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Expression of Vascular Endothelial Growth Factor and Vascular Endothelial Growth Factor Receptor-2 (KDR/Flk-1) in Ischemic Skeletal Muscle and Its Regeneration

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Vascular endothelial growth factor (VEGF) is a hypoxia-inducible endothelial cell mitogen and survival factor. Its receptor VEGFR-2 (KDR/Flk-1) mediates these effects. We studied the expression of VEGF and VEGFR-2 in ischemic human and rabbit skeletal muscle by immunohistochemistry and *in situ* hybridization. Human samples were obtained from eight lower limb amputations because of acute or chronic critical ischemia. In chronically ischemic human skeletal muscle VEGF and VEGFR-2 expression was restricted to atrophic and regenerating skeletal myocytes, whereas in acutely ischemic limbs VEGF and VEGFR-2 were expressed diffusely in the affected muscle. Hypoxia-inducible factor-1α was associated with VEGF and VEGFR-2 expression both in acute and chronic ischemia but not in regeneration. Hindlimb ischemia was induced in 20 New Zealand White rabbits by excising the femoral artery. Magnetic resonance imaging and histological sections revealed extensive ischemic damage in the thigh and leg muscles of ischemic rabbit hindlimbs with VEGF expression similar to acute human lower limb ischemia. After 1 and 3 weeks of ischemia VEGF expression was restricted to regenerating myotubes and by 6 weeks regeneration and expression of VEGF was diminished. VEGFR-2 expression was co-localized with VEGF expression in regenerating myotubes. Macrophages and an increased number of capillaries were associated with areas of ischemic muscle expressing VEGF and VEGFR-2. In conclusion, two patterns of VEGF and VEGFR-2 expression in human and rabbit ischemic skeletal muscle are demonstrated. In acute skeletal muscle ischemia VEGF and VEGFR-2 are expressed diffusely in the affected muscle. In chronic skeletal muscle ischemia and in skeletal muscle recovering from ischemia VEGF and VEGFR-2 expression are restricted to atrophic and regenerating muscle cells suggesting the operation of an autocrine pathway that may promote survival and regeneration of myocytes. *(Am J Pathol 2002, 160:1393–1403)*

Vascular endothelial growth factor (VEGF) is an angiogenic growth factor expressed in response to tissue ischemia.[1–3] VEGF increases vascular permeability, induces proliferation and migration of endothelial cells, and is a survival factor.[3–5] Many effects of VEGF are mediated by nitric oxide that is produced by eNOS and to a lesser extent by iNOS.[6] VEGF binds to three known receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and Neuropilin-1. VEGFR-2 mediates most of the mitogenic, survival, and vascular permeability effects[5–7] and has been reported to be both up- and down-regulated by hypoxia,[8,9] whereas VEGFR-1 is up-regulated.[9] VEGF is crucial for development because embryos lacking even a single VEGF allele show growth retardation and die between embryonic day 11 and 12.[10,11] Recently, VEGF has been used as a recombinant protein or gene therapy to augment vascularization defects in lower limbs and myocardium in animals and humans.[12–14]

VEGF is expressed as at least five isoforms consisting of polypeptides with 121-, 145-, 165-, 189-, or 206-amino-acid residues, differing in their extracellular matrix-binding properties.[3] VEGF expression is induced by hypoxia, hypoglycemia, inflammation, tissue repair, and malignancy, but many signal transduction pathways that regulate VEGF expression remain unknown. It is known that

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hypoxia and hypoglycemia induce VEGF expression by increasing its transcription and stabilizing VEGF mRNA.[15] Hypoxia-inducible factor-1α (HIF-1α) is the main regulator of VEGF expression under different oxygen concentrations.[16] Growth factors such as platelet-derived growth factor and fibroblast growth factor-2 also stimulate VEGF synthesis synergistically with hypoxia.[17,18]

Previously, VEGF has been thought to be an endothelial cell-specific mitogen, but recent reports show that it may have multiple roles *in vivo*. For example, it enhances chemotaxis and migration of vascular smooth muscle cells, coordinates longitudinal bone growth and endochondral bone formation, is critical for the development of retinal cells and a survival factor for renal tubular epithelial cells, and it has a direct survival effect on ischemic neuronal cells *in vitro*.[19–23] An important feature of VEGF is its chemotactic effect on circulating endothelial precursor cells and monocytes, which are inducers of vascular growth.[24,25] Thus, apart from angiogenesis VEGF may induce other effects promoting the recovery of ischemic tissues.[14]

Although several clinical trials have already been started with VEGF protein or gene therapy,[26] little information is available about the expression patterns of VEGF and VEGFR-2 in ischemic human skeletal muscle and whether animal models bear any resemblance to the human situation. Here we demonstrate the expression of VEGF, and for the first time, the expression of VEGFR-2 in skeletal myocytes of critically ischemic human lower limbs and in the rabbit model of hindlimb ischemia. It is shown that in acute ischemia widespread VEGF and VEGFR-2 expression is present whereas in chronic ischemia only atrophic and regenerating muscle cells express VEGF and VEGFR-2.

Materials and Methods

Ischemic Human Skeletal Muscle

Ischemic human skeletal muscle samples were obtained from eight patients after lower limb amputation because of critical ischemia. Characteristics of the patients and muscle samples are shown in Table 1. Six patients suffered from chronic critical limb ischemia, defined as ischemia resulting in either long-lasting rest-pain (>2 to 4 weeks) and/or tissue loss. Two patients had acute lower limb ischemia because of either an embolism or a major thrombosis with occlusive atherosclerosis. Samples were collected from two different regions of each amputee: one sample was collected from a region that represented macroscopically the healthiest part of the amputee, and the other one was chosen from a region that showed more profound ischemia on the basis of the color and integrity of muscle. Studies of human tissues were approved by the Ethical Committee of Kuopio University Hospital.

