OxLDL upregulates CXCR2 expression in monocytes via scavenger receptors and activation of p38 mitogen-activated protein kinase

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Abstract

Objective: Chemokine receptor CXCR2 has been implied to play a substantial role in pathogenesis of atherosclerosis, but the underlying molecular mechanisms remain to be clarified. In the present study, we examined the modulating effect of oxLDL on expression of CXCR2 and its functional effect in monocytes. Methods and results: OxLDL (20-\mu g protein/ml), but not LDL (80-\mu g protein/ml), upregulated the surface expression of the CXC chemokine receptor CXCR2 (measured by flow cytometry) in both human freshly peripheral blood monocytes and human monocytic U937 cells. OxLDL, but not LDL, increased CXCR2 mRNA determined by RT-PCR in both cells. Treatment of oxLDL (40-\mu g protein/ml) enhanced chemotaxis of U937 cells to IL-8 and their adhesion to an endothelial cell line, ECV304 (both \textit{P}<0.05 vs. control). Pretreatment of monocytes with scavenger receptor inhibitors, polyinosinic acid (100 \mu g/ml) and dextran sulfate (50 \mu g/ml) attenuated CXCR2 expression, but pertussis toxin or cholera toxin had no effect. OxLDL induced the activation of p38MAPK in monocytes, and this effect of oxLDL was blocked by the scavenger receptor inhibitors. Furthermore, p38 MAPK inhibitors SB203580 or SK&F86002 markedly reduced oxLDL-induced CXCR2 expression. Conclusions: This observation demonstrated that oxLDL upregulates CXCR2 expression in monocytes and promotes the chemotaxis and adhesion of monocytes. The effect of oxLDL is mediated through scavenger receptor and p38 MAPK activation. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Atherosclerosis is characterized at its early stage by the fatty streak, which is composed of cholesterol-loaded foam cells mostly derived from circulating monocytes that have taken up oxidized low-density lipoprotein (oxLDL) in subendothelial space [1,2]. Chemokines, which are classified into four distinct subfamilies as CXC, CC, C and CX\textsubscript{3}C according to the arrangement of the di-cysteine motif [3,4], have been demonstrated to be critically involved in the recruitment of monocytes into atherosclerotic lesion [5,6]. It has been shown that oxLDL induces substantial expression of CXC chemokines IL-8 in mono-

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mitogen-activated protein kinase (MAPK) appears to be a family of most essential ones. Three major subfamilies of structurally related MAPK have been identified in mammalian cells: p38 MAPK, p42/44 MAPK and JNKs/SAPKs. p38 MAPK subfamily, containing at least four members, is strongly activated in response to various stimuli, which has been implicated to play a critical role in the development of atherosclerosis [13]. We have previously demonstrated that oxLDL can activate p38 MAPK in vascular smooth muscle cells, which correlates with oxLDL-induced cytotoxicity [14].

The present study was designed to investigate the potential effect of oxLDL on the expression of CXCR2 in freshly collected peripheral blood monocytes (PBM) and U937 cells, a monocytic cell line as well as the functional role of CXCR2 expression. In addition, scavenger receptors and activation of p38 MAPK in response to oxLDL was also examined in this study.

2. Methods

2.1. Materials

FCS, 1640 RPMI and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from Gibco-BRL. IL-8, phycoerythrin-conjugated mouse antibody IgG1 (Ab\textsubscript{IgG1}), and phycoerythrin-conjugated mouse anti-human CXCR2 monoclonal antibody (Ab\textsubscript{CXCR2}) were from Pharmigen. The rabbit polyclonal phospho-p38 MAPK antibody specific for dual-phosphorylated Thr\textsuperscript{180} and Tyr\textsuperscript{182} of p38 MAPK, total-p38 MAPK antibody, and p38 MAPK assay kit were purchased from New England Biolabs Inc. Nitrocellulose membranes (Hybond) and enhanced chemiluminescence detecting system were obtained from Amersham Pharmacia Biotech. SB203580 and SK&F86002 [14,15] were from Calbiochem Inc. All other reagents, unless indicated, were from Sigma Chemical Co.

