Impairment in Postischemic Neovascularization in Mice Lacking the CXC Chemokine Receptor 3

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Abstract—Inflammatory cell infiltration is a feature of postischemic neovascularization. However, mechanisms leading to leukocyte attraction to the site of neovascularization are still undefined. We hypothesized that the CXC chemokine receptor 3 (CXCR3) may contribute to leukocyte accumulation and subsequently to blood vessel growth in the ischemic area. Ischemia induced by femoral artery ligation improved the number of CXCR3-expressing cells and the level of its ligand, CXCL10. Angiographic score, blood flow recovery measurement, and capillary density analysis showed a significant decrease of ischemic/nonischemic leg ratio in CXCR3-deficient mice when compared with controls (P<0.05), at day 21 after ischemia. Interestingly, this impairment was as important as that observed in mice deficient for the well known CC-chemokine monocyte chemoattractant protein-1 (MCP-1). At day 7 of ischemic injury, the number of CD3-positive T cells and Mac-3–positive monocytes/macrophages was 38% and 45% lower, respectively, in the ischemic leg of CXCR3-deficient mice compared with the control group (P<0.05), suggesting an important role for CXCR3 in leukocyte recruitment into the ischemic area. VEGF protein content, a classical proangiogenic factor, was also markedly reduced (80% reduction) in ischemic leg of CXCR3-deficient mice (P<0.01). Injection of bone marrow–derived mononuclear cells (BM-MNCs) isolated from wild-type animals restored the neovascularization reaction in CXCR3-deficient mice whereas BM-MNCs from CXCR3-deficient mice was ineffective. In conclusion, CXCR3 plays a key role in neovascularization and provides novel information on the mechanisms leading to leukocyte infiltration in the vessel growth area. (Circ Res. 2005;96:576-582.)

Key Words: inflammation ■ angiogenesis ■ ischemia ■ chemokine receptor

Neovascularization occurs after thrombotic vessel obstruction of the feeding artery. Three principal responses, which can be referred to as vasculogenesis, true angiogenesis, and collateral growth, are involved in this vessel growth process, although their relative importance and underlying pathways remain unclear.1 An increasing body of evidence suggest that both hypoxia and inflammation trigger the neovascularization process in the setting of ischemia. Among the actors of the inflammatory reaction, monocytes/macrophages have been shown to accumulate in the ischemic area and positively modulate vessel growth.2–3 Nude mice that lack all T cells or CD4+ T lymphocyte–deficient mice exhibit a reduction in postischemic vessel growth,4,5 suggesting that T lymphocytes are also key mediators of neovascularization. Leukocytes modulate neovascularization through the expression of angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 and the release of pro- and antiinflammatory cytokines that control the production of matrix metalloproteinases and, subsequently, matrix degradation.2,6–9

Accumulation of leukocytes at inflammatory sites is regulated by a family of small, discrete chemotactic proteins called chemokines. Four major chemokine subfamilies are distinguished based on the arrangement of conserved cystein residues within the amino acid sequences. Chemokines are potent mediators of cell adhesion and migration through their interactions with a family of G protein–coupled receptors (CCR, CXCR, or CX3CR) expressed on leukocytes. The cellular specificity of chemokines is determined by restricted expression of their chemokine receptors on various leukocyte cell types. Inflammatory cell infiltration is a feature of the post-ischemic neovascularization process. However, mechanisms leading to leukocyte attraction to the site of neovascularization are still undefined. Recently, exogenous adminis-
traction of the well-known CC-chemokine monocyte chemoattractant protein-1 (MCP-1/CC/1) or a deficiency in its receptor CCR2 have been shown to play a crucial role in monocyte recruitment in the adventitia of growing arteries after vessel occlusion.\textsuperscript{10,11} Given the complexity of the inflammatory reaction and the requirement of both monocytes and lymphocytes in the postischemic neovascularization process, it is likely that alternative CCR or CX(3)CR-mediated signal transduction pathway may contribute to leukocyte accumulation. CXC chemokine receptor 3 (CXCR3) is expressed on monocytes and activated memory Th1 lymphocytes. Furthermore, eosi

**Materials and Methods**

**Experimental Protocol**

C57Bl/6 wild-type mice, C57Bl/6 CXCR3-deficient mice, and C57Bl/6 MCP-1–deficient mice (10 weeks old) were anesthetized by isoflurane inhalation. Mice underwent surgical ligation of the proximal part of the right deep femoral artery, just below the origin of the circumflexa femoris lateralis, as previously described.\textsuperscript{7,8} The experiments have been performed in a blinded manner.

