Abstract—The subendothelial accumulation of macrophage-derived foam cells is one of the hallmarks of atherosclerosis. The recruitment of monocytes to the intima requires the interaction of locally produced chemokines with specific cell surface receptors, including the receptor (CCR2) for monocyte chemoattractant protein-1 (MCP-1). We have previously reported that monocyte CCR2 gene expression and function are effectively downregulated by proinflammatory cytokines. In this study we identified low density lipoprotein (LDL) as a positive regulator of CCR2 expression. Monocyte CCR2 expression was dramatically increased in hypercholesterolemic patients compared with normocholesterolemic controls. Similarly, incubation of human THP-1 monocytes with LDL induced a rapid increase in CCR2 mRNA and protein. By 24 hours the number of cell surface receptors was doubled, causing a 3-fold increase in the chemotactic response to MCP-1. The increase in CCR2 expression and chemotaxis was promoted by native LDL but not by oxidized LDL. Oxidized LDL rapidly downregulated CCR2 expression, whereas reductively methylated LDL, which does not bind to the LDL receptor, had only modest effects on CCR2 expression. A neutralizing anti–LDL receptor antibody prevented the effect of LDL, suggesting that binding and internalization of LDL were essential for CCR2 upregulation. The induction of CCR2 expression appeared to be mediated by LDL-derived cholesterol, because cells treated with free cholesterol also showed increased CCR2 expression. These data suggest that elevated plasma LDL levels in conditions such as hypercholesterolemia enhance monocyte CCR2 expression and chemotactic response and potentially contribute to increased monocyte recruitment to the vessel wall in chronic inflammation and atherogenesis. (Arterioscler Thromb Vasc Biol. 1998;18:1983-1991.)

Key Words: CCR2 ■ monocyte chemoattractant protein-1 ■ LDL ■ chemotaxis

The first grossly visible lesion in human and experimental atherosclerosis is the fatty streak, composed of cholesterol-loaded foam cells. Most of these cells are derived from circulating monocytes that have taken up residence in the subendothelial space.\(^1\)\(^-\)\(^4\) Increased monocyte recruitment can be explained in part by the production of chemoattractant factors and adhesion molecules by the cells of the arterial wall. LDL that has undergone minimally oxidative modifications has been shown to induce in aortic endothelial and smooth muscle cells the secretion of monocyte chemoattractant protein-1 (MCP-1),\(^5\)\(^,\)\(^6\) a member of the family of C-C or \(\beta\)-chemokines that attract mainly monocytes.\(^7\)\(^-\)\(^9\) Additional factors that can augment the recruitment of monocytes, primarily through induction of MCP-1 secretion by vascular smooth muscle and endothelial cells, include thrombin, which is generated at sites of vascular injury, macrophage colony-stimulating factor, and other inflammatory cytokines present in atherosclerotic lesions.\(^10\)\(^-\)\(^13\) Atherosclerotic lesions have a predilection for areas where the blood flow is disturbed, and hemodynamic forces may play a role in monocyte recruitment. Activation of endothelial cells by exposure to fluid mechanical forces mediates the secretion of MCP-1 and the differential expression of adhesion molecules implicated in atherosclerosis.\(^14\)\(^-\)\(^16\) The strong expression of MCP-1 in human and rabbit atherosclerotic lesions is a further indication that this chemokine might be importantly involved in monocyte recruitment during atherogenesis.\(^17\)\(^,\)\(^18\)

Monocyte recruitment is a multistep process.\(^19\) The firm adhesion to the endothelium is supported by adhesion molecules, and subsequent transendothelial migration is mediated by the interaction of locally produced chemokines with cell surface receptors.\(^9\)\(^,\)\(^20\) The receptor for MCP-1, CCR2, has been cloned recently and belongs to the family of 7 transmembrane-spanning, GTP-binding protein–coupled receptors.\(^21\) Two isoforms of the human MCP-1 receptor have been identified. Both CCR2A and CCR2B, the latter being

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the predominant form on human monocytes, are highly homologous and differ only in their carboxyl tail. Recent studies in our laboratory have demonstrated that monocyte CCR2 expression is affected by inflammatory modulators and is effectively downregulated by proinflammatory cytokines, growth factors such as granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and MCP-1 itself. Thus, factors produced by cells of the vessel wall can control monocyte responses through modulation of CCR2 expression, may prevent a disproportionate inflammatory response, and support the arrest of recruited monocytes in lesions.

