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Up-Regulated Expression of the CXCR2 Ligand KC/GRO- α in Atherosclerotic Lesions Plays a Central Role in Macrophage Accumulation and Lesion Progression

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Macrophage-mediated inflammation is central to atherogenesis. We have determined previously that the CXC chemokine receptor CXCR2 is involved in advanced atherosclerosis. We sought to determine whether one of the ligands of CXCR2, KC/GRO- α , can also modulate atherogenesis. KC/GRO- $\alpha^{-/-}$ mice were generated and mated with the atherosclerosis-prone LDLR $^{-/-}$ mice. There was a significant reduction in atherosclerosis in mice lacking KC/GRO- α ; however, this reduction was only approximately half that seen previously in mice lacking CXCR2 in the leukocyte. To determine whether CXCR2 is involved in the early formation of atherosclerosis, leukocyte-specific CXCR2 $^{-/-}$ chimeric mice on LDLR $^{-/-}$ background were generated. Early fatty streak lesion formation in these mice was not affected by leukocyte CXCR2 deficiency whereas lesions were less developed in mice lacking leukocyte CXCR2 when atherosclerosis was allowed to progress to the intermediate stage. Macrophages were relatively sparse in the lesions of leukocyte CXCR2 $^{-/-}$ mice despite robust MCP-1 expression. These studies indicate that KC/GRO- α /CXCR2 does not play a critical role in recruitment of macrophages into early atherosclerotic lesions but both arterial KC/GRO- α and leukocyte-specific CXCR2 expression are central to macrophage accumu-

lation in established fatty streak lesions. (Am J Pathol 2006; 168:1385–1395; DOI: 10.2353/ajpath.2006.040748)

A large body of evidence supports the ingress and activation of leukocytes, including T cells and macrophages, in the atherosclerotic lesion to be a central component of disease pathogenesis.¹ Although the precise role of T cells in atherogenesis still requires clarification,^{2–4} monocytes/macrophages are present at all stages of atherosclerosis and are believed to play a pivotal role in progression of early atheroma to larger, complex atherosclerotic lesions.^{1,5–8}

Complex orchestration of adhesion molecules and their receptors as well as certain chemotactic cytokines (chemokines) directs monocyte trafficking into the vessel wall.^{9–13} The low-molecular weight polypeptides comprising the chemokine superfamily include two large subgroups of polypeptides, designated as the CC family and the CXC family on the basis of the spacing of the N-terminal pair of cysteine residues.^{14–17} The principal effects of chemokines of the CC family are to chemoattract T cells, monocytes, eosinophils, and natural killer cells, whereas CXC family chemokines are best recognized for their chemotactic activities for neutrophils and T cells.^{14–17} CC and CXC chemokine receptors are predominantly members of the G protein-coupled receptor superfamily and are expressed as integral membrane proteins with seven transmembrane domains, four extracellular domains (N-terminal domain), and four intracel-

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lular domains (C-terminal domain). CC and CXC chemokine receptors each have multiple members (CCR1 to CCR11 and CXCR1 to CXCR6), and most of these receptors bind several chemokine ligands.^{14–17}

The prototypic CC chemokine, monocyte chemoattractant protein 1 (MCP-1), unequivocally plays a major role in the accumulation of macrophages in atherosclerotic lesions. Specifically, MCP-1 is abundant in lesions,^{9,10} and circulating monocytes express CCR2, which is the major receptor for MCP-1.¹⁸ MCP-1 promotes macrophage adhesion and chemotaxis *in vitro*.^{19,20} Moreover, four recent *in vivo* studies, using a variety of approaches, have demonstrated contributory roles of MCP-1 and CCR2 in atherosclerosis.^{21–24} In each study, monocyte ingress was markedly inhibited, but monocyte ingress was not totally abrogated.^{21–24} Furthermore, progression of early atherosclerotic disease occurred in apoE and CCR2 double-knockout mice on an atherogenic diet.^{21,23} Therefore, chemokines other than MCP-1 are likely contributing to progression of established lesions, a notion reinforced by the recent demonstration that CCR2 expression becomes down-regulated by monocyte differentiation into the macrophage or via activation of monocytes by a variety of inflammatory stimuli including oxidized low-density lipoprotein.²⁵

We previously observed that CXCR2, a receptor for interleukin (IL)-8 and several other CXC chemokines, has a major impact on macrophage accumulation in advanced lesions.²⁶ Using a bone marrow transplantation chimera model, we generated atherosclerosis-prone LDLR^{-/-} mice with and without the capacity for leukocyte-specific expression of CXCR2. When fed an atherogenic diet for 16 weeks, the mice that received CXCR2^{-/-} leukocytes (CXCR2^{-/-} BMT) had significantly smaller lesions, with a smaller lipid core and less smooth muscle proliferation. Furthermore, at 16 weeks, the lesions of the CXCR2^{-/-} BMT group were almost devoid of macrophages. On the other hand, at 16 weeks, the lesions from recipients of CXCR2^{+/+} leukocytes (CXCR2^{+/+} BMT) contained large numbers of CXCR2-positive macrophages.

