Chemokines in the Pathogenesis of Vascular Disease

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Abstract—Our increasing appreciation of the importance of inflammation in vascular disease has focused attention on the molecules that direct the migration of leukocytes from the blood stream to the vessel wall. In this review, we summarize roles of the chemokines, a family of small secreted proteins that selectively recruit monocytes, neutrophils, and lymphocytes to sites of vascular injury, inflammation, and developing atherosclerosis. Chemokines induce chemotaxis through the activation of G-protein–coupled receptors, and the receptors that a given leukocyte expresses determines the chemokines to which it will respond. Monocyte chemoattractant protein 1 (MCP-1), acting through its receptor CCR2, appears to play an early and important role in the recruitment of monocytes to atherosclerotic lesions and in the formation of intimal hyperplasia after arterial injury. Acute thrombosis is an often fatal complication of atherosclerotic plaque rupture, and recent evidence suggests that MCP-1 contributes to thrombin generation and thrombus formation by generating tissue factor. Because of their critical roles in monocyte recruitment in vascular and nonvascular diseases, MCP-1 and CCR2 have become important therapeutic targets, and efforts are underway to develop potent and specific antagonists of these and related chemokines. (Circ Res. 2004;95:858-866.)

Key Words: chemokine • CCR2 • vascular • atherosclerosis • monocyte chemoattractant protein 1

A preponderance of evidence from clinical and experimental studies supports the notion that inflammation plays an important role in a wide range of cardiovascular diseases1–4 and has focused attention on the signals that initiate cellular infiltration of vascular tissues. The chemokines are a family of potent chemotactic cytokines that regulate the trafficking of leukocytes and are rapidly upregulated at sites of vascular inflammation. Here, we review the role of monocyte chemoattractant protein 1 (MCP-1) and related chemokines in regulating the recruitment of monocyte/macrophages to the vessel wall and discuss how these chemokines contribute to the pathophysiology of vascular disease, with an emphasis on atherosclerosis.

Chemokines and Chemokine Receptors
Chemokines (chemotactic cytokines) are small heparin-binding proteins that direct the migration of circulating leukocytes to sites of inflammation or injury.5,6 There are ≈50 human chemokines, which are divided into three major families based on differences in their structure and function.

The largest family is known as the CC chemokines because the first two of the four conserved cysteine residues that are characteristic of chemokines are adjacent to each other. CC chemokines tend to attract mononuclear cells and are found at sites of chronic inflammation. The most thoroughly characterized CC chemokine is MCP-1 (also known as CCL2), a potent agonist for monocytes, memory T cells, and basophils.
MCP-1 has been implicated as a key player in the recruitment of monocytes from the blood into early atherosclerotic lesions, the development of intimal hyperplasia after angioplasty, as well as in vasculogenesis and in aspects of thrombosis. Other members of the CC family include RANTES (CCL5), macrophage inflammatory protein 1α (MIP-1α) (CCL3), and MIP-1β (CCL4).

The CXC family of chemokines, of which IL-8 (CXCL8) is the prototypical member, attract polymorphonuclear leukocytes and have been implicated in acute pulmonary inflammation. IL-8 also activates monocytes and may direct their recruitment to vascular lesions. CXCL16 is a single amino acid residue between the first two canonical tumor necrosis factor-binding sites. Its full-length, soluble chemokine domain fused to a mucin-like stalk and a membrane domain. Thus, unlike other soluble chemokines, it is a type 1 transmembrane protein. In its full-length, membrane-bound protein, FK is an efficient cell-adhesion receptor that can arrest cells under physiological flow conditions. FK can be cleaved from the cell membrane by tumor necrosis factor–α–converting enzyme and by the metalloprotease ADAM-10 to release a soluble protein. In this soluble form, FK is a potent chemoattractant for monocytes, T cells, and natural killer (NK) cells. Thus, depending on whether it exists as an immobilized protein or a soluble protein, FK can function as a cell-adhesion receptor or as a chemoattractant. FK is expressed in atherosclerotic lesions and has several potential roles in atherogenesis. CXCL16 also has a soluble domain linked to a mucin stalk. CXCL16 is expressed on macrophages and dendritic cells, and, in particular relevance to cardiovascular disease, it has been reported to scavenge oxidized lipids.

