CCR2 Deficiency Decreases Intimal Hyperplasia After Arterial Injury

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Abstract—Monocyte chemoattractant protein (MCP)-1 is upregulated in atherosclerotic plaques and in the media and intima of injured arteries. CC chemokine receptor 2 (CCR2) is the only known functional receptor for MCP-1. Mice deficient in MCP-1 or CCR2 have marked reductions in atherosclerosis. This study examines the effect of CCR2 deficiency in a murine model of femoral arterial injury. Four weeks after injury, arteries from CCR2−/− mice showed a 61.4% reduction (P<0.01) in intimal area and a 62% reduction (P<0.05) in intima/media ratio when compared with CCR2+/+ littermates. The response of CCR2−/− mice was not significantly different from that of CCR2+/+ mice. Five days after injury, the medial proliferation index, determined by bromodeoxyuridine incorporation, was decreased by 59.8% in CCR2−/− mice when compared with CCR2+/+ littermates (P<0.05). Although leukocytes rapidly adhered to the injured arterial surface, there was no significant macrophage infiltration in the arterial wall of either CCR2−/− or CCR2+/+ mice 5 and 28 days after injury. These results demonstrate that CCR2 plays an important role in mediating smooth muscle cell proliferation and intimal hyperplasia in a non-hyperlipidemic model of acute arterial injury. CCR2 may thus be an important target for inhibiting the response to acute arterial injury. (Arterioscler Thromb Vase Biol. 2002;22:554-559.)

Key Words: chemokines ■ intimal hyperplasia ■ arterial injury ■ monocyte chemoattractant protein 1 ■ smooth muscle proliferation

Monocyte chemoattractant protein (MCP)-1 is a CC chemokine expressed by macrophages, endothelial cells, and smooth muscle cells (SMCs) (reviewed by Gu et al1). MCP-1 antigen is not detected in normal vascular endothelium, but is found on the luminal endothelium of early human atherosclerotic lesions.2 MCP-1 expression is induced in medial SMCs during early atherosclerotic lesion development in carotid arteries of non-human primates fed a hypercholesterolemic diet.3 In advanced human atherosclerotic plaques, MCP-1 is expressed in macrophages and SMCs.2,4,5 MCP-1 antigen and mRNA are also induced in medial SMCs in animal models of arterial injury.6–8

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MCP-1 binds to CC chemokine receptor 2 (CCR2), a member of the family of G protein–coupled receptors with 7 transmembrane-spanning domains (reviewed by Charo9). Studies using MCP-1−/− and CCR2−/− mice have demonstrated that MCP-1 and its receptor are important mediators of macrophage accumulation and lesion formation in mouse models of atherosclerosis. CCR2−/− mice have defects in leukocyte adhesion,10 monocyte/macrophage recruitment,11 and impaired production of interferon-γ.12 When these mice are crossed with atherosclerosis-prone apolipoprotein E−/− mice and fed a Western diet, they display a marked decrease in macrophage accumulation and lesion size.13,14 Similarly, MCP-1−/− mice crossed with low density lipoprotein receptor (LDL-R)−/− mice and fed a Western diet have substantial reductions in macrophage accumulation and lesion size.15 In addition, overexpression of MCP-1 in apolipoprotein E−/− mice accelerates atherosclerosis through enhanced macrophage and lipid accumulation.16

Acute arterial injury results in proliferation and migration of SMCs and the development of intimal hyperplasia,17,18 a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic plaques.19 Although arterial injury is associated with the rapid and transient adherence of leukocytes to the luminal surface,20,21 in normolipemic animals, leukocytes do not accumulate in the arterial wall or become foam cells. Despite the lack of leukocyte accumulation within the injured wall, several studies have implicated macrophages and other leu-
kocytes in the development of intimal hyperplasia after arterial injury. Treatment of rabbits with an analogue of sialyl-Lewis \((x)\) to block selectins diminished intimal hyperplasia at 4 weeks.\(^{22}\) Monoclonal antibodies to the leukocyte integrin Mac-1 \((CD11b/CD18, \alpha M\beta 2)\) reduced intimal hyperplasia in balloon-injured or stented iliac arteries of normolipemic rabbits.\(^{20}\) In addition, animals lacking Mac-1 showed a reduction in intimal hyperplasia after balloon injury.\(^{23}\) Recently, antibodies to MCP-1 were shown to inhibit intimal hyperplasia in injured rat carotid arteries.\(^{6}\) Activated circulating leukocytes and Mac-1 expression have also been reported to be markers of restenosis risk in patients undergoing coronary angioplasty.\(^{24}\)