In addition to cytokines and modified forms of LDL, native LDL at concentrations typical for hypercholesterolemia has been demonstrated to affect endothelial gene expression, resulting in increased monocyte binding. Reciprocally, monocytes from hyperlipidemic subjects or animal models have been shown to be more responsive to inflammatory stimuli and have shown increased cell motility, chemotaxis, and adherence to cultured endothelial cells compared with monocytes from normolipidemic controls. However, the mechanisms by which these functional changes occur are not known.

In the present study, we examined the effects of atherogenic lipoproteins on CCR2 expression. Our data demonstrate that native plasma LDL, but not oxidized LDL, rapidly upregulates CCR2 expression and profoundly enhances the monocyte chemotactic response to MCP-1. These results indicate that elevated plasma LDL levels in hypercholesterolemia may increase monocyte chemotactic responses and cause excessive monocyte infiltration and accumulation in atherosclerotic lesions.

Methods

Materials

Cell culture medium and Hanks’ balanced salt solution were purchased from Life Technologies, Inc. FCS was from Hyclone Laboratories. Recombinant human MCP-1 and the neutralizing monoclonal anti-human MCP-1 antibody were obtained from R&D Systems Inc. The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine and dioctyl phthalate, and the radioactivity associated with the cell pellet was counted. The specific 125I-MCP-1 binding was determined by subtracting the nonspecific binding estimated in the presence of 100 nmol/L unlabeled MCP-1 from total binding. To determine the exact binding affinity as given in Table 2 and validate the estimation of Bmax by the end-point method described above, the cells were incubated with 0.07 nmol/L 125I-MCP-1 in the presence of increasing concentrations (from 0.001 to 100 nmol/L) of unlabeled MCP-1, and the data were analyzed using the LIGAND program. The receptor number

Equilibrium Binding Analysis

THP-1 monocytes incubated under various conditions were isolated by centrifugation and washed 3 times with PBS, and cell number was determined by counting an aliquot, stained with crystal violet, under a microscope. The cells were resuspended at 2 x 10⁶ cells/mL in 200 µL of binding buffer consisting of 50 mmol/L HEPES, 1 mmol/L CaCl₂, 5 mmol/L MgCl₂, and 0.5% BSA, pH 7.2, and binding assays were carried out as described. In brief, the cells were incubated for 90 minutes at 25°C with 0.07 nmol/L 125I-MCP-1 (specific activity, 2200 Ci/mmol) in the absence or presence of 100 nmol/L unlabeled MCP-1. The reaction was terminated by separating the cells from the incubation buffer by centrifugation through a 1:1 mixture (vol/vol) of dibutyl phthalate and dioctyl phthalate, and the radioactivity associated with the cell pellet was counted. The specific 125I-MCP-1 binding was determined by subtracting the nonspecific binding estimated in the presence of 100 nmol/L unlabeled MCP-1 from total binding. To determine the exact binding affinity as given in Table 2 and validate the estimation of Bmax by the end-point method described above, the cells were incubated with 0.07 nmol/L 125I-MCP-1 in the presence of increasing concentrations (from 0.001 to 100 nmol/L) of unlabeled MCP-1, and the data were analyzed using the LIGAND program. The receptor number

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determined by ligand binding was normalized to cell number and expressed as fmol/10^6 cells.

