Modulation of vascular remodeling : a role for the immune system, growth factors, and transcriptional regulation

Seghers, L.

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Expression of Vascular Endothelial Growth Factor, Stromal Cell-Derived Factor-1 and CXCR4 in human limb muscle with acute and chronic ischemia

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Arteriosclerosis Thrombosis Vascular Biology. 2007 jun;27(6):1426-32

Abstract

Objective: Vascular endothelial growth factor (VEGF)-induced stromal cell-derived factor-1 (SDF-1) has been implicated in angiogenesis in ischemic tissues by recruitment of CXCR4-positive bone marrow-derived circulating cells with paracrine functions in preclinical models. Here, evidence for this is provided in patients with peripheral artery disease.

Methods and Results: Expression patterns of VEGF, SDF-1 and CXCR4 were studied in amputated limbs of 16 patients. VEGF-A was expressed in vascular structures and myofibers. SDF-1 was expressed in endothelial and sub-endothelial cells, whereas CXCR4 was expressed in proximity to capillaries. VEGF-A, SDF-1 and CXCR4 expressions were generally decreased in ischemic muscle as compared to non-ischemic muscle in patients with chronic ischemia (0.41-, 0.97- and 0.54-fold induction (medians), respectively), whereas substantially increased in 2 patients with acute-on-chronic ischemia (3.5-65.8-fold, 3.9-19.0-fold and 4.1-30.6-fold induction, respectively). Furthermore, these gene expressions strongly correlated with capillary area. Only acute ischemic tissue displayed a high percentage of HIF-1α-positive nuclei.

Conclusions: These data suggest that VEGF and SDF-1 function as pro-angiogenic factors in patients with ischemic disease by perivascular retention of CXCR4-positive cells. Furthermore, these genes are down-regulated in chronic ischemia as opposed to up-regulated in more acute ischemia. The VEGF-SDF-1-CXCR4 pathway is a promising target to treat chronic ischemic disease.

Introduction

The stimulation of neovascularization using growth factors is a promising experimental treatment for arterial occlusive disease. Early results obtained from preclinical studies using angiogenic factors, in particular vascular endothelial growth factor (VEGF), were promising and led to great expectations. However, placebo controlled clinical trials of therapeutic angiogenesis were inconsistent [1-4]. To improve angiogenic strategies, more information is required about cellular and molecular mechanisms involved in vascular growth in ischemic tissues.

Recently, stem cells have been implicated to play a role in neovascularization [5]. This has led to some promising results using autologous bone marrow transplantation for the stimulation of collateral artery growth in patients with peripheral artery disease [6]. Furthermore, bone marrow mononuclear cells from patients with chronic ischemic heart disease have a reduced capacity to induce collateral formation in mice, which is paralleled by a reduced migratory response for bone marrow cells to stromal cell derived factor 1 (SDF-1, also known as CXCL12) and VEGF [7].

SDF-1 is implicated as a chemokine for CXCR4-positive stem cells [8]. It was recently shown that SDF-1 gene expression is regulated by the transcription factor hypoxia inducible- factor-1 (HIF-1) in endothelial cells resulting in selective *in vivo* expression of SDF-1 in ischemic tissue [9]. From the latter study, it was concluded that recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1-induced expression of SDF-1. More recently, it was demonstrated that bone marrow-derived circulating cells are retained in close proximity to angiogenic vessels by SDF-1 induced by VEGF in activated perivascular myofibroblasts in mice [10]. Other investigators reported that cytokine-mediated release of SDF-1 from platelets constitute the major determinant of neovascularization through mobilization of non-endothelial CXCR4+VEGFR1+ hematopoietic progenitor cells [11]. Moreover, SDF-1 (gene) therapy enhances ischemia-induced vasculogenesis and angiogenesis in mice, and is associated with incorporation of bone marrow cells into the vasculature [12, 13].

Here, expression patterns of VEGF, SDF-1 and CXCR4 were studied in relation to other angiogenic factors, such as VEGF-C, -D, and VEGF-receptor 1 to 3 in amputated limbs of 16 patients with peripheral arterial disease, of which 14 suffered from chronic ischemia, whereas 2 patients suffered from acute-on-chronic ischemia.

In both chronic and acute-on-chronic ischemia, SDF-1 was expressed in endothelial and sub-endothelial cells, whereas CXCR4 was expressed in proximity to capillaries. VEGF-A was expressed in vascular structures and myofibers. Interestingly, with an increased degree of ischemia, VEGF-A, SDF-1 and CXCR4 expressions were decreased or unchanged in patients with chronic ischemia, as opposed to substantially increased in

both patients with acute-on-chronic ischemia, as described for the HIF-VEGF-VEGFR-2 pathway [14].

