Absence of Chemokine (C-X-C Motif) Ligand 10 Diminishes Perfusion Recovery After Local Arterial Occlusion in Mice

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Objective—In arteriogenesis, pre-existing anastomoses undergo enlargement to restore blood flow in ischemic tissues. Chemokine (C-X-C motif) ligand 10 (CXCL10) is secreted after Toll-like receptor activation. Toll-like receptors are involved in arteriogenesis; however, the role of CXCL10 is still unclear. In this study, we investigated the role for CXCL10 in a murine hindlimb ischemia model.

Approach and Results—Unilateral femoral artery ligation was performed in wild-type (WT) and CXCL10 knockout (KO) mice and perfusion recovery was measured using laser-Doppler perfusion analysis. Perfusion recovery was significantly lower in KO mice compared with WT at days 4 and 7 after surgery (KO versus WT: 28±5% versus 81±13% at day 4; P=0.003 and 57±12% versus 107±8% at day 7; P=0.003). Vessel measurements of α-smooth muscle actin–positive vessels revealed increasing numbers in time after surgery, which was significantly higher in WT when compared with that in KO. Furthermore, α-smooth muscle actin–positive vessels were significantly larger in WT when compared with those in KO at day 7 (wall thickness, P<0.001; lumen area, P=0.003). Local inflammation was assessed in hindlimb muscles, but this did not differ between WT and KO. Chimerization experiments analyzing perfusion recovery and histology revealed an equal contribution for bone marrow–derived and circulating CXCL10. Migration assays showed a stimulating role for both intrinsic and extrinsic CXCL10 in vascular smooth muscle cell migration.

Conclusions—CXCL10 plays a causal role in arteriogenesis. Bone marrow–derived CXCL10 and tissue-derived CXCL10 play a critical role in accelerating perfusion recovery after arterial occlusion in mice probably by promoting vascular smooth muscle cell recruitment and maturation of pre-existing anastomoses. (Arterioscler Thromb Vasc Biol. 2014;34:594-602.)

Key Words: cell movement ■ chemokine CXCL10 ■ inflammation ■ interferon-γ-inducible protein of 10 kDa ■ myocytes, smooth muscle

Patients experiencing a reduced arterial flow because of atherosclerotic lesion development in middle and larger arteries often benefit from adaptive collateral artery growth, known as arteriogenesis. During arteriogenesis, pre-existing collaterals undergo enlargement triggered by increased shear stress resulting in improved tissue perfusion.1 The underlying mechanism of arteriogenesis is not yet fully understood, but important components of this process are the infiltration and extravasation of inflammatory cells and recruitment and proliferation of vascular smooth muscle cells (VSMCs). Previously, we have shown that Toll-like receptor 2 and 4 and downstream nuclear factor-xB p50 signaling are involved in the process of arteriogenesis.2,3 Disruption of the extracellular matrix by matrix metalloproteinases enables VSMC migration and outward growth of the collateral vessel.4,5 As a result of increased shear stress, activated endothelial cells start to upregulate factors that induce the regrowth of VSMCs,6 resulting in luminal vessel diameter increase as well as vessel wall maturation by recruitment and proliferation of VSMCs.7 In addition, recruitment of activated inflammatory cells to the site of arteriogenesis has been reported in many preclinical and clinical studies.8–10 Chemokines, such as monocyte chemoattractant protein-1, have shown to play a crucial role in the process of local recruitment of inflammatory cells as monocytes, macrophages, and T lymphocytes for stimulation of arteriogenesis but has also been indicated to affect migration and proliferation of VSMCs.9,11 C-X-C chemokine ligand 10 (CXCL10), also known as interferon-inducible protein 10, belongs to the C-X-C chemokine superfamily. It functions as a potent chemoattractant for monocytes and T lymphocytes12–14 and signals via the C-X-C motif receptor 3 (CXCR3).15 In response to interferon-γ after Toll-like receptor stimulation,
various cell types, such as endothelial cells, fibroblasts, monocytes, and T lymphocytes, can secrete CXCL10. In addition to its leukocyte attractant properties, CXCL10 also induces migration and proliferation of VSMCs. Previous studies demonstrated a role for both CXCR3 and interferon-γ in the field of arteriogenesis. Loss of CXCR3 in mice resulted in lower perfusion recovery after femoral artery ligation accompanied with lower capillary density in the ischemic calf muscle and less infiltration of macrophages and T lymphocytes in hindlimb muscles. Furthermore, additional studies associated hindlimb ischemia in mice with an elevation of interferon-γ plasma levels during the first 3 days after arterial occlusion. The role of CXCL10 has been studied in a mouse model for myocardial infarction (MI) where CXCL10 deficiency was associated with decreased microvascular density in the infarct and peri-infarct area after induction of MI. CXCL10 has been described as a late-phase protein during perfusion recovery after hindlimb ischemia that plays a more pronounced role in vessel maturation, which is known as a late step in neovascularization. In fact, similar patterns of mRNA levels of CXCR3, the receptor for CXCL10, and CXCL9, another ligand for CXCR3, could be observed in a murine model for hindlimb ischemia. However, the causal role for CXCL10 in hindlimb ischemia has not been studied yet. In this study, we investigated the role of CXCL10 in arteriogenesis using a mouse model for hindlimb ischemia. We hypothesized that mice lacking CXCL10 demonstrate reduced collateral vessel formation resulting in lower perfusion recovery and that influx of inflammatory cells into the surrounding tissue is hampered.