In the intima of human atherosclerotic lesions, macrophage-derived foam cells express IL-8, which is induced by certain atherogenic stimuli including modified low-density lipoprotein.^{27–30} Furthermore, peripheral blood mononuclear cells from hypercholesterolemic patients have a marked enhancement of IL-8 production.³¹ Mice do not express a peptide homologue of IL-8, but they do express one of the GRO chemokines, KC/GRO- α , in the intima of advanced atherosclerotic lesions in the chimeric LDLR^{-/-} mice.²⁶

These findings prompted further investigation of how leukocyte CXCR2 and leukocyte plus lesion KC/GRO- α expression affected monocyte recruitment to early lesions as well as macrophage retention or accumulation in established lesions. Because our previous study was limited to examination of only advanced lesions after 16 weeks of marked hyperlipidemia, the current study assessed the role of CXCR2 and GRO- α expression in early and intermediate lesions. Our results reveal that expression of KC/GRO- α in the vessel wall and lesion macrophage CXCR2 expression are central to the accumulation

of macrophages in the progression of early atherosclerotic lesions. In contrast, our results also reveal that GRO- α and CXCR2 may not be essential for monocyte ingress into early atherosclerotic lesions.

Materials and Methods

Animals and Diet

LDLR^{-/-} mice backcrossed onto the C57BL/6 background were initially obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at the Scripps Research Institute Animal Facility. Breeding pairs of the CXCR2^{+/-} animals were initially obtained from Genentech (South San Francisco, CA) and bred to generate the CXCR2^{-/-} and CXCR2^{+/+} mice used as bone marrow donors. These mice were genotyped by polymerase chain reaction using primers and conditions previously published.²⁶ The KC/GRO- α -null mice (described below) were mated with LDLR^{-/-} mice from our facility to generate double-mutant mice. Mice were weaned at 4 weeks of age and kept on a 12-hour light/dark cycle in a specific pathogen-free facility. They were fed a chow diet (diet no. 5015; Harlan Teklad, Madison, WI) *ad libitum*.

Generation of KC/GRO- α ^{-/-} Mice

Murine KC genomic clones were isolated from a 129-derived genomic library. A 3.8-kb *Xba*I-*Apa*I fragment from the 5' end of the gene was subcloned into pGem 11zf (Promega Corp., Madison, WI). The fragment was rescued from the plasmid using *Xho*I/*Not*I digestion and inserted into the *Xho*I-*Not*I site from the pPNT vector, generating pPNT-XN. A 4.8-kb *Bam*HI fragment from the 3' end of the gene was cloned into the *Bam*HI site of pPNT-XN, generating the final vector pPNTKC/ko. Orientation of the *Bam*HI insertion was confirmed by digestion with *Xho*I/*Cla*I. The targeting vector was electroporated into CJ7 ES cells and neomycin-resistant clones were isolated. Targeted clones were identified by Southern blot of genomic DNA digested overnight with *Kpn*II/*Eco*RV. Samples were fractionated in 0.7% agarose gels and transferred onto nylon membranes. The probe consisted of a 0.5-kb fragment located downstream of 3' homologous region contained in the targeting vector. Heterozygous ES cells for the targeted locus were injected into C57BL/6J blastocysts, and chimeric mice were obtained from two different ES cell clones. Chimeric males were bred to C57BL/6J females. Progeny containing heterozygous genotype for the KC locus were intercrossed. KC^{-/-} mice were obtained with the expected frequency of 25%. KC^{-/-} mice were viable and fertile and did not show any gross abnormalities.

KC/GRO- α ^{-/-}, LDLR^{-/-} Double-Mutant Mice and Study Protocol

KC/GRO- α ^{-/-} mice were mated with LDLR^{-/-} mice (both on C57BL/6 background). Genotyping for KC/GRO- α was performed by reverse transcriptase-polymer-

ase chain reaction. mRNA was isolated from the tail pieces from each animal and reverse-transcribed. Primers were designed and optimized to determine which region of the KC gene was disrupted because screening through the neo gene insertion would detect both the KC and LDLR altered alleles. The following primers were used: 5'-GAA GAC AGA CTG CTC TGA TGG CAC-3' and 5'-CCC TTC TAC TAG CAC AGT GGT TGA-3'.

