Decreased Atherosclerotic Lesion Formation in CX3CR1/Apolipoprotein E Double Knockout Mice

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Background—Fractalkine (CX3CL1), a CX3C chemokine, is expressed in the vessel wall and mediates the firm adhesion and chemotaxis of leukocytes expressing its receptor, CX3CR1. A polymorphism in the CX3CR1 gene is associated with low CX3CR1 expression and reduced risk of acute coronary disease in humans.

Methods and Results—We generated CX3CR1-deficient mice (CX3CR1<sup>−/−</sup>) by targeted gene disruption and crossed them with the proatherogenic apolipoprotein E-deficient mice (apoE<sup>−/−</sup>). Here we show that the extent of lipid-stained lesions in the thoracic aorta was reduced by 59% in CX3CR1<sup>−/−</sup>/apoE<sup>−/−</sup> double knockout mice compared with their CX3CR1<sup>+/+</sup>/apoE<sup>−/−</sup> littermates. The development of atherosclerosis in the aortic sinus was also markedly altered in the double knockout mice, with 50% reduction in macrophage accumulation. Although lesions of CX3CR1<sup>−/−</sup> mice were smaller in size, they retained a substantial accumulation of smooth muscle cells and collagen, features consistent with a stable plaque phenotype. Finally, CX3CR1<sup>+/−</sup>/apoE<sup>−/−</sup> mice showed the same reduction in atherosclerosis as the CX3CR1<sup>−/−</sup>/apoE<sup>−/−</sup> mice.

Conclusions—The CX3CR1-CX3CL1 pathway seems to play a direct and critical role in monocyte recruitment and atherosclerotic lesion development in a mouse model of human atherosclerosis. (Circulation. 2003;107:r27-r34.)

Key Words: atherosclerosis ■ leukocytes ■ receptors ■ inflammation

Atherosclerosis is a chronic inflammatory disease of the arterial wall that carries a high morbidity and mortality. One of the first events in atherogenesis is endothelial activation in an inflammatory context (oxidized lipoproteins and others), leading to close interactions with the circulating leukocytes that adhere to the inflamed endothelium and then migrate and accumulate in the intima.1,2 Trafficking of blood leukocytes to peripheral tissues is a complex physiological process under the tight control of a number of inflammatory-driven mechanisms that sequentially include selectin-mediated rolling, chemokine receptor activation leading to integrin-mediated adherence, and ultimately transendothelial migration.3 Further interactions between the recruited inflammatory cells, the extracellular matrix, and the resident vascular cells will ultimately lead to the formation of an atherosclerotic plaque.1,3,4

Chemokines are small, structurally related, disulfide-linked polypeptides that are potent mediators of cell adhesion and migration through their interactions with a family of G-protein–coupled receptors expressed on leukocytes.5 Interaction between the well known CC chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2) and its receptor CCR2 has been shown to play a critical role in monocyte recruitment into the arterial wall and the development of atherosclerosis in mice.6,7 and genetic evidence exists linking CCR2 polymorphism with coronary atherosclerosis in humans.8,9 Similarly, a role for the chemokine receptor CXCR2, which binds to a number of C-X-C chemokines, including interleukin (IL)-8, has been suggested to enhance monocyte recruitment and disease progression.10 Fractalkine (CX3CL1) is the unique member of the CX3C subfamily and is expressed in both a soluble and membrane-bound form on the surface of inflamed endothelium, which confers to it special properties among the other members of the chemokine family.11 Interestingly, whereas soluble CX3CL1 was reported to recruit lymphocytes and monocytes,11,12 immobilized forms of CX3CL1 and CX3CL1-expressing human umbilical vein endothelial cells have been shown to directly mediate the rapid capture and firm adhesion of leukocytes expressing its receptor CX3CR1 under physiological flow.
conditions. In contrast to cell adhesion mediated by the chemokine KC (murine CXCL1) through its receptor CXCR2, the CX3CL1-induced firm adhesion is uniquely mediated by direct binding of the chemokine to CX3CR1 and does not require the upregulation and activation of integrins, suggesting that CX3CL1 and CX3CR1 mediate a novel pathway for leukocyte trafficking. Apart from a role in postischemic brain injury and protection against cardiac allograft rejection in the presence of cyclosporin, mice deficient in either CX3CL1 or its receptor CX3CR1, bred in a sterile environment, were phenotypically indistinguishable from wild type mice when subjected to a number of inflammatory stimuli.