2.2. Lipoprotein isolation and oxidation

Endotoxin-free LDL and oxLDL were obtained as previously described [14]. LDL were separated from freshly drawn normal plasma by sequential ultracentrifugation and extensively dialyzed at 4°C against 0.15 mol/l NaCl and 0.01%EDTA (pH 8.0). After the EDTA was removed, LDL was undertaken to oxidative modification by Cu\textsuperscript{2+} incubation (5 µmol/l CuSO\textsubscript{4}, 20 h at 37°C). The extent of modification was assessed by the measurement of thiobarbituric acid-reactive substances (TBARS) and by determination of electrophoresis motility on agarose gels in barbital buffer at pH 8.6. The obtained oxLDL possessed a TBARS value of 25 nmol/mg of protein, whereas the LDL showed no detectable TBARS. The oxLDL moved 2–3 times faster on agarose gel electrophoresis than the LDL did.

2.3. Cell culture

Human PBM from healthy donor blood packs were obtained by Ficoll–Hypaque method and then by adherence to plastic, and cultured for 2 h at 37°C in a 5% CO$_2$ incubator. After removing the non-adherent cells, the adhered cells were 90% monocytes. A monocytic cell line, U937 [16] and an endothelial cell line, ECV304 [17], were cultured in RPMI 1640 supplemented with 10%-inactivated fetal serum, 100 u/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine.

2.4. Flow cytometric analysis

Cells were washed three times with cold buffer (PBS/1% FCS/0.1% sodium azide), resuspended in 100 µl binding buffer supplemented with 1 µg phycoerythrin-conjugated anti-CXCR2 mAb, or 1 µg phycoerythrin-conjugated isotype anti-IgG1 mAb control, and incubated on ice in the dark for 45 min. Subsequently, the cells were washed twice and analyzed using flow cytometry (Becton Dickson). Only PBM were accounted according to their light scatter and size during flow cytometry.

Mean Fluorescence Intensity was determined by subtracting the Mean Fluorescence Intensity of cells stained with Ab\textsubscript{IgG1} control from the Mean Fluorescence Intensity of cells stained with Ab\textsubscript{CXCR2} [18].

2.5. Reverse transcription-PCR (RT-PCR) analysis

Total mRNA from 10$^6$ cells was isolated with TRIzol (Gibco). First strand cDNA was then synthesized from 2 µg total RNA using reverse transcriptase (Gibco). A parallel control for DNA contamination was carried out without adding reverse transcriptase in the first strand synthesis. Primers were synthesized according to motif: CGGAATTCAAATGGAAGATTTTAACATGG (CXCR2, sense), CCGCTCGAGTTAGAGAGTAGTGGGAAATCTGAG (CXCR2, antisense), TCCATGACAATTCTTGCGACATGGTGGTGGAGTC (GAPDH, sense), and GTTGCTGGTTGAAGTCACAGGAGTC (GAPDH, antisense). cDNA was amplified by 30 cycles set to 94°C denaturation (60 s), 58°C annealing (60 s) and 72°C extension (60 s). PCR products were analyzed by 2% agarose gel electrophoresis. The specificity of the amplification was confirmed by DNA sequencing. The concentration of the reverse transcribed cDNA in the PCR mixture was adjusted to assure a linear correlation between template and product. The housekeeping gene GAPDH was used as a control template for normalizing relative changes of CXCR2 mRNA in RT-PCR.
2.6. Chemotaxis assay

Chemotaxis was assessed using a modification of transendothelial migration assay. The endothelial cell used for this assay was the endothelial cell line, ECV304. Endothelial cells were cultured on 6.5 mm diameter Transwell culture inserts (Costar) with an 8.0-μm-pore size. ECV304 (2×10⁵) were plated onto each insert of the 24-well chemotactic plate and incubated at 37°C for 48–96 h. IL-8 was added to the 24-well tissue culture plate in a final volume of 600 μL. U937 cells were resuspended in RPMI 1640 containing 1 mg/ml BSA. The plate was then incubated at 37°C in 5% CO₂/95% air for 1 h. The cells that had migrated to the bottom chamber were counted using microscope.

2.7. Static cell adhesion assays

ECV304 cells were plated in 96-well plates (3–4×10⁴ cells/well) and incubated for 48 h. U937 cells were pretreated with 40-μg protein/ml oxLDL for 24 h and then added to each well (2–3×10⁶ cells/well). The plate was incubated for additional 30 min at 37°C. Non-adherent U937 cells were removed by washing twice with PBS. Adherent U937 cells were counted in four low-powered microscopic fields for each treatment.