**Analysis of CCR2 Transcripts**

Total RNA was isolated by guanidinium thiocyanate–phenol-chloroform extraction. The mRNA was reverse-transcribed with Superscript II (Life Technologies Inc) using 2 μg of total RNA and 0.12 nmol oligo(dT)_18 primers (Boehringer Mannheim). CCR2 gene expression was then estimated by semiquantitative polymerase chain reaction (PCR). Two primers with the sequences 5'-ATGCTGTCCACATCCTCGTG (sense primer) and 5'-TTATAAACCCGAGGCACCTGGTC (antisense primer) were used to amplify the full-length cDNA of CCR2B (1083 bp) as described previously. Routinely, 25 to 30 PCR amplification cycles were used, and the concentrations of the reverse-transcribed template cDNA in the reaction mixture was adjusted to obtain a linear relation between template and product. The specificity of the amplification was confirmed by DNA sequencing of the PCR product. The amplified DNA was analyzed by agarose gel electrophoresis and stained with ethidium bromide. The intensity of the stained band was analyzed by densitometric scanning (Image Quant, Molecular Dynamics) and compared with that of the internal standard. As an internal standard, GAPDH was amplified and analyzed under identical conditions using 5'-TCGGAGTCAACGGATTTGTCGTA as the sense primer and 5'-ATGGAGCTGGTGCTAGTCCCT as the antisense primer. The expected size of the amplified cDNA was 520 bp, which was used to normalize relative changes of CCR2 mRNA. As a negative control to test for contamination with genomic DNA, RNA samples without prior treatment with reverse transcriptase were used for PCR analysis using the primers described above. All samples tested negative for genomic DNA contamination.

**Chemotaxis Assay**

THP-1 monocytes incubated under various conditions were rinsed 3 times with PBS and then suspended at a concentration of 1.5×10^6 cells/mL in chemotaxis buffer consisting of Tyrode's salt buffer (Sigma), 1% NaHCO_3, and 0.5% BSA, pH 7.4. The chemotacticants MCP-1 or N-formyl-methionyl-leucyl-phenylalanine were added to the lower chamber of the 48-well microchemotaxis Boyden chamber (Neuroprobe) at a concentration of 10 nmol/L in chemotaxis buffer. The cell suspension (5×10^5 cells/mL) was added to the upper chamber, which was separated from the lower chamber by a 5-μm-pore-size polycarbonate membrane facing the chemoattractant. Three replicate filters were used for each treatment. As a control, chemotaxis induced by MCP-1 was inhibited by including a neutralizing mouse anti–human MCP-1 monoclonal antibody.

**Determination of Cellular Cholesterol**

Cellular lipids were extracted from 10^6 cells with chloroform/methanol (2:1, vol/vol), dried under N_2, and resuspended in 50 μL isopropanol. Free and total cholesterol contents after hydrolysis of cholesterol ester with cholesterol hydrolyase were estimated by the fluorometric method, and the content of cholesterol ester was calculated by subtracting free cholesterol from total cholesterol.

**Other Analytical Procedures**

Protein was determined by the method of Lowry et al. Data are expressed as mean±SD and were analyzed by Student's t test. Plasma triglycerides and total, HDL, and LDL cholesterol were analyzed in the Lipid Analytical Laboratory of the La Jolla Lipid Clinic. The analytical procedures used have been standardized by the Centers for Disease Control and Prevention, Atlanta, Ga. Total cholesterol, HDL cholesterol after removal of other lipoproteins by heparin/manganese precipitation, and triglycerides were measured by enzymatic techniques with an Abbott VP analyzer (Abbott Laboratories) using cholesterol reagent (Boehringer Mannheim) and triglyceride reagents (Abbott Laboratories).

**Results**

**Induction of CCR2 Expression by LDL**

THP-1 monocytes were routinely grown in RPMI 1640 medium supplemented with 10% serum. The final concentration of total cholesterol in the growth medium was, on average, 80 μg/mL and that of LDL cholesterol was 30 μg/mL, which equates to ∼15 μg of LDL protein per mL. THP-1 monocytes maintained under these conditions showed a specific binding of 5.9±0.6 fmol MCP-1 per 10^6 cells. Treatment with freshly isolated human plasma LDL at 5 and 50 μg LDL protein per mL for 24 hours induced a 1.2-fold (P<0.05) and a 1.7-fold, respectively, increase in CCR2 expression (Figure 1A).