Materials and methods

Sample collection from patients

Samples of skeletal muscle were obtained after informed consent from 16 patients from November 2001 to June 2003, according to the guidelines of the Institutional Review Board. Patient characteristics are depicted in Table 1. Patients underwent below-knee or above-knee amputation because of critical ischemia without possibilities for vascular reconstruction. All patients suffered from chronic ischemia, however, patients #5 and #11 demonstrated sudden progression of ischemia by occlusion of a bypass graft leading to swift amputation, and were thus considered as acute-on-chronic ischemic. The former patient was re-admitted 1 month after urokinase thrombolysis of an occluded femoro-popliteal bypass graft (PTFE) with reocclusion of the bypass. After one unsuccessful revascularization attempt, belowknee amputation was performed 1 week later. The latter patient showed sudden onset of rest pain due to occlusion of a femoro-crural bypass graft 9 days postoperatively, which was followed by below-knee amputation 2 weeks later. To compare non-ischemic with ischemic muscle within one patient, biopsies were collected at amputation level, representing relatively non-ischemic muscle, and, more distally, near the Achilles tendon (Soleus muscle) and between the toes (Interosseus, Extensor digitorum or Adductor hallucis muscle), representing ischemic muscle (Table 1). Each biopsy was performed in duplo; one muscle sample was fixated in 4% formaldehyde and subsequently embedded in paraffin for immunohistochemistry, one muscle sample was frozen in liquid nitrogen for RNA analysis. Since in 6/16 patients biopsies could only be collected from 2 instead of 3 levels of the limb for various reasons, e.g. previous foot amputation or extensive gangrene, comparisons of gene and protein expressions were performed between 2 levels for each patient to allow valid statistics, preferably between soleus and gastrocnemius muscle to maintain constant muscle types.

Table 1. Patients characteristics

Patient #	Age/ Sex	Indication for amputation	Surgery	Diabetes	Other risk factors	Significant angiographic stenosis / occlusions	Proximal control muscle sample	Middle ischemic muscle sample	Distal ischemic muscle sample
Chronic ischemia									
1	52,F	Rest pain+necrosis	BKA	No	Smoking	AP	Gastrocnemius	-	Soleus
2	67,M	Infected ulcer	BKA	No	Smoking, hypertension	AP	Gastrocnemius	-	Soleus
3	76,F	Progressive pain+necrosis	BKA	DM II	Smoking, hypertension, dyslipidemia	Fem-Fem cross-over bypass	Gastrocnemius	-	Soleus
4	57,M	Neurogenic+myogenic damage after compartment syndrome	AKA	DM I	No	AIE, AFC, AFS	Vastus medialis	-	Gastrocnemius
6	72,M	Rest pain+necrosis	BKA	No	Smoking, hypertension, dyslipidemia	Fem-Crur bypass	Gastrocnemius	Soleus	Interossius I-II
7	52,F	Progressive necrosis with infection	BKA	DM I	dyslipidemia	AP, ATP	Gastrocnemius	-	Soleus
8	56,M	Progressive pain+non-healing ulcer	BKA	No	Smoking	AIE	Gastrocnemius	Soleus	Interossius I-II
9	54,M	Rest pain+gangrene	BKA	No	Smoking	Infrarenal Abdominal Aorta, AFC	Biceps femoris	Soleus	Adductor hallucis
10	59,F	Gangrene+restpain	BKA	No	Smoking	Abdominal Aorta, AFS	Tibialis anterior	Soleus	Interossius I-II
12*	72,M	Non-healing amputation wound	BKA	DM II	Smoking	ATA, A. Peronea	Soleus	Soleus	Interossius III-IV
13	83,F	Rest pain+necrosis	BKA	No	No	AFP, AFS, AP, no crural vessels	Gastrocnemius	Soleus	Interossius I-II
14	88,F	Non-healing toe amputation wound	BKA	DM I	Hypertension	AFS	Soleus	Soleus	Extensor digitorum IV
15	79,M	Rest pain+infection	BKA	DM II	Smoking	AFS, AFC	Gastrocnemius	Soleus	Interossius I-II
16	70,M	Rest pain+non-healing ulcer	BKA	DM II	Smoking, hypertension	ATP	Tibialis anterior	Soleus	Interossius I-II
Acute-on-chronic ischemia									
5	79,F	Rest pain, occluded Fem-Pop bypass	BKA	No	Smoking	AFP, Fem-Pop bypass	Gastrocnemius	-	Interossius I-II
11	81,F	Rest pain, occluded Fem-Crur bypass	BKA	DM	-	Fem-Crur bypass	Gastrocnemius	Soleus	Interossius I-II

Abbreviations: BKA:below-knee amputation, AKA:above-knee amputation, AIE:arteria iliaca externa, AFC:arteria femoralis communis, AFS:arteria femoralis superficialis, AFP:arteria femoralis profunda, AP:arteria poplitea, ATP:arteria tibialis posterior. *No material available for RNA analysis

Immunohistochemistry

Five μm-thick paraffin-embedded sections of skeletal muscle were re-hydrated and endogenous peroxidase activity was blocked. Immunohistochemistry was performed using the avidin-biotin-horseradish peroxidase system (DakoCytomation) [15]. Endothelial staining was performed with antibody against human CD31 (1:200, Hec 65, TNO) and monoclonal antibody against human CD34 (1:100, clone QBEnd/10, Novocastra Laboratories). For CD31 staining, sections were pre-incubated with trypsin (Fluka BioChemica) for 30 minutes at 37°C for antigen unmasking, and the immunohistochemical reaction was enhanced by tyramine amplification as described [16]. SDF-1 and CXCR4 were detected using a monoclonal antibody against human SDF-1 (1:200, clone 79018, R&D Systems) and human CXCR4 (1:200, clone 44716, R&D Systems). For SDF-1 and CXCR4 staining, heat-induced antigen retrieval was performed using citrate buffer (pH 6.0) for 10 minutes before incubation with antibodies. VEGF-A was detected using a polyclonal antibody against the 165, 189 and 121 amino-acid splice variants of human VEGF-A (1:50, clone sc-152, Santa Cruz Biotechnology). VEGF-C and D were detected with a polyclonal antibody against human VEGF-C (1:100, clone Z-CVC7, Zymed) and a monoclonal antibody against human VEGF-D (1:50, clone 78923.11, R&D Systems). In negative control incubations, the primary antibodies were omitted. The signal was detected using the NovaRED substrate kit (Vector Laboratories) and sections were counterstained by Mayer's hematoxylin.