Rabbit Model of Hindlimb Ischemia

Twenty New Zealand White rabbits were anesthetized with fentanyl-fluanisone (0.2 ml/kg, Hypnorm; Janssen, Beerse, Belgium) and midazolam (1.5 mg/kg, Dormicum; Roche, Basel, Switzerland). Unilateral ischemia was induced by surgically removing the superficial femoral artery and ligating the deep femoral and lateral femoral circumflex arteries as previously described.[27] All animals received prophylactic cefuroxim antibiotics (125 mg, Zinacef; GlaxoWellcome, Research Triangle Park, NC) before surgery. Bubrenorphine (Temgesic, 0.03 mg/kg, Schering-Plough, Kenilworth, NJ) was given as needed for postoperative pain. Rabbits were sacrificed 3 days and 1, 3, or 6 weeks after the operation ($n = 5$ in each group) and muscle samples were collected from tibialis anterior and soleus muscles in the leg and rectus and adductor muscles in the thigh for immunohistochemistry and *in situ* hybridization. Before the termination of the follow-up period selective internal iliac angiography was performed to visualize collateral artery growth. All animal experiments were approved by Experimental Animal Committee of Kuopio University.

Immunohistochemistry

Human and rabbit skeletal muscle samples were immersion-fixed in 4% paraformaldehyde and 15% sucrose (pH 7.4) for 4 hours, rinsed in 15% sucrose (pH 7.4) overnight, and embedded with paraffin.[28] Six-μm-thick sections were prepared and immunohistochemistry was done using the avidin-biotin-horseradish peroxidase system (Vector Laboratories, Burlingame, CA). Capillaries were immunostained with a monoclonal antibody (mAb) against human CD31 (dilution 1:50; DAKO, Glostrup, Denmark). Macrophages in rabbit skeletal muscle were stained with a mAb against rabbit macrophages (1:50, RAM-11; DAKO) and human macrophages with a mAb against human CD-68 (1:100, DAKO). Skeletal myocytes were immunostained with a mAb against human α-actin (1:50, HHF-35; DAKO). HIF-1α was detected in human skeletal muscle samples with a mAb against human HIF-1α (1:100, Ab-4 clone H1α67; Neomarkers, Fremont, CA). This HIF-1α antibody did not work on sections from rabbit tissues. Two antibodies from different manufacturers were used for VEGF [1:500, clone sc-7269 (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:200 (R&D Systems, Minneapolis, MN)] and VEGFR-2 [1:500, clone sc-6251 (Santa Cruz Biotechnology) and 1:200 (Research Diagnostics Inc., Flanders, NJ)].[29] In addition, the expression of VEGFR-1 (Flt-1) was studied with a polyclonal antibody against human VEGFR-1 (1:500, clone sc-316; Santa Cruz Biotechnology). Controls for immunostainings included incubations with irrelevant class- and species-matched immunoglobulins and incubations in which the primary antibody was omitted. Photographs of histological sections were taken using an Olympus AX70 microscope (Olympus Optical, Japan) with analySIS software (Soft Imaging System GmbH, Germany). Immunostainings were graded as follows: −, absent (0% of section area showed staining); +, weak (0 to 10%); ++, moderate (10 to 50%), or +++, strong immunostaining (50 to 100%).

Table 1. VEGF, VEGFR-2, and HIF-1α Expression in Patients with Acute or Chronic Critical Lower Limb Ischemia

Patient number	Age/sex	Type of critical ischemia/ amputation line	Diagnosis	Type 2 diabetes mellitus	Proximal/distal muscle sample	Histopathology/ number of macrophages	HIF-1α expression	VEGF/ VEGFR-2 expression	Source of VEGF
1	80/M	Acute femur	Thrombosis prosthesis axillobife-moralis	No	m. rectus femoris	Normal −	−	− −	−
					m. tibialis anterior	Thrombi in blood vessels, necrosis +++	+++	+++ ++	Myocytes, macrophages
2	84/M	Acute femur	Thrombosis arteria iliacae	No	m. rectus femoris	Thrombi in blood vessels, atrophy, mild necrosis +	+	+ −	Macrophages
					m. semitend.	Thrombi in blood vessels, severe necrosis ++	++	+++ +	Myocytes, macrophages
3	64/M	Chronic infrapatellar	ASO cum gangraena pedis	Yes	m. ext. dig. longus	Atrophy +	+	− −	−
					m. ext. dig. longus	Severe atrophy +++	++	++ +	Atrophic myocytes
4	69/M	Chronic infrapatellar	ASO cum gangraena pedis	Yes	m. gastrocnemius	Atrophy +	+	− −	−
					m. tibialis anterior	Severe Atrophy +++	++	++ +	Atrophic myocytes
5	97/F	Chronic femur	ASO cum gangraena pedis	No	m. rectus femoris	Thrombi in blood vessels, necrosis +++	++	++ −	Macrophages, myocytes
					m. tibialis anterior	Atrophy, regeneration +	+	+ +	Regenerating myocytes
6	66/F	Chronic infrapatellar	ASO cum gangraena pedis	Yes	m. tibialis anterior	Myocyte swelling, regeneration +	+	+ +	Regenerating myocytes
					m. tibialis anterior	Myocyte swelling, regeneration +	+	+ +	Regenerating myocytes
7	78/F	Chronic infrapatellar	ASO cum gangraena pedis	Yes	m. tibialis anterior	Atrophy, regeneration +	++	++ +	Regenerating myocytes
					m. ext. dig. longus	Atrophy, regeneration, necrosis ++	++	++ +	Regenerating myocytes
8	82/F	Chronic femur	ASO cum gangraena pedis	No	m. tibialis anterior	Atrophy +	+	+ −	Macrophages
					m. gastrocnemius	Atrophy +	+	+ −	Atrophic myocytes

−, absent; +, weak; ++, moderate; +++, strong expression/immunostaining. See Materials and Methods section for the description of the immunohistochemical grading system.