Materials and Methods

Materials and methods are available in the online-only Supplement.

Results

CXCL10 Protein Levels in Tissue and Plasma

CXCL10 protein levels in muscle tissue of the operated limb isolated from wild-type (WT) mice changed during perfusion recovery. In both adductor (nonischemic; Figure 1A) and peroneus (ischemic; Figure 1B) muscle tissue, CXCL10 protein levels reached its peak at day 4 (*P<0.05 versus baseline in peroneus tissue).

CXCL10 protein levels in plasma from WT mice also changed during perfusion recovery after femoral artery ligation, although in a different pattern. At day 1, plasma levels remained at baseline levels, but at day 2 the levels increased significantly. After day 2, plasma levels dropped again toward baseline levels (P<0.05 compared with baseline, days 1, 3, and 4). At day 7, the levels seem to increase again but did not reach statistical differences compared with baseline (Figure 1C).

Perfusion Recovery

CXCL10−/− mice showed decreased perfusion recovery when compared with WT mice at day 4 after ligation (WT versus CXCL10−/−; 81±13% versus 28±5%; P=0.003) and at day 7 (WT versus CXCL10−/−; 107±8% versus 57±12%; P=0.003; Figure 2A). To assess whether the effect of CXCL10 is mediated via bone marrow–derived cells or tissue-derived cells, we performed femoral artery ligation in chimeras. To confirm chimerization, genomic expression of CXCL10 DNA was assessed 6 weeks after irradiation and bone marrow transplantation. An expression of the corresponding genotype of >85% was acceptable for continuation of the experiments (Figure 2B).

Compared with WT, corresponding chimeras (CXCL10−/− bone marrow in WT mice) showed significantly lower
CXCL10−/− mice was significantly lower than in WT at day 4. However, the number of α-SMA–positive vessels in the adductor muscle of the operated hindlimb (total number of α-SMA–positive vessels per section) at baseline, no differences could be observed between WT and CXCL10−/− during arteriogenesis, we calculated the percentage of desmin-positive collateral vessels. During arteriogenesis, in WT mice, more desmin-positive vessels were lost at day 4 compared with CXCL10−/− (WT versus CXCL10−/−; vessel wall area: 241±21 versus 87±20 μm², P<0.001; vessel wall thickness: 3.57±0.23 versus 2.23±0.23 μm, P<0.001; vessel lumen area: 268±37 versus 109±27 μm², P<0.01; outer perimeter: 92±4.8 versus 59±5.8 μm, P<0.001; and inner perimeter: 69±4.4 versus 47±4.6 μm, P<0.01; Figure 5A–5E). To identify the changes in VSMC subtypes between WT and CXCL10−/− during arteriogenesis, we calculated the percentage of desmin-positive collateral vessels. During arteriogenesis, in WT mice, more desmin-positive vessels were lost at day 4 compared with CXCL10−/− (WT versus CXCL10−/−: 18.5% versus 32.5%; P<0.01). Simultaneously, no significant changes in percentage desmin-positive collateral vessels could be observed in CXCL10−/− mice in time (Figure 5G). These data suggest that only in the WT mice transient change in VSMC subtype expression occurred during collateral vessel maturation (arteriogenesis). Collateral vessels from both WT and CXCL10−/− mice positive for α-SMA also express the receptor for CXCL10 (CXCR3) at all time points. Figure 5H shows a representative image of CXCR3 staining by an α-SMA–positive vessel.