When annealed at 52°C for 40 cycles, a 272-bp product (302 to 574 of the mRNA sequence) was yielded. A cohort of 14, 6- to 8-week-old male LDLR^{-/-}, KC/GRO- α ^{-/-} mice and 14 age-matched male LDLR^{-/-}, KC/GRO- α ^{+/-} controls were fed a high-fat diet (HFD) that contained 15.8% fat, 1.25% cholesterol, and no cholate (diet no. 94059, Harlan Teklad) for 16 weeks to induce atherosclerosis. Blood was drawn immediately before the start of the HFD regimen and every 4 weeks thereafter. After 16 weeks the mice were sacrificed and processed exactly as described below for the CXCR2 bone marrow chimeric mice.

Generation of CXCR2 and KC/GRO- α Bone Marrow Chimeric Mice

For both sets of chimeric mice, 24 6-week-old male LDLR^{-/-} mice were subjected to 1000 rads of total body irradiation to eliminate most of the endogenous bone marrow-derived cells as well as stem cells. For controls, half the irradiated mice were reconstituted with bone marrow cells isolated from KC/GRO- α ^{+/-} (KC/GRO- α ^{+/-} BMT) or CXCR2^{+/-} mice (CXCR2^{+/-} BMT mice) and the other half with marrow from KC/GRO- α ^{-/-} (KC/GRO- α ^{-/-} BMT) or CXCR2^{-/-} mice (CXCR2^{-/-} BMT mice) as experimental mice exactly as described previously.²⁶ The mice consumed a chow diet for 4 weeks while they were allowed to be repopulated with the donor bone marrow. The KC/GRO- α chimeric mice were fed the HFD for 16 weeks to induce advanced atherosclerosis. To observe different degrees of atherosclerosis in the CXCR2 chimeric mice one group of six mice was fed the HFD for 3 weeks, whereas a second group of six mice was fed this diet for 6 weeks. At week 0 (before BMT) and at every 3 weeks (CXCR2 chimeric mice) or at every 4 weeks (KC/GRO- α chimeric mice), blood was drawn via the retro-orbital plexus after an 8-hour fast. Plasma was obtained by centrifuging the blood at 5000 $\times$ *g* for 10 minutes at 4°C. Total plasma cholesterol was measured with an enzymatic kit from Sigma (St. Louis, MO).

Another group of 16 6- to 8-week-old male LDLR^{-/-} mice was irradiated, and one half was reconstituted with marrow from CXCR2^{-/-} mice and the other half with marrow from wild-type mice as described above. This was done to determine whether there were differences in the number of circulating leukocytes because of their CXCR2 status. Blood was taken at 4 weeks after BMT while the mice were consuming a chow diet. The mice were fed the HFD for an additional 3 weeks before being bled again. The neutrophils, monocytes, and lymphocytes were counted from Wright-stained blood smears at the Scripps Research Institute core pathology facility by a technician who was blinded

to the identities of the mice. All procedures were in accordance with institutional guidelines.

Peripheral Blood Leukocyte Expression of CXCR2

To assess if peripheral blood leukocyte expression of CXCR2 was enhanced by hyperlipidemia, eight 6- to 8-week-old male LDLR^{-/-} mice were divided into two groups. One group was fed the chow diet, whereas the other group was given the HFD for 16 weeks. During this time the mice were bled at 0, 8, 12, and 16 weeks and their peripheral blood cells were analyzed for CXCR2 expression. The blood (0.1 ml) was centrifuged at 3000 $\times$ *g* for 5 minutes at 4°C and the cell pellet washed with phosphate-buffered saline containing 2% fetal bovine serum. The 0.1 ml cell suspension was incubated at 4°C with 1 ml of Fc receptor-blocking solution (Fc block:CD16/32, clone 2.4G2; PharMingen, La Jolla, CA) to prevent nonspecific Ig binding. The cells were stained for 30 minutes at 4°C with phycoerythrin-labeled Gr-1 (PharMingen) and Cy-labeled anti-CD11b (Serotec, Oxford, UK) antibodies to identify neutrophils (CD11b⁺, Gr-1⁺ cells) and monocytes (CD11b⁺, Gr-1⁻ cells). Subsequently the cells were incubated with rabbit anti-mouse CXCR2 antibody (a generous gift of Dr. N. Mukaida, Kanazawa University, Japan) for 30 minutes followed by incubation with fluorescein isothiocyanate-labeled rabbit IgG secondary antibody. The stained cells were analyzed on a Becton Dickinson (Mountain View, CA) fluorescence-activated cell sorting (FACS) system equipped with CellQuest software, and the percentages of neutrophils and monocytes staining positively for CXCR2 were determined.

