Collateral Artery Growth (Arteriogenesis) After Experimental Arterial Occlusion Is Impaired in Mice Lacking CC-Chemokine Receptor-2

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Abstract—Arteriogenesis has been associated with the presence of monocytes/macrophages within the collateral vessel wall. Induced macrophage migration in vivo is driven by the binding of monocyte chemoattractant protein-1 (MCP-1, or CCL2 in the new nomenclature) to the CCR2-chemokine receptor on macrophages. To determine whether the CCL2-CCR2 signaling pathway is involved in the accumulation of macrophages in growing collateral vessels, we used mice that are deficient in CCR2 in a model of experimental arterial occlusion and collateral vessel growth. In an in vitro CCL2-driven chemotaxis assay, mononuclear cells isolated from wild-type BALB/c mice exhibited CCL2 concentration–dependent migration, whereas this migration was abolished in cells from CCR2−/− mice on a BALB/c genetic background. In vivo, blood flow recovery as measured by laser Doppler (LDI) and MRI (MRI) was impaired in CCR2−/− mice on either the BALB/c or C57BL/6 genetic backgrounds. Three weeks after femoral artery ligation, LDI perfusion ratio of operated versus nonoperated distal hindlimb in BALB/c wild-type mice increased to 0.45±0.06 and in CCR2−/− animals only to 0.21±0.03 (P<0.01). In C57BL/6 mice, ratio increased to 0.96±0.09 and 0.85±0.08 (P<0.05), respectively. MRI at 3 weeks (0.76±0.06 versus 0.62±0.01; P<0.05) and hemoglobin oxygen saturation measurements confirmed these findings. Active foot movement score significantly decreased and gastrocnemius muscle atrophy was significantly greater in CCR2−/− mice. Morphometric analysis showed a lesser increase in collateral vessel diameters in CCR2−/− mice. Importantly, the number of invaded monocytes/macrophages in the perivascular space of collateral arteries of CCR2−/− animals was dramatically reduced in comparison to wild-type mice. In conclusion, our results demonstrate that the CCR2 signaling pathway is essential for efficient collateral artery growth. (Circ Res. 2004;94:671-677.)

Key Words: collateral artery growth • monocytes • CCL2 • CCR2 • hindlimb ischemia

Collateral artery growth (arteriogenesis) is most likely the only form of vascular growth leading to the formation of large conductance blood vessels compensating for blood flow deficits caused by relevant stenosis of major arteries.1–4 However, the arteriogenic mechanisms are yet not completely understood. After arterial occlusion, the pre- versus postocclusive pressure differences provokes a sudden increase of the collateral blood flow through preexisting connections. This leads to an enhanced shear stress on the collateral wall which is considered to be the initial trigger of arteriogenesis.5,6 Multiple cellular responses like activation of vascular endothelial cells are induced leading to a subsequent increase in the expression of surface adhesion molecules (eg, ICAM-1 and VCAM-1).7

Initially, only correlative studies revealed the potential link between collateral artery growth and blood leukocytes. Monocytes/macrophages were found adhered to the activated collateral endothelium8 and accumulated in the adventitia or the perivascular space.9 Recently, we provided functional proof for the critical participation of circulating blood monocytes in arteriogenesis: When the blood monocyte concentration was pharmacologically increased, arteriogenesis was accelerated. Conversely, when monocytes were depleted from the blood, growth of collateral arteries was almost completely abolished.10,11 Because the mechanisms leading to attraction of monocytes to the collateral endothelium are not yet clarified, we now investigated the signal transduction molecules guiding blood monocytes to the activated collateral arteriole.

Local infusion of the CC-chemokine monocyte chemoattractant protein-1 (MCP-1, now denominated CCL2,12,13 has been demonstrated to enhance collateral artery growth.4 The CC-chemokine receptor-2 (CCR2), a member of a group of structurally related 7-transmembrane–spanning protein recep-
tors, has been shown to be the major receptor for MCP-1, although other members of the MCP subfamily may also be ligands of CCR2. Studies in CCR2-deficient mice revealed that this receptor is of importance for leukocyte adhesion and monocyte extravasation during inflammatory responses.

We used an ischemic hindlimb model to determine whether the MCP-1-CCR2 pathway is involved in monocyte migration activities during the early processes of collateral artery growth. Our data show that CCR2 gene–deficient (CCR2−/−) mice have impaired blood flow recovery after femoral artery ligation as assessed by laser Doppler imaging and MRI flow measurement. Furthermore, we demonstrate in vivo and in vitro that this impairment is based on a substantially reduced monocyte migration activity.