The above studies suggest MCP-1–mediated macrophage/leukocyte recruitment to injured arteries is important for the development of intimal hyperplasia. To further test this hypothesis, we used a model of transluminal femoral arterial injury\(^{21}\) in CCR2\(^{+/−}\) mice. Here, we report that CCR2\(^{+/−}\) mice had a 61.4% reduction in intimal area at 4 weeks and 59.8% reduction in medial DNA synthesis at 5 days compared with CCR2\(^{−/−}\) controls. This study implicates CCR2 in the early response to arterial injury, and suggests MCP-1 and CCR2 are important for the development of intimal hyperplasia.

**Methods**

**Animals**

Mice were housed at the Center for Laboratory Animal Sciences at the Mount Sinai Medical Center, New York, NY. Procedures and animal care were approved by the Institutional Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals. Littermate mice were bred from CCR2\(^{+/−}\) pairs and genotyped as previously described.\(^{13}\) The original breeding pairs were backcrossed 8 times into a C57BL/6J background. Mice were weaned at 4 weeks and fed standard rodent chow (PMI Nutrition International) and water ad libitum. At age 12 to 16 weeks, 32 mice (10 CCR2\(^{−/−}\), 12 CCR2\(^{+/−}\), 10 CCR2\(^{+/+}\)) underwent bilateral femoral artery transluminal injury by passage of a 0.25-mm diameter angioplasty guidewire, as previously described.\(^{21}\) Mice were sacrificed at the times indicated by pentobarbital overdose.

**Immunohistochemistry and Morphometry**

Femoral arteries were perfusion-fixed with 4% paraformaldehyde at 100 mm Hg as described.\(^{21}\) Three 5-μm-thick sections, cut 100 μm apart from each other, were stained with combined Masson’s trichrome elastic. The three sections obtained from each arterial segment were analyzed by using computerized morphometry (Image Pro Plus software, Media Cybernetics), and the average values were calculated. Measurements included luminal area, medial area, intimal area, vessel area, and the lengths of the internal elastic lamina and external elastic lamina. The intima-to-media (I/M) ratio was calculated as previously described.\(^{21}\) All sections were analyzed separately by two investigators blinded to the study design. Four CCR2\(^{+/−}\) and two CCR2\(^{−/−}\) arteries were not included in the analysis because of either damage to the media with severe medial necrosis or occlusive thrombosis. We have previously demonstrated that the injury technique results in complete endothelial denudation.\(^{21}\) To assure that this remained true in this study, arteries harvested before injury and immediately after injury (3 animals per group) were stained with antibody to ICAM-1. Low-profile cells with central nuclei on the luminal surface that were positively stained were identified as endothelial cells (Figure 1A).

For immunohistochemical analysis, representative sections were stained for MCP-1 (goat anti-mouse MCP-1/IE, R&D Systems, 1:300 dilution), SMC (alkaline phosphatase conjugated monoclonal anti-smooth muscle α-actin, Sigma, 1:100 dilution), macrophages (MOMA-2, rat anti-mouse macrophages/monocytes, Biosource International, 1:400 dilution; rat anti-mouse CD68 antibodies, Serotec, Inc, dilution 1:100), and endothelial cells (rat anti-mouse ICAM-1, Seikagaku America, 1:200 dilution). Negative controls were prepared by substitution of the primary antibody with an irrelevant antibody of the same isotype.

**5-Bromo-2-Deoxyuridine Incorporation**

Four days after arterial injury, mice were injected intraperitoneally with 3 doses of bromodeoxyuridine \((\text{BrdU}, \text{Boehringer Manheim}, 30 \text{ mg/kg})\) at 8-hour intervals before euthanasia.\(^{25}\) Arteries were harvested on day 5 after injury as described above and 2 sections per injured artery were immunostained with an anti-BrdU antibody (Clone BMG 6H8, Boehringer Mannheim, 1:10 dilution). The proliferation index was calculated as the percentage of the ratio between BrdU-stained nuclei over the total number of cells in the media.

