultaneously, and was expressed as a percentage of the contralateral limbs.

2.8. Histological assessment

Muscle tissues were obtained as transverse sections from the quadriceps and adductor muscles of the ischemic limb after hemodynamic examination. Frozen sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells, as described previously [25]. Capillary density was evaluated by histological examination of five randomly selected fields of one muscle section, and the number of capillaries was counted (mean number of capillary per square millimeter).

2.9. Statistical analysis

Results are expressed as mean±S.E.M. Statistical significance was evaluated by unpaired Student's *t*-test for comparisons between two means, and one-way ANOVA combined with Fisher's PLSD test for more than two means. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. AAV-mediated VEGF gene transfer in vitro

We initially performed in vitro transduction of the human $VEGF_{165}$ gene by AAV vectors. HEK-293 cells were incubated in a medium containing AAV-VEGF (1.0×10^5 particles/cell) for 24 h, and subjected to Western blotting. Immunoblotting using anti-human VEGF anti-

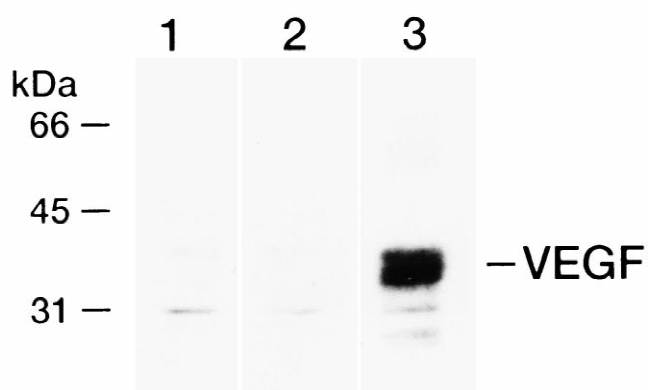

Fig. 1. Expression of human $VEGF_{165}$ protein in HEK-293 cells. HEK-293 cells were transduced with AAV-VEGF or AAV-LacZ and lysed 24 h after transduction. The VEGF protein was separated by 12% polyacrylamide gel electrophoresis and blotted onto a membrane. 42 kDa VEGF protein was evident in VEGF-transduced HEK-293 cells (lane 3), while no VEGF protein was expressed in both untransduced (lane 1) and LacZ-transduced (lane 2) HEK-293 cells.

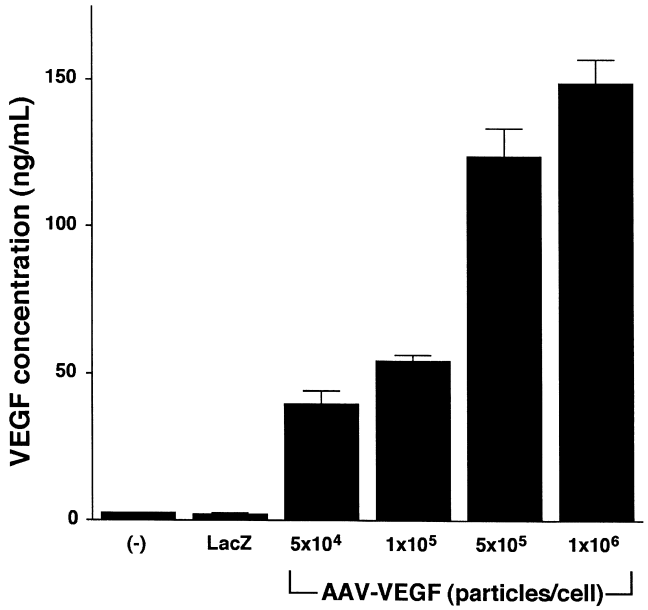

Fig. 2. VEGF concentrations in the culture supernatant of AAV-transduced HEK-293 cells. HEK-293 cells were exposed to increasing titers of AAV-VEGF or AAV-LacZ for 24 h. Then, 24 h after replacement of the culture medium, the VEGF concentration was measured by ELISA. The VEGF concentration is shown as the mean value±S.E.M. of measurements from one experiment ($n=4$), representative of three different experiments.

bodies clearly demonstrated 42 kDa VEGF protein expression in the VEGF-transduced HEK-293 cells (Fig. 1).