Assessment of Atherosclerosis

Extent of atherosclerosis in the mice was assessed by quantitative analysis of the lesions in the aortic valve as well as on the aortic surface of each mouse, as detailed previously.²⁶ Briefly, the OCT-embedded, frozen aortic valves were sectioned serially at 10 μ m thickness for a total of 300 μ m beginning at the base of the aortic valve where all three leaflets were first visible. Every fourth section for a total of five sections from each animal was stained with Oil Red O to reveal the lipid-rich lesions. The stained areas were quantified using a computer-assisted video imaging system, and the mean area of the five sections from each animal was used to compare the lesion areas of the groups. Aortas were cleaned and stripped of fat on the adventitia before being excised from the animal. The longitudinally opened aortas were pinned on wax and stained with Sudan IV to reveal the lesions on the surface. The lesions were quantitated by calculating the percentage of the total surface area that was covered with lesion using computer-assisted morphometry.

Immunohistochemistry

Detailed staining methods are described in our previous publication.²⁶ The mouse aortic valve lesions were ana-

Figure 1. Representative photomicrographs of aortic surface lesions (A–F) stained with Sudan IV and aortic valve lesions (G–L) stained with Oil Red O. The mice were sacrificed at 22 to 24 weeks of age, after consuming the HFD for 16 weeks, and the tissues were prepared as described in Materials and Methods. Original magnifications, $\times 40$.

lyzed with the following antibodies: anti-MOMA-2 (Sero-
tec) for the detection of intimal macrophages; anti-mouse
CXCR2²⁶ for detection of leukocyte CXCR2; and anti-KC/

GRO- α (R&D Systems, Minneapolis, MN) and anti-mouse
MCP-1 (R&D Systems) for the detection of specific
murine chemokines. The frozen tissue sections were

blocked with 5% normal sera and incubated overnight at 4°C with the primary antibody (1 to 10 $\mu\text{g/ml}$). The sections were blocked for endogenous peroxidase activity with Peroxo-Block (Zymed, South San Francisco, CA) and incubated with the appropriate secondary antibody (5 $\mu\text{g/ml}$) for 1 hour. The washed sections were incubated for 30 minutes with Vectastain ABC Elite solution (Vector Laboratories, Burlingame, CA). At this point, the staining protocol for the chemokines was enhanced by incubating the sections in 1:100 Tyramide signal amplification solution (New England Nuclear, Beverly, MA), followed by another 30-minute incubation with Vectastain ABC solution. All sections were developed with 9-amino-3-ethylene-carbazole (AEC) (Vector Laboratories) and counterstained with hematoxylin. Negative control sections for KC/GRO- α and MCP-1 were prepared by using appropriate dilutions of the normal serum of the species in which the primary antibodies were made.

Statistical Analysis

Results are given as mean $\pm$ SD unless otherwise noted. Student's *t*-test was used to compare the percentage of CXCR2⁺ cells obtained by FACS analysis as well as plasma cholesterol levels and the leukocyte counts between the treatment groups. Mann-Whitney *U*-test was used to compare the Oil Red O-stained aortic valve lesion areas.

Results

Specific Role of KC/GRO- α in Atherogenesis

To test the role of KC/GRO- α in atherosclerosis, KC/GRO- α ^{-/-} mice were generated as described in Materials and Methods, and these mice, which were observed at a gross level to be phenotypically normal, were mated with LDLR^{-/-} mice. The resulting double-knockout mice also showed no gross phenotypic abnormality and they bred normally. A cohort of 6- to 8-week-old male LDLR^{-/-},KC/GRO- α ^{-/-} mice and matched control littermates deficient only in LDLR (LDLR^{-/-},KC/GRO- α ^{+/+}) were fed an atherogenic diet (as described in Materials and Methods) for a period of 16 weeks. The mice were bled before and several times during the dietary regimen to determine their lipid profile. Total plasma cholesterol and triglyceride levels were similar between the two groups of mice at all time points (data not shown). On sacrifice, the hearts and aortas were excised from the mice to examine the extent of atherosclerosis in these tissues. Representative examples of both aortic surface and aortic valve lesions are shown in Figure 1. Although differences in lesion formation between the two groups of mice were modest, Sudan IV-stained surface lesion in the aortas was nevertheless significantly smaller in the LDLR^{-/-},KC/GRO- α ^{-/-} mice ($14.2 \pm 3.3\%$) compared to the lesions in the LDLR^{-/-},KC/GRO- α ^{+/+} mice ($17.1 \pm 2.2\%$) (Figure 2). Similarly, Oil Red O-stained atherosclerotic lesion area in the serial sections of the aortic valve was significantly lower in the LDLR^{-/-},KC/GRO- α ^{-/-} mice ($186,423 \pm$

Figure 2. Graphic depiction of the extent of aortic surface and aortic valve lesions in LDLR^{-/-},KC/GRO- α ^{-/-} versus LDLR^{-/-},KC/GRO- α ^{+/+} mice after consuming the HFD diet for 16 weeks. Aortic surface lesions were calculated by measuring the Sudan IV-stained areas as well as the entire inner surface area of the aorta and expressing the data as percentage of entire area covered by lesions. Aortic valve lesions were measured as areas covered by Oil Red O staining. The means of each group are shown adjacent to the individual values as **filled triangles**.