To determine whether plasma LDL affects gene transcription or translation, THP-1 monocytes were treated with LDL (5 and 50 μg protein per mL) for 24 hours, and total RNA was isolated from the untreated as well as LDL-treated cells. The steady-state level of CCR2 transcripts was estimated by semiquantitative PCR analysis of reverse-transcribed mRNA as described under Methods. As an internal control, the housekeeping gene GAPDH was estimated under identical conditions and used to normalize relative changes in CCR2 mRNA levels. The presence of human LDL in the culture medium had a profound effect on CCR2 expression and induced a 2.5- to 3-fold increase of CCR2 transcripts in THP-1 cells (Figure 1B).

To demonstrate that LDL-mediated CCR2 expression was not peculiar to THP-1 cells, we determined CCR2 mRNA levels in freshly isolated human blood monocytes. Four normolcholesterolemic females (LDL cholesterol <100 mg/dL) and 11 hypercholesterolemic females (LDL cholesterol >130 mg/dL) matched for age were studied. In all cases, the monocyte CCR2 expression, estimated by semiquantitative PCR amplification of reverse-transcribed mRNA, was higher in individuals of the hypercholesterolemic group than in the ones of the control group (Figure 1C). The plasma lipid profile and monocyte CCR2 expression of all subjects studied are summarized in Table 1. On average, the CCR2 expression in the hypercholesterolemic group was ∼2-fold higher compared with the control group. Analysis of the data suggested a significant correlation between the LDL cholesterol level and CCR2 mRNA expression.

The time course of the effect on CCR2 expression was examined by incubating THP-1 monocytes with LDL (50 μg of protein per mL) for as long as 72 hours. Surface expression was determined by 125I–MCP-1 binding analysis at the various time points. By 6 hours, the binding sites for MCP-1 on the plasma membrane were increased by 20% (P<0.006) and by 48 hours, a doubling of MCP-1 binding was observed (Figure 2). The results from CCR2 mRNA expression and these binding studies suggested that the LDL-mediated increase in CCR2 expression was regulated primarily at the level of gene transcription.

Receptor surface expression was determined by end-point ligand binding analysis at saturating ligand concentrations and by assuming an unchanged binding affinity of ∼0.5
Effect of Increased CCR2 Expression on Chemotaxis

The functional relevance of the increased density of CCR2 on the cell surface was analyzed by chemotaxis assays. THP-1 monocytes were incubated for 24 hours in culture medium supplemented with increasing concentrations of freshly isolated LDL, and the cells’ chemotactic response to MCP-1 and N-formyl-methionyl-leucyl-phenylalanine was examined. As in previous experiments, human LDL added at concentrations as low as 5 μg protein per mL induced a 20% increase in CCR2 surface expression (Figure 1). However, this rather small increase in receptor number had a profound effect on chemotaxis, almost doubling the response to 10 nmol/L MCP-1 (Figure 3). A close correlation between LDL concentration in the medium, the number of cell surface receptors for MCP-1, and the chemotactic responsiveness was observed.

