Granulocyte/macrophage Colony-stimulating Factor Plays an Essential Role in Oxidized Low Density Lipoprotein-induced Macrophage Proliferation

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We and other groups have recently demonstrated that oxidized low density lipoprotein (Ox-LDL) induces proliferation of macrophages in vitro. Since previous immunohistochemical studies demonstrated that macrophages and macrophage-derived foam cells proliferated in situ in atherosclerotic lesions, it seems reasonable to expect that the Ox-LDL induced macrophage proliferation might be linked to the development of atherosclerotic lesions. Thus, clarification of the molecular cascades of Ox-LDL-induced macrophage proliferation is expected to enhance our knowledge of the pathogenesis of atherosclerosis. Recently, we demonstrated that the activation of PKC leads to release into the culture medium of granulocyte/macrophage colony-stimulating factor (GM-CSF) which plays an important role in Ox-LDL-induced macrophage proliferation. In this review article, we mainly show the role of GM-CSF in the Ox-LDL-induced macrophage proliferation. Moreover, based on our recent findings, we summarize the Ox-LDL-induced signaling pathway for macrophage proliferation. J Atheroscier Thromb, 2000; 7: 14-20.

Key words: Macrophage, Ox-LDL, Atherosclerosis, GM-CSF, proliferation

Background

A massive cluster of macrophage-derived foam cells observed in the subendothelial spaces is one of the characteristic features of the early stage of atherosclerotic lesions (1). Macrophages take up chemically modified low density lipoproteins (LDL), such as oxidized LDL (Ox-LDL) and acetylated LDL (Ac-LDL) through the so-called scavenger receptors, and become foam cells in vitro (2-4). Foam cells produce various bioactive molecules, such as cytokines and growth factors, which play essential roles in the development and progression of atherosclerotic lesions (1).

One of the characteristic events in the atherosclerotic lesion is the proliferation of cellular components of arterial walls. Several morphological studies emphasize the presence of macrophages and macrophage-derived foam cells proliferating in the early stage of atherosclerotic lesions (5-7). Gordon et al. (6) demonstrated that 27% of PCNA (proliferating cell nuclear antigen)-positive cells in atherosclerotic lesions of human coronary arteries corresponded to macrophages. In the aortae of both the Watanabe heritable hyperlipidemic rabbits (WHHL) and cholesterol-fed rabbits, Rosenfeld and Ross (5) demonstrated that 30% of cells incorporating [3H]thymidine were derived from macrophages and 12% of these labeled cells showed foam cell morphology. Spagnoli et al. (7) demonstrated that foam cells of the rabbit atherosclerotic plaque arrested in metaphase by colchicine showed a macrophage phenotype. Since macrophage-derived foam cells play an crucial role in the development of atherosclerotic lesions, it seems reasonable to expect that macrophage proliferation may enhance the progression of atherosclerosis. Thus, to elucidate the pathogenesis of atherosclerosis, it seems reasonable to examine the mechanisms of macrophage proliferation. In this regard, we (8-17) and other groups (18-20) recently demonstrated...
that Ox-LDL induced macrophage proliferation in vitro. These in vitro observations strongly imply that Ox-LDL might act as a proliferation inducer for macrophages in vivo. In this review article, we focus on the role of granulocyte/macrophage colony-stimulating factor (GM-CSF) in macrophage proliferation. Moreover, based on our recent findings, the Ox-LDL-induced signaling pathway for macrophage proliferation is summarized.

Involvement of a soluble factor(s) in Ox-LDL-induced macrophage proliferation

There are two possible mechanisms for macrophage proliferation induced by Ox-LDL. One is that Ox-LDL-induced mitogenic stimulus directly leads to macrophage proliferation. The other is that Ox-LDL stimulates the induction of certain growth factor(s) which leads to macrophage proliferation. To determine whether the proliferation-promoting activity of Ox-LDL is due to its direct effect on macrophage proliferation, or due to its indirect effect, we tested the effect of medium exchange on Ox-LDL-induced proliferation of murine peritoneal macrophages. Incubation with Ox-LDL for 5 days without medium exchange induced a significant thymidine incorporation (Fig. 1). However, when cells were incubated with Ox-LDL for 5 days replacing the medium at day 1 or 2 by fresh medium containing the same concentration of Ox-LDL, thymidine incorporation was markedly reduced by 80% or 70%, respectively (Fig. 1). In contrast, replacement of the medium at day 3 or 4 by fresh medium containing the same concentration of Ox-LDL, did not change the thymidine incorporation (Fig. 1). A cell-counting assay also showed that medium exchange at day 1 or 2 significantly inhibited Ox-LDL-induced macrophage proliferation (Table 1). These results suggest that a soluble factor(s) released from macrophages into the medium during day 1 to 2 may be involved in the induction of macrophage proliferation by Ox-LDL.