We next investigated whether VEGF was successfully released from the transduced HEK-293 cells. Cells were incubated with the vector-containing medium for 24 h, and the concentration of VEGF in the cultured medium was measured by ELISA. Fig. 2 demonstrates the relationship between AAV-VEGF vector concentrations and VEGF levels in the culture supernatant. VEGF levels in the culture supernatant increased in a titer-dependent manner (5×10^4–1×10^6 particles/cell). Conversely, the culture supernatant from non-transduced cells and LacZ-transduced cells contained quite low levels of VEGF.

3.2. VEGF gene transfer into skeletal muscles

We next performed in vivo gene transfer into the rat skeletal muscle using AAV vectors. To determine whether an AAV vector could be used to efficiently and stably transduce rat muscle tissues, 1.0×10^{13} particles of AAV-LacZ were injected into the tibialis anterior muscle. X-gal histochemical staining revealed efficient β-galactosidase expression (the majority of muscle fibers in the area of injection) at least 12 weeks after injection (data not shown). We then performed in vivo transduction of the human $VEGF_{165}$ gene by AAV vectors (1.8×10^{13} particles/site). To confirm human $VEGF_{165}$ gene expression in transduced rat tibialis anterior muscles, RT-PCR using human VEGF-specific primers was performed. VEGF gene expression was consistently observed 4 and 10 weeks after injections (Fig. 3). On the other hand, no gene expression was observed in AAV-LacZ-injected tibialis anterior muscles. No human $VEGF_{165}$ mRNA could be detected by RT-PCR from remote organs (brain, heart, liver, spleen, kidney, and testes) in AAV-VEGF-treated rats 4 weeks after injection (Fig. 3).

We further examined VEGF secretion from transduced muscle tissues. AAV vector-injected tibialis anterior muscle tissues were excised and cultured in serum-free DMEM/F-12 medium, and the VEGF concentrations of the culture supernatant were measured by ELISA. Ten weeks after gene transfer, significant amounts of VEGF (up to 5.3±1.5 ng/g tissue/24 h) were detected in the culture supernatant

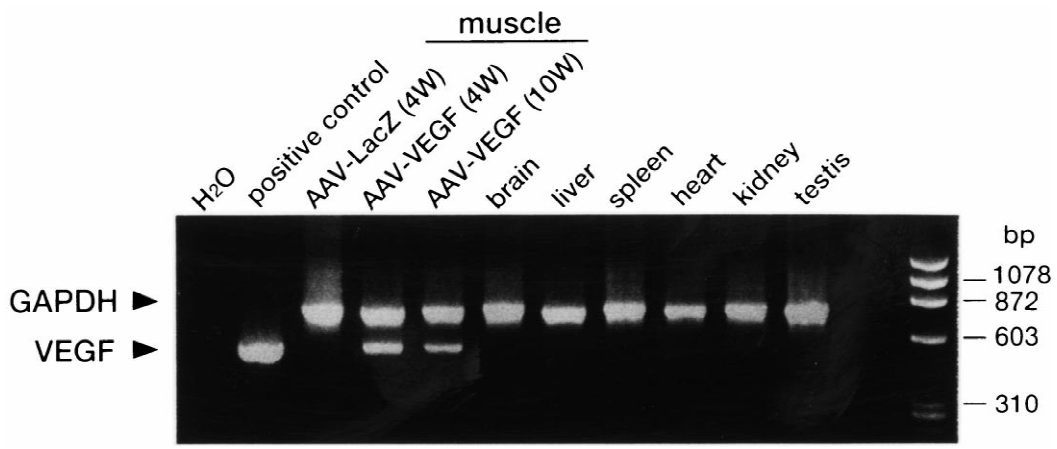

Fig. 3. Expression of VEGF mRNA in vector-injected muscle tissues and remote organs. Muscle tissues and other organs were isolated at 4 and 10 weeks after intramuscular injection and total RNA was extracted. After DNase-I treatment, RT-PCR using human VEGF-specific primers was performed. The sizes of PCR products for rat GAPDH and human VEGF were 747 and 531 bp, respectively. VEGF expression plasmid (pCMV-VEGF, 1 ng) was used as a positive control. GAPDH mRNA served as an internal standard. The PCR products were electrophoresed on ethidium bromide-stained 2.0% agarose gels. Three independent experiments yielded identical results.