**Statistical Analysis**

Because there were no significant differences in any of the morphometric values between males and females, data from all animals was combined. Numerical data are expressed as mean ± SEM. A two-tailed, unpaired t test was used to compare data among CCR2\(^{+/−}\),

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**Figure 1.** Histochemical analysis of femoral arteries from CCR2\(^{+/−}\) and CCR2\(^{−/−}\) mice. CCR2\(^{+/−}\) and CCR2\(^{−/−}\) mice were subjected to femoral arterial injury. Arterial sections were stained with (A) anti-ICAM antibodies before arterial injury of CCR2\(^{+/−}\) (magnification, ×40; arrow points to ICAM-positive endothelial cells); (B) anti-ICAM antibodies immediately after injury of CCR2\(^{+/−}\) (magnification, ×40); (C) Combined Masson’s trichrome-elastic 28 days after injury of CCR2\(^{+/−}\) (magnification, ×40); (D) Combined Masson’s trichrome-elastic 28 days after injury of CCR2\(^{−/−}\) (magnification, ×40); (E) anti-actin antibodies 5 days after injury of CCR2\(^{+/−}\) (magnification, ×40); (F) anti-BrdU antibodies 5 days after injury of CCR2\(^{+/−}\) (magnification, ×40); (G) anti-actin and anti-BrdU antibodies 5 days after injury of CCR2\(^{+/−}\) (magnification, ×10; arrows point to BrdU-positive nuclei in actin-positive cells); and (H) anti-actin antibodies 4 weeks after injury of CCR2\(^{+/−}\) (magnification, ×40). Panels B, E, and H are representative of sections from injured CCR2\(^{+/−}\) and CCR2\(^{−/−}\) animals. EEL indicates external elastic laminar; IEL, internal elastic laminar.
CCR2+/+ and CCR2−/− mice. Probability values <0.05 were considered significant.

**Results**

**CCR2 Deficiency Is Associated With Decreased Proliferative Response**

To assess the role of CCR2 in mediating intimal hyperplasia, CCR2+/+, CCR2++/−, and CCR2−/− mice underwent femoral artery injury. The severity of injury was similar in each group, as judged by complete endothelial denudation (Figure 1A and 1B) and minimal damage to the internal elastic lamina (<3% of the internal elastic lamina disrupted). Four weeks after injury, CCR2−/− mice had markedly smaller intimal lesions than their CCR2+/+ littermates (Figure 1C and 1D). The mean intimal area and the I/M ratio of CCR2−/− mice were reduced by 61.4% (P<0.01) and 62% (P<0.05), respectively, compared with CCR2+/+ mice; there was no significant difference in medial area (Figure 2). No significant differences were observed between CCR2+/+ and CCR2−/− mice.

The response to balloon arterial injury is thought to begin with early proliferation of medial SMCs (peaking at 48 to 72 hours in rodent models) and migration of these cells into the intima.17,26 Agents that inhibit intimal hyperplasia, such as heparin or antibodies to basic fibroblast growth factor,27,28 have frequently been shown to reduce this early medial SMC proliferation. To assess whether CCR2 deficiency inhibited this early medial SMC proliferation, mice were injected with BrdU 4 days after injury and their femoral arteries examined on day 5 after injury. The medial BrdU staining was confined to α-actin–positive cells (Figure 1E and 1G). CCR2−/− mice displayed a 59.8% reduction in proliferation index (19.9±3.1 versus 8.0±1.5 for CCR2+/+; P<0.01) (Figure 3).

**Expression of MCP-1 and Accumulation of Macrophages in the Injured Arterial Wall**

Five days after injury, the media from CCR2+/+ and CCR2−/− mice stained diffusely for MCP-1 antigen (Figure 4A and 4B). MCP-1–positive cells appeared to be SMCs as determined by staining for α-actin (see Figure 1E). No differences

![Figure 3. Analysis of DNA synthesis in balloon-injured mouse femoral arteries, proliferation of injured mouse femoral arteries. CCR2+/+ and CCR2−/− mice were subjected to femoral arterial balloon injury. Four days after injury, mice were injected intraperitoneally with BrdU 3 times at 8-hour intervals. Twenty-four hours after the first injection, arteries were harvested and immunostained with an anti-BrdU antibody. The proliferation index was calculated as percentage of the ratio between BrdU-stained nuclei over the total number of cells in the vessel wall. *P<0.01 compared with CCR2+/+.](image)

