Expression of Vascular Endothelial Growth Factor, Stromal Cell-Derived Factor-1, and CXCR4 in Human Limb Muscle With Acute and Chronic Ischemia

Vincent van Weel, Leonard Seghers, Margreet R. de Vries, Esther J. Kuiper, Reinier O. Schlingemann, Ingeborg Bajema, Jan H.N. Lindeman, Pien M. Delis-van Diemen, Victor W.M. van Hinsbergh, J. Hajo van Bockel, Paul H.A. Quax

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 subendothelial cells, whereas CXCR4 was expressed in proximity to capillaries. VEGF-A, SDF-1, and CXCR4 expressions were generally decreased in ischemic muscle as compared with nonischemic muscle in patients with chronic ischemia (0.41-fold, 0.97-fold, and 0.54-fold induction [medians], respectively), whereas substantially increased in 2 patients with acute-on-chronic ischemia (3.5- to 65.8-fold, 3.9- to 19.0-fold, and 4.1- to 30.6-fold induction, respectively). Furthermore, these gene expressions strongly correlated with capillary area. Only acute ischemic tissue displayed a high percentage of hypoxia-inducible factor-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 downregulated in chronic ischemia as opposed to upregulated in more acute ischemia. The VEGF-SDF-1-CXCR4 pathway is a promising target to treat chronic ischemic disease. (Arterioscler Thromb Vasc Biol. 2007;27:1-2.)

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. 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. 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. 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. SDF-1 is implicated as a chemokine for CXCR4-positive stem cells. 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. 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. Other investigators reported that cytokine-mediated release of SDF-1 from platelets constitute the major determinant of neovascularization through mobilization of nonendothelial CXCR4 "VEGFR1" hematopoietic progenitor cells. Moreover, SDF-1 (gene) therapy enhances ischemia-induced vasullogenesis and angiogenesis in mice, and is associated with incorporation of bone marrow cells into the vasculature.
Here, expression patterns of VEGF, SDF-1, and CXCR4 were studied in relation to other angiogenic factors, such as VEGF-C, VEGF-D, and VEGF receptors 1 to 3 in amputated limbs of 16 patients with peripheral arterial disease, of which 14 had chronic ischemia, whereas 2 patients had acute-on-chronic ischemia. In both chronic and acute-on-chronic ischemia, SDF-1 was expressed in endothelial and subendothelial 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 from 16 patients from November 2001 to June 2003 are depicted in the Table. Patients underwent below-knee or above-knee amputation because of critical ischemia without possibilities for vascular reconstruction. All patients had 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 cases. The former patient was re-admitted 1 month after urokinase thrombolysis of an occluded femoro-popliteal bypass graft with re-occlusion of the bypass. After an unsuccessful revascularization attempt, below-knee amputation was performed 1 week later. The latter patient showed sudden onset of rest pain caused by occlusion of a femoro-crural bypass graft 9 days postoperatively, which was followed by below-knee amputation 2 weeks later.

To compare nonischemic with ischemic muscle within 1 patient, biopsies were performed at amputation level, representing relatively nonischemic muscle and, more distally, near the Achilles tendon (Soleus muscle) and between the toes (Interosseus, Extensor digitorum IV). Each biopsy was performed in duplo; one muscle sample was fixated in 4% formaldehyde and subsequently embedded in paraffin for immunohistochemistry, and one muscle sample was frozen in liquid nitrogen (LN) for RNA analysis. Because in 6 of 16 patients the biopsy samples could only be collected from 2 instead of 3 levels of the limb for various reasons, eg, 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.

**Immunohistochemistry**

Immunohistochemistry was performed on 5-μm-thick paraffin-embedded sections of skeletal muscle using antibodies against CD31, CD34, SDF-1, CXCR4, VEGF-A, VEGF-C, and VEGF-D, and VEGF...
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 biopsy samples derived from distal levels of the limb (most ischemic area) that were not apparent at the amputation level (relatively nonischemic area) (Figure 1A and 1B). 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 1D), which became significant at the level of interosseous muscle of the foot as compared with both amputation level and soleus muscle (239.6 ± 29.5 as compared with 151 ± 19.0 and 153.7 ± 18.3 capillaries/mm², respectively; P = 0.01; n = 10; Figure 1E). In addition, area per capillary was increased in ischemic interosseus muscle as compared with at the amputation level (102.2 ± 5.0 µm² as compared with 81.2 ± 6.4 µm², respectively; P = 0.03; n = 10; Figure 1F).