Expression of GM-CSF in macrophages

Three types of cytokines are known to regulate the proliferation or differentiation of the monocyte/macrophage lineage, including interleukin-3 (IL-3) (21), macrophage colony-stimulating factor (M-CSF) (22, 23) and GM-CSF (24, 25). To determine which factor is responsible for Ox-LDL-induced macrophage proliferation, neutralizing antibodies against these factors were tested for their effects on Ox-LDL-induced macrophage proliferation. Anti-M-CSF and anti-IL-3 antibodies had no effect on Ox-LDL-induced thymidine incorporation, whereas anti-GM-CSF antibody significantly suppressed it by 90% in a dose-dependent manner (Fig. 2). The cell-counting assay also showed that the Ox-LDL-induced increase in the cell number was suppressed by 85% by neutralizing antibody against GM-CSF (Table 2). RT-PCR analysis showed that Ox-LDL induced a transient expression of GM-CSF mRNA, with the peak occurring 1 hour after the addition of Ox-LDL (Fig. 3). Moreover, the addition of Ox-LDL at >20 μg/ml significantly enhanced the secretion of GM-CSF in a dose-dependent manner (Fig. 4). The concentrations of GM-CSF in the medium reached a peak level at 4 h, followed by a time-dependent decrease to 24 h. The highest concentration of GM-CSF (2 pM) occurred at 4 h, produced by using 40 μg/ml of Ox-

![Fig. 1. Effect of medium exchange on Ox-LDL-induced macrophage proliferation. Peritoneal macrophage monolayers from DDY mice (5 x 10⁴ cells/well in 24-well tissue culture plates) were incubated with or without 20 μg/ml of Ox-LDL. At indicated times (days 1, 2, 3 and 4), cultured wells were replaced with 1 ml of fresh medium containing the same concentration of Ox-LDL and incubated for a total of 5 days. During the last 18 h of incubation, cells in each well were chased with [³H] thymidine, harvested, and cellular radioactivity was determined. Data represent the mean±SD of three experiments.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>Ox-LDL no medium exchange at day 1</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td>medium exchange at day 2</td>
<td>4.8±0.3</td>
</tr>
<tr>
<td>at day 3</td>
<td>4.9±0.2</td>
</tr>
<tr>
<td>at day 4</td>
<td>5.8±0.6</td>
</tr>
<tr>
<td>at day 4</td>
<td>5.6±0.3</td>
</tr>
</tbody>
</table>

*p<0.01, compared with no medium exchange (Student’s t-test).
LDL (Fig. 4). These results indicate that the increase in GM-CSF mRNA by Ox-LDL was linked to the release of GM-CSF into the medium and the subsequent macrophage proliferation. Ox-LDL-induced GM-CSF expression was also reported by Rajavashisth et al. (26) who demonstrated that minimally oxidized LDL induced expression of GM-CSF mRNA in vivo as well as endothelial cells in vitro.

Role of protein kinase C (PKC) in expression of GM-CSF

We previously demonstrated that activation of membrane PKC was involved in the Ox-LDL-induced macrophage proliferation (15). Thus, we next examined the effect of a PKC inhibitor, calphostin C, on the Ox-LDL-induced both macrophage proliferation and GM-CSF release. Figure 5A shows that calphostin C inhibited the Ox-LDL-induced thymidine incorporation into macrophages. Moreover, Ox-LDL-induced increase in GM-CSF release was effectively inhibited by calphostin C in a dose-dependent manner, suggesting the involvement of certain types of PKC activation in Ox-LDL-induced GM-CSF expression (Fig. 5B). This notion was supported by a following report. Phorbol 12-myristate 13-acetate (PMA) and A23187 (a calcium ionophore) were reported to increase GM-CSF mRNA levels through activation of PKC in a human Jurkat T cell line (27).

In addition to this pathway, the recent study by Martens et al. (18) showed the involvement of the phosphatidylinositol-3 kinase (PI3K) pathway in Ox-LDL-induced macrophage proliferation. Although PI3K inhibitors such as wortmannin and LY294002 had no appreciable effect on Ox-LDL-induced GM-CSF release from macrophages, these inhibitors significantly inhibited recombinant GM-CSF-induced thymidine incorporation into macrophages (28, 29). It is therefore likely that Ox-LDL-induced GM-CSF release is independent of PI3K activation and that PI3K might be involved in macrophage proliferation after GM-CSF expression.

Effect of recombinant GM-CSF on macrophage proliferation

To confirm the role of GM-CSF in Ox-LDL-induced macrophage proliferation, we examined the effect of recombinant murine GM-CSF on murine macrophage proliferation. Incubation of macrophages with 1 pM recombinant GM-CSF led to a significant increase in thymidine incorporation (from 150 to 2,000 cpm/well). In addition, thymidine incorporation induced by >10 pM recombinant GM-CSF reached a plateau level (14,000 cpm/well). Under our experimental conditions, incubation of these macrophages with 1 pM recombinant murine GM-CSF did not lead to a significant increase in cell

Table 2. Effects of neutralizing antibodies on Ox-LDL-induced macrophage proliferation determined by cell counting assay.