Materials and Methods

Animals
CCR2−/− mice were generated on a mixed C57BL/6× strain 129 genetic background as described previously. The disrupted CCR2 allele was backcrossed to the C57BL/6 and BALB/c genetic backgrounds for eight generations. Experimental wild-type and CCR2−/− animals were used at an age of 8 to 12 weeks. All experimental protocols were performed after approval of the State of Hessen, Regierungspraesidium Darmstadt, according to Section 8 of the German Tierschutzgesetz, and also in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

In Vitro Chemotaxis Assay
Anticoagulated peripheral blood was harvested from wild-type and CCR2−/− mice, and the mononuclear cells were isolated by density gradient centrifugation. The cells were then washed twice with phosphate-buffered saline (PBS). Chemotaxis assays were performed using 24-well transwell chambers (Costar). Murine MCP-1 (JE: Peprotech) was dilution in RPMI1640 (Life Technologies) to a concentration between 0 and 200 ng/mL and placed into bottom wells. Cells were diluted in RPMI1640 medium to a concentration of 106 cells/mL, and 100 μL were added to each top well. Chambers were incubated for 3 hours in a humidified incubator with 5% CO2. The number of migrated cells was determined using a CASYII cell counter (Schaefer Systems).

Ischemic Hindlimb Model
Surgery
The surgical procedure was performed as previously described. Briefly, the right femoral artery was exposed and ligated just distally to the origin of the arteria profunda femoris.

Laser Doppler Imaging
Relative blood flow to the foot was measured under standardized conditions by laser Doppler imaging (LDI) as described. Measurements were performed before, immediately after surgery, and on postoperative days 3, 7, 14, and 21. The right-to-left (R/L) ratio (operated versus nonoperated leg) was calculated for each animal (n=9 for each group).

Oxygen Saturation of Hemoglobin
Oxygen saturation of hemoglobin in the foot was determined by the hemoglobin absorption spectrum as previously described. Measurements were performed immediately after LDI. R/L ratio was calculated for each mouse.

Active Foot Movement Score
To assess functional recovery of the limbs, we developed a scoring system based on active foot movement. Single mice were placed in a cage. Movement of the occluded hindlimb was scored in one of four different categories: group 1 (use of the leg only), group 2 (active foot use), group 3 (full use of the foot but no spreading of toes), and group 4 (unrestricted active movement). Scores were ascribed in a randomized and blinded fashion directly before anesthetizing mice before LDI, starting 3 days after surgery.

MRI
MRI (MRI) was performed directly after LDI using a temperature-controlled magnetic resonance probe. Three axial slice planes were acquired over the length of the right and left calf muscles. A gradient echo sequence was used to acquire slices of 0.62 mm with an in-plane resolution of 100 μm using a 90° slice selection pulse and a fast repetition time of 20 ms. Vessels in calf muscle images were selected by defining a region of interest around the vessel location and setting a threshold of twice the standard deviation of the noise from the surrounding tissue. The R/L ratio of the vessel area intensity was calculated for each slice. Three slices were averaged for each mouse at each time point.

Tissue Sampling and Morphometry of Collateral Arteries
Tissue sampling and morphometric analysis of collateral artery growth were performed as described. Briefly, the hindlimbs were perfusion fixed using 2% paraformaldehyde, rinsed with buffer, and for easier identification filled with contrast agent using a constant pressure. Thereafter, collateral arteries of the adductor muscle were harvested, cryopreserved and sectioned with a CM3000 cryostat (Leica). Serial sections (6 μm thick) were incubated with a FITC-conjugated monoclonal antibody (mab) against smooth muscle actin and with TRITC-conjugated BS1-lectin (Sigma-Aldrich). Nuclear staining was performed using DAPI (Mobitec). Transversal sections of the collateral arteries were photographed with a Leica DMLD digital camera on a Leitz DMRB fluorescence microscope. Collateral artery diameters were calculated using ImageJ (NIH).

Immunohistochemistry
For monocyte/macrophage identification, 6-μm thick sections of collateral arteries (n=4 per group) were fixed in acetone (10 minutes) and incubated with 3% H2O2 in methanol for 10 minutes to block endogenous peroxidase activity. After washing with PBS, sections were incubated for 30 minutes in 1% bovine serum albumin in PBS, then stained for 12 hours at 4°C with a rat monoclonal antibody against murine Mac-3 (1:100; BD Biosciences). After washing, sections were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated donkey anti rat IgG (Dianova). Immunoperoxidase signals were visualized by incubation with diaminobenzidine. Nuclei were counterstained with hematoxylin. Mac-3–positive cells were quantified in the adventitia and perivascular space of two defined collateral arteries in the adductor muscles. For each animal, five sections were counted.