In chimeric mice, the mean vessel geometry of α-SMA–positive vessels was also assessed and showed a small difference between the chimeras, in which WT→CXCL10−/− chimeras displayed larger geometry compared with CXCL10−/−→WT

We therefore assessed the number of CD31-positive vessels in the ischemic peroneus muscle, expressed per muscle fiber. At baseline, no differences could be observed between WT and CXCL10−/−. Again CXCL10−/− mice had significantly fewer vessels compared with WT at day 4, but this difference diminished at day 7 (WT versus CXCL10−/−: 1.75±0.05 versus 1.21±0.09 at day 4; P<0.001 and 1.00±0.09 versus 0.93±0.10 at day 7; P=0.63; Figure 3B). These data indicate that, although the initial angiogenic response was slowed down in CXCL10−/− mice, angiogenic-related vascular growth was compensated at later time points because of degradation of angiogenic vessels in WT mice.

To further identify the role of bone marrow–derived and circulating CXCL10, vessel numbers were assessed in chimeric mice. The number of α-SMA–positive vessels is higher in WT→CXCL10−/− chimeras compared with CXCL10−/−→WT chimeras, although not significant (Figure 4A). This is in concordance with the perfusion recovery data. About the angiogenic response, no difference could be observed between the chimeras (Figure 4B).

**Collateral Vessel Geometry**

A hallmark of arteriogenesis is the enlargement of collateral vessels, where VSMCs in the vessel wall are recruited and start to accumulate and proliferate to ensure permanent vessel diameter and wall thickness enlargement. We assessed the mean vessel geometry of α-SMA–positive vessels at baseline and 4 and 7 days after surgery. Vessel geometry increased over time in both groups, but in WT this increase was more pronounced with significantly larger vessels compared with CXCL10−/− at day 7 (WT versus CXCL10−/−; vessel wall area: 241±21 versus 87±20 μm², P<0.001; vessel wall thickness: 3.57±0.23 versus 2.23±0.23 μm, P<0.001; vessel lumen area: 268±37 versus 109±27 μm², P<0.01; outer perimeter: 92±4.8 versus 59±5.8 μm, P<0.001; and inner perimeter: 69±4.4 versus 47±4.6 μm, P<0.01; Figure 5A–5E). To identify the changes in VSMC subtypes between WT and CXCL10−/− during arteriogenesis, we calculated the percentage of desmin-positive collateral vessels. During arteriogenesis, in WT mice, more desmin-positive vessels were lost at day 4 compared with CXCL10−/− (WT versus CXCL10−/−: 18.5% versus 40%; P<0.001). At day 7, the percentage increased in WT mice, indicating collateral vessel maturation (WT versus CXCL10−/−: 54% versus 32.5%; P<0.01). Simultaneously, no significant changes in percentage desmin-positive collateral vessels could be observed in CXCL10−/− mice in time (Figure 5G). These data suggest that only in the WT mice transient change in VSMC subtype expression occurred during collateral vessel maturation (arteriogenesis). Collateral vessels from both WT and CXCL10−/− mice positive for α-SMA also express the receptor for CXCL10 (CXCR3) at all time points. Figure 5H shows a representative image of CXCR3 staining by an α-SMA–positive vessel.

**Vessel Number in Adductor and Peroneus Tissue**

Because perfusion recovery depends on SMC-positive conductance vessels that bypass the occluded naïve artery, we assessed the number of α-smooth muscle actin (α-SMA)–positive vessels in the adductor muscle of the operated hindlimb (total number of α-SMA–positive vessels per section). At baseline, no differences could be observed. Compared with baseline, the number of α-SMA–positive vessels was higher in both WT and CXCL10−/− at 4 days after ligation. However, the number of α-SMA–positive vessels in CXCL10−/− mice was significantly lower than in WT at day 4 (WT versus CXCL10−/−: 102±7 versus 67±5; P<0.001) and at day 7 (WT versus CXCL10−/−: 88±9 versus 60±6; P=0.02; Figure 3A).

Although arteriogenesis in the used mouse model occurs mostly in the upper hindlimb (adductor), angiogenesis takes place in the ischemic area, that is, distal from the occlusion.
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(Figure 6). Interestingly, the collateral vessel geometry data fully represent the differences in the perfusion recovery in all experimental groups.

Influx of Inflammatory Cells

Influx of inflammatory cells into the local muscle tissue plays a crucial role in vascular remodeling and perfusion recovery. Therefore, we analyzed the number of macrophages and T lymphocytes in the perivascular space of collateral arteries in the adductor muscle at baseline and days 4 and 7 after surgery. Indeed, compared with baseline, both macrophage and T-lymphocyte numbers increased during perfusion recovery, but no significant differences could be observed between WT and CXCL10−/− (Figure 3C and 3D).