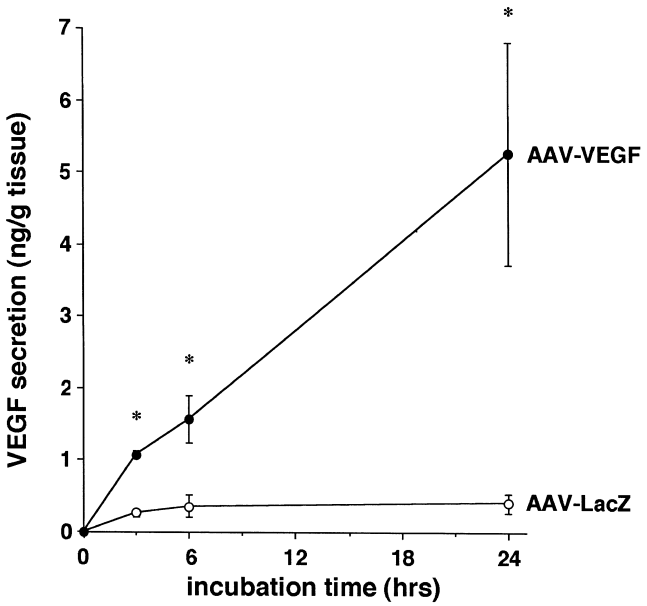

Fig. 4. VEGF secretion from AAV vector-injected tibialis anterior muscles. Ten weeks after gene transfer, muscle tissues were excised and cultured in serum-free DMEM/F-12 medium at 37 °C. After incubation for various periods as indicated on the *X*-axis, the VEGF concentration of the culture supernatant was measured by ELISA. VEGF concentrations were normalized to the protein content per dish and are shown as mean values±S.E.M. of measurements from one experiment ($n=4$), representative of two different experiments. *$P<0.05$ compared with values of AAV-LacZ-transduced muscles.

of AAV-VEGF-injected (1.8×10^{13} particles/site) muscle tissues (Fig. 4), while no substantial amount of VEGF was detected in AAV-LacZ-injected tissues. These results suggest constitutive expression and release of VEGF from AAV-VEGF-transduced muscles.

3.3. VEGF levels in the systemic circulation

Blood samples were also obtained from the peripheral veins of rats 1, 2, 4, and 8 weeks after AAV-VEGF transduction and analyzed with ELISA for human VEGF to detect VEGF levels in the systemic circulation. Serum VEGF levels were lower than the detectable level of the assay kit (15.6 pg/ml) at each time point.

3.4. VEGF gene transfer to a rat hindlimb ischemia model

We next investigated the therapeutic effects of AAV-mediated VEGF gene transfer. We performed AAV-mediated gene transfer by intramuscular injection into rat ischemic hindlimbs. Six weeks after gene transfer, we assessed the blood flow of rat hindlimbs using an ultrasound flowmeter. As shown in Fig. 5, the mean blood flow