Statistical Analysis
Data pairs were analyzed by Student’s t test. A value of P<0.05 was considered to be significant. Data are expressed as mean±SEM.

Results
Chemotaxis Assay
We performed a chemotaxis assay using freshly isolated blood mononuclear cells (MNCs). As shown in Figure 1, the migration rate of wild-type (WT)–derived MNCs depended on the concentration of the recombinant murine MCP-1 (rmMCP-1), which had been added to the lower compartment of the transwell chamber. A maximum was achieved at an
rmMCP-1 concentration of 200 ng/mL. In contrast, when mononuclear cells from CCR2<sup>−/−</sup> mice were used, cell migration was almost completely abolished.

**Hindlimb Perfusion Measurements**

We next quantified blood flow in the distal mouse hindlimbs at six time points after experimental femoral artery stenosis using laser Doppler imaging (LDI) (Figure 2A). Figure 2B shows quantitative analysis of LDI data. In both CCR2<sup>−/−</sup> and WT mice with the BALB/c background, right-to-left (R/L) ratio substantially decreased immediately after occlusion [1.02±0.07 to 0.02±0.005 (control) versus 1.01±0.064 to 0.18±0.006 (CCR2<sup>−/−</sup>), NS]. From day 3 on, a continuous recovery in blood flow was observed in control animals during the whole observation period of 3 weeks, whereas in CCR2<sup>−/−</sup> mice the recovery was severely reduced [R/L ratio, 0.45±0.06 (control) versus 0.21±0.03 (CCR2<sup>−/−</sup>), NS]. In wild-types of the C57BL/6 strain, an approximately 80% recovery of foot blood flow was observed at postoperative day 7 in wild-type mice (R/L ratio, 0.83±0.06) as well as in CCR2<sup>−/−</sup> animals (R/L ratio, 0.84±0.08) (Figure 2C). However, the CCR2<sup>−/−</sup> mice did not further improve (R/L ratio, 0.85±0.08, 3 weeks after surgery), whereas a continuous improvement of blood flow recovery was observed in the control group during the whole observation period (R/L ratio, 0.96±0.09, 3 weeks after surgery; P<0.05). These data were confirmed by MRI measurements (0.76±0.06 in controls versus 0.62±0.1 in CCR2<sup>−/−</sup> 3 weeks after surgery; P<0.05).

**Hemoglobin Oxygen Saturation Measurements**

The pedal oxygen saturation of hemoglobin was measured on the mouse foot at an equivalent position on the operated and nonoperated limb. Immediately after ligation, as shown in Figure 3, in BALB/c mice, a similar drop of oxygen saturation was observed on the occluded side in CCR2<sup>−/−</sup> and in wild-type mice [0.13±0.01 (CCR2<sup>−/−</sup>) versus 0.10±0.009 (WT), NS]. From day 3 onward, the rate of oxygen saturation recovery was significantly higher in wild-type mice than in the CCR2<sup>−/−</sup> group. The maximal difference was observed at day 7 [0.25±0.035 (CCR2<sup>−/−</sup>) versus 0.44±0.04 (WT); P<0.05]. In contrast, in C57BL/6 mice, hemoglobin oxygen saturation deficits were almost compensated 3 days after ligation and at no time a difference between WT and CCR2<sup>−/−</sup> mice was observed (not shown).

**Active Foot Movement Score**

As a functional parameter to assess the progress of collateral artery growth we applied a score to classify the active foot movements in the BALB/c strain (Figure 4A). Starting from day 14, a significantly lower (P<0.05) active movement of the foot on the occluded side was observed in the CCR2<sup>−/−</sup> group when compared with wild-type animals.

**Gastrocnemius Atrophy**

The reduced blood supply after occlusion of the femoral artery led to ischemic damage and muscle atrophy in the gastrocnemius muscle as reflected by the decreased muscle weight (Figure 4B). In CCR2<sup>−/−</sup> mice, atrophy of the gastrocnemius muscle was more pronounced compared with wild-type animals [68.5±13.04% of left side (WT) versus 50.4% of left side (CCR2<sup>−/−</sup>); P<0.01].