In Situ *Hybridization*

The expression of VEGF and VEGFR-2 mRNA were studied in human and rabbit ischemic muscles by *in situ* hybridization either using radioactive [^{33}P]UTP-labeled or nonradioactive digoxigenin-labeled riboprobes. A riboprobe covering the whole coding region of human $VEGF_{165}$ cDNA and a 377-bp fragment of human VEGFR-2 cDNA were used with corresponding sense probes as controls as previously described.[28] For nonra-

Table 2. VEGF Expression in Ischemic Rabbit Hindlimb Muscles after the Removal of the Femoral Artery

	Nonischemic limb	Time point after operation			
		Day 3	Week 1	Week 3	Week 6
Source of VEGF					
Mononuclear infiltrate	−	+++	++	+	−
Skeletal myocytes	−	+++	+++	++	+
Microscopical observation	Normal	Infiltration of macrophages, necrosis of myocytes	Necrosis, regeneration of myocytes	Regeneration, fibrosis	Regeneration ended, adipose cell infiltration, fibrosis

−, absent; +, weak; ++, moderate; +++, strong immunostaining. See Materials and Methods section for the description of the immunohistochemical grading system.

dioactive *in situ* hybridization cDNAs were transcribed using the DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany) to get digoxigenin-labeled run-off transcripts. Positive signal was then detected in paraffin-embedded sections using NBT/BCIP as the color substrate as described.[30]

Magnetic Resonance Imaging (MRI)

MRI was done for rabbits with hindlimb ischemia induced 1 or 6 weeks earlier using Varian ^{UNITY}INOVA (Varian Inc., Palo Alto, CA) imaging console interfaced to a 4.7 T horizontal magnet (Magnex Scientific Ltd., Abingdon, UK) with actively shielded gradients (Magnex Scientific Ltd.) and a custom-built surface coil (50 mm in diameter) placed between the thighs. MRI data were acquired using a flow compensated T_2*-weighted three-dimensional gradient-echo sequence (FOV, 6 × 8 × 6 cm^3; matrix, 256 × 128 × 64; TR, 25 ms; TE, 80 ms) with gadodiamide-contrast medium (GdDTPA-BMA; Omniscan, Nycomed, Norway). GdDTPA-BMA was injected via ear vein as a bolus (0.25 mmol/kg) and the acquisition was started 3 minutes after the injection. It has been previously shown that a GdDTPA-enhanced region of MRI encompasses both viable peri-infarction and nonviable infarction zones in myocardial ischemia.[31]

Results

VEGF and VEGFR-2 Are Expressed Diffusely in Acute Human Skeletal Muscle Ischemia

In acute ischemia because of the occlusion of the femoral artery or prosthesis (patients 1 and 2 in Table 1) little VEGF or VEGFR-2 and relatively normal muscle histology was found in the proximal control samples (Figure 1; a to d). In the distal muscle samples histological sections revealed thrombus formation in blood vessels and interstitial edema consistent with acute ischemia and vascular permeability induced by strong VEGF expression (Figure 1, e and f). By immunohistochemistry, VEGFR-2 was also found in skeletal myocytes of the distal muscle samples (Figure 1g). VEGF immunostaining was mostly cytoplasmic whereas VEGFR-2 was located on the cell membranes. As shown by CD31 immunostaining, a high number of capillaries and large endothelial cell-covered vessels without visible media or adventitia layers were observed in the same areas (Figure 1f). Abundant nuclear HIF-1α immunostaining suggests that the widespread VEGF expression in acute lower limb ischemia is mediated by HIF-1α (Figure 1h).

VEGF and VEGFR-2 Expression in Chronically Ischemic Human Skeletal Muscle

In patients with chronic critical lower-limb ischemia (patients 3–8 in Table 1), VEGF protein and mRNA were found in atrophic but not in normal skeletal muscle cells by immunohistochemistry (Figure 2c) and *in situ* hybridization (Figure 1, i and j). VEGFR-2 was also expressed in atrophic skeletal muscle cells confirmed by two different monoclonal antibodies used against VEGFR-2 (Figure 1m and Figure 2d) and *in situ* hybridization (Figure 1, k and l). As a rule, the more atrophic were the myocytes the stronger was the VEGF and VEGFR-2 expression. Adipose cell- and monocyte-macrophage infiltration was associated with atrophic skeletal muscle cells (Figure 2b). Furthermore, an increased number of blood vessels were co-localized with regions of atrophic muscle and VEGF expression (Figure 2e). In patients 3 and 4 there was a significant difference in the degree of ischemia between control and ischemic samples both in the macroscopic and microscopic examination, whereas in the samples from patients 5 to 8 only a small difference could be observed. Consequently, in patients 3 and 4 more VEGF immunoreactivity with more extensive atrophy was observed in the distal samples compared to the proximal samples. In addition to atrophic myocytes, VEGF expression was found in regenerating human skeletal muscle cells of patients 5 to 7 with chronic critical ischemia, especially in fibers where regeneration had recently begun (Figure 1, n and o). Regenerating skeletal muscle fibers can be distinguished from normal fibers by their small size and central nuclei. HIF-1α was associated with VEGF expression in atrophic but not with regenerating myocytes of chronically ischemic legs (Figure 1p). However, HIF-1α expression was diminished in atrophic myocytes as compared to acutely ischemic muscle cells. Expression of VEGFR-1 was restricted to endothelium both in acute and chronic skeletal muscle ischemia (data not shown).