![Figure 1: LDL-induced expression of monocyte CCR2. A, 125I-MCP-1 binding analysis. For the final 24 hours before analysis, THP-1 monocytes were cultured in complete growth medium supplemented with the indicated amounts of freshly isolated native human LDL. After treatment with LDL, the cells were collected by centrifugation and washed with PBS, and 125I-MCP-1 binding analysis was performed as described under Methods. Shown is the specific binding of 125I-MCP-1 calculated by subtracting nonspecific binding, determined in the presence of 100 nmol/L unlabeled MCP-1, from total binding. Data are expressed as percent specific binding relative to that of untreated control cells (100%), which bound 5.9±0.6 fmol 125I-MCP-1 per 10⁶ cells. The results represent the mean±SD of 3 independent experiments. B, Effect of LDL on CCR2 mRNA. THP-1 monocytes were treated with LDL as described above. Total RNA was isolated, reverse-transcribed, amplified by PCR, and analyzed by gel electrophoresis as described under Methods. For PCR, primers were chosen to yield the full-length coding region of CCR2B (1083 bp) and a 520-bp fragment of GAPDH that was included as an internal standard. Lanes 1 and 4, untreated control cells; lanes 2 and 5, cells treated with LDL at 5 μg of protein per mL; and lanes 3 and 6, cells treated with LDL at 50 μg protein per mL LDL. C, Monocyte CCR2 expression in hypercholesterolemic patients and normocholesterolemic controls. Monocytes were isolated from 4 controls with LDL cholesterol (LDL-C) levels <100 mg/dL and from 11 patients with LDL-C levels >130 mg/dL. Total RNA was prepared, and CCR2 expression was determined as described above and analyzed by gel electrophoresis. Lanes 1 through 4 represent controls, and lanes 5 through 7 are a representative selection of the 11 patients with LDL-C levels >130 mg/dL. CCR2 is shown in the upper panel and GAPDH, included as an internal control, is displayed in the lower panel.

To determine whether LDL induced any changes in binding affinity, we generated complete binding curves with untreated THP-1 cells and with THP-1 cells that had been treated with LDL (50 μg of protein per mL) for 24 and 48 hours. As shown in Table 2, the binding affinities remained essentially unchanged. Consistent with the data reported above, the number of binding sites for MCP-1 increased 1.7-fold after 24 hours and 2.1-fold after 48 hours. These studies demonstrated that LDL induces a time-dependent increase in monocyte CCR2 expression without affecting the binding affinity.

![Figure 2: Kinetics of MCP-1 receptor expression. THP-1 monocytes were preincubated for the indicated time periods with freshly isolated, native, human LDL at a concentration of 50 μg protein per mL. Specific 125I-MCP-1 binding analysis was performed as described in Figure 1. Data are expressed as percent specific binding relative to that of control cells (100%), which bound 4.9±0.2 fmol 125I-MCP-1 per 10⁶ cells. The results represent the mean±SD of 3 independent experiments.

![Image](http://atvb.ahajournals.org/)

**TABLE 1. Lipid Profile and Monocyte CCR2 Expression in Normocholesterolemic and Hypercholesterolemic Subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normocholesterolemic Group (n=4)</th>
<th>Hypercholesterolemic Group (n=11)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>50.8±3.8</td>
<td>55.6±5.1</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.7±2.7</td>
<td>24.5±2.3</td>
<td>NS</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>159.8±15.8</td>
<td>240.1±26.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>169.5±96.1</td>
<td>197.8±60.4</td>
<td>NS</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>46.5±13.8</td>
<td>40.4±3.8</td>
<td>NS</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>79.4±9.8</td>
<td>167.0±20.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CCR2, % of GAPDH</td>
<td>45.7±25.3</td>
<td>105.0±37.1</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; TC, total cholesterol; and TG, triglycerides. Monocytes were isolated from female donors with LDL cholesterol levels <100 mg/dL (normocholesterolemic group) and from those with LDL cholesterol levels >130 mg/dL (hypercholesterolemic group). The donors were matched for age, and all were nonsmokers. CCR2 expression was estimated by semiquantitative PCR as described under Methods and expressed as % of GAPDH levels estimated under identical conditions.

*Analyzed by independent Student's t test.
When the basal concentration of LDL in the medium (≈15 μg LDL protein per mL, which equates to ≈30 μg LDL cholesterol per mL) was increased by 500 μg of LDL protein per mL (≈1 mg LDL cholesterol per mL), the chemotactic response of the treated THP-1 monocytes to 10 nmol/L MCP-1 increased ~3-fold compared with the untreated controls. This response was almost completely abolished by a neutralizing mouse anti–human MCP-1 antibody. The chemotaxis induced by MCP-1 in cells treated with 500 μg LDL protein per mL was identical to that induced by 10 nmol/L N-formyl-methionyl-leucyl-phenylalanine. This peptide, a synthetic analogue of naturally occurring products derived from bacteria during infection, is commonly used to study activation of neutrophils and monocytes through the receptor for N-formyl peptides. The chemotactic activity in response to the N-formyl peptide was not affected by LDL.

