CXCL16/SR-PSOX Is an Interferon-γ–Regulated Chemokine and Scavenger Receptor Expressed in Atherosclerotic Lesions

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Objective—Atherosclerosis is an inflammatory disease. Several chemokines are important for monocyte/macrophage and T-cell recruitment to the lesion. CXCL16 is a recently discovered chemokine that is expressed in soluble and transmembrane forms, ligates CXCR6 chemokine receptor, and guides migration of activated Th1 and Tc1 cells. It is identical to scavenger receptor SR-PSOX, which mediates uptake of oxidized low-density lipoprotein. We investigated whether CXCL16 expression is controlled by interferon-γ (IFN-γ) cytokine-abundant in atherosclerotic lesions.

Methods and Results—CXCL16 and CXCR6 expression was identified by polymerase chain reaction and histochemistry in atherosclerotic lesions from humans and apolipoprotein-E–deficient mice. In vitro IFN-γ–induced CXCL16 in human monocyctic THP-1 cells and primary human monocytes, which led to increased uptake of oxidized low-density lipoprotein in THP-1 cells, which could be blocked by peptide antibodies against CXCL16. In vivo IFN-γ induces CXCL16 expression in murine atherosclerotic lesions.

Conclusions—We demonstrate a novel role of IFN-γ in foam cell formation through upregulation of CXCL16/SR-PSOX. CXCR6 expression in the plaque confirms the presence of cells able to respond to CXCL16. Therefore, this chemokine/scavenger receptor could serve as a molecular link between lipid metabolism and immune activity in the atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: atherosclerosis ■ chemokines ■ interferon-γ ■ oxidized low-density lipoprotein ■ scavenger receptors

Growing body of evidence shows that atherosclerosis can be considered as an inflammatory disease. Infiltrates of activated macrophages and T cells are prominent in human and experimental atherosclerotic lesions. Recruitment of these cells to lesions is guided by endothelial leukocyte adhesion molecules and chemoattractants. Once present in the subendothelial space, macrophages engulf oxidatively modified low-density lipoproteins (ox-LDL) through their scavenger receptors and develop into cholesterol-filled foam cells, a hallmark of atherosclerotic lesion formation. Therefore, scavenger receptors play a crucial role in the pathogenesis of atherosclerosis. T cells recruited to forming lesions are largely of the Th1 effector cell type: they recognize endocytosed antigens, secrete interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and other proinflammatory cytokines, activate macrophages, and promote a delayed-type hypersensitivity-like reaction.

The scavenger receptor for phosphatidyl serine and ox-LDL (SR-PSOX) is a recently discovered macrophage receptor that mediates internalization of ox-LDL and phosphatidylserine-coated particles such as apoptotic bodies. It was recently found to be expressed in human atherosclerotic lesions. Surprisingly, the sequence of SR-PSOX is identical to a recently discovered chemokine, CXCL16. The latter exists in a transmembrane as well as in a soluble form. CXCL16 was found to ligate CXCR6/Bonzo, an important co-receptor for HIV. CXCL16 is expressed by dendritic cells in the T-cell zone of lymph nodes and by sinusoid-associated cells of the splenic red pulp and has been associated with the guidance of activated IFN-γ–producing Th1 and Tc1 cells to areas on infection.

Because IFN-γ–producing T cells are abundant in atherosclerotic lesions and because SR-PSOX is identical to the transmembrane form of CXCL16, we decided to investigate the role of IFN-γ on CXCL16/SR-PSOX expression and its effects on cholesterol accumulation and atherosclerosis.

Methods

Human Plaque Samples and Cells

Advanced human atherosclerotic plaques were obtained from patients undergoing carotid endarterectomy surgery because of symp-
omatocatonic carotid stenosis. Control specimens from mammary arteries and veins were obtained from patients undergoing coronary bypass surgery. All samples were obtained after informed consent, and the protocol was approved by the local ethical committee of Karolinska Hospital. Peripheral blood mononuclear cells were isolated from human buffy coats obtained from healthy donors by centrifugation over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). To obtain primary monocyteic cells, peripheral blood mononuclear cells (20×10^6 cells/well) were allowed to adhere for 2 hours, nonadherent cells were removed, and adhered monocytes were cultured in RPMI-1640 medium containing 10% fetal calf serum (Gibco, Life Technologies, Rockville, Md). THP-1 cells were obtained from the American Type Culture Collection (Manassas, Va) and maintained in the same medium.