Recent studies suggested a potential role for CX3CL1/ CX3CR1 in atherosclerosis. CX3CL1 has been shown to be expressed on inflamed endothelium and in macrophages of atherosclerotic lesions. In addition, recent studies have reported a significant association between coronary vascular endothelial dysfunction in humans and a polymorphism in the CX3CR1 gene that affects receptor expression and ligand-binding affinity. Most important is the strong association established data, in the light of the available data reviewed above, we planned to assess the direct role of CX3CR1 expression in atherosclerosis. We crossed CX3CR1 knockout mice with atherosclerosis-prone apolipoprotein E (apoE)-deficient mice and examined the effects of CX3CR1 deficiency on the development and composition of atherosclerotic lesions. Our studies identified a critical role for CX3CR1 in macrophage accumulation in atherosclerotic lesions of apoE knockout mice.

Methods

Generation of CX3CR1/apoE Double Knockout Mice

The cloning of the CX3CR1 gene from a 129/Sv mouse genomic library has been described previously. A 6 kb KpnI-MunI fragment was subcloned into the multicloning site of the Bluescript plasmid (Stratagene) and excised with NotI-XhoI for transfer into the pPNT plasmid. A 2.2 kb KpnI-EcoRI fragment was directly subcloned into plasmid pPNT. Both fragments were cloned on both sides of a neomycin resistance cassette (~1.8 kb) and reconstitute a CX3CR1 gene that lacks a 0.6 kb MunI-KpnI fragment containing the CX3CR1 start codon. The construct was linearized with EcoRI and 25 μg DNA was electroporated into 10 million mouse embryonic stem cells of the R1.211 cell line. Clones resistant to G418 (350 μg/mL) and to ganciclovir (2 μmol/L) were selected and expanded on feeder layer cells in 24 well plates. Two homologous recombinants were identified by Southern blot hybridization of genomic DNA digested with EcoRI and hybridized with a probe outside of the gene; HSV TK, and Herpes simplex virus thymidine kinase. Dotted lines delineate potential regions of homologous recombination. Also indicated is the location of the probe used for ES cell lines and mice genomic screening. B, Southern blot analysis of genomic DNA from mice obtained from F2 offspring of heterozygous (+/-) parents. Targeted allele (~12 kb) is distinguished from the wild-type allele (~10 kb) using the probe indicated in A. C, RT-PCR analysis of total RNA from spleen. HPRT amplification ensured equal retrotranscription of the RNA.
to produce CX3CR1+/−apoE−/−. These mice were then intercrossed to generate homozygous apoE−/− mice bearing combination of CX3CR1+/+; +/−; and −/−. Mice were generated at the National Institutes of Health (NIH) and then transferred to the Nouvelle Animalerie Commune of Pitié-Salpêtrière under specific pathogen-free conditions. We chose to feed the mice with regular rodent chow diet to avoid the induction of a very severe hypercholesterolemia, which has its own consequences on the immune system23 and is not typical of the human situation. All experiments were done according to NIH and Pitié-Salpêtrière animal experimental ethics committee guidelines.

Genotyping

A genomic polymerase chain reaction (PCR) assay was performed using the primers set (WTP:GGCCTGGTTATTTGGCCGACAT and ASP:TGGGGTGACGCCACTAAGAT) to amplify the wild type allele and the primers set (KOP:GACCGCTTCCTGGTGCTTTA and ASP) for the mutated allele. The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Reverse Transcription-PCR Amplification of CX3CR1

Cellular RNA was prepared from splenocytes using the RNeasy kit (QIAGEN). After reverse transcription, cDNA were amplified using 2 sets of primers, CX3CR1 ORF and neomycin resistance cassette. Amplification reactions were performed in a solution (20 µL) containing 20 pmol each of the appropriate primer, ~1 µL of cDNA, 2 µL 10× buffer, 0.2 µmol/L dNTP, and 1 U of Taq polymerase (Boehringer Mannheim). Hypoxanthine phosphoribosyltransferase (HPRT) was used to normalize the amount of mRNA between different cell types.