$43,874 \mu\text{m}^2$) than in the LDLR^{-/-},KC/GRO- α ^{+/+} mice ($238,636 \pm 43,757 \mu\text{m}^2$) (Figure 2).

Immunohistochemical staining of the aortic valve sections with an antibody specific for mouse macrophage (MOMA-2) revealed that there was less macrophage accumulation overall in the lesions of LDLR^{-/-},KC/GRO- α ^{-/-} mice compared to the LDLR^{-/-},KC/GRO- α ^{+/+} mice (Figure 3). Under these conditions, KC/GRO- α staining was abundant in the valve sections of control mice but confirmed to be absent in the sections of double-knockout mice (Figure 4).

In contrast to the findings for double-knockout mice, the extent of atherosclerosis in the aortic valves and aortas of irradiated chimeric LDLR^{-/-} mice receiving KC/GRO- α ^{-/-} bone marrow ($186,440 \pm 65,543 \mu\text{m}^2$ and $13.5 \pm 4.6\%$) did not significantly differ from chimeric LDLR^{-/-} recipients of KC/GRO- α ^{+/+} bone marrow

Figure 3. Representative photomicrographs showing macrophage accumulation in the lesion. Aortic valve sections were immunohistochemically stained with MOMA-2 antibody to reveal the red-stained macrophage present within the lesions of LDLR^{-/-}, KC/GRO- α ^{+/+} mice (**A, C**) and LDLR^{-/-}, KC/GRO- α ^{-/-} mice (**B, D**). Original magnifications, $\times 100$.

($208,317 \pm 41,895 \mu\text{m}^2$ and $13.8 \pm 3.5\%$) after 16 weeks of consuming an atherogenic diet. It should be emphasized that although the lesion sizes between the previously mentioned LDLR^{-/-}, KC/GRO- α ^{-/-} mice and LDLR^{-/-} mice receiving KC/GRO- α ^{-/-} bone marrow

were very similar, because the chimeric mice were subjected to irradiation and BMT whereas the double-knock-out mice were not, the lesions between these groups of animals cannot be directly compared. The fact that lesions were significantly smaller (albeit modestly) in

Figure 4. Aortic valve sections from LDLR^{-/-}, KC/GRO- α ^{+/+} (**A**) and LDLR^{-/-}, KC/GRO- α ^{-/-} (**B**) mice stained for KC/GRO- α . The sections were immunohistochemically stained with an antibody against mouse KC/GRO- α . Original magnifications, $\times 250$.

LDLR^{-/-},KC/GRO- α ^{-/-} mice compared to LDLR^{-/-},KC/GRO- α ^{+/+} mice but not in LDLR^{-/-} mice repopulated with KC/GRO- α ^{-/-} marrow compared to LDLR^{-/-} mice repopulated with wild-type marrow suggests that perhaps the cellular sources of KC/GRO- α that contribute to atherosclerosis are cells in the vasculature other than bone marrow-derived leukocytes. We next turned our attention to defining the disease stage at which leukocyte expression of the KC/GRO- α receptor was modulating lesion progression.

Leukocyte CXCR2 Expression in Early Lesion Formation and the Progression of Atherosclerosis

CXCR2 is robustly expressed in macrophages that accumulate in the subendothelial space of advanced atherosclerotic lesions in hyperlipidemic mice.²⁶ Therefore, we first determined if hyperlipidemia alters CXCR2 expression on circulating mononuclear cells. To do so, a group of four LDLR^{-/-} mice were fed a chow diet and a group of equal size was fed the HFD for a total of 16 weeks during which time the level of CXCR2 expression was measured in their blood leukocytes. FACS analysis revealed that only 2 to 3% of the circulating monocytes (identified as CD11b⁺ and Gr-1⁻ cells) expressed CXCR2 initially. After 8 weeks of consuming their respective diets, the chow-fed mice showed a slight increase in CXCR2⁺ monocytes ($4.1 \pm 1.0\%$), whereas the HFD-fed group showed no changes ($2.2 \pm 0.6\%$). After 12 weeks of consuming the HFD both groups had a minor increase in their CXCR2⁺ monocytes ($8.9 \pm 2.2\%$ in HFD group versus $5.9 \pm 1.2\%$ in chow group). This increase was more pronounced at week 16 ($10.5 \pm 0.6\%$ in HFD group versus $6.9 \pm 1.3\%$ in chow group). However, none of the differences between the two groups was significant. The percentage of neutrophils positive for CXCR2 was similar between the chow- and HFD-fed mice (60 to 70% positive) at all time points up to week 12. At week 16 there was a significant increase in the CXCR2⁺ neutrophils only in the chow group (from $69.7 \pm 0.7\%$ at week 12 to $90.6 \pm 1.6\%$ at week 16). As with monocytes, none of the differences in neutrophil expression of CXCR2 observed between the two groups was significant. These results indicated that the fraction of CXCR2⁺ mononuclear leukocytes in the LDLR^{-/-} mice was not influenced by hyperlipidemia.