2.8. Western blotting

U937 cells, after different treatment, were lysed with the SDS sample buffer containing 62.5 mmol/l Tris (pH 6.8), 2% SDS (w/v), and 10% glycerol. Sample were heated at 95°C for 5 min and centrifuged (13 000 g, 5 min) at 4°C, and supernatant (20 μg protein/lane) was analyzed by SDS–PAGE in a 10% acrylamide gel. Proteins were transferred to nitrocellulose membranes and the membranes were blocked with 5% non-fat dry milk in TBST (20 mmol/l Tris [pH 8.0], 0.1% tween-20, and 150 mmol/l). The membranes were blotted with the primary antibody (phospho-p38 MAPK or total p38 MAPK) [19], then horseradish peroxidase-conjugated antibody, and detected by enhanced chemiluminescence detecting system according to the manufacturer’s instruction. For repeated immunoblotting, membranes were stripped in 62.5 mmol/l Tris (pH 6.7), 2% SDS, 0.1% tween-20, and 0.1 mmol/l 2-mercaptoethanol for 30 min at 70°C.

2.9. Statistical analysis

All data represent the mean±S.D. of at least three independent experiments. Student t-test was used for the statistical analysis of the results. Values of P<0.05 were considered to significant.

3. Results

3.1. OxLDL upregulated expression of CXCR2 in U937 cells

As shown in Fig. 1A, unstimulated U937 cells expressed CXCR2 at the lowest detectable level. Treatment of U937 with oxLDL, but not LDL, induced the upregulation of CXCR2 in a concentration- and time-dependent manner (Fig. 1B and C). As shown in Fig. 1D, treatment of U937 cells with oxLDL significantly induced expression of CXCR2 mRNA compared with control group. In contrast, the expression of CXCR2 remained unchanged in LDL-treated U937 cells.

3.2. OxLDL upregulated expression of CXCR2 in PBM

Consistent with the results obtained from U937 cells, treatment of PBM with oxLDL stimulated expression of CXCR2 in concentration- and time-dependent manners as compared to the unstimulated control (Fig. 2A, B, C). CXCR2 mRNA was also significantly upregulated by oxLDL treatment, while the untreated PBM expressed no detectable CXCR2 mRNA (Fig. 2D). Similarly, treatment of PBM with LDL did not affect the expression of CXCR2 either at protein or mRNA level (Fig. 2). These results indicated that the upregulatory effect of oxLDL on CXCR2 expression was not limited to U937 cells, which can thus serve as a good model for PBM study.

3.3. OxLDL enhanced chemotaxis of U937 cells to IL-8

The physiological role of increased CXCR2 expression was tested using a sensitive transendothelial chemotaxis assay by assessing the response of U937 cells to IL-8. IL-8 induced chemotaxis of unstimulated U937 cells to a maximal response at a concentration of 500 ng/ml. To determine the effect of oxLDL on chemotactic activity, treatment of U937 cells with oxLDL (40-μg protein/ml for 24 h), which significantly upregulated the surface CXCR2 as shown in Fig. 1, resulted in an enhanced chemotactic response at 500 ng/ml IL-8 about 2–3 folds as compared with that in the unstimulated cells (Fig. 3). Again, LDL treatment did not lead to any enhancement of chemotaxis of U937 cells to IL-8 (Fig. 3).

3.4. OxLDL enhanced adhesion of U937 cells to endothelial cells

Adhesion of U937 cells to endothelial ECV304 cells was determined to assess the functional role of CXCR2 upregulation by oxLDL. As shown in Fig. 4, exposure of U937 cells to oxLDL (40-μg protein/ml for 24 h) resulted in a marked increase in number of the U937 cells bound to ECV304 cells. Preincubation of the oxLDL-treated U937
cells with CXCR2 antibodies significantly reduced the oxLDL's effect (by about 50%), while addition of the control IgG1 antibodies exhibited no effect on the U937 cell binding. Our data suggested that upregulated CXCR2 by oxLDL was responsible for most of the enhanced binding of U937 cells to endothelial cells.
3.5. Scavenger receptors likely mediated the oxLDL-induced effects

To test whether the oxLDL-induced upregulation of CXCR2 was mediated by Scavenger receptors (SR), U937 cells were pretreated with SR inhibitors (polyinosinic acid (polyI) or dextran sulfate) before addition of oxLDL. The results showed that polyI and dextran sulfate (Fig. 5A) diminished CXCR2 upregulation induced by oxLDL to 56% and 41%, respectively. Pretreatment with either Gs...
protein inhibitor cholera toxin (CTX) or Gi/o protein inhibitor pertussis toxin (PTX) did not affect the oxLDL-induced CXCR2 upregulation (Fig. 5A), indicating an involvement of SR but not G protein-coupled receptors in the oxLDL-induced upregulation of CXCR2 in U937 cells.