Chemokine Binding Studies

Binding experiments were carried out using 0.2 nmol/L 125I-CX3CL1 and CCL5 (specific activity = 2250 Ci/mmol protein) purchased from Amersham. One million splenocytes were incubated in duplicate with 125I-labeled chemokine in the presence or absence of a 1000 fold excess of unlabeled recombinant human chemokine (PeproTech) in binding medium (HBSS with 1 mg/mL bovine serum albumin, 1 mmol/L CaCl2, and 1 mmol/L MgCl2). Fluorescence in the bottom well was measured using a Packard Fusion microplate analyzer (PerkinElmer Life Sciences Inc). Results are expressed as a chemotactic index representing the number of cells migrating in response to CX3CL1 relative to the number of cells migrating in absence of chemokine. All conditions were tested in triplicate. Medium-induced migration represents 5% to 10% of cells that were subtracted for each assay.

Cytokine Assays

Mouse splenocytes (1 million/mL) were cultured in the presence of concanavalin A (1 µg/mL) for 2 days. Murine cytokines (IL-2, IL-4, IL-5, interferon-γ, and tumor necrosis factor-α) were measured in supernatants using the mouse Th1/Th2 cytokine cytometric bead array kit according to the manufacturer’s instructions (BD Biosciences).

Assessment of the Extent of Atherosclerosis in Aortas and Aortic Sinus

To examine the effect of CX3CR1 deficiency on the development of advanced atherosclerotic lesions, CX3CR1+/−/apoE−/− mice and their CX3CR1+/−/apoE−/− littermates were studied at 25 weeks of age. Plasma cholesterol and high-density lipoprotein were measured with a commercially available cholesterol kit (Sigma). Morphometric and immunohistochemical studies were performed in the aortic sinus and the thoracic aorta (spanning from the brachiocephalic artery to the renal arteries and including the first 3 mm of the brachiocephalic artery) as previously described.24 A goat polyclonal antibody against mouse CX3CL1 (Santa Cruz) was used at a dilution of 1:30 to assess CX3CL1 expression. Irrelevant immunoglobulins were used as negative controls. At least 4 sections per animal were analyzed for each immunostaining.

Statistical Analysis

Data are expressed as mean±SEM. Statistical significance was determined by use of ANOVA. A value of P<0.05 was considered to be statistically significant.

Results

Specific Decrease in CX3CL1-Binding and Chemotactic Activities in Monocytes of CX3CR1+/apoE Double Knockout Mice

To selectively inactivate CX3CR1, a ~600 bp MunI-KpnI fragment of the CX3CR1 gene was replaced with a neomycin resistance cassette by homologous recombination in 129/Sv embryonic stem (ES) cells (Figure 1A). The deleted fragment contains 220 bp of untranslated region, the start codon, and 382 bp of open reading frame, and its absence should preclude any expression of CX3CR1. ES clones were selected for antibiotic resistance, screened for homologous recombination by Southern blot, and injected into blastocysts to generate chimeric mice. The chimeric mice were then mated with C57Bl/6 mice. Heterozygous offspring were then mated to produce litters of each genotypes (+/+; +/+−; and −−/−). Genotypes were determined by Southern blot analysis using a 5′ probe (Figure 1A) external to the construct (Figure 1B). Transcripts coding for CX3CR1 were not amplified in CX3CR1+/− mice, whereas they were found in wild type animals (Figure 1C). Reciprocally, neomycin-resistance cassette was amplified in CX3CR1+/− mice, but not in wild type mice. Expectedly, both genes could be amplified in heterozygous mice. CX3CR1+/− were viable and fertile and exhibited no overt developmental, morphological, or behavioral abnormalities as described previously.16,17 To investigate potential alternative receptors for CX3CL1 in the absence of CX3CR1, we measured binding (Figure 2A) and chemotactic (Figure 2B) properties of splenocytes in response to CX3CL1. CCL5 was tested as a positive control. Splenocytes from CX3CR1+/− mice bound efficiently CX3CL1 and CCL5, whereas only CCL5 specifically bound to splenocytes from CX3CR1+/− mice. No binding of C3XCL1 could be detected. Furthermore, CX3CR1+/− splenocytes migrated in response to both ligands, whereas CX3CR1+/− cells were sensitive to CCL5, but not to CX3CL1. These data indicated that CX3CR1 is not expressed in CX3CR1+/− mice and suggested that there is no other functional receptor for CX3CL1 in these cells.