HIF-1α staining was performed as described by Bos et al [17]. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide. After antigen retrieval, a cooling-off period of 20 minutes preceded the 30 min incubation of the primary antibody for HIF-1α (1:500, Abcam). Thereafter, the catalyzed signal amplification system (DAKO, Glostrup, Denmark) was used for HIF-1α staining according to the manufacturer's instructions.

Stainings were developed with diaminobenzidine. Before the slides were mounted, all sections were counterstained for 45 seconds with hematoxylin and dehydrated in alcohol and xylene. Appropriate negative controls (obtained by omission of the primary antibody) and positive controls (human kidney cell carcinoma) were used throughout.

For VEGF receptor staining, air-dried serial cryostat sections (5 μm-thick) were fixed in cold acetone for 10 minutes, and subsequently stained using the following antibodies: monoclonal antibodies Flt-19 (against VEGFR-1, 1:400), KDR-1 (against VEGFR-2, 1:400), and 9D9F9 (against VEGFR-3, 1:1500), as described.18 Flt-19 and KDR-1 were kindly provided by Dr HA Weich, National Research Centre for Biotechnology, Braunschweig, Germany; 9D9F9 by Prof K Alitalo, Haartman Institute, Helsinki, Finland. To study general skeletal muscle morphology, sections were stained by the hematoxylin phloxin saphrane (HPS) technique.

Quantification of immunostained sections

Capillary density and area per capillary were quantified from randomly photographed sections (3-6 images per section) using image analysis (Qwin, Leica). Intensity of staining for the various angiogenic factors was studied at the different levels of each amputated limb in a single blinded fashion. For HIF-1α staining, only cells with completely stained nuclei were regarded as positive, and this nuclear staining was interpreted as an increased level; cytoplasmic staining, observed occasionally, was ignored because active HIF-1α is located only in the nucleus. The fraction of nuclei with an increased level of HIF-1α positivity was estimated visually by two observers (L. Seghers and N. Pires) in 10 fields per section.

RNA analysis

Total RNA was extracted from frozen muscle using the RNeasy fibrous tissue midi kit (QIAGen) according to the manufacturer's protocol. To prevent contamination of genomic DNA in PCR, RNA samples were treated with DNAse prior to cDNA synthesis using RNAse-free DNAse (QIAGen) according to the manufacturer's protocol. One microgram of total RNA was reversed transcribed into cDNA in a final volume of 233 ml using Ready-To-Go You-Prime First-Strand Beads (Amersham) according to the manufacturer's protocol. Samples were stored at –20°C until PCR analysis. Primers pairs and probes for studying expression of human SDF-1 and human CXCR4 by real-time RT-PCR were purchased (Applied Biosystems, Hs00171022_m1 and Hs00237052_m1, respectively). Primer sets for human VEGFA were 5'-GCCCACTGAGGAGTCCAACA-3' (sense), 5'-TCCTATGTGCTGGCCTTGGT-3' (anti-sense), 5'-FAM-CACCATGCAGATTATGCGGA TCAA A CC-3' (probe), as designed using the specific criteria of the primer express software (Perkin Elmer). Samples were normalized to GAPDH housekeeping gene expression (Perkin Elmer). PCR was performed using Q-PCR mastermix (Eurogentec) in a 25ml reaction volume. After 2 minutes of incubation at 50°C the enzyme was activated by incubation at 95°C for 10 minutes followed by 40 PCR cycles consisting of 15 seconds denaturation at 95°C and hybridization at 60°C for 1 minute.

Statistical analysis

Results are expressed as mean±SEM or median with 95% confidence interval (CI). Comparisons between means were performed using one-way ANOVA test with LSD post-hoc analysis. Comparisons of immunostaining intensity between different levels of limbs were performed with the Sign-test (single-blinded), as described [19]. Pearson correlations were used to study relationships. P-values <0.05 were considered statistically significant.

Results

Vessel density and size parallel the degree of ischemia in human skeletal muscle

Marked morphological features of skeletal muscle, characteristic for chronic ischemia, were observed in muscle biopsies derived from distal levels of the limb (most ischemic area) that were not apparent at the amputation level (relatively non ischemic area) (Figure 1A and B). They consisted of disorganized muscle composition, adipose cells within muscular tissue, regenerating and atrophic myofibers, and infiltrating inflammatory cells. Capillary density increased with the degree of ischemia (Figure 1C and D), which became significant at the level of interosseus muscle of the foot as compared to both amputation level and soleus muscle (239.6±29.5 as compared to 151±19.0 and 153.7±18.3 capillaries/mm^2 respectively, p=0.01, n=10) (Figure 1E). In addition, area per capillary was increased in ischemic interosseus muscle as compared to at the amputation level (102.2±5.0 μm^2 as compared to 81.2±6.4 μm^2 respectively, p=0.03, n=10) (Figure 1F).