Chemokines exert their cellular effects by activating seven-transmembrane–domain G-protein–coupled receptors. Whether a leukocyte responds to a particular chemokine is determined by its complement of chemokine receptors. Chemokine binding activates a signal transduction cascade that activates phosphatidylinositol-3 kinase, increases levels of inositol triphosphate and intracellular calcium, activates Rho protein B, and mitogen-activated protein kinases, and eventually leads to a localized infiltration of monocyte/macrophages to atheroma. Thus, overexpression of MCP-1 in specific tissues causes a localized infiltration of monocyte/macrophages. In bone marrow transplantation studies, overexpression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis. Deletion of MCP-1 in LDL receptor-null mice attenuated the progression of dietary-induced atherosclerosis. Similar results were reported in MCP-1–null mice expressing human apolipoprotein B.

CCR2 is the only established functional receptor for MCP-1 on hematopoietic cells, and its deletion in apoE-deficient mice afforded significant protection from both macrophage accumulation and atherosclerotic lesion formation in response to a high-fat diet (Figure 2). Similar studies in mice fed a regular chow diet showed that CCR2+/− mice were more resistant to the development of atherosclerosis than wild-type mice. In contrast, mice deficient in CCR5, which is activated by MIP-1α and RANTES but not by MCP-1, were not protected against atherosclerosis. These studies provide strong evidence that activation of CCR2, presumably by MCP-1, contributes to foam cell formation, one of the earliest manifestations of atherosclerosis. It should be noted that CCR2+/− and MCP-1−/− mice have distinct immunologic properties and respond differently to fem-
oral arterial injury. In addition, MCP-3 and MCP-5 activate CCR2. Therefore, it is possible that the results obtained with the CCR2−/− mice may not be solely mediated by MCP-1. Although most work has focused on MCP-1, other chemokines may also play a role. For example, MetRANTES, a chemokine receptor antagonist that blocks CCR1 and CCR5, significantly reduced lesion progression in atherosclerotic mice.

The importance of inflammation in the later stages of atherosclerosis, especially plaque rupture, has been emphasized in several recent reviews. The traditional view that lesion growth is driven by constant division and migration of smooth muscle cells is being supplanted by models in which cytokines and proteases within lesional inflammatory cells, particularly macrophages, contribute directly to plaque growth and rupture. Underscoring the potential importance of inflammation in atherogenesis is the recent finding that plasma levels of the acute-phase reactant C-reactive protein are a stronger predictor of cardiovascular events than LDL-cholesterol levels. The extent to which chemokines such as MCP-1 contribute to the retention and activation of macrophages in advanced lesions is unclear. However, as an early response gene that is robustly induced in macrophages and vascular wall cells by tumor necrosis factor-α, platelet-derived growth factor, and thrombin, MCP-1 is almost surely present in significant amounts. Through its ability to activate tissue factor, MCP-1 might also contribute to the thrombotic aspects of advanced atherosclerotic lesions. The subsequent elaboration of thrombin would provide potent positive feedback for the local synthesis of additional MCP-1.

Arterial Injury

Chemokines and their receptors have also been implicated in the response of the arterial wall to injury. Animal models of arterial injury are characterized by the development of intimal hyperplasia caused by migration and replication of smooth muscle cells. Intimal hyperplasia is an important component of the atherosclerotic plaque and is thought to be critical to the development of restenosis after coronary artery angioplasty and stenting. Smooth muscle cell chemoattractants and mitogens are key mediators of intimal hyperplasia. The source of these molecules is likely to include the major cells of the arterial wall, such as endothelial cells, smooth muscle cells, and adventitial fibroblasts, as well as the circulation. In addition, leukocytes accumulating at the surface or migrating into injured or atherosclerotic vessels are rich in growth factors and cytokines that can activate smooth muscle cells.