**Mouse Tissues and Cells**

Female apolipoprotein-E–deficient (ApoE−/−) mice on the C57BL/6 background (strain C57BL/6H-ApoETM1UNC129) were used for experiments. One group of mice was fed a “Western” diet with 0.15% cholesterol from age 6 weeks and euthanized at age 16 or 26 weeks. These mice received a single intraperitoneal injection of 200 ng recombinant mouse IFN-γ (Peprotech, London, UK; specific activity 10^7 U/mg) in 10% apoE−/− mouse serum as vehicle (n=8) or vehicle only (n=6). Eight hours after injection, animals were euthanized and aortas were isolated. Atherosclerotic areas were dissected under a microscope and immediately frozen. Total mRNA was isolated and analyzed for expression of CXCL16 and CD68 as described. All animal experiments were in accordance with national guidelines and approved by the local ethical committee.

**Anti-CXCL16 Peptide Antibodies**

For the analysis of CXCL16 protein expression and studies of ox-LDL uptake, human CXCL16 peptides 59 to 73 and 200 to 214 were synthesized with Fmoc chemistry, conjugated to keyhole limpet hemocyanin, and used with Freund adjuvant for hyperimmunization of New Zealand White rabbits (AgriSera, Vanss, Sweden). Peptide antibodies were affinity-purified on peptide-conjugated Sepharose columns and used for experiments.

**Immunohistochemistry: ApoE−/− Mice**

Cryostat sections of the proximal aorta from 5-26-week-old mice were stained with an affinity-purified rabbit anti-mouse CXCL16 antibody (a generous gift from Drs M. Matloubian and J. G. Cyster) and with rat-anti-Cd4 (Clone H129.19; PharMingen, San Diego, Calif), followed by biotin-conjugated goat F(ab')2 anti-rat IgG (Southern Biotechnology Associates, Birmingham, Ala), or with biotinylated rat anti-mouse CD11b (Clone M1/70; PharMingen). Visualization was accomplished with an avidin–horseradish peroxidase conjugate (Vector Laboratories, Burlingame, Calif) as previously described. Sections were counterstained with hematoxylxylin. CXCL16 staining was controlled by application of a blocking peptide before peptide antibody, CXCR6 by normal rabbit serum as negative control, and CD4 and CD68 staining by using monoclonal antibodies of the same isotype (IgG1), but directed against an irrelevant antigen.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA from cell lines or mRNA from mouse aortas was extracted and quantified by real-time polymerase chain reaction (PCR) as previously described using the following primers and probes: mouse CXCL16, FW: 5-TCTTTTCTTGTGCGCCCTGT-3; RW: 5-CAGGCAACTGTC CCTGG T-3, and TM: 5-TGACCTTG GCCAGGGATGCC-3; mouse CXCR6, FW: 5-CCTTTTTGGG-CTATGAC-3; RW: 5-ATGCTCTGAGAAGTTCGAC-3, and TM: 5-CACCTATGAGTGGGTCTTTGGC-3; mouse CXCL16, FW: 5-CAAGGGATCC AGGGAGGTTGTGAG-3; RW: 5-CCAAAGTGTAACGTCGCAATAAGGA-3, and TM: 5-CCG- TACCCATCCCCACTGCTCTCCTC-3; mouse IFN-γ, FW: 5-TCAATGCACTAGATGTTGAAAGAA-3, RW: 5-TGGGCTCG- CAGGATTTCATCAG-3, and TM: 5-TACACATCTTTTGGG- CATGTTCTCCAG-3; mouse β-actin, FW: 5-AGAGGGAAATCCTG- TGCGTACG-3, RW: 5-CAATAGTGATGACCTGGCCGT-3, and TM: 5-CACTGGCCTCACTCTCTCCTCC-3, human CXCL16, FW: 5-GGGCCACCAGAAAGGCTTAC-3, RW: 5-CTGAGATGGGC CCCCCTCTGAG-3, and TM: 5-CCTACGCCACCCAAAATTTTCT CAGG-3; human CXCR6, FW: 5-ATGCGATCGAAGCCCTTAC-3, RW: 5-TTAAGCAGCCGCTCTCCAGTA-3, and TM: 5-AC- CATCATTGGTGACAGGCCCACATG-3; human β-actin, FW: 5-CTGGCTCGTCTGACCCGAGG-3, RW: 5-GAAGTCCTCACAATGACCTGGT-3, and TM: 5-CCTGAAACCCAAAGCCACCG-3. All data are presented normalized to β-actin mRNA expression.