Influx of inflammatory cells was different between the chimeric mice. Although CXCL10−/−→WT chimeras showed

Figure 3. Histology on hindlimb muscles for vessel density and inflammatory cell influx in wild-type (WT) and CXCL10−/− mice. Histology was performed at baseline, days 4 and 7 in both WT (n=10; black bars) and CXCL10−/− mice (n=9; white bars). Total numbers of α-smooth muscle actin (SMA)–positive vessels per section were determined in the adductor muscle of the operated hindlimb (A). Number of CD31-positive vessels corrected for muscle fiber was determined in the ischemic calf muscle (peroneus) of the operated hindlimb (B). Macrophage and T-lymphocyte number was determined in the perivascular space around the growing collateral vessels in the adductor muscle of the operated hindlimb (C and D). Data are presented as means±SEM; *P=0.02, **P<0.001; WT vs CXCL10−/−. Photos are representative images of WT and CXCL10−/− mice at day 7 per analysis. Scale bar, 100 μm.

Figure 4. Histology of hindlimb muscles for vessel density and inflammatory cell influx in chimeric mice. Histology was performed at day 7 in both wild-type (WT) bone marrow→CXCL10−/− mice (n=15; black bars) and CXCL10−/− bone marrow→WT mice (n=15; white bars). Total numbers of α-smooth muscle actin (SMA)–positive vessels per section were determined in the adductor muscle of the operated hindlimb (A). Number of CD31-positive vessels corrected for muscle fiber was determined in the ischemic calf muscle (peroneus) of the operated hindlimb (B). Macrophage and T-lymphocyte number was determined in the perivascular space around the growing collateral vessels in the adductor muscle of the operated hindlimb (C and D). Data are presented as mean±SEM; *P=0.01.
Figure 5. Vessel geometry and desmin and C-X-C motif receptor 3 (CXCR3) expression in collateral vessels. Collateral vessel geometry was analyzed in α-smooth muscle actin (SMA)–positive vessels in the adductor muscle and expressed as the mean of 3 to 6 randomly chosen vessels per section per mouse. Analysis was performed at baseline and days 4 and 7 in both wild-type (WT; n=10; black bars) and CXCL10−/− mice (n=9; white bars). Vessel wall thickness is calculated as the average diameter of the vessel wall and expressed in micrometer (A). Vessel wall area and lumen area per vessel are expressed in micrometer square (B and C). Outer and inner perimeter is expressed in micrometer (D and E). Photos are representative images of α-SMA staining for WT and CXCL10−/− mice at baseline and day 7 (F). Desmin-positive collateral vessels were calculated as a percentage of α-SMA–positive vessels at baseline, days 4 and 7 in both WT and CXCL10−/− mice. Percentage of desmin-positive vessels in WT was significantly lower at day 4 and higher at day 7 compared with CXCL10−/− (similar levels at all time points; G). Representative image of CXCR3 expression in α-SMA–positive vessel wall (red indicates CXCR3 staining; green indicates α-SMA staining; blue indicates nuclei; H). Data are presented as mean±SEM; *P<0.01, **P<0.001. Scale bar, 100 µm.
similar macrophage influx levels compared with CXCL10−/− mice, WT→CXCL10−/− chimeras showed significantly lower levels (lower than WT), suggesting a greater effect for resident CXCL10 than circulating CXCL10 in attraction of macrophages. The difference between both chimeric groups was significant (WT→CXCL10−/− chimeras versus CXCL0−/−→WT chimeras; 1.7±0.24 versus 2.7±0.26; P=0.01). T-lymphocyte influx showed a similar pattern, although no significant differences could be observed (Figure 4C and 4D).

**In Vitro Migration of VSMCs**

To evaluate the paracrine effects of CXCL10 on the migration of SMCs, human aortic VSMCs were seeded on a Transwell membrane and allowed to migrate for 18 hours in a gradient of recombinant human CXCL10 in different concentrations (0, 10, and 100 ng/mL). Compared with control (0 ng/mL), relative migration of VSMCs toward recombinant human CXCL10 was significantly higher for both 10 and 100 ng/mL (P<0.05 versus control; Figure 7A). To evaluate the autocrine effects of CXCL10 on the migration of SMCs, human aortic VSMCs were seeded on a Transwell membrane and allowed to migrate for 18 hours in a gradient of 5% fetal calf serum. Knockdown of CXCL10 expression by siRNA in VSMCs significantly decreased the total number of migrated cells (Figure 7B). The chemotactic effect of CXCL10 secretion by endothelial cells on the migration of VSMCs was also evaluated. Knockdown of CXCL10 in human umbilical cord endothelial cells (HUVECs), seeded in the lower chamber of a Transwell membrane, significantly reduced the migration of VSMCs from the upper chamber to the HUVECs in the lower chamber (Figure 7C). Knockdown of CXCL10 was evaluated in transfected HUVECs and VSMCs with semi-quantitative polymerase chain reaction (PCR) on CXCL10 at 24 hours after transfection. Gel analysis of the PCR products revealed knockdown of CXCL10 after siRNA treatment compared with siSham in both VSMCs (Figure 7D) and HUVECs (Figure 7E).