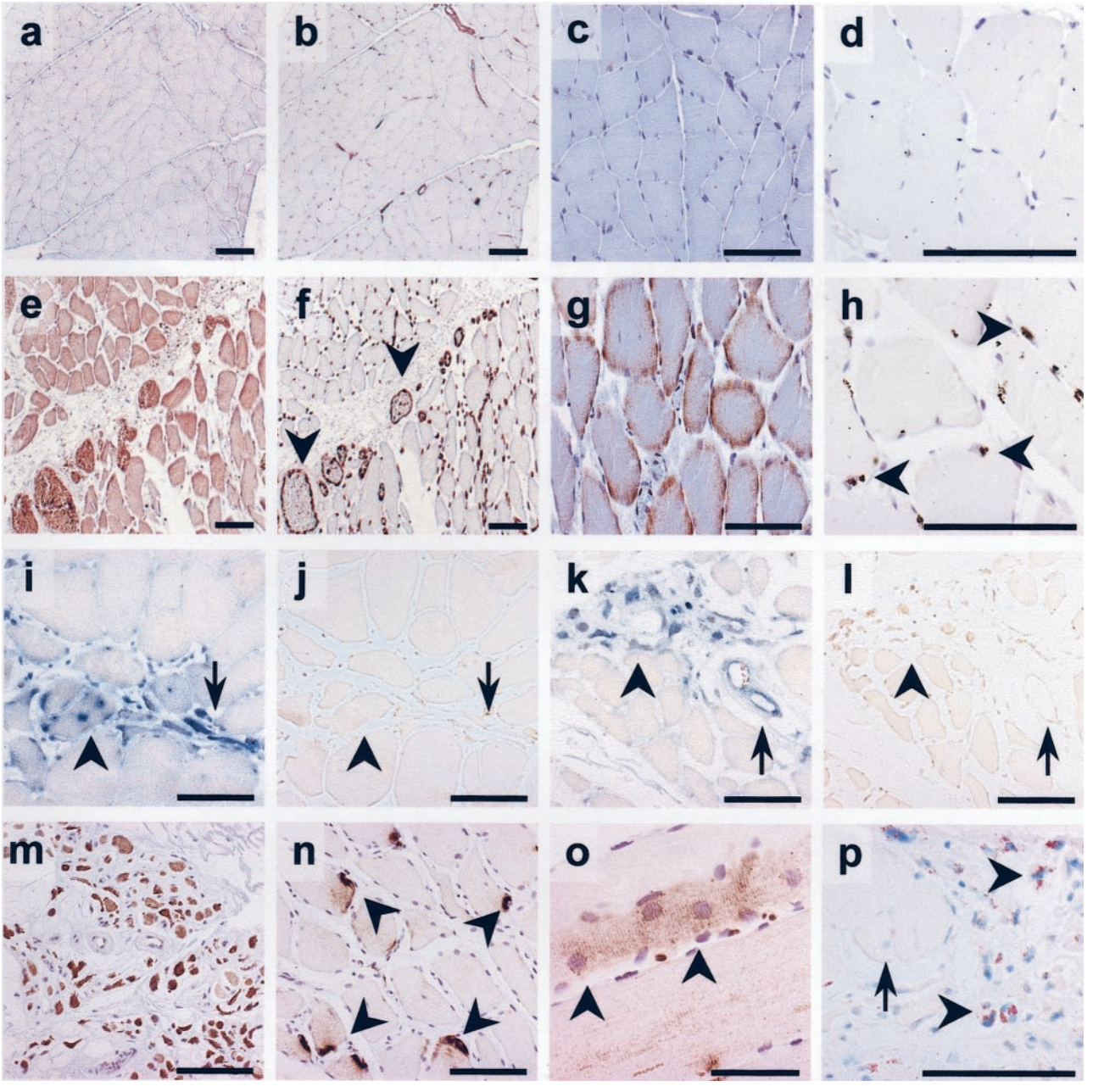

Figure 1. VEGF, VEGFR-2, and HIF-1α expression in acute and chronic human lower-limb ischemia and skeletal muscle regeneration. **a–h:** Sections from patient 1 with acute limb ischemia because of thrombosis of prosthesis axillobifemoralis. Nonischemic rectus femoris muscle shows normal histology in VEGF (Santa Cruz) (**a**), CD31 (**b**), VEGFR-2 (RDI) (**c**), and HIF-1α (**d**) immunostainings. **e:** Ischemic tibialis anterior muscle shows a strong and mostly cytoplasmic VEGF immunostaining (Santa Cruz) and interstitial edema. **f:** A high number of capillaries, thrombus formation in blood vessels consistent with acute ischemia (**arrowheads**), and large vessels without visible media or adventitia layers (CD31). **g:** VEGFR-2 immunostaining on the cell membranes of ischemic skeletal muscle cells (anti-VEGFR-2 from RDI) and HIF-1α in nuclei of myocytes (**arrowheads**). **i–l:** *In situ* hybridization of chronically ischemic human skeletal muscle using nonradioactive DIG-labeled riboprobes. **i:** VEGF anti-sense. Signal is detected around nuclei of a regenerating myofiber (**arrowhead**) and in atrophic skeletal muscle cells (**arrow**). **j:** VEGF sense control. **Arrow** and **arrowheads** point to the same areas as in **i**. **k:** VEGFR-2 mRNA is detected in atrophic skeletal myocytes (**arrowhead**) and on endothelium (**arrow**). **l:** VEGFR-2 sense control. **Arrow** and **arrowheads** point to the same areas as in **k**. **m:** Atrophic skeletal myocytes are strongly positive for VEGFR-2 immunostaining (anti-VEGFR-2 from RDI). **n:** VEGF at the edges of skeletal muscle cells where regeneration starts with satellite cell activation (**arrowheads**, anti-VEGF from Santa Cruz). **o:** In a regenerating myofiber with central nuclei (**arrowheads**) VEGF immunostaining is cytoplasmic (anti-VEGF from Santa Cruz). In neighboring nonregenerating fibers with marginal nuclei VEGF is absent. **p:** HIF-1α immunostaining demonstrates significant HIF-1α expression only in atrophic skeletal myocytes (**arrowheads**) but not in normal myocytes (**arrow**) in chronically ischemic muscle. Scale bars, 100 μm (**a–n**, **p**); 50 μm (**o**).