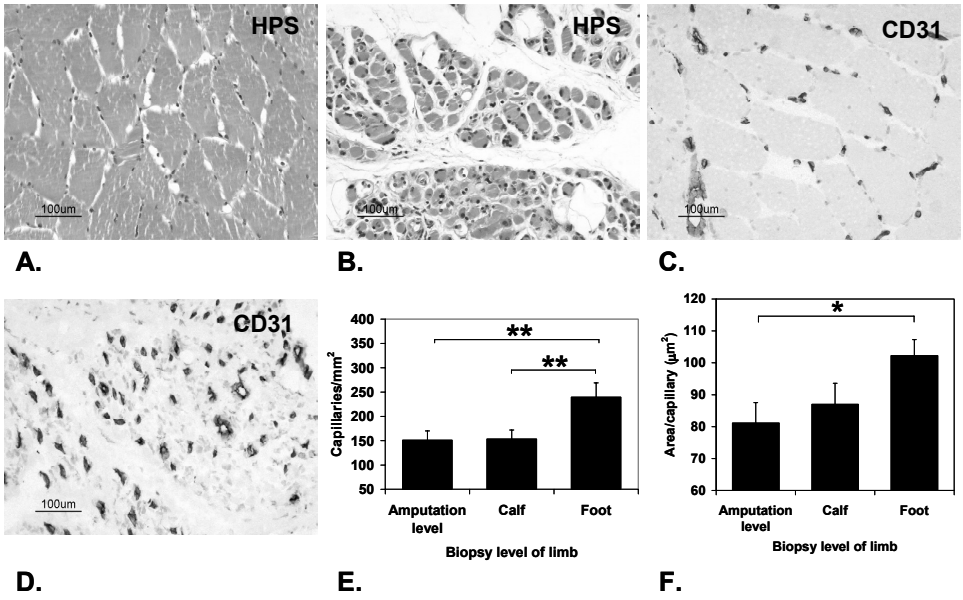


Figure 1. A-D. HPS and CD31 staining of gastrocnemius muscle at amputation level (A and C) and ischemic interosseus muscle (B and D) (patient #6). (x150) **E-F.** Quantification of capillary density and area in muscle sections at the level of amputation, calf or foot, depicted as capillaries/mm^2 and μm^2, respectively (n=10 patients). *P<0.05, **P<0.01. *See color figure on page 229.*

Expression patterns of VEGFs and SDF-1, and their receptors in human ischemic skeletal muscle

VEGF-A was expressed in cytoplasm of myofibers, in endothelial cells, subendothelial pericytes, and adventitial (angiogenic) capillaries in ischemic muscle of nearly all patients (Figure 2A). Especially in muscle with acute-on-chronic ischemia VEGF-A expressing inflammatory cells were visible between myofibers (Figure 2B).

In chronically ischemic muscle, atrophic myofibers, regenerating polynuclear myofibers and satellite cells all strongly stained for VEGF-A (Figure 2C). There was no expression of VEGF-C in any muscle sample (Figure 2D), whereas expression was evident in control colon tissue (data not shown). VEGF-D was expressed in both non-ischemic and ischemic muscle and was located in cytoplasm of myofibers, and in close proximity to capillaries between myofibers (Figure 2E). VEGF receptor -1, -2 and -3 were expressed in nearly all micro-vessels adjacent to myofibers (Figure 2FH).