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 (angio-

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 with nonischemic tissues (fold inductions: VEGF-A, median 0.41 [95% CI, 0.18 to 0.85]; SDF-1, median 0.97 [95% CI, 0.44 to 1.36]; CXCR4, median 0.54 [95% CI, 0.18 to 1.23]; n = 13; Figure 4). Significant downregulation (fold induction ≤0.5) occurred in 9 of 13

Statistical Analysis

Results are expressed as mean ± SEM or median with 95% CI. Comparisons between means were performed using 1-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. Pearson correlations were used to study relationships. P < 0.05 was considered statistically significant.

RNA Analysis

Total RNA was extracted from frozen muscle and reversed transcribed into cDNA. Real-time reverse-transcription polymerase chain reaction was performed using primers and probe sets for human SDF-1 and CXCR4 (Applied Biosystems), and human VEGF-A (designed with Perkin Elmer primer express software), normalized to GAPDH housekeeping gene (Perkin Elmer).
patients, 4 of 13 patients, and 8 of 13 patients for VEGF-A, SDF-1, and CXCR4, respectively. Only 2 of 13 patients with chronic limb ischemia showed significant upregulations (fold induction ≥2.0); 1 patient for both VEGF-A and CXCR4, but not SDF-1 (patient 9, 2.1-fold, 3.9-fold, 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-fold, 19.0-fold, and 30.6-fold inductions between ischemic and nonischemic muscle for VEGF, SDF-1, and CXCR4, respectively. For patient 11, these values were 3.5-fold, 3.9-fold, 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 (supplemental Table I). 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 nonischemic 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, respec-
tively; $P=1$), 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 for 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-1 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 (supplemental Figure IIA). 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, and 0.8 for patients 1, 4, 6, 8, and 9, respectively; supplemental Figure IIB).
was determined by mRNA analysis using reliable quantitative real-time polymerase chain reaction with an inpatient control and was, for the first time, correlated to the degree of ischemia as reflected by the amount of angiogenesis. VEGF, SDF-1, and CXCR4 expressions were highly correlated with capillary area, but not with capillary density, suggesting involvement of these factors in the enlargement of neovessels, as reported for VEGF.22,23 Furthermore, locations of muscle biopsies within one level of the limbs were kept constant, if possible, to limit variations in expression caused by differences in muscle type. Finally, our cohort was divided in chronic ischemic or acute-on-chronic ischemic patients. In the latter group, VEGF, SDF-1, and CXCR4 upregulation in ischemic tissue was higher when muscle biopsy was performed 1 week as compared with 2 weeks after graft occlusion. Correspondingly, in previous mouse studies, SDF-1 and CXCR4 upregulations were transient after induction of hind limb ischemia.24,25

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 with control muscle at the level of amputation, and were abundantly expressed in atrophic myofibers and satellite cells, as reported.18 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 downregulation. This hypothesis is strengthened by our HIF-1α expression data, suggesting an inability of hypoxic tissues to express sufficient HIF-1α, and thus downstream 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.23 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 endothelial tissue, was expressed in nearly all microvessels throughout ischemic limbs, suggesting VEGF-receptor 3 expression on activated endothelial cells, as described.26 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 peri-endothelial cells, probably of fibroblastic or smooth muscle nature.10,12 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 neovascularure is still debated, ranging from minor28–31 to major,32,33 in previous studies. Here, CXCR4-positive cells were only scarcely observed in arterial walls without evidence of incorporation in endothelium. However, 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 biopsy samples collected at different levels of the amputated limbs not only differ in the degree of ischemia but also differ 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 lie in that global gene expression varies between glycolytic and oxidative skeletal muscle, although not reported for SDF-1 and CXCR4.34

In conclusion, we provide evidence in human ischemic skeletal muscle for a role of VEGF and SDF-1 in adult neo-vascularization 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 optimized angiogenic treatments.

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Disclosures
None.

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Materials and Methods

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). 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. 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. 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. 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 µl 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 VEGF-A were 5'-GCCCACTGAGGAGTCCAACA-3' (sense), 5'-TCCTATGTGCTGGCCTTGGT-3' (anti-sense), 5'-FAM-CACCAGCTTGGGATCAAAACC-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 25µl 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.
Results

Negative controls of the immunohistochemistry data

CD31

Control

VEGF-A

Control

VEGF-C

Control
**Figure I.** Immunoreactivity of tissues stained with the different specific antibodies (CD31, VEGF-A, VEGF-C, VEGF-D, VEGFR1, VEGFR2, VEGFR3, SDF-1, CD34, CXCR4, HIF-1α) as compared to their negative control incubations with pre-immune serum in the absence of primary antibodies.
Figure II. 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.
Table I.

**Pearson correlations between ischemic/non-ischemic fold inductions of VEGF-A, SDF-1 and CXCR4 expression and capillary density and area**

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<th>SDF-1</th>
<th>CXCR4</th>
<th>Cap Density</th>
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<td>0.993**</td>
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*P<0.05, **P<0.01.
References