Potential Mechanisms of Lipoprotein-Induced CCR2 expression

To determine whether the effect on CCR2 expression was specific for native LDL or whether modified forms of LDL had similar effects, we incubated THP-1 monocytes with mildly and fully oxidized LDL (50 μg protein per mL) for as long as 48 hours.

### Table 2. Effect of LDL Treatment on Receptor Expression and Binding Affinity

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Bmax, fmol/10⁶ Cells (Mean ± SD)</th>
<th>Affinity, nmol/L (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.9 ± 0.6</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>24</td>
<td>10.0 ± 1.8</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>48</td>
<td>12.2 ± 0.7</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

125I–MCP-1 binding analysis was performed on THP-1 monocytes treated with LDL at a concentration of 50 μg protein per mL for 0, 24, or 48 hours as described under Methods. Maximal binding sites (Bmax) and binding affinities were determined by using the LIGAND program.

![Figure 3. Effect of LDL on monocyte chemotaxis. THP-1 monocytes were treated for 24 hours with LDL at the indicated concentrations. MCP-1 or N-formyl-methionyl-leucyl-phenylalanine (FMLP) was added to the lower compartment of the Boyden chamber at a concentration of 10 nmol/L. The cells treated under the various conditions were placed in the upper chamber, and the chemotaxis assays were conducted as outlined under Methods. A neutralizing mouse anti–human MCP-1 monoclonal antibody (anti MCP-1) was added at 5 μg/mL together with the chemokine in control experiments. The number of migrated cells was determined by counting 5 random high-power microscope fields and expressed as the mean number of cells per field. Data represent the mean ± SD of 3 individual experiments.](http://atvb.ahajournals.org/)

![Figure 4. Effect of oxidized LDL on CCR2 expression. THP-1 monocytes were incubated for the indicated time periods with modified LDL at a concentration of 50 μg protein per mL. The cells were then isolated by centrifugation and washed with PBS, and CCR2 expression was determined by 125I–MCP-1 binding analysis as described in Figure 1. A, THP-1 monocytes were treated with fully oxidized LDL. Untreated cells bound 4.2 ± 0.6 fmol 125I–MCP-1 per 10⁶ cells, which was taken as 100%, and binding by the treated cells was expressed relative to that of the control cells. B, THP-1 monocytes were treated with mildly oxidized LDL. Binding of 125I–MCP-1 to untreated cells was 4.5 ± 0.8 fmol/10⁶ cells and was taken as 100%. The results represent the mean ± SD of 3 independent experiments.](http://atvb.ahajournals.org/)

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To determine whether the effect on CCR2 expression was specific for native LDL or whether modified forms of LDL had similar effects, we incubated THP-1 monocytes with mildly and fully oxidized LDL (50 μg of protein per mL) for as long as 48 hours. The extent of oxidation of mildly oxidized LDL, prepared by brief incubation with copper, was similar to that of mildly oxidized LDL described by others and contained ≈4 nmol TBARS per mg protein. Fully oxidized LDL prepared by extensive oxidation with copper contained ≈55 nmol TBARS per mg protein. No cytotoxicity was observed at the concentrations of oxidized LDL used, and the proliferation rate and viability of the THP-1 monocytes remained unchanged. Fully oxidized LDL rapidly downregulated CCR2 expression, and by 6 hours, the binding sites for MCP-1 were reduced by ~60% (Figure 4A). After
24 hours, essentially no cell surface expression of CCR2 was detected, and only residual binding of MCP-1 was observed. Mildly oxidized LDL had no effect on monocyte CCR2 expression, and the binding of 125I–MCP-1 remained unchanged (Figure 4B).