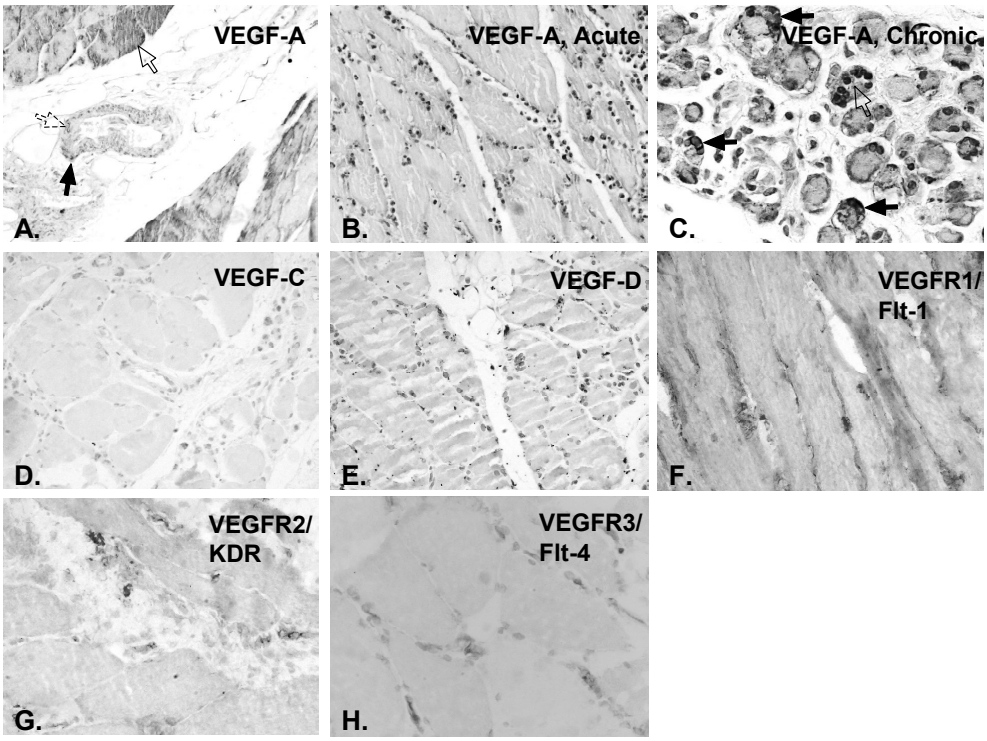


Figure 2. A. VEGF-A staining of ischemic interosseus muscle (patient #10) (x150). VEGF-A was expressed in myofibers (open arrow), adventitial capillaries (dotted arrow) and pericytes (solid arrow). **B.** VEGF-A staining of acute-on-chronic ischemic interosseus muscle (patient #5) showing infiltrating VEGF-expressing cells (x150). **C.** VEGF-A staining of chronically ischemic interosseus muscle (patient #6) showing VEGF expression in atrophic myofibers associated with satellite cells (solid arrows) and regenerating myofibers (open arrow) (x200). **D-H.** Staining of ischemic muscle for VEGF-C (D, x150), VEGF-D (E, x150), VEGF-receptor 1 (F, x200), VEGF-receptor 2 (G, x200), and VEGF-receptor 3 (H, x200). *See color figure on page 230.*

SDF-1 antigen was present both in capillaries adjacent to myofibers (Figure 3A) and in larger vessels (Figure 3B+C). SDF-1 was located at endothelial cells, as indicated by co-localization with CD31 and CD34 endothelial staining (Figure 3D and E), as well as at sub-endothelial pericytes. CXCR4 was mainly expressed in proximity to capillaries between myofibers (Figure 3F). Only sporadically, CXCR4 expression was observed in the wall of larger vessels. This was most obviously encountered in relatively non-ischemic gastrocnemius muscle at the amputation level in a patient with chronically ischemic disease (Figure 3G). Importantly, CXCR4 expression was not observed in endothelial cells, indicating that there was no incorporation of CXCR4-positive cells in endothelium (Figure 3G, arrowheads). No staining was observed in negative control incubations for all antibodies used (data not shown).

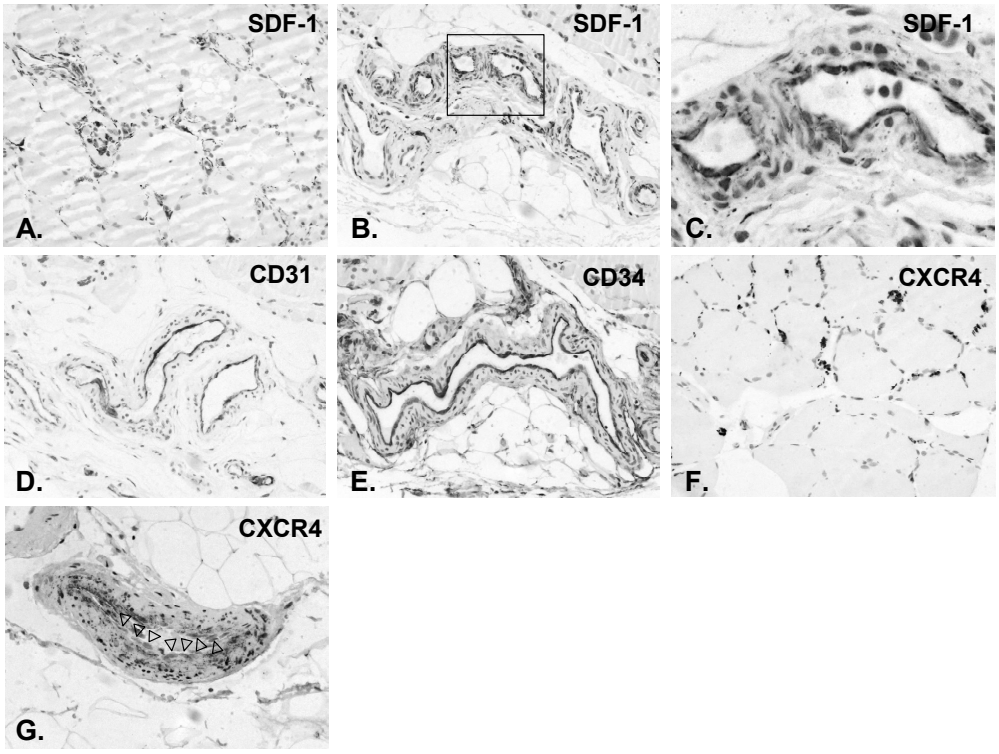
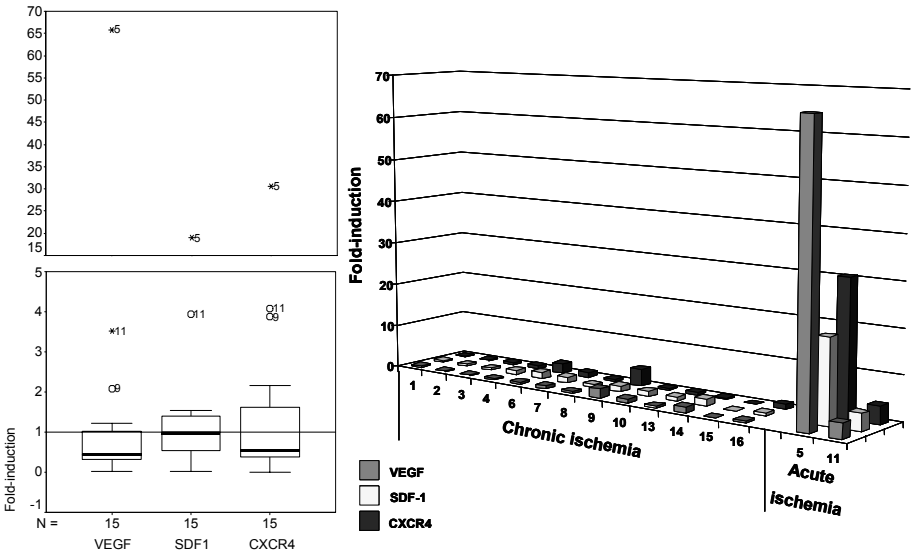