Extensive Ischemic Muscle Damage and VEGF Expression in the Rabbit Hindlimb Ischemia Model

Complete removal of femoral artery combined with the ligation of lateral femoral circumflex and deep femoral arteries has been frequently used as a model for hindlimb ischemia.[27,32] The procedure leads to a lack of arteries supplying major muscles of the thigh, the quadriceps femoris and adductor muscles (Figure 3a). As a consequence, as shown by GdDTPA-BMA-enhanced MRI 7

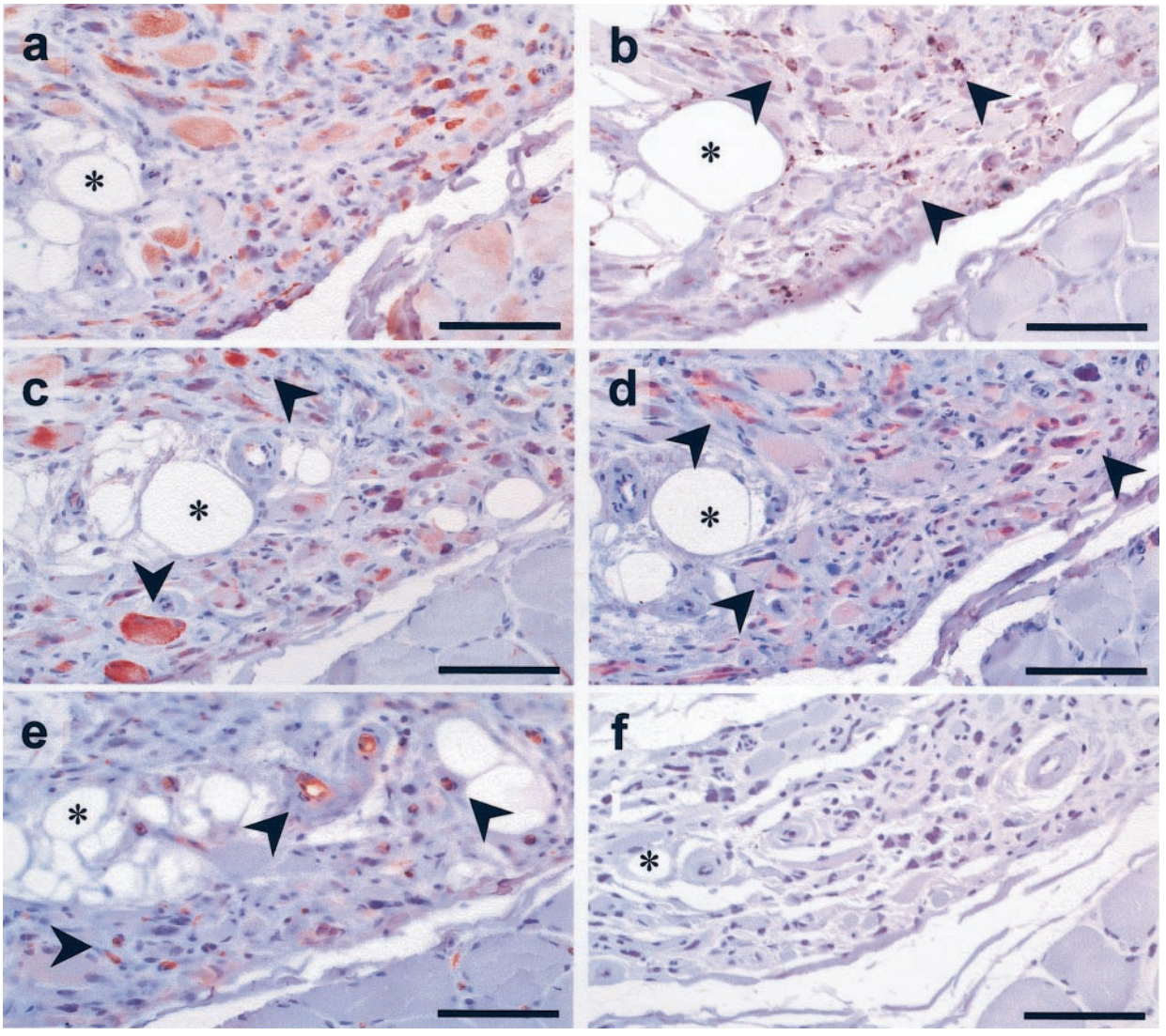

Figure 2. VEGF and VEGFR-2 in atrophic skeletal muscle cells in chronically ischemic human lower-limb muscle. **a–f:** Serial sections from extensor digitorum longus muscle of patient 3. **a:** α-Actin immunostaining (HHF-35). **b:** As a demonstration of chronic ischemia, adipose cell (**asterisks** in all figures) and macrophage infiltration (**arrowheads**) are associated with skeletal muscle atrophy (CD68 immunostaining). **c** and **d:** Atrophic (**arrowheads**) but not normal skeletal muscle cells in the **bottom right** corner show immunostaining for VEGF (anti-VEGF from Santa Cruz) (**c**) and for VEGFR-2 immunostaining (anti-VEGFR-2 from Santa Cruz) (**d**). **Arrowheads** indicate positive cells. **e:** More blood vessels (**arrowheads**) are seen in the area of atrophic muscle expressing VEGF than in the region of normal muscle (CD31 immunostaining). **f:** Control immunostaining in which the first antibody was omitted. Scale bars, 100 μm.

days after induction of ischemia, extensive ischemic damages are often present in these muscles (Figure 3b). Six weeks after the removal of the femoral artery limb muscles have regenerated because the collateral-dependent blood flow to the limb has improved (Figure 3c). However, pathological changes and muscle atrophy are still visible in MRI as indicated by extravasation of GdDTPA-BMA (Figure 3c). Histological sections at the same time point reveal sustained low VEGF expression and increased vascularization indicating a role for VEGF in increasing capillary density and vascular permeability (Figure 3, h and i).

On the basis of VEGF expression and histopathology, it was found that the rabbit model has similarities with acute human peripheral ischemia (Table 2). In animals killed on day 3 VEGF was expressed diffusely throughout the affected muscles accompanied by mononuclear cell infiltrates and necrotic skeletal muscle cells (Figure 3, d and e). However, as opposed to acute human skeletal muscle ischemia, little VEGFR-2 was found in ischemic rabbit myocytes at this time point. Both after 1 and 3 weeks of ischemia, regenerating myocytes were the main source of VEGF (Figure 3f and Figure 4a). At 3 weeks fibrosis was also often present in the ischemic limb muscles. By 6 weeks regeneration had ended in most cases, and adipose tissue infiltrate and fibrosis were observed. At this time point only regenerated myofibers contained small amounts of cytoplasmic and nuclear VEGF immunostaining (Figure 3h).