Next, we assessed for potential differences in circulating numbers of monocytes attributable to leukocyte-specific CXCR2 deficiency. To do so, two groups of LDLR^{-/-} mice were irradiated and repopulated with marrow from either CXCR2^{+/+} or CXCR2^{-/-} mice exactly as described earlier. Their blood was taken at 4 weeks after BMT and again after 3 weeks on the HFD to perform leukocyte counts using the standard Wright stain method. Monocyte numbers between the two groups were similar whether the animals were fed chow or HFD. However, after consumption of HFD both groups exhibited a reduction in monocyte numbers from 581 ± 140 to 214 ± 101 per mm³ for the CXCR2^{+/+} BMT mice and from $420 \pm$

Figure 5. Quantitation of atherosclerotic lesion areas in the aortic valves of individual mice. After sacrifice, the hearts were removed, embedded in OCT, and frozen. Serial cryosections were taken and stained with Oil Red O to reveal the lipid-laden aortic valve lesions. Computer-assisted morphometry was used to quantitate the areas that were stained with Oil Red O. Each symbol represents the mean lesion area of five sections of the aortic valve taken 40 μ m apart from the base of the valve where all three leaflets were visible from a single mouse. The means of each group are shown adjacent to the individual values as **filled triangles**.

271 to 227 ± 173 per mm³ for the CXCR2^{-/-} BMT mice. No differences in the neutrophil numbers between the CXCR2^{+/+} BMT and CXCR2^{-/-} BMT mice were observed in mice fed chow (2145 ± 970 versus 3055 ± 1522 per mm³) or HFD (3340 ± 816 versus 3684 ± 1314 per mm³). Lymphocyte numbers were significantly lower in the CXCR2^{-/-} BMT mice on both chow (7261 ± 1380 versus 3026 ± 1155 per mm³) and HFD ($12,025 \pm 1612$ versus 5240 ± 2011 per mm³).

We next determined if leukocyte-specific CXCR2 modulated the early stages of atherosclerosis. To do so, LDLR^{-/-} mice chimeric with CXCR2^{-/-} mice generated by marrow transplantation were fed the HFD for 3 or 6 weeks. As previously observed,²⁶ CXCR2^{-/-} BMT mice on the HFD had ~20 to 25% lower plasma cholesterol levels ($P < 0.05$) just before sacrifice than their CXCR2^{+/+} BMT counterparts (data not shown). After 3 weeks of consuming the HFD, the lesion area of the aortic valve sections was minimal and was not statistically different between the CXCR2^{-/-} BMT ($23,773 \pm 10,295$ μ m²) and CXCR2^{+/+} BMT ($28,791 \pm 18,414$ μ m²) mice (Figure 5). However, after 6 weeks on the HFD, lesion areas were significantly smaller in the CXCR2^{-/-} BMT mice ($91,891 \pm 54,791$ μ m²) than in the CXCR2^{+/+} BMT mice ($162,132 \pm 41,844$ μ m²) ($P < 0.05$) (Figure 5).

Using immunohistochemical staining with MOMA-2 antibody (Figure 6), we determined that the early (3 weeks of HFD) fatty streak lesions of both CXCR2^{+/+} BMT (Figure 6, A and G) and CXCR2^{-/-} BMT (Figure 6, D and J) mice were comprised almost exclusively of subendothelial macrophages. Moreover, the lesions appeared grossly similar, consistent with a comparable degree of early macrophage ingress into the intima in both groups

Figure 6. Immunohistochemical staining of representative aortic valve sections for macrophages with MOMA-2 antibody. **A** and **C**, **B** and **H**, and **G** and **I** represent sections from CXCR2^{+/+} BMT mice at 3, 6, and 16 weeks after HFD, respectively. **D** and **J**, **E** and **K**, and **F** and **L** represent sections from CXCR2^{-/-} BMT mice at 3, 6, and 16 weeks after HFD, respectively. Macrophages are seen in the early fatty streak lesions (3 weeks after HFD) of both groups. After 6 weeks of HFD, macrophages were more abundant in CXCR2^{+/+} BMT sections than in the CXCR2^{-/-} BMT sections. However, 16 weeks after HFD, CXCR2^{-/-} BMT lesions were nearly devoid of macrophages, whereas the lesions of CXCR2^{+/+} BMT mice contained numerous macrophages. The heart sections 16 weeks after HFD from mice fed the diet for 16 weeks were obtained from a previous study published elsewhere.²⁶ Original magnifications: $\times 100$ (**A–F**); $\times 250$ (**G–L**).