**Quantification of Neovascularization**

Twenty-one days after ischemia, vessel density was evaluated by three different methods, as previously described.\textsuperscript{7,8}

**Microangiography**

Mice were anesthetized (isoflurane inhalation) and longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta to inject contrast medium (Barium sulfate, 1 g/mL). Angiography of hindlimbs was then performed and images (two per limb, and mice were placed on a heating plate at 37°C to minimize temperature variation. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, perfusion was simulated in the foot and expressed as a ratio of ischemic to nonischemic leg.

**Capillary Density Analysis**

Frozen tissue sections (7 \( \mu \)m) from calf muscle were incubated with rabbit polyclonal antibody directed against total fibronectin (dilution 1:50) to identify capillaries. The capillary-to-myocytes ratio was determined in both ischemic and nonischemic legs. Results are then expressed as ischemic to nonischemic ratio.

**Laser Doppler Perfusion Imaging**

Briefly, excess hairs were removed by depilatory cream from the limb, and mice were placed on a heating plate at 37°C to minimize temperature variation. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, perfusion was calculated in the foot and expressed as a ratio of ischemic to nonischemic leg.

**Determination of CXCL10, CXCL9, and GAPDH mRNA level by RT-PCR**

Total RNA was extracted from ischemic and nonischemic calf according to the Trizol reagent protocol (Life technologies). Reverse transcription–polymerase chain reaction (RT-PCR) was then performed. Briefly, total RNA was reverse-transcribed with a 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 50 minutes at 42°C. For PCR amplification, the resulting cDNA was amplified using 2.5 \( U \) Taq DNA polymerase (Invitrogen) and 7.5 nmol/L primers giving a DNA fragment of 200 bp for CXCL10 or 212 bp for CXCL9 (Specific PCR primer kit, R&D Systems). Thirty-five amplification cycles were undertaken as follows: denaturation at 95°C for 45 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. After PCR amplification, PCR products were loaded on a 5% agarose gel and fluorescence was analyzed using a computer-based imaging system (Fuji Bas 1000, Fuji Medical Systems, Clichy, France). For GAPDH PCR amplification, the cDNA was amplified using 80 nmol/L primers (genome express) giving a DNA fragment of 983 bp. The antisense primer was 5′-CAGTTAAGGCCATGAGGTCCACCAC-3′ and the sense primer was 5′-TGAAAGTCCGGTTGACCGATTGGG-3′.

**Immunohistochemistry**

Staining for inflammatory cells was performed 7 and 21 days after ischemia. Frozen tissue sections (7 \( \mu \)m) from calf or thigh muscle were incubated with rabbit polyclonal anti-CXCR3 antibody (1:50, Santa-Cruz), goat anti-mouse CRG-2/CXCL10 antibody (15 \( \mu \)g/mL, R&D Systems), rat monoclonal antibody directed against Mac-3 (1:50, BD pharmingen), and with goat anti-CD3\( \alpha \) antibody (1:100, Santa-Cruz). Immunostains were visualized by using avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit elite, Vector Laboratories, Biovalley, Marne La Vallee, France) and then analyzed in randomly chosen fields using Histolab software. CD-3–positive cells were quantified in the whole ischemic area, and Mac-3–positive cells were counted in the adventitia and perivascular space of arteries.

**In Vitro Chemotaxis Assay**

Cell migration was evaluated by using a 96-well microchemotaxis chamber assay (ChemoTx, Neuroprobe Inc). Briefly, splenocytes were washed twice with PBS, resuspended in serum-free RPMI 1640 containing 0.1% BSA, then labeled 30 minutes at 37°C with 5-(and-6)-carboxyfluorescin diacetate, succinimidyl ester (CFSE) (Molecular Probes) in PBS. Cells were then washed in PBS and resuspended in RPMI-1640 (1 million cells/mL). The lower well of a chemotaxis chamber was filled with 30 mL of chemokine solution or medium (HBSS with 0.1% BSA, 1 mmol/L CaCl\(_2\), and 1 mmol/L MgCl\(_2\)). A 3-mm pore size was layered over the wells, and the splenocyte suspension (50 000 in assay medium) was seeded on the top surface of the filter. The chemotaxis chamber was incubated at 37°C, 100% humidity, 5% CO\(_2\) for 1 hour. The nonmigrated cells were washed from the top surface with PBS and the chamber was centrifuged 5 minutes at 1500 rpm. Fluorescence in the bottom well was measured using a Packard Fusion microplate analyzer (PerkinElmer Life Sciences Inc). Results are expressed as a chemotactic index representing the number of cells migrating in response to chemokines relative to the number of cells migrating in absence of chemokine. All conditions were tested in quadruplicate. Medium-induced migration represents 5% to 10% of cells that were subtracted for each assay.