VEGF, VEGFR-2, and Macrophages Are Associated with Skeletal Muscle Regeneration

One week after excision of the femoral artery, strong VEGF expression was found in regenerating skeletal

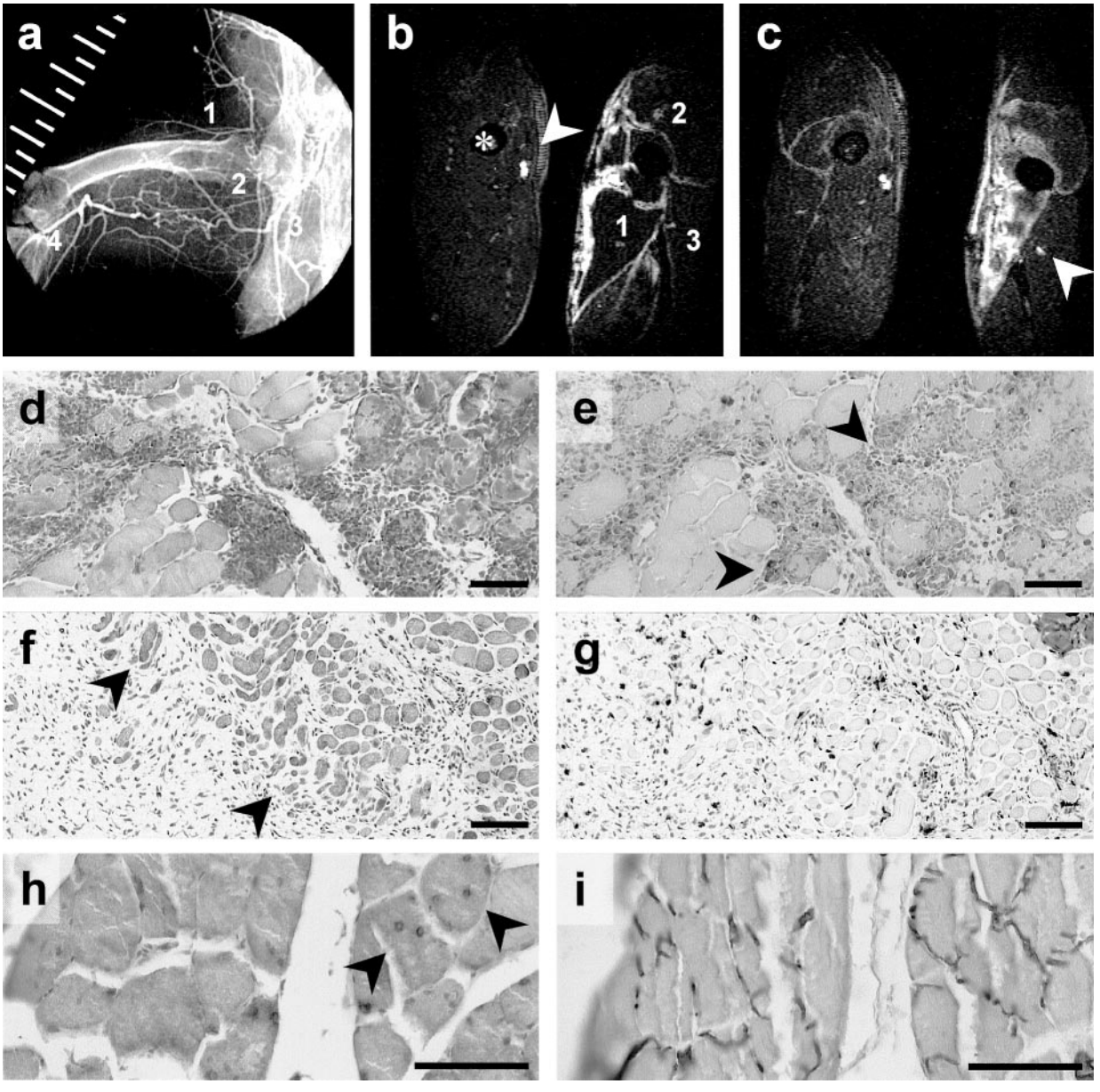

Figure 3. In the rabbit model of hindlimb ischemia the complete removal of the femoral artery results in acute ischemia featuring necrosis in major muscles of the thigh and strong VEGF expression. **a:** Internal iliac angiography of an ischemic rabbit hindlimb 6 weeks after operation shows that after the ligation of lateral circumflex femoral (**1**) and deep femoral (**2**) arteries blood flow to the ischemic limb is dependent on collateral vessels originating from the internal iliac artery (**3**). Re-entry sites for corkscrew collaterals are the caudal femoral and popliteal arteries (**4**). **b:** Representative transversal GdDTPA-BMA enhanced T_2*-weighted MRI of an ischemic rabbit thigh 1 week after excision of the femoral artery. Inner parts of the ischemic adductor (**1**) and quadriceps femoris (**2**) muscles are darker than healthy muscles of the contralateral limb and they are surrounded by bright GdDTPA-BMA contrast suggesting severe ischemia and necrosis, whereas the abductor muscles (**3**), which are supplied by collaterals from the internal iliac artery, are viable. **Asterisk** indicates the femur bone and the **arrowhead** the femoral artery and vein of the healthy contralateral limb. **c:** MRI of the same rabbit thigh as in **b** 6 weeks after the operation. Ischemic adductor and quadriceps femoris muscles show bright GdDTPA-BMA contrast demonstrating the extravasation of GdDTPA-BMA in these muscles. **Arrowhead** indicates a collateral artery. **d–i:** Histological sections of ischemic rabbit skeletal muscles at different time points. **d**, **f**, and **h:** VEGF immunostaining (anti-VEGF from Santa Cruz). **e** and **g:** Macrophage immunostaining (RAM11). **i:** Endothelial immunostaining (CD31). Three days after the onset of ischemia VEGF is diffusely expressed in skeletal myocytes (**d**) and infiltrated leukocytes (**e, arrowheads**). **f:** Three weeks after the induction of ischemia VEGF is expressed in atrophic and regenerating myofibers (**arrowheads**). **g:** Macrophages and fibrosis are present at this time point. **h:** Regeneration and VEGF expression have diminished 6 weeks after acute ischemia, although some staining is still observed near the central nuclei of the regenerated myofibers (**arrowheads**). **i:** Increased capillary density co-localizes with VEGF expression and regenerated myocytes. Scale bars, 100 μm.