MCP-1 mRNA and antigen are rapidly induced in the arterial media in a variety of normolipemic and hyperlipemic models of arterial injury. The induction of MCP-1 does not always correlate with macrophage accumulation. For example, in balloon and wire injury models of normal rodent arteries, few macrophages are found in the arterial wall. In hypercholesterolemic animals such as cholesterol-fed pigs and rabbits or ApoE−/− mice, however, arterial injury elicits abundant macrophage infiltration. Interestingly, macrophages accumulate abundantly in normal rodent arteries after stenting. Thus, although MCP-1 may recruit macrophages to sites of arterial injury, it might not be sufficient to assure the development of macrophage-rich lesions.

The response to arterial injury appears to be mediated by MCP-1 and CCR2. Antibodies to MCP-1 attenuate intimal hyperplasia in a rat model of carotid artery injury. In addition, a mutant form of MCP-1 with “dominant negative” properties inhibited intimal hyperplasia in a primate model of femoral arterial injury. A CCR2 blocking antibody provided significant protection against in-stent restenosis; the protection was as good as that of an antibody against CD18, which blocks leukocyte adhesion. We recently examined the effects of CCR2 and MCP-1 gene deletions on the development of intimal hyperplasia in mouse models of wire-induced femoral arterial injury. Four weeks after injury, arteries from CCR2−/− mice had substantially smaller (∼62%) intimal areas and lower intima/media ratios than CCR2+/- littermates. Five days after injury, the medial proliferation index was decreased by ∼60% in CCR2−/− mice. Interestingly, the effect of MCP-1 deletion was less pronounced, showing ∼30% reductions in intimal area and intima/media ratio and no change in the medial proliferation index. These data suggest that MCP-1 and CCR2 deficiencies may have different effects on the arterial wall and raise the possibility that MCP-1 may affect smooth muscle cell migration directly or indirectly. Targeting CCR2 may prove more effective than targeting MCP-1.

As noted, in many animal models used to examine the effect of MCP-1 and CCR2 on intimal hyperplasia, macrophages do not accumulate within the arterial wall. A number of studies, however, have raised the possibility that MCP-1 plays a direct role in activating smooth muscle cells. MCP-1 induced tissue factor, the initiator of coagulation and a critical mediator of arterial thrombosis, in human and rat smooth muscle cells. This induction was dependent on activation of protein kinase C and mobilization of Ca2+.

Figure 2. Impaired monocyte recruitment in CCR2−/− mice. In response to a high-fat, Western-type diet, ApoE−/− mice rapidly recruit blood monocytes into the subendothelial space, where they accumulate lipids and become foam cells. Macrophage accumulation and foam cell formation are markedly reduced in CCR2−/− mice. Reproduced from Boring et al. with permission from Nature.
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in rat smooth muscle cells. Several studies have also suggested that MCP-1 induces smooth muscle cell proliferation, whereas others have suggested that it is inhibitory or has no effect. It is therefore possible that the benefits of inhibiting MCP-1 on the development of intimal hyperplasia are attributable to direct effects on smooth muscle cells rather than to the inhibition of macrophage accumulation. The induction of tissue factor by MCP-1 was also intriguing because CCR2 was not detected in the human smooth muscle cells studied, even by RT-PCR, consistent with CCR2-independent responses to MCP-1 and perhaps with the presence of a second receptor. In support of this possibility, MCP-1 induced tissue factor in CCR2−/− mice.