Because the nature of the immune response may affect lesion development,25 we examined T-cell cytokine profiles
in cultured splenocytes from both groups. As expected from the pattern of CX3CR1 expression, CX3CR1 deficiency did not affect concanavalin A-induced T cell production of interferon-γ, IL-4, IL-2, IL-5, or tumor necrosis factor-α (data not shown).

Fractalkine Is Expressed in Murine Atherosclerotic Plaques

Although CX3CL1 has been shown to be expressed in human atherosclerotic plaques, no study has examined its expression in experimental models of atherosclerosis. Therefore, using immunohistochemical techniques, we looked at the expression of CX3CL1 in atherosclerotic plaques of the apoE knockout mice used in this study. As shown in Figure 3, immunoreactive CX3CL1 was detected in leukocytes infiltrating the early fatty streaks, in acellular areas surrounding macrophages in the lipid core of advanced atherosclerotic plaques, in some luminal endothelial cells, and occasionally in some medial smooth muscle cells.

Effects of CX3CR1 Deficiency on Atherosclerotic Lesion Size and Composition

Animal weights, plasma cholesterol levels, and leukocyte counts were similar in the 2 groups (Table). We first examined the thoracic aortas for differences in lipid staining. We found a marked reduction in the extent of surface area stained with Oil Red O in CX3CR1−/− mice compared with controls (Figure 4). Quantitative computer-assisted image analysis showed an important 55% decrease in the percentage of aortic surface area positively stained with Oil Red O (3.6±0.05% versus 7.9±0.9% in CX3CR1 deficient and control mice, respectively, *P<0.0003) (Figure 4). The effect of CX3CR1 deficiency on aortic lesion size was observed in both males and females (Figure 4). We also assessed the effect of CX3CR1 deficiency on lesion development in the aortic sinus, a predilection site for accelerated plaque formation. Again, we found a substantial decrease in atherosclerotic lesion size of both males and females deficient for CX3CR1 compared with controls (185 393±24 878 μm², n=11 versus 353 707±18 984 μm², n=9, respectively; *P<0.0001) (Figure 5). Therefore, our results point to a critical role of CX3CR1-mediated processes in the development and progression of atherosclerosis. These effects are likely to result from the modulation of monocyte recruitment into the lesions, as CX3CR1-deficient mice exhibited a marked 50% reduction in sinus surface area with monocyte-macrophage markers (MOMA)-2–positive macrophages (50 606±6908 μm² versus 101 768±8600 μm², respectively; *P=0.0003). CD3-positive T-lymphocyte staining was detected within the le-
sions of both groups of mice and showed similar distribution (data not shown).

We then determined the smooth muscle cell and collagen contents of the plaques (n=11 for CX3CR1<sup>+/+</sup>/apoE<sup>−/−</sup>; n=9 for CX3CR1<sup>−/−</sup>/apoE<sup>−/−</sup>), these 2 constituents being instrumental in ensuring plaque stability. Interestingly, the smaller atherosclerotic plaques of CX3CR1-deficient mice retained substantial accumulation of smooth muscle cells (6.6±1.0%) and collagen (23.1±3.1%) that was comparable to that observed in control plaques (6.8±0.07% and 24.6±3.1%, respectively), suggesting a preservation of the plaque “stable” phenotype.

**Effect of Single Allele CX3CR1 Deficiency on Atherosclerotic Lesion Size**

We also analyzed the extent of atherosclerosis in the thoracic aortas of 7 CX3CR1<sup>+/+</sup>/apoE<sup>−/−</sup> mice at 25 weeks of age. Weights and plasma cholesterol levels (data not shown) were similar to those reported in the CX3CR1<sup>+/+</sup>/apoE<sup>−/−</sup> and...
CX3CR1pared with within the arterial wall of controls, a substantial decrease in macrophage infiltration activities of CX3CR1 deficient monocytes compared with presence of CX3CL1 in murine atherosclerotic plaques, atherosclerosis. The results of this study clearly show the CX3CR1 deficiency on the development and composition of CX3CR1

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figure 5. Reduced atherosclerotic lesion size in the aortic sinus of CX3CR1/apoE double knockout mice. Representative photomicrographs show sections of advanced aortic sinus plaques from CX3CR1++/++ (a) or 2CX3CR1++/- (b) apoE−/− mice. c. Quantitative analysis of intimal lesion area. Black circles represent females and gray circles represent males. Bar indicates the mean value for each group.