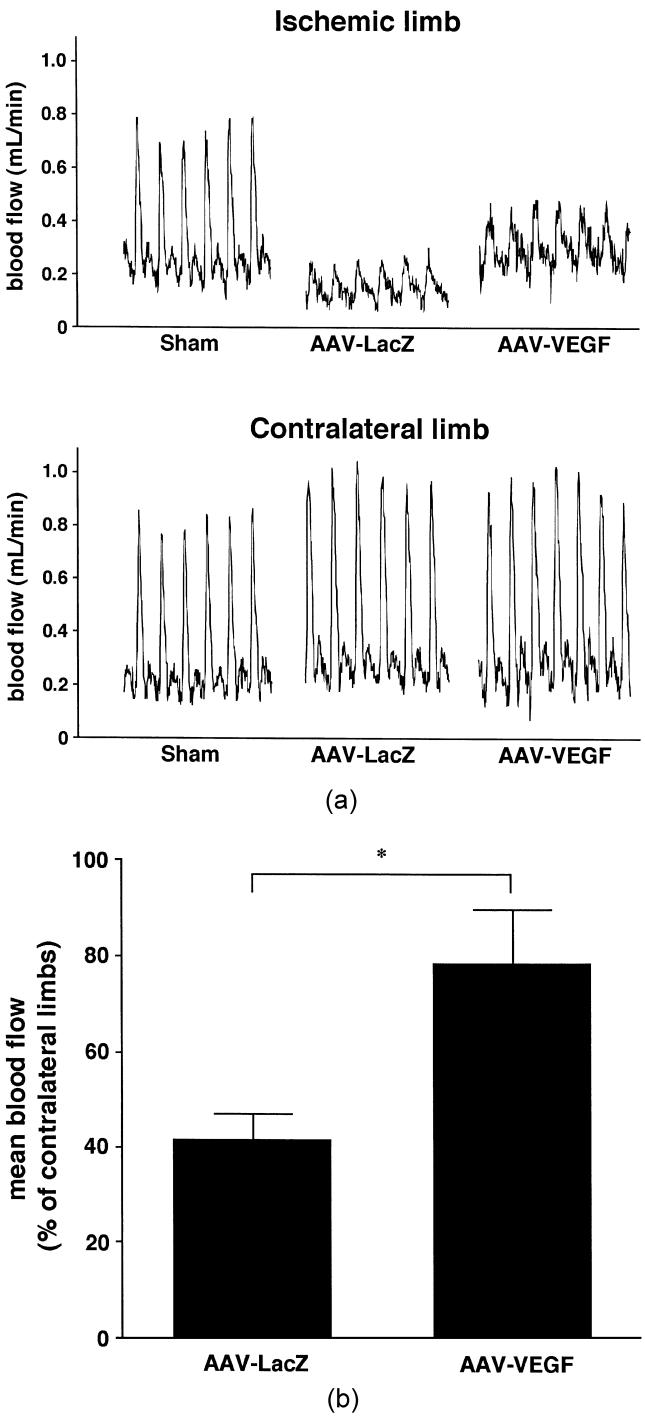

Fig. 5. Blood flow in the rat hindlimb was measured by a transit-time ultrasound flowmeter 6 weeks after gene transfer. After anesthetization, blood flow of both ischemic and contralateral limbs was recorded simultaneously in sham-operated ($n=3$), AAV-LacZ-transduced (1.5×10^{13} particles/body; $n=8$) and AAV-VEGF-transduced (2.0×10^{13} particles/body; $n=8$) rats. (A) Representative record of blood flow. (B) Mean blood flow in the ischemic limbs. Values are expressed as a percentage of contralateral limbs and are shown as mean values±S.E.M. *$P<0.01$.

in AAV-VEGF-transduced ischemic limbs (78.2±11.3% of contralateral limbs) was significantly higher than that in AAV-LacZ-transduced ischemic limbs (41.5±5.4%).

To assess the improved perfusion of ischemic limbs, we measured the skin temperature of the rat hindlimbs with infrared thermography. The skin temperature of the LacZ-transduced ischemic limb was about 2 °C lower than that of the contralateral limb. Conversely, AAV-mediated VEGF gene transfer brought about a restoration of the skin temperature in the ischemic limb (Fig. 6).

The thigh muscles of the ischemic limb were examined histologically 6 weeks after gene transfer. As shown in