of animals. In contrast, at the intermediate stage (6 weeks of HFD), macrophages were dispersed throughout the lesion of CXCR2^{+/+} BMT (Figure 6, B and H) mice but were fewer in number and tended to be concentrated toward the luminal side of the neointima in the CXCR2^{-/-} BMT (Figure 6, E and K) mice. The neointima of CXCR2^{-/-} BMT lesions was relatively hypocellular and composed primarily of lipid pools within extracellular matrix that contained an occasional MOMA-2-negative cell (Figure 6, E, F, K, and L). Using antibodies specific for both CD4 and CD8 (Pharmingen), the same sections were stained for lymphocytes, but no lymphocytes were detectable (data not shown). Under these conditions, we confirmed²⁶ that after 16 weeks on the HFD, the lesions of CXCR2^{-/-} BMT (Figure 6, F and L) mice were almost devoid of macrophages compared to the CXCR2^{+/+} BMT (Figure 6, C and I) mice.

Intimal macrophages in both early and more advanced lesions expressed CXCR2, and we observed that at least one CXCR2 ligand, KC/GRO- α , was expressed in the intima of lesions in both CXCR2^{-/-} BMT and CXCR2^{+/+} BMT mice at all time points (Figure 7, A–H). A related CXCR2 ligand that is a more potent neutrophil chemoattractant, the GRO- β , γ homologue MIP-2, was not detected in any of the lesions (data not shown). But intimal MCP-1 staining was diffuse and present at all three stages of atherosclerosis in both groups of mice (Figure 7, I–P). Therefore, peripheral blood leukocyte expression of CXCR2, which was not a critical determinant of mononuclear leukocyte adhesion and migration into early lesions in the vessel wall in hypercholesterolemic mice, also did not modulate atherogenesis by grossly affecting lesion intima MCP-1 expression.

Figure 7. Immunohistochemical staining of aortic valve lesions for KC/GRO- α and MCP-1. Both CXCR2^{+/+} BMT (**A–C**) and CXCR2^{-/-} BMT (**E–G**) sections at all time points stained positive for KC/GRO- α . **D** and **H**: Sections incubated with a nonimmune serum to serve as negative controls. Because of the known involvement of the CC chemokine, MCP-1, and its receptor in atherogenesis, the aortic valve sections were stained with an antibody specific for mouse MCP-1. Both CXCR2^{+/+} BMT (**I–K**) and CXCR2^{-/-} BMT (**M–O**) tissues at all time points showed diffuse positive staining with the antibody, suggesting a role for MCP-1 in atherosclerosis. **L** and **P**: Negative control sections incubated with nonimmune serum. The 16-week tissue sections after HFD were from a previous study published elsewhere.²⁶ Original magnifications, $\times 250$.

Discussion

Atherosclerosis is critically mediated by monocyte recruitment followed by differentiation of recruited monocytes into macrophages, by M-CSF-mediated macrophage growth,^{6,8} macrophage scavenger receptor function,⁷ and progressive macrophage-mediated inflammation.¹ Chemokines are best recognized for their effects on leukocyte adhesion and chemotaxis. The prototypic CC chemokine MCP-1, acting via CCR2, is a central promoter of monocyte recruitment to atherosclerotic lesions.^{9,10,18–24} In this study, we have evaluated the role of the prototypic CXC chemokine, KC/GRO- α , on atherosclerosis. Our study was the first to evaluate a disease phenotype in KC/GRO- α -null mice, which demonstrated no gross phenotype under normal conditions and bred normally. We observed a moderate but significant attenuation in atherosclerotic lesion formation after 16 weeks of consuming the HFD in the LDLR^{-/-}, KC/GRO- α ^{-/-} mice. The reduction in atherosclerotic lesion size in the LDLR^{-/-}, KC/GRO- α ^{-/-} mice was approximately half that of the attenuation in lesion size previously

seen (and confirmed here) in mice with leukocyte-specific deficiency of CXCR2,²⁶ suggesting perhaps that CXCR2 ligand(s) other than KC/GRO- α may have compensated somewhat for the loss of KC/GRO- α in enhancing the CXCR2-mediated atherogenic functions of macrophages. Our study also showed that leukocyte-specific deficiency of KC/GRO- α did not alter the extent of atherosclerosis in bone marrow-transplanted chimeric mice. Although it is tempting to conclude from this that the source of KC/GRO- α in the vasculature that plays a key role in atherosclerosis is cells other than leukocytes, the relatively modest differences in atherosclerosis in the double-knockout mice and the chimeric model indicates a need for further studies to definitively answer this question.