**Postmortem Determination of Collateral Blood Vessel Size**

To evaluate collateral vessel size, we performed histological morphometry of collateral arteries from the adductor muscle (Figures 5A and 5B). As shown in Figure 5C, in BALB/c wild-type animals, the mean area of the collateral arteries was increased to 167±15% of the nonligated left side, whereas in CCR2<sup>−/−</sup> mice only an increase to 125±12.8% was observed. These differences reached statistical significance. Mean area of collateral arteries on the untreated left side did not differ between control and CCR2<sup>−/−</sup> mice, and postmortem angiograms showed no visible difference in the density of preexisting collateral vessels (data not shown).

**Macrophages in the Adductor Muscles**

We next examined whether monocyte invasion of the collateral wall area was intact in wild-type and CCR2-deficient mice. To do this, we counted the number of Mac-3<sup>+</sup>-positive cells in the adductor muscle after femoral artery ligation (Figure 6). The number of monocytes/macrophages in the area of interest of collateral arteries from CCR2<sup>−/−</sup> animals (1.6±0.9 cells/section) was dramatically reduced compared with the number in wild-type animals (8.77±1.97 cells/section; P<0.001). In adductor muscles of nonligated hindlimbs, monocytes/macrophages were detected only sporadically (data not shown).

**Discussion**

We have investigated the effect of chemokine receptor-2 (CCR2) gene deficiency on mononuclear cell chemotaxis and on collateral artery growth (arteriogenesis) after femoral artery occlusion. Our results from the in vitro chemotaxis assays confirmed the results of previous studies showing that CCL2 is a potent chemoattractant for monocytes, that mono-
cyte migration is dependent on CCL2 concentration, and that the migration of murine CCR2-deficient monocytes in response to CCL2 is almost completely abrogated.\textsuperscript{12} Consistent with these results from in vitro assays, in CCR2-deficient mice, we found a sharp decrease in the number of monocytes/macrophages in the adventitia and perivascular space of collateral arteries after femoral artery occlusion. This paucity of arterial macrophages correlated with a marked perturbation in collateral artery growth, leading to a reduction in active foot movement, atrophy of the calf muscle, and slower recovery of blood flow and hemoglobin oxygen saturation in the distal hindlimb.

At inflammatory sites, leukocyte accumulation is regulated by chemokines.\textsuperscript{23,24} The chemokine monocyte chemoattractant protein-1 (MCP-1, now denominated as CCL2\textsuperscript{13}) has been demonstrated to be a potent stimulator of collateral artery growth when infused in the rabbit hindlimb after femoral artery occlusion.\textsuperscript{4} Because leukocyte, and particularly monocyte, accumulation in the adventitia of growing collateral arteries is a constant feature in all animal models of arteriogenesis, including dog, pig, rat, rabbit, and mouse,\textsuperscript{25} we hypothesized that the CCL2-CCR2 signal transduction pathway may form the basis for this phenomenon. The principle of this concept was confirmed by the results of our in vitro chemotaxis assay showing the absence of migratory activity in CCR2$^{-/-}$ monocytes. Thus, as the next step, we tested CCR2 gene-deficient mice in the hindlimb ischemia model.

Active foot movement was significantly lower in CCR2-deficient mice relative to wild-type animals during the entire observation period after arterial occlusion. At the end of the study, atrophy in the gastrocnemius muscles from the occluded limbs was observed in all animals; however, the loss in muscle weight in CCR2$^{-/-}$ mice was
50%, whereas wild-type mice exhibited a weight reduction of only 23%.

Perfusion measurements using laser Doppler imaging (LDI) showed an early and significant impairment of blood flow recovery in the hindlimbs of CCR2−/− in relation to wild-type BALB/c mice. Because the impaired blood flow recovery in CCR2−/− mice was maintained at each time point during the 3-week observation, an increasing gap in recovery with respect to wild-type mice was observed.

In another functional readout, hemoglobin oxygen saturation, CCR2-deficient mice show significantly lower R/L ratios than wild-type mice beginning at day 7 after surgery. This difference is even more noteworthy considering that in our model, oxygen consumption determinations are less sensitive because measurements were performed on anesthetized mice under resting conditions. In summary, we provide a set of in vivo functional data demonstrating impaired blood flow recovery after femoral artery occlusion in CCR2−/− mice.

To test whether the impaired blood flow recovery of CCR2−/− mice was indeed due to a reduced collateral artery growth, we performed histomorphometrical analysis of collateral vessel diameters in adductor muscles. Whereas in wild-type animals a 60% increase in mean collateral vessel diameter was observed 3 weeks after arterial occlusion, in CCR2−/− mice, the mean collateral vessel diameter increased by only 17%, thus showing that collateral artery growth is defective in mice lacking the CCR2−/− receptor.