**Flow Cytometry Analysis and Uptake of ox-LDL**

THP-1 (0.2×10^6 cells/well) and human peripheral blood mononuclear cells (2×10^6 cells/well) were incubated with IFN-γ (10 ng/mL) in RPMI-1640 containing 10% human serum for 4 days. Cells were removed by scraping, washed in PBS 0.5% bovine serum albumin (BSA), and incubated with 100 μg/mL anti-CXCL16 antibodies in 100 μL PBS 0.5% BSA at 4°C for 20 minutes and washed twice with PBS. Staining was detected with FITC-labeled goat anti-rabbit antibodies (1:20) (Dakopatts, Dakotap, Denmark). For analysis of ox-LDL uptake, THP-1 cells (0.2×10^6 cells/well) were incubated for 4 days with IFN-γ (10 ng/mL). Analysis of ox-LDL uptake was performed as previously described. For blocking experiments, equimolar concentrations of both anti-CXCL16 peptide antibodies (100 μg/mL in total) were added and incubated for 30 minutes at room temperature before the addition of ox-LDL. FACS-Calibur and a FASCStar (Becton Dickinson) with CellQuest software were used for acquisition and data analysis.

**Statistical Analysis**

Results are expressed in means±SEM. Student t test was used to assess differences between groups. Holm correction for multiple comparisons was performed in Figure 1. Differences were considered significant at P<0.05.

**Results**

**Expression of CXCL16 and CXCR6 in Atherosclerotic Lesions**

We studied the expression of CXCL16 in human endarterectomy specimens and also in a murine model of atherosclerosis, the apoE−/− mouse. Using real-time PCR, very low levels of CXCL16 mRNA expression could be detected in normal mammary arteries and veins. Much higher levels of CXCL16 mRNA were observed in atherosclerotic plaques (Figure 1). Importantly, mRNA levels for the CXCL16 receptor, CXCR6, were also elevated in human plaques (Figure 1).
To localize CXCL16 protein, a peptide antibody preparation was produced against amino acids 200 to 214 of the human CXCL16 peptide sequence. Staining of serial sections of human atherosclerotic lesions with this antibody showed the presence of CXCL16 in regions rich in CD68+ macrophages and CD4+ T cells (Figure 2). Preincubation of antibody with the peptide used to raise this antibody completely abrogated staining, confirming the specificity of the antibody reaction. The same regions of the affected vessels stained positively for CXCR6 (Figure 2). Similarly, preincubation of anti-CXCR6 antibody with the specific peptide completely abrogated staining (data not shown).

Elevated levels of CXCL16 mRNA were also detected in atherosclerotic aortas of apoE−/− mice when compared with the aortas of nonatherosclerotic, age-matched C57BL/6 mice (Figure 3). High levels of CXCL16 mRNA were found in 16-week-old apoE−/− mice fed 0.15% cholesterol for 10 weeks, with further increases in mRNA expression during disease progression (Figure 3). In parallel, increasing mRNA levels were registered for the macrophage marker, CD68, implying macrophage accumulation in lesions (Figure 3). CXCR6 mRNA was also expressed in these samples, indicating the presence in the tissue of cells capable of responding to CXCL16 (Figure 3).

Immunohistochemical analysis of mouse lesions revealed that CXCL16 protein was expressed in areas rich in CD11b+ macrophages and CD4+ T cells (please see online Figure I at http://atvb.ahajournals.org).