**Discussion**

Arteriogenesis is known as an inflammatory process in which recruitment and proliferation of both inflammatory cells and VSMCs are important for maintaining tissue perfusion after local arterial occlusion. Chemokines are well-known key players in this process. CXCL10 has been described previously as a potent chemokine for attracting inflammatory cells, especially monocytes and T lymphocytes but has also been associated with angiogenesis in the ischemic myocardium after induction of MI in mice. In this study, we investigated the causal role of CXCL10 for perfusion recovery in a mouse model for hindlimb ischemia.

To elucidate the role for CXCL10 in perfusion recovery, we first measured CXCL10 plasma levels and protein levels
CXCL10 levels increased significantly in ischemic muscle tissue at day 4. In nonischemic tissue, a similar trend was observed. CXCL10 plasma levels increased significantly already on day 2. Based on the general short life of chemokines, this suggests that circulating CXCL10 is involved particularly in the initiation process during the early phase after femoral artery occlusion, whereas tissue CXCL10 is involved at a later stage. To establish causality, we performed a unilateral femoral artery ligation in WT and CXCL10−/− mice. In line with the plasma and tissue levels, perfusion recovery was reduced in CXCL10−/− mice as early as day 4 compared with WT. This difference was still evident at day 7, when WT mice reached full perfusion recovery. This is in concordance with the study of Waeckel et al.19 using CXCR3 −/− mice in a hindlimb ischemia model.

To determine whether the role of CXCL10 relies on circulating or resident cells, we performed bone marrow transplantsations in WT and CXCL10−/− mice followed by unilateral femoral artery ligation. Transplanting CXCL10−/− bone marrow into WT mice led to comparable reductions of perfusion recovery demonstrated by total CXCL10−/− mice. When WT bone marrow was transplanted into CXCL10−/− mice, perfusion recovery was significantly improved. Yet, WT bone marrow transplantation was not able to fully restore perfusion to comparable amounts as in WT mice. This indicates that CXCL10 from both circulating and resident cells is important in perfusion recovery, which is in line with the fact that leukocytes as well as endothelial cells, VSMCs, and fibroblasts can be a source for CXCL10.16–18

To investigate the role of CXCL10 in attracting inflammatory cells during perfusion recovery, we assessed local...
macrophage and T-lymphocyte accumulation around collateral vessels in WT and CXCL10−/− mice at different time points during perfusion recovery. Although we observed the expected increased influx of these cells after hindlimb ischemia, no significant differences could be observed in macrophage and T-lymphocyte accumulation between WT and CXCL10−/− mice at any time point. Previous studies in ischemic tissues showed decreased leukocyte accumulation in CXCR3−/− and CXCL10−/− mice in a murine MI model. 

In the murine hindlimb ischemia model, arteriogenesis in the upper limb is initiated by shear stress and not ischemia, which might explain the differences in leukocyte recruitment. Chimeric experiments revealed a greater effect for CXCL10 expressed by tissue resident cells in the attraction of macrophages and T lymphocytes toward the tissue because CXCL10 deficiency in bone marrow alone does not influence cell influx at least in the last phase of perfusion recovery (day 7). Furthermore, transplantation of WT bone marrow in CXCL10−/− even resulted in lower influx. Therefore, CXCL10 (day 7). Furthermore, transplantation of WT bone marrow in CXCL10−/− even resulted in lower influx. Therefore, CXCL10 deficiency in bone marrow alone does not influence cell influx at least in the last phase of perfusion recovery (day 7). It has been shown that bone marrow transplantation of WT bone marrow results in a hematopoietic chimera, which might explain the differences in leukocyte recruitment.

CXCR3−/− and CXCL10−/− mice in a murine MI model. 19,21 Because CXCL10 is known to act on both endothelial cells and SMCs,16–18 we analyzed the number of α-SMA–positive vessels and measured collateral vessel dimensions in the hindlimb muscle. WT mice showed more α-SMA–positive vessels after femoral artery ligation than CXCL10−/− mice. Concomitantly, enlargement of collateral vessels in WT mice during perfusion recovery is more prominent compared with CXCL10−/− mice pointing out a role for CXCL10 in migration of SMCs. The direct effect of CXCL10 on SMCs is plausible because we demonstrated the expression of CXCR3 by SMCs in the hindlimb tissue.