Figure 3. A-E. Immunohistochemical stainings of ischemic interosseus muscle sample derived from patient #6; staining for SDF-1 (A, x150), staining of sequential sections of large vessels for SDF-1 (B, x125, and C, higher magnification of the boxed area in (B), x400), for CD31 (D, x125), and for CD34 (E, x125). **F-G.** Staining for CXCR4 of muscle samples derived from patient #21; ischemic interosseus muscle tissue (F, x150), large vessel in gastrocnemius muscle tissue (G, x200). Endothelial cells did not express CXCR4 (arrowheads). *See color figure on page 231.*

Differential expression of VEGF-A, SDF-1 and CXCR4 between acute and chronic ischemia

In chronically ischemic limbs, there was a decreased or unchanged RNA expression of VEGF-A, SDF-1 and CXCR4 in ischemic as compared to non-ischemic tissues (fold inductions: VEGF-A, median 0.41 (95% CI 0.18-0.85); SDF-1, median 0.97 (95% CI 0.44-1.36); CXCR4, median 0.54 (95% CI 0.18-1.23); n=13) (Figure 4).

Significant down-regulation (fold induction ≤0.5) occurred in 9/13 patients, 4/13 patients and 8/13 patients for VEGF-A, SDF-1 and CXCR4, respectively. Only 2/13 patients with chronic limb ischemia showed significant up-regulations (fold induction ≥2.0); one patient for both VEGF-A and CXCR4, but not SDF-1 (patient #9, 2.1-, 3.9- and 1.4-fold induction, respectively) (Figure 4), another patient for only CXCR4 (patient #6, 2.2-fold induction). On the contrary, in the acute-on-chronic ischemic limbs there was an overall increased RNA expression for VEGF-A, SDF-1 and CXCR4 in ischemic muscle, most evidently in patient #5, with 65.8-, 19.0- and 30.6- fold inductions between ischemic and non-ischemic muscle for VEGF, SDF-1 and CXCR4, respectively. For patient #11, these values were 3.5-, 3.9- and 4.1-fold, respectively (Figure 4). In addition, there was a strong significant correlation between the ischemia-related changes of VEGF-A, SDF-1 and CXCR4 expressions within each patient (Table 2).



A. **B.**

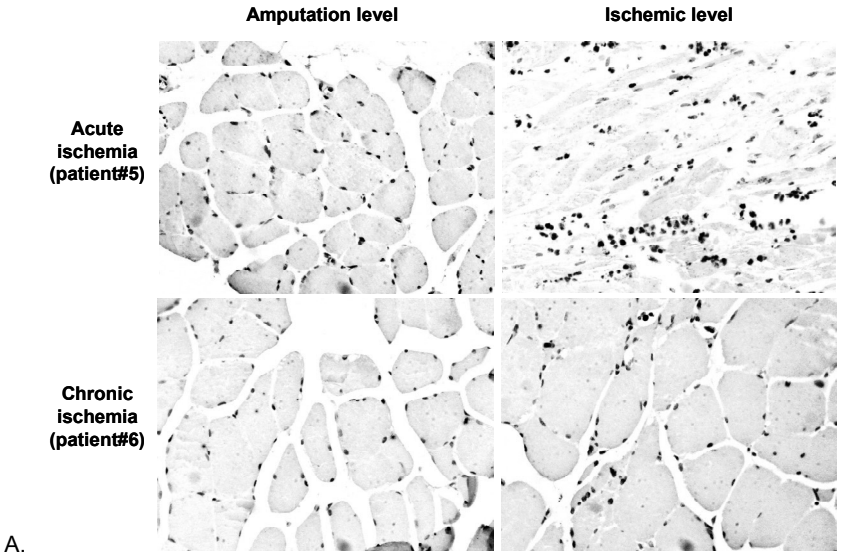
Figure 4. A. Box plot of mRNA expressions determined in muscle samples by real-time RT-PCR for VEGF-A, SDF-1 and CXCR4 in fold inductions between ischemic and non-ischemic muscle (medians, N=15, *5=patient #5, o9=patient #9, o11=patient #11). **B.** Bar graph representing mRNA expressions of VEGF, SDF-1 and CXCR4 in fold inductions between ischemic and non-ischemic muscle for each patient. *See color figure on page 232.*

Table 2. Pearson correlations between ischemic/non-ischemic fold inductions of VEGF-A, SDF-1 and CXCR4 expression and capillary density and area. *P<0.05; **P<0.01.