CX3CR1+/−/apoE−/− mice. CX3CR1+/+/apoE−/− mice showed the same reduction in atherosclerosis (3.8 ± 0.9% ; P = 0.0003 compared with controls) as the CX3CR1+/−/apoE−/− mice.

Discussion

On the basis of data from the literature showing that the CX3CR1/CX3CL1 pathway is involved in leukocyte-endothelial interactions and that alterations in CX3CR1 expression and function are associated with the extent and severity of coronary artery disease in humans, we generated CX3CR1+/−/apoE−/− mice to study the direct role of CX3CR1 deficiency on the development and composition of atherosclerosis. The results of this study clearly show the presence of CX3CL1 in murine atherosclerotic plaques, a specific alteration in CX3CL1-binding and chemotactic activities of CX3CR1 deficient monocytes compared with controls, a substantial decrease in macrophage infiltration within the arterial wall of CX3CR1+/−/apoE−/− mice compared with CX3CR1+/+/apoE−/− mice, and a marked reduction in atherosclerotic lesion size at 2 different atherosclerosis-prone sites (aortic sinus and thoracic aorta) in the mice lacking the CX3CR1 gene. These results were observed despite similar plasma cholesterol levels and leukocyte blood counts between the 2 groups. In addition, only 1 muted CX3CR1 allele was sufficient to induce the full atherosclerosis phenotype. Thus, our data imply a direct and major role for CX3CR1/CX3CL1 pathway in the development of macrophage-rich atherosclerotic lesions.

Intralesional macrophages are known to be derived from blood-borne monocytes. Monocyte recruitment into the arterial wall has been shown to be stimulated, at least in part, by the intimal accumulation and oxidation of low-density lipoproteins, and it occurs very early in the course of fatty streak formation. The causal and critical role of blood-borne monocytes in atherogenesis has been elegantly demonstrated by Smith et al., who showed a huge reduction in macrophage infiltration and fatty streak formation despite very high cholesterol levels in mice with decreased numbers of circulating monocytes due to a deficiency in macrophage colony-stimulating factor. In mice, trafficking of monocytes into the subendothelium seems to be controlled by a crosstalk of signals derived from both the vessel and the circulation. Chemokines, essentially produced within the “inflamed” arterial wall, have been shown to play important roles in this setting through their interactions with receptors expressed on the surface of circulating blood cells. Among the large families of chemokines and chemokine receptors, only 2 pathways have been shown to be directly involved in atherosclerosis. CCL2 knockout mice exhibited an 80% reduction in macrophage infiltration and lesion formation, and those deficient for its receptor CCR2 had nearly 50% decrease in lesion size. On the other hand, the lack of CXCR2 expression in bone marrow cells, a receptor for an array of chemokines including mCXCL1 (an ortholog of IL-8), has been shown to be responsible for an almost 50% reduction in lesion development in mice fed a high-fat atherogenic diet. Our study extends our understanding of the role of chemokines in atherogenesis and shows that mice deficient for 1 or 2 CX3CR1 alleles have a 50% to 55% reduction in arterial macrophage infiltration and lesion formation, pointing for the first time to a critical role for another chemokine receptor in this setting.