In addition, we further elucidated on the VSMC subtypes by analyzing desmin expression in α-SMA–positive vessels, a marker for contractile VSMCs. We observed that WT mice showed an early decrease in desmin expression (more synthetic than contractile VSMCs), which is a hallmark of arteriogenesis. However, desmin expression in CXCL10−/− mice hardly changed. These data suggest that only the WT mice showed transient changes in VSMC subtype expression during collateral vessel maturation (arteriogenesis), a phenomenon not occurring in CXCL10−/− mice. This points to a role of CXCL10 in the regulation of VSMC phenotype switch, already described by Wallace et al.23

Our data showed that CXCL10 stimulated migration of VSMCs in vitro. Furthermore, knockdown of CXCL10 by siRNA in VSMCs and HUVECs resulted in reduced VSMC migration, indicating that CXCL10 is both intrinsically and extrinsically involved in VSMC migration. However, we were unable to directly show either the effect of CXCR3 on VSMC migration or the effect of CXCL10 on HUVEC migration. Yates-Binder et al24 already showed an inhibiting effect of an interferon-inducible protein 10 peptide on endothelial cell motility and tube formation, although showing this in a different cell type (human microvascular endothelial cells versus HUVECs).

However, the arteriogenic response in the mouse model is of greater importance than the angiogenic response.

Although we did not further investigate the intracellular effect of CXCL10 on VSMCs, these intracellular effects of CXCL10 on VSMCs have already been described previously. CXCL10 effects are mediated through its specific receptor CXCR3(α). CXCR3 is a 7-transmembrane–spanning G-protein–coupled receptor. Binding of CXCL10 to CXCR3 leads to the exchange of GTP to GDP, followed by dissociation of the regulatory Gα subunit from the catalytic Gβγ subunit dimer. On activation, the G-protein subunits can activate different enzymes leading to the production of inositol phosphates, protein kinase activation, an increase in intracellular Ca2+ production and actin reorganization. Activation of the CXCR3 by CXCL10 leads to different cellular actions, such as degranulation, respiratory burst, and phagocytosis.15,25,26

Finally, the role of CXCL10 has been described extensively in different mouse models for cardiovascular diseases, for example, atherosclerosis, aneurysm formation, or MI. Although this study shows a beneficial effect of CXCL10 on arteriogenesis, it should be mentioned that other animal models have shown detrimental effects of CXCL10 on atherosclerotic plaque formation,14 aneurysm formation,27 or MI.21

Taken together, our in vivo and in vitro data imply that a significant part of the beneficial effect of CXCL10 in perfusion recovery is mediated via regulation of vascular cells. In summary, our findings strongly point toward a predominant action of CXCL10 on VSMCs rather than on leukocyte infiltration in arteriogenesis.

In conclusion, CXCL10 is involved in perfusion recovery after unilateral femoral artery ligation in mice. CXCL10, from both circulating and resident cells, is involved in the process of perfusion recovery. CXCL10 stimulates α-SMA–positive vessel formation and growth but does not influence the influx of inflammatory cells during hindlimb ischemia. This study identifies a causal role for CXCL10 during arteriogenesis in a murine hindlimb ischemia model, probably via stimulation of α-SMA–positive cell recruitment to the collateral vasculature.

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Disclosures
None.
Chemokines are powerful chemotactic molecules and key players during inflammatory responses in cardiovascular diseases to balance immune responses. Because of their potent actions and small molecular size, they are often proposed as therapeutic targets. Chemokine (C-X-C motif) ligand 10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation. 2006;113:2301–2312.


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Materials and methods

CXCL10 measurements
Adductor and peroneus tissue from the operated hind limb and plasma were obtained from wildtype mice (C57Bl/6J) at baseline, day 1, 2, 3, 4 and 7 after surgery, as published before. Muscle tissue was grinded and protein was isolated using Tris isolation (Roche) according to manufacturer’s instructions. Blood was isolated and centrifuged at 300 G. Plasma was isolated and stored immediately at -80 °C. CXCL10 protein levels were determined using Simplex assay (ProcartaPlex™ Luminex Mouse Immunoassays, eBioscience) according to manufacturer’s instructions. CXCL10 plasma levels were determined using a Quantikine ELISA (R&D Systems, cat # MCX100) according to manufacturer’s instructions.