		VEGF-A	SDF-1	CXCR4	Cap Density	Cap Area
VEGF-A	Pearson Correlation	1	0.989**	0.993**	0.421	0.848**
	N	15	15	15	8	8
SDF-1	Pearson Correlation	0.989**	1	0.992**	0.421	0.834*
	N	15	15	15	8	8
CXCR4	Pearson Correlation	0.993**	0.992**	1	0.364	0.812*
	N	15	15	15	8	8
Cap Density	Pearson Correlation	0.421	0.421	0.364	1	0.627
	N	8	8	8	8	8
Cap Area	Pearson Correlation	0.848**	0.834*	0.812*	0.627	1
	N	8	8	8	8	8

Moreover, VEGF-A, SDF-1 and CXCR4 expressions were correlated with capillary area (p=0.008, 0.010 and 0.014 respectively), but not with capillary density. No significant correlations with risk factors such as diabetes, smoking, hypertension or dyslipidemia were observed. On a protein level, immunohistochemical staining intensity was not significantly different between ischemic and non-ischemic muscle for the chronic ischemic group, for VEGF-A (9 and 3 signs, respectively, P=0.15), VEGF-D (5 and 7, respectively, P=0.77), VEGF-receptor 1 (6 and 7, respectively, P=1), VEGF-receptor 2 (9 and 4, respectively, P=0.27), VEGF-receptor 3 (7 and 6, respectively, P=1), SDF-1 (8 and 6, respectively, P=0.79) and CXCR4 (6 and 8, respectively, P=0.79). For the acute-on-chronic ischemia group, the number of 2 patients was too small to perform the Sign-test for statistical comparison. Finally, to test whether the difference in angiogenic expressions between acute and chronic ischemia is hypoxia-related, immunohistochemistry for HIF-1α was performed in a selection of patients with acute-on-chronic (n=2) and chronic (n=5) ischemia. Nuclear HIF-1α staining was observed in kidney carcinoma (positive control) and in part of cells of muscle specimens. HIF-1α nuclear staining was absent or limited at the amputation level, while it was very profound in muscle of acute hypoxic legs (patients #5, #11; nuclei of inflammatory cells brightly positive, those of endothelial cells and some muscle cells moderately positive). An intermediate staining (mainly inflammatory cells) was observed in chronic hypoxic specimens (Figure 5A). The percentage of HIF-1α stained nuclei of total nuclei strongly increased with the level of ischemia in more acute ischemic limbs (ratios between ischemic and amputation level 6.6

and 2.6 for patients #5 and #11, respectively), whereas no significant upregulation was observed in chronically ischemic limbs (ratios 0.9, 1.2, 0.6, 0.7, 0.8 for patients #1, #4, #6, #8, #9, respectively) (Figure 5B).



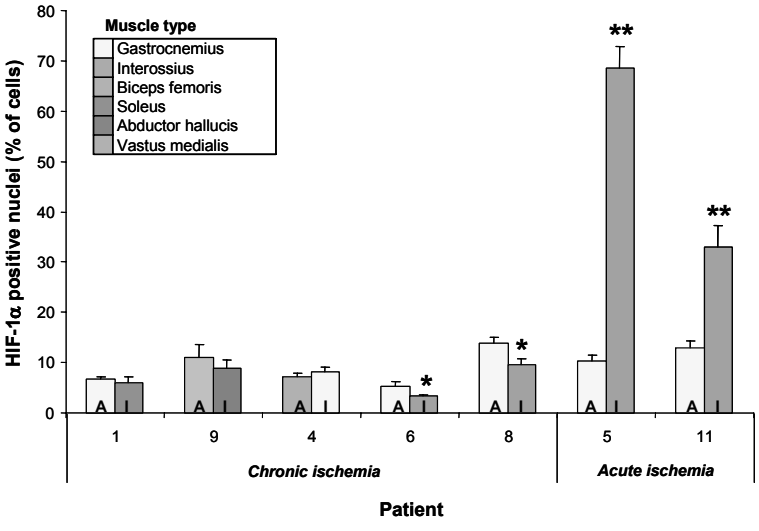


Figure 5. A. Representative images of muscle sections stained for HIF-1α at amputation level and distal ischemic level in a patient with acute-on-chronic ischemia (patient #5) and a patient with chronic ischemia (patient #6) (x150). **B** Quantification of HIF-1α staining at the amputation level (A) and distal ischemic level (I) in patients with chronic ischemia (n=5) or acute ischemia (n=2), expressed as HIF-1α positive nuclei (% of cells). *P<0.05, **P<0.01. *See color figure on page 233.*