There are many reasons to suggest that the different chemokine pathways act in a coordinated, sequential manner and interact with other signaling pathways to achieve complete monocyte recruitment into the subendothelium. In an ex vivo model of monocyte arrest on perfused mouse carotid arteries, Huo et al. have ascribed chemokine-mediated monocyte arrest to mCXCL1 and not to CCL2. Because almost 80% of lesion size is lost in CCL2 deficient mice, the role of mCXCL1/CXCR2 in monocyte migration seems to be very modest, suggesting that mCXCL1/CXCR2 and CCL2/CXCR2 act in a sequential manner to ensure monocyte infiltration, the former being responsible for monocyte adhesion and the latter controlling monocyte migration through the endothelium. However, although a functional mCXCL1/CXCR2 pathway is required for full monocyte infiltration to occur, mice repopulated with CXCR2-deficient bone marrow could still mount a significant macrophage infiltration, suggesting that pathways other than CXCR2 may also be responsible for monocyte adhesion processes. The results of the present study point to a potential candidate for this task by demonstrating that CX3CR1 greatly affects the development of macrophage-rich atherosclerotic lesions. Moreover, it is likely that CXCR2 and CX3CR1 pathways act independently from one another. Indeed, CXCR2-mediated monocyte arrest on endothelium has been shown to be integrin-dependent and
involves the upregulation of α4β1 affinity, leading to vascular cell adhesion molecule-1 (VCAM-1)-dependent adhesion.20 In contrast, CX3CR1-mediated firm adhesion to endothelium is unique in that it does not require integrin activity.13,14 Therefore, our results seem to offer a potential explanation for the residual infiltration in CXCR2-deficient mice and would also explain, at least in part, the residual 60% macrophage infiltration in mice with a profound reduction in VCAM-1 expression and function.30 Conversely, it is likely that the persistent macrophage infiltration observed in CX3CR1 knockout mice of the present study reflects the contribution of the other signaling pathways discussed above. It seems that, as elegantly formulated by Rollins,28 the labor of monocyte infiltration has been divided by the chemokine system to ensure the highest productivity.

Severe clinical manifestations of atherosclerosis (myocardial infarction, stroke) are related to both the instability and extent of atherosclerotic disease. Unstable atherosclerotic plaques characterized by increased accumulation of macrophages and decreased smooth muscle cell and collagen content are more prone to undergo rupture and vessel thrombosis than are stable plaques with reduced numbers of macrophages and increased accumulation of smooth muscle cells and collagen.9 Therefore, in our study, we also assessed the composition of atherosclerotic plaques. We found that atherosclerotic lesions of CX3CR1−/− mice retained accumulation of smooth muscle cells and collagen, 2 major determinants of plaque stability, comparable to lesions of CX3CR1+/+ mice. Although the murine model of atherosclerosis used here does not allow a direct evaluation of the plaque vulnerability to rupture, our results suggest that CX3CR1 deficiency did not affect the relative accumulation of smooth muscle cells and collagen and thus is unlikely to alter plaque stability.

On the other hand, the clinical severity of atherosclerosis is related to the extent of the disease. Segments of coronary arteries that contain a severe stenosis at angiography are several-fold more likely to occlude than segments without an angiographic stenosis.31 However, because latter are far more numerous, they account for the bulk of coronary occlusions. The major finding of the present study is the marked reduction in the extent of atherosclerotic lesions in CX3CR1-deficient mice compared with controls, which was documented in 2 different atherosclerosis-prone sites, the aortic sinus and the thoracic aorta. Although we did not analyze lesion development in the coronary vasculature, the thoracic aorta with its cerebral and systemic branches is a major atherosclerotic site in humans. Most importantly, our results offer a pathophysiological explanation for a recent finding in humans that showed that a polymorphism in CX3CR1 gene (M280 allele) associated with reduced CX3CR1 expression and function is a genetic risk factor for coronary artery disease.20,21 Indeed, CX3CR1 M280 heterozygosity has been shown to be associated with a markedly reduced risk of acute coronary ischemic events independent of established risk factor.21 This protective effect is likely to be due to the reduced prevalence and lower severity of coronary angiographic stenosis observed in patients with the CX3CR1 M280 polymorphism.20 Our finding that CX3CR1 deficiency, even when restricted to one allele, preferentially affects the extent of atherosclerotic lesions in apoE knockout mice, may help explain the clinical results and provides a direct demonstration of a role of the CX3CR1/CX3CL1 pathway in atherosclerosis in the mouse.

In conclusion, this study identifies a critical role for CX3CR1 in the development of macrophage-rich atherosclerotic lesions in apoE knockout mice and suggests that strategies aimed at the blockade of the CX3CR1 receptor on circulating leukocytes may be of potential benefit in atherosclerosis.

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