The studies involving early and intermediate lesions in CXCR2^{-/-} chimeric mice indicated that leukocyte-specific CXCR2 is generally proatherogenic and that deficiency of this chemokine receptor results in substantial inhibition of atherosclerosis. Our studies ruled out monocytopenia as a contributor to altered atherogenesis in the

presence of leukocyte-specific CXCR2 deficiency. Hence, proatherogenic effects of monocyte lineage CXCR2 expression are distinct from the monocytopenic effects of M-CSF.⁶ Furthermore, marked hyperlipidemia did not affect mouse monocyte CXCR2 expression in peripheral blood, in contrast to the capacity of hyperlipidemia to up-regulate both monocyte CCR2 expression and adhesion.^{32,33}

These CXCR2 deficiency studies also indicated that CXCR2 expression by intimal macrophages was both an early and persistent feature of atherosclerosis, suggesting that CXCR2 expression on the lesion macrophage was acquired sometime during their transformation into macrophage foam cells. CXCR2 expression can be up-regulated *in vitro* as monocytes make contact with endothelial cells,³⁴ thereby modulating monocyte-endothelial cell adhesion. But that intimal MOMA-2⁺ macrophage recruitment and lesion areas were comparable between the CXCR2^{+/+} BMT and CXCR2^{-/-} BMT groups in fatty streak lesions suggests that it is the up-regulation of CXCR2 expression by differentiating macrophages in the intima, rather than effects of GRO- α and other CXCR2 ligands on monocyte-endothelial adhesion and leukocyte entry into early lesions, that most likely accounted for the robust expression of CXCR2 associated with mouse lesion leukocytes. However, because the lesion sizes were relatively small, it is also possible that any effect of leukocyte-specific CXCR2 deficiency on lesion formation may not have been clearly revealed. Nevertheless, we speculate that CXCR2 expression becomes up-regulated as the emigrated monocytes undergo transformation into activated macrophages. In this regard, CXCR2 expression is highly regulated by cytokines such as IL-4, IL-10, and IL-13 and by other factors that may regulate vascular inflammation *in vivo*.^{35–38}

In the intermediate atherosclerosis stage, macrophages in the lesions of the CXCR2^{-/-} BMT mice were located more superficially on the luminal side of the intima rather than dispersed throughout the intima as in the CXCR2^{+/+} BMT mice. Moreover, in the advanced stages of atherosclerosis, the lesions of the CXCR2^{-/-} BMT mice demonstrated little progression beyond the intermediate stage with a relatively marked decline in lesion macrophage staining. Under these conditions, intimal MCP-1 expression was not qualitatively different in lesions of CXCR2^{+/+} BMT and CXCR2^{-/-} BMT mice. In accordance with this, we have observed that both CXCR2-deficient and normal mouse bone marrow-derived macrophages, isolated by culture in the presence of M-CSF mice, produce comparable amounts (~20 to 30 ng per ml) of MCP-1 (R.A. Terkeltaub, unpublished observations). As in other forms of leukocyte-mediated inflammation, the perpetuation and progression of atherosclerosis may require the continuing ingress of leukocytes from peripheral blood. But the association of deficient lesion macrophage CXCR2 expression with marked inhibition of both macrophage retention and disease progression in established atherosclerosis appeared to reflect the direct activities of CXCR2 ligands on macrophages rather than indirect effects on monocyte/macrophage recruitment, retention, and activation

through MCP-1 or M-CSF. Direct effects of CXCR2 ligands with the potential to promote retention and activation of recruited intimal CXCR2⁺ macrophages include stimulation of leukocyte integrin activation that results in enhanced adhesion to extracellular matrix constituents including fibronectin, which is abundant in established atherosclerotic lesions.³⁹ Importantly, chemokines can promote fibronectin-integrin interactions that modulate cell migration, spreading, and differentiation that may conceivably regulate cell retention in atherosclerotic lesions.^{40,41}

In summary, deficiencies of either KC/GRO- α or macrophage CXCR2 expression are associated with a loss of intimal macrophages and attenuated disease progression throughout time within established fatty streak lesions. These findings identify a potentially unique therapeutic role in atherosclerosis for inhibitors of the expression or action of CXCR2 ligands. For example, potential inducers of GRO- α and/or IL-8 in atherosclerotic lesion cells include minimally modified or oxidized low-density lipoprotein, thrombin, and CD40-CD40 ligand (CD40L) interaction.^{11,27,42,43} Thus, suppression of hyperlipidemia, thrombosis, and CD40-CD40L interactions may suppress the progression of established disease by acting in part through suppression of CXCR2 ligand expression. The results presented in this study indicate the need for future investigation of the effects of specific pharmacological and immunological inhibitors of CXCR2, GRO- α , and possibly of other individual CXCR2 ligands on the course of atherosclerosis.

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