**VEGF Protein Content**

VEGF protein expression was determined by Western blot in ischemic and nonischemic legs, as previously described.\textsuperscript{7,8}

**Infusion of Bone Marrow–Derived Mononuclear Cells Isolated From Wild-Type and CXCR3-Deficient Animals in Mice Lacking CXCR3**

For rescue experiments, bone marrow cells were obtained by flushing tibias and femurs of wild-type or CXCR3-deficient mice. Low-density mononuclear cells were then isolated by density-gradient centrifugation with Ficoll, as previously described.\textsuperscript{9} Five hours after hindlimb ischemia, CXCR3-deficient animals received...
Figure 1. A, Representative photomicrographs of CXCR3-positive cells in ischemic calf and thigh muscle of wild-type and CXCR3-deficient mice, at 7 days (D7) and 21 days (D21) after ischemia. Arrows indicate representative examples of CXCR3-positive cells. B, Representative photomicrographs of CXCL-10 positive staining in ischemic calf muscle of wild-type mice at 7 days (D7) and 21 days (D21) after ischemia. Arrows indicate representative examples of CXCL-10-positive staining. Sections stained with IgG control were used as negative control. C, Representative photomicrographs and quantitative evaluation of CXCL10 and CXCL9 mRNA levels in ischemic calf of wild-type animals. Values are mean±SEM, n=4. **P<0.01 vs day 7.
intravenous injection of $1 \times 10^6$ BM-MNCs. Animals were euthanized at day 10 after ischemia.

**Statistical Analysis**

Results are expressed as mean±SEM. One-way analysis of variance ANOVA was used to compare each parameter. Post hoc Bonferroni t test comparisons were then performed to identify which group differences account for the significant overall ANOVA. A value of $P<0.05$ was considered significant.

**Results**

**Expression of CXCR3 and CXCL-10 in Ischemic Hindlimb**

We first assessed the expression of CXCR3 and its ligands, CXCL-10 and CXCL-9, in the ischemic leg.

**CXCR3 Protein Expression**

CXCR3-positive cells were mainly localized in the adventitia and perivascular space of arteries in the ischemic calf, at day 7 after hindlimb ischemia (Figure 1A). CXCR3-expressing cells were also localized in the perivascular space of arteries in the thigh (Figure 1A). Staining was undetectable in the nonischemic leg. As expected, we could not detect CXCR3-positive cells in CXCR3-deficient animals. Only a small amount of arterioles displayed CXCR3-positive staining in ischemic calf (Figure 1A), at day 21 after hindlimb ischemia.

**CXCL-10 and CXCL-9 Expression**

CXCL-10–positive staining was observed in interstitial space around myocytes and vascular structure in the ischemic calf, at day 7 after hindlimb ischemia (Figure 1B). CXCL-10–positive staining was also evidenced, at day 21 after hindlimb ischemia (Figure 1B). In addition, we detected CXCL-10 gene expression in ischemic calf muscle, at day 7 after ischemia. CXCL-10 mRNA was 3-fold increased in ischemic compared with nonischemic calf muscle ($P<0.01$) (Figure 1C). This CXCL-10 mRNA upregulation was markedly reduced at day 21 after the onset of ischemia ($3.08 \pm 0.36$ versus $1.30 \pm 0.26$ for the ischemic/non ischemic ratio at day 7 versus that of day 21, respectively; $P<0.01$). Similarly, CXCL-9 gene expression was measured in ischemic tissue. CXCL-9 mRNA levels tended to be increased at day 7 compared with day 21, however this difference did not reach statistical significance ($1.27 \pm 0.26$ versus $0.67 \pm 0.18$ for the ischemic/nonischemic ratio at day 7 versus that of day 21, respectively; $P>0.05$) (Figure 1C). Overall, these results suggest that endogenous CXCR3/CXCL-10 related pathway may play a role in vascular-related processes in the setting of ischemia.