Smooth muscle cells respond to a number of chemokines other than MCP-1 and possess a variety of chemokine receptors, including CCR3, CCR5, CCR8, and CXCR4. CCR5 is present on human aortic smooth muscle cells, and its ligands MIP-1α and MIP-1β mobilize intracellular calcium and induce tissue factor. Tissue factor-mediated thrombosis is widely regarded as a key factor in the pathogenesis of acute coronary syndromes, such as unstable angina, myocardial infarction, and sudden death. The induction of tissue factor by MIP-1β can be blocked by inhibitors of intracellular calcium mobilization, protein kinase C, and mitogen and p42/44 mitogen-activated protein kinase. Thus, in smooth muscle cells, CCR5 may transduce signaling pathways known to have protein manifestations and to be associated with smooth muscle cell activation. Recently, Met-RANTES, an inhibitor of CCR5 and CCR1, was shown to inhibit intimal hyperplasia after wire arterial injury in Apoe−/− mice. Role of CCR5 in vasculogenesis is not clear. However, these data raise the interesting possibility that MCP-1 acts through a receptor other than CCR2. As noted, there is evidence that vascular smooth muscle cells, which do not express CCR2, express tissue factor in response to MCP-1.

Few studies in humans have addressed the role of MCP-1/CCR2 in atherosclerosis. However, subjects with hypercholesterolemia tended to express higher levels of CCR2 on monocytes, and CCR2 expression correlated positively with plasma LDL cholesterol levels and inversely with plasma high-density lipoprotein levels. Furthermore, in postmenopausal women receiving estrogen replacement therapy, which raised high-density lipoprotein cholesterol levels and lowered LDL-cholesterol levels, CCR2 expression on monocytes was reduced. These findings suggested that high cholesterol levels increase the sensitivity of monocyte/macrophages to MCP-1, thereby increasing their movement into early atherosclerotic lesions. The subsequent downregulation of CCR2, as monocytes differentiate into macrophages, might serve to retain the cells within the lesion.

A relatively common genetic variant in CCR2 has been identified in which valine at position 64 is changed to isoleucine. Although in vitro studies have failed to reveal significant abnormalities in receptor signaling or MCP-1 binding, the 64I mutation is associated with reduced risk of HIV/AIDS, pulmonary sarcoidosis, and acute renal transplant rejection. Information on the 64I mutation and the risk for cardiovascular disease is limited and inconsistent. In healthy subjects with a family history of heart disease, both
men and women with two copies of the Ile allele were less likely to have significant coronary artery calcification than those with two copies of the Val allele, suggesting a protective role for the polymorphism.\textsuperscript{76} In another study, the 64I mutation was associated with myocardial infarction or reduced left ventricular function in patients aged 65 years or younger, suggesting that the mutation may be deleterious.\textsuperscript{77} However, the 64I allele was not associated with coronary artery atherosclerosis, which may indicate that the effects of CCR2 are more related to plaque stability than to plaque size as measured by angiography.

Evidence for a role for MCP-1 in ischemic heart disease comes from a study of plasma MCP-1 levels in healthy volunteers and patients with acute coronary syndrome.\textsuperscript{78} Although the MCP-1 levels overlapped considerably between the two groups, acute coronary syndrome patients with the highest levels of MCP-1 (the top quartile, corresponding to the 90th percentile in the healthy normal population) had a significantly increased risk of death or myocardial infarction over 10 months of follow-up. Although high MCP-1 levels were associated with other traditional risk factors, high levels correlated with poor outcomes, even after adjustment for plasma levels of C-reactive protein. It remains to be established whether these high MCP-1 levels contribute to exacerbation of the disease through continued monocyte/macrophage recruitment and activation or simply reflect the presence of macrophages and other MCP-1-producing cells in established lesions.

Taken together, these studies suggest possible roles for CCR2 and MCP-1 in determining risk for atherosclerosis, myocardial infarction, and left ventricular function, but larger prospective studies will be needed to fully address this important question.