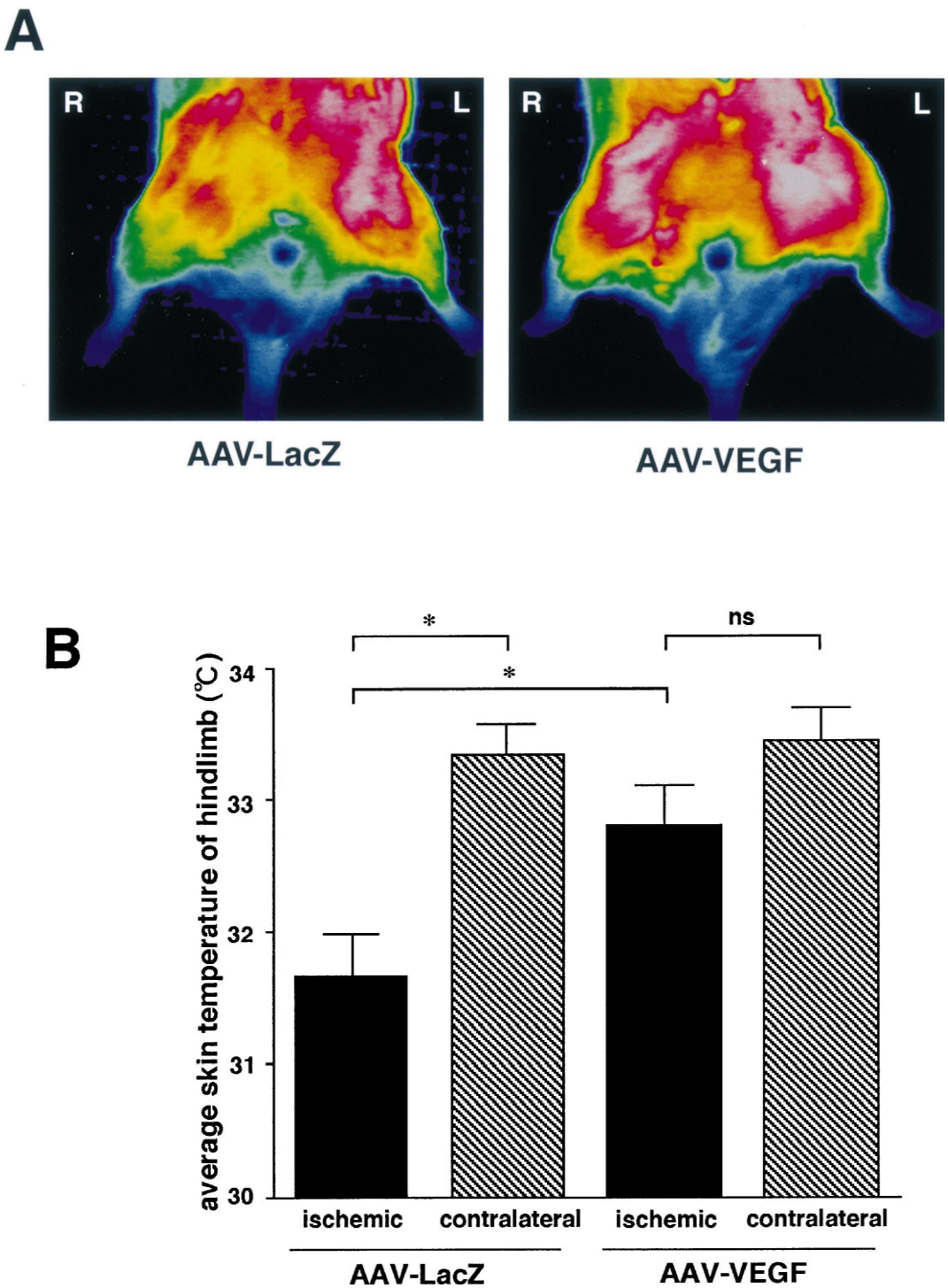

Fig. 6. Thermography of rat hindlimbs was performed 6 weeks after gene transfer. Rats were anesthetized and their lower body coats were shaved. The skin temperature of each rat hindlimb was measured with infrared thermography. (A) Color images of infrared thermography. R, ischemic limbs; L, contralateral limbs. (B) Average skin temperature (°C) of ischemic and contralateral hindlimbs in AAV-LacZ- (1.5×10^{13} particles/body; $n=4$) or AAV-VEGF- (2.0×10^{13} particles/body; $n=4$) transduced rats. *$P<0.05$.