Figure 1. CXCL16 and CXCR6 mRNA expression in human atherosclerosis. Samples from human atherosclerotic carotid plaques (n=8) or mammary arteries (n=6) and veins (n=6) were analyzed for CXCL16 and CXCR6 mRNA expression by real-time reverse-transcription PCR. Both transcripts were significantly upregulated in the carotid lesions when compared with nonatherosclerotic arteries. Data are presented in mean±SEM; *P<0.05 vs nonatherosclerotic mammary arteries.

Figure 2. Immunohistochemical analysis of human atherosclerotic lesions in serial sections. Antibody binding was visualized by the avidin-biotin-peroxidase detection system. Sections were counterstained with hematoxylin. Original magnifications ×50 (left column) and ×400 (right column). CXCL16 staining shows positive labeling of areas rich in macrophages (CD68), T cells (CD4), and CXCR6-positive cells. Preincubation of CXCL16 antibody with the peptide used to raise this antibody completely abrogates staining. Staining for BONZO (CXCR6) shows positive labeling in areas rich in macrophages (CD68), T cells (CD4), and CXCL16.
CXCL16 Is Induced by IFN-γ and Mediates Uptake of ox-LDL

The coexistence of CXCL16-expressing macrophages and T cells in atherosclerotic lesions led us to examine the effects of the T-cell cytokine, IFN-γ, on the expression of CXCL16 in macrophages in vitro. Addition of 10 to 100 ng/mL IFN-γ to human monocytic THP-1 cells induced a 3- to 5-fold increase in expression of CXCL16 mRNA, and a modest increase was observed already at 0.1 ng/mL of IFN-γ (Figure 4A). CXCL16 mRNA levels were significantly higher compared with control after 24 hours of stimulation and increased continuously for up to 96 hours (Figure 4B).

Next, we analyzed CXCL16 protein expression after addition of IFN-γ (Figure 5). Similar to the findings on the mRNA level, incubation of THP-1 cells with IFN-γ for 4 days resulted in an increased cell surface CXCL16 expression, as revealed by fluorescence-activated cell sorter (FACS) analysis after staining with a peptide anti-human CXCL16 antibody (amino acid residues 59 to 73) (Figure 5A). CXCL16 mRNA levels were significantly higher compared with control after 24 hours of stimulation and increased continuously for up to 96 hours (Figure 4B).

In Vivo Treatment With IFN-γ Increases CXCL16 Expression in Atherosclerotic Lesions

Finally, we wanted to elucidate whether IFN-γ treatment affects the expression of CXCL16 in vivo. Eighteen-week-old ApoE−/− mice fed normal chow were injected once intraperitoneally with IFN-γ 8 hours before euthanization. CXCL16 gene expression was analyzed in atherosclerotic parts of the aorta and compared with mRNA levels in nonatherosclerotic parts (please see online Figure II at http://atvb.ahajournals.org). Administration of IFN-γ tended to increase CXCL16 mRNA in the atherosclerotic lesions (mean±SEM, 5.23±0.90 versus 3.03±0.53 for vehicle-treated controls), whereas no change was observed in the nonatherosclerotic parts (0.31±0.05 versus 0.45±0.04 for vehicle). Because macrophages are the predominant CXCL16-expressing cells,
we normalized CXCL16 mRNA levels for variations in the number of macrophages between lesions and mice using the constitutive macrophage marker, CD68. This showed a significant increase in CXCL16 per macrophage in the atherosclerotic (1.45 ± 0.17 versus 0.93 ± 0.15 for vehicle), but not in the nondiseased aorta (0.88 ± 0.11 versus 1.06 ± 0.08 for vehicle) (please see online Figure II). Therefore, IFN-γ increases CXCL16 expression in atherosclerotic lesions in vivo.