To test whether the response to LDL required interaction with the LDL receptor, THP-1 monocytes were incubated for as long as 48 hours in complete medium supplemented with human LDL (50 μg protein per mL) in the absence or presence of anti–LDL receptor IgG C7 (1:200 dilution), an antibody that effectively blocks the interaction of LDL with its receptor.47 This antibody completely prevented the LDL-induced expression of CCR2 (Figure 5). To further test whether the effect was mediated by the LDL receptor, we incubated THP-1 monocytes with reductively methylated LDL (50 μg of protein per mL) for as long as 48 hours. Reductive methylation of 30% or more of the lysine residues prevents the binding of LDL to surface receptors.36 About 80% of the lysine residues were methylated in our preparation. Although so modified LDL was not recognized by the LDL receptor, it induced a small increase in expression of CCR2 (Figure 5). This increase of LDL-derived intracellular cholesterol was completely blocked by the presence of the anti–LDL receptor IgG C7 (Figure 6B), which also prevented any LDL-induced CCR2 expression (Figure 5). Although modification of lysine...
residues retards the clearance rate of LDL in animals, it is eventually removed from the plasma through unknown mechanisms, perhaps receptor-independent pinocytosis. Methylated LDL may be taken up by THP-1 monocytes by a similar mechanism, and this possibility might account for the slightly increased intracellular cholesterol content (Figure 6C). The magnitude of this rise paralleled the increase in CCR2 expression (Figure 5), suggesting that raising cellular cholesterol levels might trigger CCR2 gene expression.

To test this hypothesis, we incubated THP-1 monocytes with 25 μg/mL of free cholesterol for 24 hours. This treatment increased the cellular pool of cholesterol ~4-fold (Figure 7A) and caused a 2-fold increase in CCR2 expression (Figure 7B), which was not affected by the anti–LDL receptor IgG C7 (data not shown). Interestingly, CCR2 surface expression remained elevated, even after the cholesterol-loaded cells were returned to normal growth medium without supplemental cholesterol and after the cellular cholesterol had dropped significantly.

**Discussion**

Most studies on monocyte recruitment have focused on lipoprotein-induced changes in cells of the arterial wall, whereas the effects of lipoproteins on gene expression in the monocyte have received little attention. Gerrity et al showed that a chemotactic factor isolated from atherosclerotic lesions strongly stimulated chemotaxis in monocytes isolated from hypercholesterolemic swine but only weakly in monocytes from control swine. The reported molecular mass of the chemotactic factor was ~6000 Da, similar to that of human MCP-1, now known to be synthesized and secreted by cells in atherosclerotic lesions. Similar studies in human subjects indicated that monocytes isolated from hypercholesterolemic patients are functionally different and adhere in larger numbers to cultured endothelial cells than do monocytes from matched controls.

Our findings suggest that the increased monocyte accumulation in the vessel wall during arterogenesis may result in part from an enhanced chemotactic response due to upregulated CCR2 gene expression. THP-1 monocytes express, on average, 5000 MCP-1 receptors per cell, which is comparable to the number established for freshly isolated human monocytes. A finely tuned network of cytokines regulates the expression of CCR2. The transformation of monocytes from an MCP-1–responsive state to a state in which monocytes no longer respond to MCP-1 is caused by a reduction of CCR2 expression triggered by certain proinflammatory cytokines. Analysis of CCR2 message indicated that the primary factors for the increased CCR2 expression include augmented rate of gene transcription and changes in processing or stability of the mRNA. Translational regulation, receptor sequestration, and recycling from intracellular stores represent other possible mechanisms by which receptor surface density can be varied. By 6 hours of treatment with LDL, the number of CCR2 molecules on the cell surface increased by ~20%. De novo protein synthesis characteristically requires more time, and these receptors most likely represent a population that was recruited from intracellular stores. However, the major increase of CCR2 protein, measured by MCP-1 binding, was observed during the subsequent 24 to 48 hours, which is characteristic for de novo protein synthesis.