Animal procedures
The present study was approved by the Utrecht University animal experimental committee following the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). Male C57Bl/6J mice and breeding pairs of the CXCL10-/− strain were obtained from Jackson Laboratories (strain name: B6.129S4-Cxcl10tm1Adl/J). The CXCL10-/− animals were housed for breeding at our university. At 10-12 weeks of age, twenty-five C57Bl/6J (WT) mice and twenty-four CXCL10-/- mice underwent unilateral permanent femoral artery ligation as described previously. Fifteen WT and fifteen CXCL10-/- mice underwent bone marrow transplantation prior to the surgery (see below). Mice were anesthetized with Dormicum (2 mg/kg) and medetomidine (0.15 mg/kg) and analgized with fentanyl (0.02 mg/kg) via intraperitoneal injection. After surgery, the mice were antagonized by a subcutaneous injection of Anexate (1.0 mg/kg) and Atipam (5.0 mg/kg) and received 2 injections of buprenorphine (0.15 mg/kg).

Paw perfusion was assessed using Laser-Doppler Perfusion Imaging (LDPI) (Moor Instruments, Ltd, Devon, UK) before and after surgery. In addition, paw perfusion was assessed at day 4 and 7, without the use of fentanyl to avoid potential changes in vasotonus. Perfusion recovery in the ischemic limb is expressed as a percentage of the contralateral non-ischemic limb perfusion. Mice were terminated at baseline, day 4 or day 7.

Bone marrow transplantation
Before bone marrow transplantation WT and CXCL10-/- mice were lethally irradiated at the age of 6 weeks with a dose of 700 cGray (1030 motor units) before transplantation with respectively CXCL10-/- bone marrow and WT bone marrow cells. Bone marrow was isolated from five age matched CXCL10-/- donor mice and five age matched WT donor mice by flushing the humeral and femoral bones with sterile RPMI 1640 medium (Invitrogen). Each mouse received 5 million cells in 250 µl RPMI medium via tail vein injection. After a recovery period of 6 weeks, mice underwent femoral artery ligation, followed by assessment of paw perfusion as described above. Before surgery, blood was drawn via cheek puncture to check transplantation efficacy. Genomic DNA was isolated automatically using GeneMole (Mole Genetics) and Mole strips (Mole Genetics, MGK20-10-102). QPCR was performed using primers specific for WT and CXCL10-/- mice as provided by Jackson Laboratories. A success rate of >85% of phenotype expression was acceptable for continuation of analysis. Chimeric mice were terminated at day 7.

Immunohistochemistry
Immunohistochemistry was performed on the adductor and peroneus muscles at baseline, 4 and 7 days after surgery in WT and CXCL10-/- mice. For chimeric mice,
Analysis was performed at day 7 only. Muscles were embedded in paraffin and 4µm thick sections were cut. Prior to staining of the adductor muscle, sections were boiled in Sodium Citrate Tribasic Hydrate (10mM, pH6.0) as antigen retrieval solution.

Tissue macrophages were identified by staining for Mac-3 (1:200, BD Pharmingen, 550292), followed by a biotin labeled goat anti-rat antibody (1:200, Southern Biotech) and streptavidin-HRPPO (1:1000, Southern Biotech, 7100-05). T lymphocytes were identified by staining for CD3 (1:100, DAKO, A0452), followed by Powervision anti-rabbit-HRPO (ImmunoVision Technologies, DPVM-110HRP). All sections were developed using 3-Amino-9-ethylcarbazole (AEC) and counterstained using haematoxylin. To assess alpha-Smooth Muscle Actin (α-SMA) positive vessels in adductor tissue, the sections were incubated with a FITC-labeled α-SMA antibody (1:400, Sigma, F3777). Nuclei were counterstained with Hoechst. Desmin positive vessels were detected by staining for Desmin (1:200, Millipore, clone Y66) followed by a goat anti-rabbit Alexa 555 (1:1000, Invitrogen, A21458). Collateral vessels were identified by incubation with a FITC-labeled α-SMA antibody (1:400, Sigma, F3777).

CXCR3 positive vessels were detected by staining for CXCR3 (1:50, R&D Systems, MAB1685), followed by a biotin labeled goat anti-rabbit antibody (1:200, Southern Biotech, 3052-08) and streptavidin-Alexa 555 (1:1000, Invitrogen, S21381).