myocytes in ischemic rabbit hindlimb muscles (Figure 4, a and e). Both VEGF immunohistochemistry and *in situ* hybridization for VEGF mRNA gave similar results. According to immunohistochemistry VEGF was mostly cytoplasmic, but also the nuclei of the regenerating myotubes stained intensively (Figure 4a). VEGFR-2 was strongly expressed in the same regenerating myotubes (Figure 4; b and g). Expression of both VEGF and VEGFR-2 was strictly restricted to regenerating myocytes whereas regions with no regeneration were absent of VEGF and VEGFR-2 (Figure 4, a and b). An increased number of capillaries, which were also notably viable and dilated

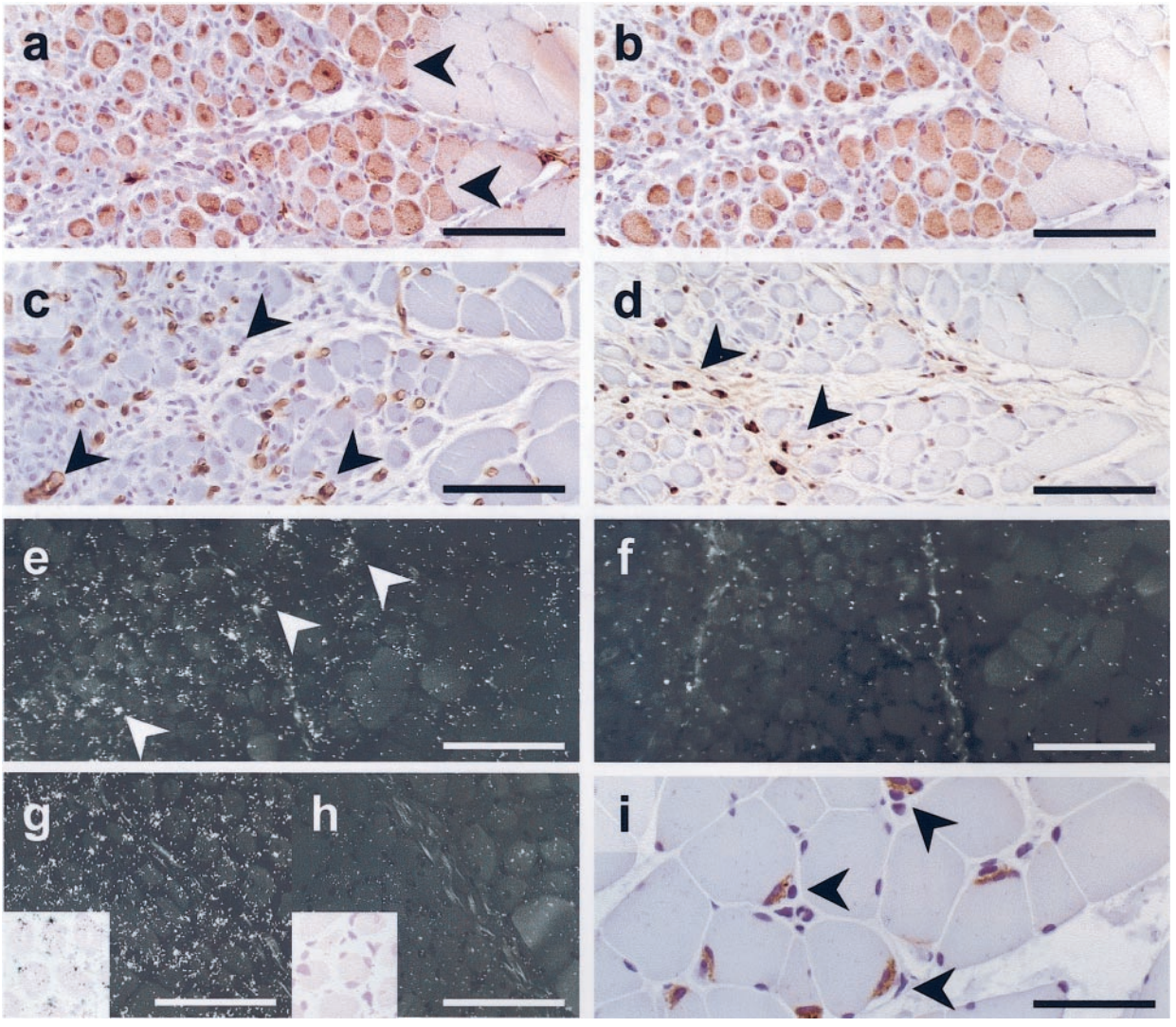

Figure 4. VEGF, VEGFR-2, and macrophages are associated with skeletal muscle regeneration. **a–h:** Sections from regenerating rabbit tibialis anterior muscle where ischemia was induced 7 days earlier by excision of femoral artery. **a:** Strong VEGF immunostaining in regenerating myocytes on the **left** (**arrowheads**) but not in nonregenerating muscle cells on the **right** (anti-VEGF from Santa Cruz). **b:** The same regenerating myocytes are also positive for VEGFR-2 immunostaining (anti-VEGFR-2 from Santa Cruz). **c:** Unlike in the nonregenerating area, capillaries are dilated (**arrowheads**) and increased in number near regenerating and VEGF-expressing myofibers (CD31 immunostaining). **d:** Macrophages (**arrowheads**) are also associated with skeletal muscle regeneration and VEGF expression (RAM11 immunostaining). **e:** *In situ* hybridization with [^{33}P]UTP-labeled VEGF anti-sense riboprobe. Signal from VEGF mRNA is found in the same area as VEGF protein (**arrowheads**). **f:** Corresponding sense control riboprobe. **g:** *In situ* hybridization with [^{33}P]UTP-labeled VEGFR-2 anti-sense riboprobe showing signal from regenerating myocytes. Bright-field image in the box. **h:** Corresponding sense control riboprobe. **i:** VEGF is present at the edges of myocytes in early skeletal muscle regeneration (**arrowheads**) in adductor muscle 7 days after induction of ischemia (anti-VEGF from Santa Cruz). Scale bars, 100 μm.

with up-regulation of CD31, was associated with VEGF expression (Figure 4c). Furthermore, macrophages had infiltrated into areas of regeneration (Figure 4d). Similar to human samples, in sections where regeneration had been recently begun, VEGF was present at the edges of myocytes (Figure 4i) where regeneration starts with satellite cell activation.