In current study, it was shown that exposure of U937 cells to oxLDL could significantly enhance the activation of p38 MAPK but not the protein level of total p38 MAPK (Fig. 5B and C), and the p38 MAPK activation was in a dose-dependent manner (data not shown). Experiments...
oxLDL-induced activation of p38 MAPK plays an important role in signaling transduction.

According to earlier reports, U937 cells migration was consistently greater in the presence of IL-8 at 50 ng/ml, although this did not reach statistical significance ($P = 0.01$) [20]. We reported that IL-8 at 500 ng/ml induced an increase in U937 cells chemotaxis with a statistical significance, which was comparable to that of human PBM [10]. The absence of high-affinity binding sites on the monocytes might explain why higher concentration IL-8 was needed during monocytes chemotaxis by IL-8. Further functional studies will be necessary to assess the potential role of IL-8 in monocyte chemotaxis.

GRO$\alpha$ has the capacity to modulate monocyte-endothelial adhesion in vitro [7]. The effects of GRO$\alpha$ on monocytes appear to be mediated in part by rapid stimulation of the activation states and the ability to bind selected matrix constituents of specific leukocyte integrins [21,22]. OxLDL (Fig. 5B, C), demonstrating that SR likely mediated oxLDL-enhanced activation of p38 MAPK. SR-AI/IId, CD36, SR-BI, macrosialin/CD68, LOX-1, and SREC [25]. SRAI/SRAII, the first scavenger receptor to be fully characterized, can bind a broad range of modified lipoproteins and other ligands, including PolyI or dextran sulfate. Studies showed that the SR-AI/SRAII/apoE double-knockout mice developed smaller atherosclerotic lesions as compared to the apoE knockout mice [26]. These findings provide direct evidence that SR-A is involved in the development of atherosclerosis. The studies also showed that the other scavenger receptors, such as CD36, play important role in atherosclerosis [27] In the present study, we found that oxLDL upregulates CXCR2 gene expression that leads to chemotaxis of monocytes and monocyte adhesion to endothelial cells. Importantly, we found that these effects of oxLDL were mediated by its scavenger receptors. This became evidence since polyI or dextran sulfate inhibited oxLDL-induced CXCR2 upregulation.

Since it was shown that oxLDL effectively induced p38 MAPK activation that was mediated by SR, next we tested whether oxLDL-induced p38 MAPK activation was critical for its upregulation of CXCR2 expression. Two specific p38 MAPK inhibitors SB203580 and SK&F86002, which have been widely used in the study of p38 MAPK, were applied in this study. Our results demonstrated that incubation of U937 cells with either SB203580 or SK&F86002 dose-dependently diminished oxLDL-induced upregulation of CXCR2 expression (Fig. 6). Additional results showed that SB203580 or SK&F86002 dose-dependently blocked p38 MAPK activation at similar potency (data not shown). Our data thus indicated that p38 MAPK was indeed critically involved in the mediation of oxLDL-induced CXCR2 upregulation.

4. Discussion

In the present study, we demonstrated that oxLDL upregulates the expression of CXCR2 mRNA and protein in monocytes. The upregulation of CXCR2 causes chemotaxis of monocytes and increases monocyte adhesion to endothelial cells. The effect of oxLDL is mediated by scavenger receptors on monocytes since scavenger receptor blockers attenuate the action of oxLDL. In this process, oxLDL-induced activation of p38 MAPK plays an important role in signaling transduction.

Inhibition of p38 MAPK activation by its specific inhibitors SB203580 or SK&F86002 significantly reduced oxLDL-induced CXCR2 upregulation in monocytes. The
role of p38 MAPK activation in oxLDL signaling pathway was further confirmed by SR-A antagonists, poly1 or dextran sulfate that inhibited the activation of p38 MAPK in response to oxLDL. Many studies [31–33] have shown that oxLDL induces the activation of p38 MAPK and p42/p44 MAPK that further trigger transcription factor NF-kappAB activation leading to gene expression.

In summary, we demonstrate that oxLDL upregulated CXCR2 expression in human PBM and U937 cells in concentration- and time-dependent manners, and oxLDL’s effect was mediated through SR and activation of p38 MAPK. The upregulated expression of CXCR2 by oxLDL was functionally associated with oxLDL-enhanced chemotaxis of monocytes to IL-8 and their adhesion to endothelium.

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