![Figure 4. Immunohistochemical analysis of MCP-1 and macrophages in femoral arteries. A) Anti–MCP-1 antibodies 5 days after injury of CCR2+/+ (magnification, ×40); (B) anti–MCP-1 antibodies 5 days after injury of CCR2−/− (magnification, ×40); (C) anti–MOMA-2 antibodies 1 hour after injury of CCR2+/+ (magnification, ×40); (D) anti–MOMA-2 antibodies 1 hour after injury of CCR2−/− (magnification, ×40); (E) anti–MOMA-2 antibodies 5 days after injury of CCR2+/+ (magnification, ×40); and (F) anti–MOMA-2 antibodies 5 days after injury of CCR2−/− (magnification, ×40). Inset in C shows higher power magnification (×200) of leukocytes with typical neutrophil morphology and absence of staining with anti–MOMA-2 antibodies and inset in D shows bone marrow staining with anti–MOMA-2 as a positive.](image)
in intensity or distribution of MCP-1 staining were seen in CCR2$^{-/-}$ and CCR2$^{+/+}$ mice.

Previous models of rodent balloon arterial injury have not been associated with prominent macrophage accumulation within the arterial wall.17,18 To examine the presence of macrophages in the murine femoral artery injury model, arteries were isolated 1 hour, 5 days, and 4 weeks after arterial injury. As previously described, 1 hour after injury, the denuded surface was covered with platelets and leukocytes.20,21 Virtually all leukocytes were identified by nuclear morphology as neutrophils. In addition, MOMA-2–positive macrophages were not noted (Figure 4C and 4D). Five days after injury, only rare MOMA-2–positive cells (<2/section) were present on the surface or within the arterial wall of CCR2$^{+/+}$ or CCR2$^{-/-}$ mice (Figure 4E and 4F). Similarly, at 4 weeks, virtually no MOMA-2–positive cells were present in the intima or media (not shown) of CCR2$^{+/+}$ or CCR2$^{-/-}$ mice. Identical results were obtained with an antibody to the macrophage antigen, CD68 (not shown). It should be noted that, at this time point, the intima of both CCR2$^{+/+}$ and CCR2$^{-/-}$ mice is composed almost exclusively of α-actin–positive cells (Figure 1H).

**Discussion**

MCP-1 is a potent monocyte chemoattractant that is induced as an early response to growth factors and cytokines in a variety of cells, including SMCs, endothelium, macrophages, and fibroblasts.1 MCP-1 is also rapidly induced in the arterial wall in response to injury.3,6,7 This study demonstrates that mice lacking CCR2, the receptor for MCP-1, have a markedly diminished proliferative response to transluminal arterial injury.

Leukocyte recruitment and foam cell accumulation are critical to the development of atherosclerotic plaques.19 Studies using atherosclerosis-prone apolipoprotein B$^{0,0}$, apolipoprotein E$^{0,0}$, or LDLR$^{-/-}$ mice have demonstrated that the lack of MCP-1 or CCR2 results in a marked decrease in macrophage-derived foam cell accumulation and lesion size in response to an atherogenic diet.13,15,16,29 These studies have highlighted the importance of macrophage accumulation in atherosclerosis and have provided strong evidence that MCP-1 and its receptor have a prime role in regulating macrophage accumulation in models of atherosclerosis.

In contrast to chronic atherosclerosis, models of balloon arterial injury in normolipemic animals are not associated with leukocyte or foam cell accumulation in the arterial wall.17,18 The present study, however, suggests that even in the absence of prominent accumulation in the arterial wall, leukocytes may be important in regulating SMC proliferation and migration in response to acute arterial injury. Leukocytes adhere to the luminal surface immediately after injury.20,21 Inhibition of this transient accumulation is associated with a decrease in intimal hyperplasia and SMC proliferation.20,22,23,30 MCP-1 has been implicated in promoting firm adhesion of monocytes to endothelium,31 and it has been shown to mediate monocyte transmigration: neutralizing antibodies against MCP-1 inhibited passage of monocytes across endothelial cell monolayers.32