Discussion
The results of the present study show that: (1) both CXCL16 and its receptor, CXCR6/Bonzo, are expressed in human and murine atherosclerotic lesions; (2) the T-cell cytokine IFN-γ induces CXCL16 expression in macrophages in vitro and in atherosclerotic lesions in vivo; and (3) IFN-γ–dependent CXCL16 upregulation leads to increased oxLDL uptake in macrophages. Together, these findings identify CXCL16 as an IFN-γ regulated scavenger receptor/chemokine that may be important for foam cell formation and T-cell recruitment to the atherosclerotic plaque.

IFN-γ is expressed in atherosclerotic lesions in humans as well as in experimental models. It is known to inhibit the uptake of modified LDL through downregulation of the scavenger receptors, SR-A and CD36. It was therefore surprising that apoE−/− mice lacking IFN-γ receptor exhibit reduced atherosclerosis and lesion lipid accumulation. Analogously, treatment of apoE−/− mice with recombinant IFN-γ increases disease. The present data shed light on this paradox by showing that novel scavenger receptor, CXCL16, is upregulated by IFN-γ.

We have previously demonstrated that IFN-γ reduces the uptake of acetyl-LDL in macrophages. The present finding that IFN-γ increases ox-LDL uptake is in apparent contradiction to our previous data. However, it should be noted that CXCL16 does not recognize acetyl-LDL. Therefore, the previous study probably reflects the action of IFN-γ on SR-A, whereas the current one suggests that IFN-γ induces upregulation of CXCL16 as an important pathway by which IFN-γ promotes foam cell formation.

Our detection of CXCL16 in human atherosclerosis confirms the recent demonstration of SR-PSOX in plaques. The finding, in the present work, that IFN-γ upregulates CXCL16 in lesions in the apoE−/− mouse model and increases ox-LDL uptake in macrophages, suggests that the scavenger receptor activity of CXCL16 may be functional in the lesion, which is known to contain IFN-γ. However, CXCL16 has dual effects. In addition to its role for ox-LDL uptake, CXCL16 acts as a chemokine that promotes the recruitment of T cells. The latter effect depends on the expression of the CXCL16 receptor, CXCR6/Bonzo, by the T cells. We now show, for the first time to our knowledge, that CXCR6/Bonzo is expressed in atherosclerotic lesions. Interestingly, IL-2 and IL-15, two cytokines known to upregulate CXCR6/Bonzo, are also present in lesions.

The finding that the T-cell–derived cytokine IFN-γ increases CXCL16, which in turn promotes the recruitment of T cells, suggests a vicious circle that may act to increase...
inflammatory/immune activity in atherosclerotic lesions. In addition, some of these T cells are known to recognize ox-LDL presented to them after uptake by scavenger receptor-expressing macrophages. Together, these effects may add to the proatherogenic role of scavenger receptors and immune cells.

In conclusion, the finding that IFN-γ upregulates expression of CXCL16/SR-PSOX, a bifunctional protein that can act as a scavenger receptor and a chemokine, may provide a molecular link between foam cell formation, immune cell recruitment, and cytokine signaling in the development of atherosclerotic lesions.

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References
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Figure legends

**Figure I.** CXCL16 protein expression in mouse atherosclerotic lesions. Aortic roots of 26 week-old ApoE^{-/-} mice fed a Western diet for 20 weeks were analyzed for CXCL16, CD11b and CD4 protein by immunohistochemistry (representative sample out of n=5). CXCL16 was expressed in macrophage (CD11b) and T cell (CD4) rich areas.

**Figure II.** IFN-γ induces mRNA expression of CXCL16 in atherosclerotic areas of aortas from ApoE^{-/-} mice in vivo. Mice (age 18 weeks) were injected intraperitoneally with 200ng/animal IFN-γ (n=8) or PBS with 10% ApoE^{-/-} mouse serum as a vehicle (n=6). Eight hours after injection atherosclerotic aortic areas were dissected out and mRNA for CXCL16 and CD68 quantified by real-time reverse-transcription PCR. Data are presented for each transcript normalized to β-actin mRNA (upper two graphs), and also for the CXCL16/CD68 mRNA ratio (lower graph). Data is shown as means ± SEM, *P<0.05 vs. vehicle.
Figure I
**Figure II**

CXCL16

mRNA (normalized vs. β-actin)

CD68

mRNA ratio

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