Discussion

In this study, we provide evidence that VEGF-A, SDF-1 and CXCR4 are expressed by or in close proximity to angiogenic vessels in ischemic muscle of patients with peripheral arterial disease. Expression patterns of these genes were highly correlated with each other and with capillary area, suggesting interactions and angiogenic functions, respectively.
Moreover, VEGF-A, SDF-1 and CXCR4 gene expressions were increased in acute on-chronic limb ischemia, whereas generally decreased in chronic limb ischemia, as quantified by real-time PCR. Scoring of immune stained sections showed, however, no significant regulation in protein expressions by ischemia. This may be explained by that groups were too small to determine differences using the Sign-test, which is known as a crude statistical test. Another explanation may be that analysis of expression in a quantitative way based on immunohistochemistry is not reliable.
Nevertheless, a recent study confirms our observation that expression of VEGF protein does not correlate with VEGF mRNA expression in an ischemic hind limb model in rats [20]. In studies using similar muscle sampling methods from human ischemic legs as reported here, angiogenic expression patterns were highly variable, ranging from up-regulation in ischemic tissues of HIF-1 [21] or VEGF [22, 23], to upregulation of FGF, but not VEGF [24], to no regulation of pro-angiogenic factors [25], to restricted up-regulation of the HIF-VEGF-VEGFR2 pathway in acute-on-chronic, but not chronic ischemia [14].
These inconsistencies may be explained by differences in methods used to determine expression, in studied tissue type (muscle or skin), in location of sampling (muscle type), and in patient selection. Here, expression of angiogenic factors was determined by mRNA analysis using reliable quantitative real-time PCR with an inpatient control, and was, for the first time, correlated to the degree of ischemia as reflected by the amount of angiogenesis.
Furthermore, locations of muscle biopsies within one level of the limbs were kept constant, if possible, to limit variations in expression due to differences in muscle type. Finally, our cohort was divided in chronic- or acute-on-chronic- ischemic patients. In the latter group, VEGF, SDF-1 and CXCR4 up-regulation in ischemic tissue was higher when muscle biopsy was performed 1 week as compared to 2 weeks after graft occlusion. Correspondingly, in previous mouse studies, SDF-1 and CXCR4 up-regulations were transient after induction of hind limb ischemia [28, 29]. In chronically ischemic muscle, VEGF-A mRNA expression was significantly downregulated, whereas both VEGF-A and VEGF-receptor 2 protein expression tended to be increased as compared to control muscle at the level of amputation, and were abundantly expressed in atrophic myofibers and satellite cells, as reported [22]. One explanation may be that, together with an increased VEGF-A expression in atrophic myofibers, there is a relative loss of muscular tissue that

is replaced by fatty tissue. Furthermore, we hypothesize that in chronically ischemic muscle VEGF-A accumulates within atrophic myofibers, becomes dysfunctional and leads to gene silencing or down-regulation. This hypothesis is strengthened by our HIF-1α expression data suggesting an inability of hypoxic tissues to express sufficient HIF-1α, and thus down-stream VEGF and SDF-1, in chronic ischemia as opposed to acute-on-chronic ischemia.

VEGF-C has been restrictedly implicated in lymphangiogenesis, whereas VEGF-D is both a potent angiogenic and lymphangiogenic factor [27]. Correspondingly, in the present study VEGF-C was not expressed in ischemic muscle, whereas VEGF-D was abundantly expressed adjacent to capillaries. Interestingly, VEGF-receptor 3, often used for the detection of lymphatic endothelium, was expressed in nearly all microvessels throughout ischemic limbs, suggesting VEGF-receptor 3 expression on activated endothelial cells, as described [30]. The location of SDF-1 expression *in vivo* remains controversial. In some studies, SDF-1 expression was localized in endothelial cells [9], whereas others showed that SDF-1 mainly co-localized with periendothelial cells, probably of fibroblastic or smooth muscle nature [10, 31]. Here, SDF-1 expression was located both in endothelial cells and in close proximity to the endothelium. Sequential sections stained for CD31 and CD34 confirmed endothelial localization. Furthermore, the contribution of incorporating bone marrow-derived cells to adult neo-vasculature is still debated, ranging from minor [32-35] to major [36, 37] in previous studies. Here, CXCR4-positive cells were only scarcely observed in arterial walls without evidence of incorporation in endothelium. On the other hand, there was a close relationship between CXCR4-positive cells and small capillaries between ischemic myofibers, suggesting peri-endothelial localization around newly sprouting vessels.

Finally, it should be noted that muscle biopsies collected at different levels of the amputated limbs not only differ in the degree of ischemia but also in muscular composition. For example, gastrocnemius muscle mainly consists of fast-twitch, glycolytic myofibers, whereas soleus muscle consists of slow-twitch, oxidative myofibers. A limitation of our methods may lay in that global gene expression varies between glycolytic and oxidative skeletal muscle, although not reported for SDF-1 and CXCR4 [38].

In conclusion, we provide evidence in human ischemic skeletal muscle for a role of VEGF and SDF-1 in adult neovascularization via retention of CXCR4-positive cells. Moreover, VEGF, SDF-1 and CXCR4 were differentially expressed between acute on- chronic and chronic ischemia. Future experiments should focus on differences in angiogenic expression profile between acute and chronic hypoxic conditions, potentially leading to optimised angiogenic treatments.

Acknowledgements
We acknowledge Kees van Leuven (TNO, Leiden) for his technical assistance.

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