There are several possible explanations for the marked reduction in intimal hyperplasia and SMC proliferation in the CCR2$^{-/-}$ mice, despite the lack of an obvious reduction in the number of macrophages in the vessel wall. First, the small number of macrophages present in the vessels of the wild-type mice makes it technically difficult to detect differences between the genotypes, should they exist. It should be noted that we are, of necessity, looking at selected points in time and that differences in the kinetics of macrophage trafficking into and out of the vessel wall might well be missed. It should also be noted that small numbers of infiltrating leukocytes could have profound effects on intimal proliferation. These small numbers could be reduced to near zero in CCR2$^{-/-}$ mice despite the inability to detect them with MOMA-2 staining.

CCR2 has been found to transduce a variety of intracellular signals associated with cell growth and migration, including mobilization of intracellular Ca$^{2+}$,33 activation of p42/44 mitogen-activated protein kinase,34 and activation of Janus kinase 2.35 Bone marrow cells from CCR2$^{-/-}$ mice showed increased apoptosis, suggesting a possible role for CCR2 in mediating cell survival.36 These studies raise the possibility that CCR2 might directly mediate SMC growth, migration, or survival. CCR2 has not been reported on SMCs, either in vivo or in vitro. In addition, we have previously reported that human SMCs respond to MCP-1 with the induction of tissue factor but do not contain CCR2 mRNA by Northern blot analysis or reverse-transcriptase polymerase chain reaction (RT-PCR).37 More recently, we have found that cultured mouse SMCs also do not contain CCR2 mRNA by RT-PCR and that MCP-1 induces tissue factor in SMCs from CCR2$^{-/-}$ mice (A.D. Schecter, A.B. Berman, L. Yi, C.M. Daly, K. Soejima, B.J. Rollins, I.F. Charo, M.B. Taubman unpublished data, 2002). Although it remains possible that in vivo, medial and intimal SMCs express CCR2 at levels not readily detectable, the above studies suggest that it is not likely that the effect of CCR2 deletion on medial proliferation and intimal hyperplasia is the result of direct CCR2 signaling on SMCs.

The lack of CCR2 might effect lesion development in an indirect way. CCR2$^{-/-}$ mice have been found to have higher levels of MCP-1 than wild-type mice after allogenic implantation (R. Gladue, I.F. Charo, unpublished data, 2002). As referenced in the previous paragraph, MCP-1 can activate human and mouse SMCs. The effect of MCP-1 on SMC proliferation is controversial; one study reported an inhibitory effect on rat SMC growth,38 whereas another reported a stimulatory effect.39 Given the problems in assessing local MCP-1 concentrations within the arterial wall and the lack of consensus of in vitro studies on MCP-1 effects on SMCs, the possibility that local MCP-1 upregulation is responsible for attenuating intimal hyperplasia in CCR2$^{-/-}$ mice is quite real and would be best addressed by using MCP-1 null mice. Similarly, the use of experiments using transfer of leukocytes from CCR2$^{+/+}$ to CCR2$^{-/-}$ mice and vice versa might provide a means for further assessing the role of macrophages in mediating the effects seen on intimal hyperplasia in this model.
CCR2 and its ligand MCP-1 have been shown to regulate macrophage accumulation and the immune response in a variety of disease states involving the vasculature, lung, central nervous system, and kidney. Studies in atherosclerotic-prone mice in particular have placed considerable attention on MCP-1 and CCR2 as targets to inhibit atherosclerosis. This study suggests that CCR2 may play a role in mediating the response of the arterial wall to injury even in the absence of hyperlipidemia or a cholesterol-rich diet. It should be noted that, although animal models of arterial injury are useful for studying SMC proliferation and intimal hyperplasia, they do not necessarily replicate the events that occur in the setting of chronic atherosclerosis or even restenosis after percutaneous interventions in humans. For example, this study was performed on normal vessels in nor malopimemic mice, whereas angioplasty is performed on already abnormal atherosclerotic vessels in humans with much higher levels of cholesterol. A number of chemokine and chemokine receptor inhibitors are under development for use in clinical trials. This study provides further rationale for testing these inhibitors as adjunctive therapy in acute intravascular interventions.

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