Chemotaxis is one of the main responses of monocytes triggered by MCP-1. The initial binding of MCP-1 is followed by transmembrane signaling, amplification of the primary stimulus, and subsequent induction of cellular responses. CCR2 expression in monocytes is low compared with the 50 000 to 100 000 receptors for N-formyl-methionyl-leucyl-phenylalanine. Although the receptor occupancy necessary for optimal chemotactic response to MCP-1 is unknown, if one considers the relatively low basal expression of monocytes to cultured endothelial cells than do monocytes from matched controls.
CCR2 (5000 receptors per cell), small changes in receptor number can profoundly affect chemotactic activity. In a recent study, we have demonstrated negative control of CCR2 gene expression by proinflammatory cytokines, which is associated with a decrease in chemotactic activity. In contrast, a 20% increase of CCR2 on the cell surface, induced by the lowest LDL concentration tested, almost doubled the chemotactic response of THP-1 monocytes to MCP-1. A close correlation between LDL concentration in the medium, the number of receptors for MCP-1 on the cell surface, and the chemotactic response was observed. Up to 3-fold increases in monocyte chemotactic activities were recorded at increasing LDL concentrations. Interestingly, the chemotactic response of the cells to N-formyl-methionyl-leucyl-phenylalanine remained unchanged by the LDL treatment. The receptor for this chemotactic peptide is either not affected by LDL or the basal receptor expression, which is at least 10-fold higher than that of CCR2, is already sufficient for optimal chemotaxis.

Leukocyte extravasation is a multistep process that involves reversible rolling, firm adhesion, and transendothelial migration. Several of the 7 transmembrane-spanning chemotractant receptors have been shown to support integrin-mediated firm adhesion of leukocytes to the endothelium. Thus, an increase in CCR2 expression as seen after exposure to high plasma LDL levels may support sustained, firm adhesion of monocytes to the vessel wall and cause excessive monocyte recruitment. This hypothesis was confirmed by results from a recent study on CCR2-deficient mice, demonstrating that CCR2 is not only essential for monocyte chemotaxis but also might play an important role in the firm adhesion of monocytes to the endothelium preceding diapedesis.

Recent studies have demonstrated that LDL is capable of eliciting intracellular signaling responses in a variety of cells. However, the effect of LDL on CCR2 expression appears independent of such signaling events and is primarily caused by the cellular levels of cholesterol. The exact mechanism by which cholesterol controls the biosynthesis of CCR2 is still unknown and will be the topic of future studies. Analysis of exon 1 of CCR2 revealed the presence of a sterol-regulatory element-like sequence, CAACGCAC, in the 5′ untranslated region, suggesting that sterols may play an important role in CCR2 gene regulation.

Oxidized LDL, found mainly in atherosclerotic lesions, is rapidly taken up by monocytes/macrophages through scavenger receptor–mediated endocytosis. Although this process causes massive accumulation of cellular cholesterol, it had the opposite effect on CCR2 expression. In contrast to LDL, oxidized LDL induced a rapid loss of monocyte CCR2. Oxidized LDL has been shown to stimulate monocyte-macrophage differentiation, probably by inducing cytokine secretion. From a previous study, we know that cytokine-induced activation or differentiation of monocytes resulted in a reduction of CCR2 expression, which may explain the effect of oxidized LDL. The extent to which LDL was oxidized largely determined its effect on CCR2 expression, and mildly oxidized LDL did not influence CCR2 expression. The effect of cholesterol on CCR2 expression may be blunted by some other components of mildly oxidized LDL, and no change in CCR2 expression was observed.

In summary, we have demonstrated that levels of LDL that are characteristic for hypercholesterolemia cause increased CCR2 expression and chemotactic activity in monocytes. Cytokines modulate the immune response by regulating the secretion of chemokines as well as by controlling CCR2 expression. High LDL concentrations in the plasma may disturb the delicate regulation of CCR2 expression by these cytokines and may result in excessive accumulation of monocytes in the arterial wall.

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References


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