**Other Chemokines Implicated in Vascular Disease**

At least three other chemokines—IL-8, FK, and CXCL16—have been linked to the development of early atherosclerotic lesions. Perhaps the best characterized of the neutrophil chemoattractants, IL-8 is also a monocyte agonist and is present in macrophage-rich atherosclerotic plaques.\textsuperscript{9,10} In irradiated LDL receptor-deficient mice that were fed an atherogenic diet after receiving bone marrow transplants of cells that either lacked or expressed the murine IL-8 receptor (the homolog of human CXCR2), mice lacking the IL-8 receptor had less accumulation of macrophages and smaller lesions.\textsuperscript{79} IL-8 also triggers the arrest of monocytes under flow conditions.\textsuperscript{8,9} This effect did not correlate with chemotaxis or the induction of an intracellular calcium flux and may therefore be mediated by novel signaling pathways. In addition, IL-8-mediated capture of monocytes occurred under physiological conditions and was VLA-4-dependent.\textsuperscript{8} These data provide evidence for a potential role of IL-8 in monocyte capture and the initiation of atherosclerosis.

FK and CXCL16 are novel chemokines composed of a chemokine-like domain fused to a mucin stalk. Both have transmembrane domains and exist as full-length immobilized proteins and, after cleavage at a site(s) near the plasma membrane, as a soluble proteins. FK is the only chemokine with three amino acids between the first two cysteine residues and is thus designated CX3CL1 (Figure 3). Full-length transmembrane FK is an efficient cell-adhesion molecule that can capture cells expressing its cognate receptor (CX3CR1) under physiologically relevant flow conditions.\textsuperscript{13,14} In humans, CX3CR1 is expressed on monocytes, NK cells, and CD3\(^+\) T cells. CX3CR1 may be preferentially expressed on CD14\(^+\)CCR2\(^+\)CD16\(^-\) monocyte/macrophages, which are long-lived resident cells.\textsuperscript{80,81} The notion that CCR2\(^+\) monocytes are “inflammatory” and short-lived, whereas CX3CR1\(^+\) monocytes are destined to become resident cells, is intriguing and has potentially important therapeutic implications. Soluble FK also activates cells via CX3CR1, one result of which is the induction of integrin-dependent binding to ICAM-1 and VCAM-1.\textsuperscript{182,83} These two forms of FK-mediated cell adhesion may work in concert to capture CX3CR1 positive cells.

FK is present in human atherosclerotic lesions,\textsuperscript{84} and McDermott et al have found that the presence of a polymorphism in CX3CR1 (V280M) correlated with protection from coronary artery disease.\textsuperscript{85,86} Haskell et al have made a CX3CR1 knockout mouse and demonstrated a role for FK in organ transplantation. In a heterotopic cardiac allograft model using donor hearts that were mismatched for both MHC class I and class II, CX3CR1\(^{−/−}\) recipients rejected the grafts more slowly than wild-type recipients, particularly in the presence of subtherapeutic levels of cyclosporin A.\textsuperscript{87} Recently, Lesnik et al demonstrated expression of FK in atherosclerotic lesions in mice (Figure 4) and found that, like CCR2\(^{−/−}\) mice, apoE-deficient CX3CR1\(^{−/−}\) mice were protected against diet-induced atherosclerosis. Similar results were reported by Combadie`r e et al.\textsuperscript{89} Further investigation of the V280M polymorphism in CX3CR1 showed that although this single amino acid change had little effect on the binding of soluble FK, it almost completely prevented the
CXCL16, a chemokine domain fused to a mucin stalk, was independently cloned as a scavenger receptor for phosphatidylyserine and oxidized lipids and as a chemokine. CXCL16 is expressed on macrophages and dendritic cells and, at lower levels, on T cells. Like FK, CXCL16 can capture cells bearing its cognate receptor, CXCR6, and is cleaved by the metalloprotease ADAM-10 to release a soluble form that has chemotactic activity. The receptor for CXCL16, CXCR6, is expressed on subsets of T cells and NKT cells.

The roles of CXCL16 in vivo are not well understood, but recent work has shown that it is present in both human and murine atherosclerotic lesions and is upregulated by interferon γ. CXCL16 may thus contribute to atherosclerosis by capturing CXCR6+ cells and by scavenging oxidized lipids. CXCL16 may also promote interactions between dendritic cells and CXCR6+ T cells, particularly Th1-polarized T cells, which express high levels of CXCR6. Bone marrow plasma cells express CXCR6, and CXCL16 is expressed constitutively by bone marrow stromal cells, suggesting a function in plasma cell development or localization. CXCL16 is also found in the thymus, suggesting a possible role in the development of T cells and/or CXCR6+ NKT cells. A more complete understanding of the significance of CXCL16/CXCR6 in cardiovascular disease and immune cell development awaits further work with mice deficient in either the chemokine or its receptor.