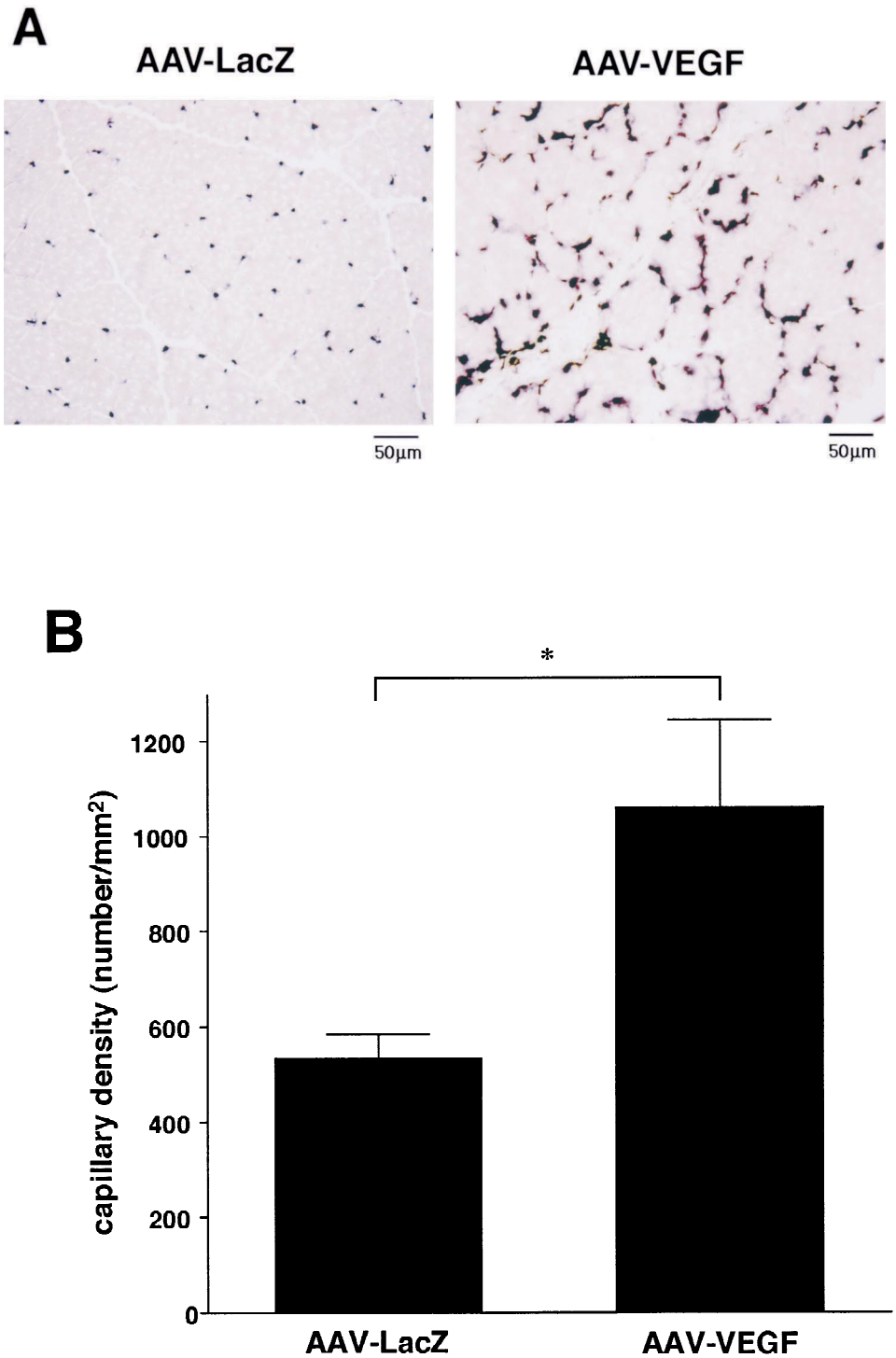

Fig. 7. (A) Representative images of rat muscle tissues using histochemical staining for alkaline phosphatase. (B) Capillary density in rat muscle tissues. Muscle tissues were obtained from the ischemic limbs of AAV-LacZ-transduced ($n=4$) and AAV-VEGF-transduced ($n=4$) rats. The number of capillaries was counted in five different fields of one muscle section, and capillary density was calculated. Data are shown as mean values±S.E.M. *$P<0.001$.

Fig. 7, capillary density was significantly higher in AAV-VEGF-injected muscle tissues ($1062\pm75/mm^2$) than that in AAV-LacZ-injected muscle tissues ($532\pm24/mm^2$). There was no angioma-like structure or inflammatory-cell infiltration observed in either ischemic or contralateral limbs.

4. Discussion

The present study was designed to examine whether the AAV vector is able to transfer the VEGF gene efficiently and safely. In the in vitro study, we revealed that AAV-mediated VEGF gene transfer into HEK-293 cells induced

the expression and secretion of VEGF. We also demonstrated that significant amounts of VEGF were detected in the culture supernatant of AAV-VEGF-injected muscle tissues, suggesting constitutive expression and release of VEGF from the muscle in vivo. However, serum VEGF levels in the systemic circulation were lower than the detectable level at each time point. This may be due to the very short biological half-life of VEGF; it may be secreted and function only in the local area of injection. Furthermore, no evidence of human $VEGF_{165}$ mRNA expression was observed at remote organs (brain, heart, liver, spleen, kidney, and testes) in AAV-VEGF-treated rats. These findings suggest that AAV-mediated gene transfer is safe without ectopic expression for administration of VEGF.