Discussion

Previously, VEGF and HIF-1α expression has been studied in the ischemic human myocardium.[33] However, the expression patterns of HIF-1α, VEGF, and VEGFR-2 in ischemic human and animal skeletal muscle have remained unclear. We demonstrate here two patterns of VEGF and VEGFR-2 expression in skeletal muscle ischemia. In acute ischemia VEGF and VEGFR-2 are expressed diffusely, VEGF originating from muscle cells and macrophages and VEGFR-2 being present on the cell membranes of ischemic myocytes. In chronic skeletal muscle ischemia and in skeletal muscle recovering from ischemia VEGF and VEGFR-2 expressions are restricted to atrophic and regenerating muscle cells. In both cases VEGF expression is co-localized with macrophage infiltration and an increased number of blood vessels. Similarly with VEGF and VEGFR-2 expression, HIF-1α expression was widespread in acutely ischemic muscles but only atrophic myocytes expressed detectable amounts of HIF-1α in chronically ischemic skeletal muscle. On the other hand, HIF-1α was not associated

with VEGF and VEGFR-2 expression in skeletal muscle regeneration. In accordance with our results the increase in HIF-1α levels was shown to be transient compared to more persistent VEGF expression in evolving myocardial infarction.[33] Furthermore, *in vitro* experiments have shown that that there are HIF-1α-independent signaling pathways to induce VEGF expression.[34] Thus, high level VEGF expression in regenerating myotubes is probably because of the activation of signaling pathways other than HIF-1α.

Previously expression of VEGF and its receptors has been shown to be up-regulated in response to various tissue injuries, but its role has been considered to be mainly restricted to angiogenesis and re-endothelialization of blood vessels.[3,35,36] For example, after an acute phase of ischemia with diffuse expression of VEGF and its receptors, Li and colleagues[36] demonstrated sustained expression of VEGF and VEGFR-2 in regions bordering the infarct zone in rat myocardium. After mechanical arterial injury VEGF and VEGFR-2 are up-regulated in arterial smooth muscle cells contributing to the repair of vascular endothelium.[29,35] The present study shows for the first time that VEGFR-2 is expressed in skeletal myocytes. This novel finding suggests that the VEGF/VEGFR-2 system may have functions distinct from blood vessel formation in the survival and regeneration of skeletal muscle affected with ischemia. In addition, macrophages were co-localized with muscle atrophy, regeneration, and VEGF expression both in acutely and chronically ischemic skeletal muscle. Macrophages express VEGFR-1[37] and thus they are potentially recruited by VEGF in the regions of muscle regeneration and atrophy. In addition to their function in scavenging cell debris, macrophages play an essential role in triggering muscle regeneration, potentially through a variety of secreted cytokines and growth factors.[38,39]

Like VEGF in the current study, hepatocyte growth factor is expressed in a transient manner during postischemic muscle regeneration, with no expression in normal muscle.[40] In fact, the similarities of hepatocyte growth factor with VEGF expression in skeletal muscle regeneration seem so striking that it is likely that there are common regulators of these growth factors in regenerating myocytes. It is also remarkable that VEGF, a secreted growth factor, and VEGFR-2 are found not only on the cell membrane but also in cytoplasm and nuclei of regenerating and atrophic skeletal muscle cells. Several lines of evidence now demonstrate that VEGF can be internalized after binding to VEGFR-2, and this ligand receptor complex travels to the nucleus leading to the up-regulation of VEGFR-2.[41,42] Furthermore, the internalization of VEGF by VEGFR-2 results in phenotypical changes at least in endothelial cells.[42,43] Interestingly, it has been shown that the activation of the phosphatidylinositol-3-OH-kinase/protein kinase Akt (PI3K/Akt) signal transduction pathway leads to the up-regulation of VEGF in an autocrine manner, induces myogenic differentiation and angiogenesis, and mediates the inhibitory effects of VEGF on apoptosis.[5,44,45] Thus, an autocrine stimulation of the VEGF/VEGFR-2 system may have an important role in cell differentiation, such as in the conversion of myoblasts into myocytes in regenerating skeletal muscle. In chronically ischemic skeletal muscles, the anti-apoptotic properties of VEGF may contribute to the survival of muscle cells.

In the rabbit model, which is frequently used to study therapeutic effects of growth factors and genes in hindlimb ischemia, extensive ischemic damage and diffuse VEGF expression was detected in the thigh and leg muscles 3 days after induction of ischemia. After the acute phase abundant VEGF/VEGFR-2 expression occurred in regenerating myocytes as studied 1 to 6 weeks after surgery. Thus, it is possible that strong endogenous VEGF/VEGFR-2 expression and muscle necrosis in this model may confound the results of a therapeutic intervention, especially if the treatments are given shortly after surgery.

Animal and human data of this study suggest that VEGF and VEGFR-2 expression is attenuated in chronic skeletal muscle ischemia compared to acute ischemia possibly because of diminished expression of HIF-1α. Excess VEGF provided by therapeutic means seems justified especially in the regions of chronically ischemic limbs where endogenous VEGF expression is low. Because VEGF is apparently involved in skeletal muscle regeneration and survival, the administration of exogenous VEGF by various approaches may be useful for the recovery of ischemic skeletal muscle or myocardium both via angiogenesis-dependent and -independent mechanisms. It is concluded that in acute ischemia VEGF and VEGFR-2 are expressed diffusely whereas in chronic ischemia only atrophic and regenerating muscle cells express VEGF and VEGFR-2.

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