**Effect of CXCR3 Deficiency on Postischemic Neovascularization**

We next analyzed the role of the CXCR3/CXCL-10 signaling in postischemic neovascularization using CXCR3-deficient animals.

**Microangiography**

Angiographic score (Figure 2A) showed a significant 40% decrease of ischemic/nonischemic leg ratio in CXCR3-deficient mice when compared with controls ($0.41 \pm 0.03$ versus $0.56 \pm 0.04$, respectively; $P<0.05$). Interestingly, this impairment was similar to the one observed in MCP-1–deficient mice ($0.38 \pm 0.015$; $P<0.01$ versus control).

**Capillary Density**

Results obtained with microangiography were confirmed by capillary density evaluation (Figure 2B). In CXCR3-deficient mice, the ratio of ischemic to nonischemic leg capillary density was 1.4-fold lower than the ratio of the control group ($P<0.05$). MCP-1 deficiency also resulted in a 30% decrease in capillary density ($P<0.05$ versus control).
Laser Doppler Perfusion Imaging
The impairment in both angiographic score and capillary density observed in mice deficient for CXCR3 was associated with a significant alteration in foot perfusion (Figure 2C). CXCR3 deficiency hampered blood flow recovery compared with control group (0.43 ± 0.01 versus 0.60 ± 0.05, respectively \( P<0.05 \)). Similarly, foot perfusion was also abrogated in MCP-1–deficient mice (0.44 ± 0.06, \( P<0.05 \) versus control).

Mechanisms Associated With the Impairment of Postischemic Neovascularization in CXCR3-Deficient Mice

Chemotaxis Effect
We performed a chemotaxis assay using splenocytes. As shown in Figure 3A, the migration rate of wild-type-derived splenocyte depended on the concentration of the CXCR3 ligand, CXCL-10. In contrast, when cells isolated from CXCR3-deficient mice were used, CXCL10-induced cell migration was abrogated. The migration induced by the CCR5 ligand, CCL5 was unaffected in CXCR3-deficient cells (Figure 3A).

Inflammatory Reaction
We assessed the number of lymphocytes in the ischemic area using CD3 immunostaining. At day 7 of ischemic injury, we found a significant 38% decrease in CD3-positive T cells in the ischemic calf muscle of CXCR3-deficient mice compared with the control group (Figure 3B), suggesting an important role for CXCR3 in lymphocytes recruitment into the ischemic area. Interestingly, a marked decrease in the number of CD3-positive cells was also observed in the thigh muscle of CXCR3-deficient animals (Figure 2B). The difference was no longer significant at day 21 (Figure 3C).

We next examined monocyte/macrophage infiltration. At day 7 of ischemic injury, the number of Mac-3–positive monocytes/macrophages was 45% lower in the calf muscle of CXCR3-deficient mice compared with control animals (4.12 versus 6.4 ± 0.7 cells/section; \( P<0.05 \)). This number was also

Figure 3. A, Chemotaxis assay. Splenocytes were isolated from control (Cont) and CXCR3-deficient mice (CXCR3KO). Assays were performed in microchemotaxis chamber. Recombinant murine CCL5 and CXCL10 were placed in lower chambers, splenocytes in upper chambers. B, top, Representative photomicrographs of CD3- and Mac3-positive cells in calf and thigh muscle of wild-type and CXCR3-deficient mice, 7 days after ischemia. Bottom, Quantitative evaluation of CD3- and Mac3-positive cells in calf muscle. C, Representative photomicrographs and quantitative evaluation of CD3- and Mac3-positive cells in calf muscle of wild-type and CXCR3-deficient mice, 7 days after ischemia. Arrows indicate representative examples of CD3- or Mac3-positive cells. D, Representative photomicrographs and quantitative evaluation of VEGF protein level. Values are mean ± SEM, n=7. *\( P<0.05 \), **\( P<0.01 \) vs wild-type mice.
substantially reduced in the thigh muscle of CXCR3-deficient animals (Figure 3B). No significant changes were observed in the nonischemic muscle (1.5 ± 0.2 versus 1.8 ± 0.1 cells/section in CXCR3-deficient mice and wild-type animals, respectively). In addition, the difference was no longer significant at day 21 both in the calf (Figure 3C) and in the thigh muscle (data not shown).