**Therapeutics: Where Do We Stand Today?**

Chemokines are important therapeutic targets, and most of the initial efforts in this area have been directed toward the development of chemokine receptor antagonists. Currently, the efficacy of CCR1 antagonists for the treatment of rheumatoid arthritis and multiple sclerosis is being evaluated in phase I or phase II clinical trials. A monoclonal antibody that blocks the binding of MCP-1 to CCR2 is being used in phase II trials for rheumatoid arthritis, and CCR5 antagonists that block HIV entry into cells are being used in advanced clinical trials as adjuvant treatments for AIDS. Small-molecule inhibitors of CX3CR1 are being used in phase I/II trials for psoriasis and rheumatoid arthritis, and CXCR4 antagonists are being evaluated for efficacy in rheumatoid arthritis and cancer. Chemokine receptors have thus proven to be tractable targets, and early efforts have largely focused on traditional inflammation-mediated diseases. Human clinical trials of chemokine antagonists for vascular indications have been more difficult to organize.

Although studies in mice and rats established the importance of chemokines, particularly MCP-1, in the development of atherosclerosis, their roles in more advanced stages of the disease are less clear. Specifically, it is unclear whether chemokine or chemokine receptor antagonists could either halt the progression or cause regression of complicated lesions. In addition, it remains to be determined whether studies performed in rodents can be extrapolated to human diseases. A central issue in the development of chemokine antagonists for atherosclerosis is the lack of good surrogate markers of disease. Simply put, if we had a safe and potent CCR2 or MCP-1 antagonist, what would we measure clinically to ascertain the correct dose or to demonstrate disease reduction? Short of large-scale studies with hard end points such as myocardial infarction or death, it is difficult to envision the use of chemokine antagonists to treat cardiovascular disease until surrogate markers are developed and validated. Advanced imaging techniques seem the most promising candidates, especially if it can be shown that changes in peripheral vessels, such as the carotid artery, correlate well with changes in the coronary arteries. Other possible biomarkers include plasma levels of C-reactive protein or MCP-1, if they could be validated as measures of vascular inflammation or plaque stability. Similar considerations would apply to measures of endothelial cell dysfunction, such as nitric oxide production. It will be interesting to see to what extent statins exert their anti-inflammatory actions by inhibiting the production of MCP-1 and other chemokines.

Restenosis may be a more apt target for chemokine antagonists than atherosclerosis. The extent of restenosis can be quantified at the time of the procedure, and symptomatic patients will likely undergo cardiac catheterization. Unlike atherosclerosis, which is a slow insidious process, restenosis occurs relatively quickly after the procedure. As noted, there...
is reason to believe that MCP-1-dependent recruitment of macrophages is important in restenosis. The introduction of drug-eluting stents dramatically reduced the incidence of restenosis in recent studies and it remains to be seen whether additional therapeutic modalities, such as chemokine receptor antagonists, will be needed.

In summary, the past few years have witnessed a rapid increase in our understanding of the role of chemokines in the recruitment of leukocytes to sites of inflammation and the importance of inflammation in the pathogenesis of atherosclerosis and other vascular diseases. Most studies have focused on the formation of early lesions. Determining whether chemokine antagonists can stabilize established atherosclerotic plaques or cause them to regress in experimental animals will likely be required before planning of human clinical trials for such antagonists. Human trials will also likely require the validation of novel imaging techniques or biomarkers to quantify lesion size or stability. Given the breadth of vascular diseases in which chemokines have been shown to play important roles, and given the success in developing potent chemokine therapeutics, it seems likely that this area will remain a focus for basic and clinical scientists for some time to come.

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