Leukocytes and monocytes in particular are important mediators of arteriogenesis. Following our hypothesis, a deficient recruitment of monocytes to the collateral wall is the main cause of the defective collateral artery growth in CCR2−/− mice. Our in vitro experiments support this hypothesis, showing that monocytes from CCR2−/− mice cannot respond to a chemoattractant CCL2 gradient. In addition, the
number of monocytes/macrophages in the perivascular space of collateral arteries after femoral artery occlusion was drastically reduced in CCR2−/− when compared with wild-type animals. These results indicate that CCR2 is required for proper monocyte recruitment to the collateral vessel wall during arteriogenesis, and CCL2 is most likely the chemoattractant trigger. However, additional cell types may be involved in the arteriogenic process. In immunohistological studies, we observed increased numbers of T-cells around growing collateral arteries.26 This is concordant with the observation that arteriogenesis is reduced in mice gene-deficient in the T-cell antigen CD4.27 Moreover, a recent study suggested that tissue macrophages are involved in arteriogenesis.28 Our histological data do not necessarily support this idea because we detected only few macrophages in the adductor muscle of the nonoperated leg with no support this idea because we detected only few macrophages via the blood stream.10

Figure 6. Quantification of macrophage numbers in the adventitia and the perivascular space of collateral arteries. A and B, Microphotographies of collateral arteries from wild-type (A) and CCR2−/− (B) animals, 4 days after femoral artery ligation were stained with monoclonal antibodies against Mac-3. Note the decreased number of monocytes/macrophages (arrows) in the adventitia of collateral vessels in the CCR2−/− group. C, Quantitative analysis of monocyte/macrophage counts in the adventitia of collateral arteries 4 days after femoral artery ligation. *P<0.01.

In a previous study, we have shown that monocytes/macrophages that have accumulated in the perivascular space of growing collateral arteries in C57BL/6 mice produce high levels of vasoactive growth factors and chemokines, including FGF-2, VEGF, and CCL2.26 The local release of these factors by macrophages suggests a multistep model for collateral vessel growth. Vascular endothelial cells in collateral arterioles are activated by shear stress to release CCL2.26,29 Mechanical forces might also induce smooth muscle cells in the vessel wall to produce CCL2.30–33 The released CCL2 would serve to lure circulating CCR2-bearing cells, most prominently monocytes, to the subendothelial regions of collateral arterioles.9,34 Activation of these cells by CCL2 and perhaps by other local molecules would lead to the release of vasoactive factors that would promote collateral artery growth. The release of chemokines also during this process would attract the other leukocyte types found in the walls of collateral arteries, such as lymphocytes and mast cells.26,35 Similarly, in angiogenesis, the CCR2 was proposed to be required for corneal and choroidal neovascularization,35,36 presumably by inducing chemotaxis of endothelial cells.37,38 Another CCR2 ligand, the chemokine CCL16, has also been shown to have strong angiogenic activity, but this effect of CCL16 is mediated through binding to CCR1.39 Nevertheless, these results highlight the critical role of a variety of chemokines and chemokine receptors in angiogenesis39 and these other interactions might account for the residual collateral artery growth in CCR2-deficient mice in our model.

We have also previously shown that flow recovery via growth of collateral arteries differ in different inbred mouse strains.40,41 C57BL/6 mice have a more developed preexisting collateral network compared with other mouse strains, which may account for the 80% recovery of foot blood flow (as measured by LDI) in this strain in the first week after arterial occlusion and the complete recovery within 3 weeks.41 In contrast, BALB/c mice do not reach complete recovery even during the 3-week observation period. Blood flow recovery at 14 and 21 days after surgery in CCR2-deficient mice on the C57BL/6 genetic background did not improve beyond the level reached at day 7. Data obtained by MRI confirmed these findings. These results suggest that CCR2 signaling plays an important role in both the early and late stages of collateral artery growth, at least in the C57BL/6 strain. Moreover, our data may also point to a partially shared pathway between arteriogenesis and atherosclerosis: CCR2−/− mice exhibit a markedly reduced ability to develop collateral arteries but are, on the other hand, almost immune against models of atherosclerosis.42,43

In conclusion, we have presented evidence to support the hypothesis that the CCL2-CCR2 signaling pathway in macrophages and perhaps endothelial cells is required for efficient collateral artery growth. Thus, our study provides an important missing piece of the puzzle emphasizing the important role of monocytes in the mechanisms involved in arteriogenesis.

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