**VEGF Protein Level**

Changes in the number of inflammatory cells were associated with modulation of VEGF protein content. Indeed, after 7 days of ischemia, VEGF protein level was markedly downregulated (80% reduction) in ischemic calf of CXCR3-deficient mice compared with that of control animals (P < 0.01) (Figure 3D). VEGF protein content was still decreased after 21 days of ischemia in CXCR3-deficient animals but this did not reach statistical significance, suggesting that VEGF content tended to return to basal level (Figure 3D).

**Rescue Experiments With Bone Marrow–Derived Cells in CXCR3-Deficient Mice With Hindlimb Ischemia**

To examine whether the impaired development of collateral flow observed in CXCR3-deficient mice was caused by a deficient supply in CXCR3-positive cells with proangiogenic potential, we performed a rescue experiment in which we infused bone marrow–derived mononuclear cells (BM-MNCs) into mice lacking the CXCR3. We evidenced that BM-MNCs from CXCR3-deficient mice was unable to improve the neovascularization reaction in CXCR3-deficient animals. In contrast, BM-MNCs from control mice fully restored blood vessel growth (Figure 4).

**Discussion**

The main result of this study is that CXCR3 expression positively modulates ischemia-induced neovascularization, likely through modulation of inflammatory cell infiltration within the ischemic area. This study also extends previous results on the role of MCP-1 in this process and shows a significant impairment in postischemic neovascularization in MCP-1–deficient mice.

Classically, inflammatory cells have been shown to promote neovascularization through various mechanisms, including production of angiogenic factors, secretion of proinflammatory cytokines, and increased matrix degradation.7–9 LPS-induced monocyte accumulation promotes vessel growth, whereas absence of macrophages is associated with a
deficient neovascularization response. A specific role of T cells in this setting was also suggested in Nude mice and in CD4-deficient mice, which exhibit a marked reduction in the angiogenic/arteriogenic process. In this study, we showed that CXCR3 and its ligands CXCL-10 and CXCL-9 were expressed in ischemic tissue. CXCR3 is a potent chemoattractant receptor, as previously described. Consistent with these data, we observed a sharp reduction in the number of T lymphocytes and monocytes in the ischemic hindlimb of CXCR3-deficient mice suggesting that CXCR3 plays a key role in the signal transduction pathways responsible for leukocyte trafficking to the neovascularization area. This reduction in inflammatory cell number correlated with a decrease in postischemic neovascularization. In addition, mononuclear cells from CXCR3-deficient mice was unable to improve blood vessel growth in ischemic area suggesting that CXCR3 plays a functional role in this setting.

CXCR3 is mainly involved in T lymphocytes attraction. However, monocyte infiltration was also affected in CXCR3-deficient mice (Figure 3B). Interestingly, the effect of CD4+ cells in vessel development are causally related, in part, to their capacity to attract the monocyte/macrophage in the ischemic area, which subsequently trigger the neovascularization reaction. Our results support the concept that mechanisms contributing to the T cell–dependent neovascularization appear to reside in the ability of these cells to induce monocyte/macrophage accumulation. VEGF protein content was also decreased in ischemic hindlimb of CXCR3-deficient mice. In foci of inflammatory cell infiltration, tissue macrophages have been shown to produce VEGF. Because CXCR3 controls, albeit indirectly, macrophage recruitment to the site of active blood vessel growth, this would in turn affect VEGF synthesis and subsequently collaterals development.

Alternatively, CXCR3 is also expressed on endothelial cells and pericytes, suggesting that its vascular related effects may participate to the vessel growth process. CXCR3 ligands have been shown to exert angiostatic activities on human microvascular endothelial cells, whereas they activate phosphatidylinositol 3-kinase/Akt pathway, cell migration, and proliferation of human vascular pericytes, suggesting that CXCR3 may have cell-type–specific effects. These opposite functions are likely explained by the interactions of CXCR3 ligands with two distinct receptors, CXCR3-A and CXCR3-B. This latter receptor may account for the angiostatic effect of CXC chemokines. Informations on CXCR3 receptor subtypes expression and on CXCR3 signaling in both vascular cells and T lymphocytes are likely to provide further information on the relation between signaling events and the biological actions linked to activation of this receptor.

In conclusion, this study reports, for the first time, that CXCR3 expression plays a key role in postischemic neovascularization and provides new information on the mechanisms involved in leukocyte infiltration within the vessel growth area. Our data also suggest that modulation of endogenous expression of MCP-1 or CXCR3-related pathways could provide novel proangiogenic strategies for the treatment of ischemic diseases.

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References