Collateral vessels were identified by incubation with a FITC-labeled α-SMA antibody (1:400, Sigma, F3777). In the peroneus tissue, vessels were identified by incubation with CD31 (1:1500, Santa Cruz, sc-1506-R), followed by a biotin labeled goat anti-rabbit antibody (1:200, Vector, BA1000) and streptavidin-Alexa 555 (1:1000, Invitrogen, S21381). All immunohistochemical stainings were analyzed in a blinded fashion using Cell^P analysis software (Olympus).

Collateral vessel size measurements

The lumen area and vessel wall dimensions of collateral vessels were assessed at baseline, 4 and 7 days after surgery from three to six randomly chosen α-SMA positive vessels per section and expressed per vessel. For chimeric mice, analysis was performed at day 7 only. Maximal vasodilatation was assured by flushing with a vasodilator (nitro-glycerine) during perfusion fixation of the hind limbs. Measurements were analyzed using Cell^P analysis software (Olympus). Lumen area and vessel wall area are expressed in square µm (µm²); vessel wall thickness, outer and inner perimeter in µm.

In vitro migration of vascular smooth muscle cells

Human Aortic Vascular Smooth Muscle Cells (VSMCs; Lonza) and Human Umbilical Vein Endothelial Cells (HUVECs; Lonza) were cultured on gelatin-coated plates in SMGM2 medium (SMBM2 medium supplemented with SMGC2 bullet kit and 5% FCS; Lonza) and EGM2 medium (EGM2 medium supplemented with EGM2 bullet kit and 2% FCS; Lonza) respectively, in 5% CO₂ at 37 °C. The experiments were performed with cells at passage 3-5. In order to investigate the effect of CXCL10 on VSMC migration, control VSMCs were plated in the upper chambers of Transwell culture inserts (FluoroBlokTM cell culture inserts, 24-well 8.0 µm pore size, Corning) at a density of 0.5 x 10⁵ cells/well in 250 µl SMBM2 medium with 0.5% FCS. The lower chambers were filled with 750 µl SMBM2 with 0.5% FCS together with three different concentrations of recombinant human CXCL10 (0 ng/ml, 10 ng/ml and 100 ng/ml) (Peprotech, cat # 300-12). Cells were left for 18h incubation in 5% CO₂ at 37 °C for migration. CXCL10 knockdown in VSMCs and HUVECs was achieved by cell transfection of a pool of 4 targeting siRNA sequences specific for CXCL10 (siCXCL10) (ThermoScientific). Control cells were transfected with a pool of 4 non-targeting siRNA sequences (siSHAM) (ThermoScientific). In order to investigate the role of endogenous CXCL10 on the migration of VSMCs in a FCS gradient, the VSMCs were plated in the upper chambers of Transwell cell culture inserts (FluoroBlokTM cell culture inserts, 24-well 8.0 µm pore size, Corning) 24h after...
transfection at a density of $0.5 \times 10^5$ cells/well in 250 µl SMBM2 medium with 0.5% FCS. The lower chambers were filled with 750 µl SMBM2 with 5% FCS. To examine the role of endothelial cell derived CXCL10 in VSMC migration, control VSMCs were plated in the upper chambers of Transwell cell culture inserts (FluoroBlokTM cell culture inserts, 24-well 8.0 µm pore size, Corning) at a density of $0.5 \times 10^5$ cells/well in 250 µl SMBM2 medium. In the lower chambers, HUVECs were seeded 24h after transfection at a density of $1.0 \times 10^5$ cells/well in 750 µl SMGM2. After 18h incubation in 5% CO$_2$ at 37 °C, cells that have migrated through the filter and were located at the bottom of the inserts were stained with calcein-AM for 30 minutes at 5% CO$_2$ at 37 °C, followed by visualization by fluorescence microscopy. The ability of the VSMCs to migrate was determined by the total number of cells (>50µm$^2$) crossing the polycarbonate membranes (analysis in ImageJ). Total RNA was isolated 24h after transfection using RNAeasy kit (Qiagen) and reversed transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad). CXCL10 mRNA levels were assessed by semi-quantitative PCR method using CXCL10 primers (sense: 5’-CTCCAGTCAGCACCATGA-3’; and antisense: 5’-CAAAATTGGCTTGCAGGAAT-3’). Primers for the housekeeping gene β-actin (sense: 5’-TCCCTGGAGAAGAGCTACGA-3’; and antisense: 5’-AGCACTGTGTTGGCGTACAG-3’) were used as a quantitative control.

Statistical analysis

SPSS version 20.0 (Chicago, IL, USA) was used for statistical analyses. Comparisons between means were analyzed with an independent T test for two groups and a one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction for comparisons with more than two groups. Differences between means with p-values <0.05 were regarded as statistically significant. Data are expressed as mean ± SEM.

References