For therapeutic angiogenesis, several different strategies have been examined to deliver VEGF in animal models. In some cases, the recombinant protein was tested. In others, VEGF gene transfer using naked DNA or adenoviral vectors was performed. The use of naked DNA is simple and well tolerated by the recipient organism due to the low toxicity and immune response. However, the transduction efficiency is significantly lower compared with other methods. The adenovirus has frequently been the vector of choice for gene transfer because it is able to transduce a variety of cells with high efficiency. However, the major limitations of adenoviral vectors are lack of sustained expression, antigenicity against viral proteins by both humoral and cytotoxic T-lymphocytes, and possible toxicity at high doses. Recently, clinical trials on therapeutic angiogenesis using VEGF have been initiated. Arterial or intramuscular gene transfer of naked DNA encoding $VEGF_{165}$ in patients with severe limb ischemia produced angiographic and histological evidence of angiogenesis [7,26]. Clinical trials using naked VEGF DNA or adenovirus-mediated gene transfer in severe myocardial ischemia patients are also now ongoing [8,27]. However, a placebo-controlled phase II study, in which recombinant $VEGF_{165}$ was delivered as a single intracoronary infusion, followed by three intravenous infusions, did not demonstrate any clinical benefit [9], possibly due to short-term, transient expression of VEGF with that transfer method.

On the other hand, AAV vectors have a number of attractive features, including lack of cytotoxicity, ability to transduce both dividing and non-dividing cells [10], and longer-term transgene expression compared with plasmid or adenovirus-based methods [11–13,28]. In the present study, VEGF expression in rat skeletal muscles persisted for at least 10 weeks after AAV-mediated gene transfer without inflammatory response. We also demonstrated that AAV-mediated VEGF gene transfer promoted capillary growth in the ischemic limb. Furthermore, we clarified that the mean blood flow in VEGF-transduced ischemic limbs was significantly higher than that in LacZ-transduced limbs, and that the average skin temperature of the former was significantly higher than that of the latter. These findings showed that AAV-mediated VEGF gene transfer had therapeutic effects in a rat hindlimb ischemia model.

The formation of hemangioma is a considerable problem associated with prolonged and high-level expression of VEGF. Springer et al. [29] reported that high levels of serum VEGF (200 μg/ml) caused hemangiomas in skeletal muscles and that low serum levels (30 μg/ml) did not cause vascular malformations, but were sufficient to induce angiogenesis in ischemic muscles. It was also shown that retroviral vector-mediated VEGF gene transfer to murine hearts induced hemangioma formation [30]. At the vector dose we used, AAV-VEGF could induce angiogenesis in the local ischemic environment; however, there was no angioma-like structure observed in any of the ischemic or contralateral limbs. Very recently, Byun et al. [31] reported AAV-mediated VEGF transfer into a rat hindlimb ischemia model. Regional blood flow and capillary density were significantly increased with an increase in tissue VEGF mRNA levels, but there was no detectable increase in serum VEGF levels. Su et al. [32] reported AAV-mediated VEGF gene transfer into the mouse ischemic heart. AAV-mediated VEGF transfer induced angiogenesis in the ischemic heart. There was no angioma observed in the heart and no VEGF was detected in the mouse serum. These and our findings suggest that AAV-mediated VEGF expression is not as high as those mediated by adenoviral and retroviral vectors, yet it is sufficient to induce new vascular formation in ischemic tissue. Thus, with an appropriate dose, AAV may represent the ideal vector for VEGF delivery.

In summary, the present study demonstrated that AAV-mediated gene transfer into rat skeletal muscles caused efficient and stable gene expression without ectopic expression. We also demonstrated that AAV-mediated VEGF gene transfer stimulates angiogenesis and thereby improves blood flow in a rat hindlimb ischemia model. These findings suggest that AAV-mediated VEGF gene transfer may be useful in the treatment of ischemic diseases.

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