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Sample</th>
<th>Cell number (×10⁴/well)</th>
<th>(×10⁴/well)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DDY mice</td>
<td>Medium alone</td>
<td>3.5 ± 0.2</td>
<td>(100%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ox-LDL</td>
<td>5.8 ± 0.5</td>
<td>(166%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ anti-GM-CSF IgG</td>
<td>3.6 ± 0.3</td>
<td>(103%)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ anti-M-CSF IgG</td>
<td>5.6 ± 0.5</td>
<td>(160%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ anti-IL-3 IgG</td>
<td>5.7 ± 0.2</td>
<td>(163%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ non-immune IgG</td>
<td>5.5 ± 0.2</td>
<td>(166%)</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.01, compared with Ox-LDL (Student’s t-test).
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number when determined by the cell-counting assay. Parallel incubation at concentrations higher than 1 nM showed a significant increase in the cell number (from 3.5 x 10⁴ to 5.8 x 10⁴ cells/well).

Conclusions

In this review article, based mainly on our findings, we emphasized an important role of GM-CSF in the Ox-LDL-induced macrophage proliferation. An anti-GM-CSF antibody significantly inhibited the Ox-LDL-induced macrophage proliferation. Moreover, Ox-LDL significantly induced the expression of GM-CSF in macrophages and subsequent release, with the maximal concentration being 1-2 pM. However, the concentration of recombinant GM-CSF required to produce an increase in cell number was >1 nM. In contrast, recombinant GM-CSF at 1 pM exhibited a significant increase in thymidine incorporation. Thus, a 1,000 times higher concentration is required for cell division. It is generally accepted that cell growth is regulated by four phases of the cell cycle: G1, S, G2 and M phase (30). Extensive studies using Saccharomyces cerevisiae have shown the presence of two checkpoints in each phase (G1/S and G2/M checkpoints) and both checkpoints must be driven forward for cell division (30). Based on the results mentioned above, it is likely that GM-CSF is required for the first checkpoint, whereas another cytokine(s) might act on the second checkpoint, from S phase to M phase, thus leading finally to the proliferation of macrophages.

We previously demonstrated that endocytic uptake of lysophosphatidylcholine in Ox-LDL through the scavenger receptor type AI/AlII plays an important role in macrophage proliferation (8-10). Our subsequent studies also demonstrated that a rise in intracellular calcium released from endoplasmic reticulum and activation of PKC resulted in macrophage proliferation (15, 16). These phenomena were inhibited by the presence of pertussis toxin, suggesting the presence of a pertussis toxin-sensitive small G-protein–coupled receptor for Ox-LDL (15). Moreover, our recent study demonstrated that PI3K was involved in Ox-LDL-induced macrophage proliferation after the release of GM-CSF (28). From these findings, we postulated the Ox-LDL-induced signaling pathway for macrophage proliferation in Figure 6.

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Fig. 5. Effects of calphostin C on Ox-LDL-induced thymidine incorporation (A) and GM-CSF release from macrophages (B).

A. Peritoneal macrophage monolayers from DDY mice (5 × 10⁴ cells/well in 24-well tissue culture plates) were incubated for 5 days with 20 μg/ml of Ox-LDL in the presence of indicated concentrations of calphostin C. During the last 18 h of incubation, cells in each well were chased with [³H] thymidine, harvested, and radioactivity was determined. Data represent the mean ± SD of three experiments.

B. Peritoneal macrophage monolayers from DDY mice (5 × 10⁶) in 10-cm dish were incubated with 40 μg/ml of Ox-LDL in the presence of indicated concentrations of calphostin C. Aliquots (300 μl) of the culture medium were taken at 4 h after incubation with Ox-LDL. The supernatants were obtained by brief centrifugation and the level of GM-CSF was determined by ELISA. Data represent the mean ± SD of three experiments.

Fig. 6. Schematic representation of the Ox-LDL-induced signaling pathways for macrophage proliferation. Oxidized low density lipoprotein (Ox-LDL) initiates a rise in intracellular calcium, which is derived from endoplasmic reticulum. This phenomenon may be mediated by the activation of phospholipase C via a signal transmitted by pertussis toxin-sensitive small G-protein-coupled certain receptor for Ox-LDL. Increase in calcium with diacylglycerol and/or endocytosed lysophosphatidylcholine through the MSR-AI/AII results in activation of protein kinase C (PKC). Subsequently, macrophages release granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF released from macrophages induces macrophage proliferation in autocrine or paracrine fashion. Phosphatidylinositol-3 kinase (PI3K) may be involved in macrophage proliferation after GM-CSF expression.

Ox-LDL; oxidized low density lipoprotein, lyso-PC; lysophosphatidylcholine, PKC; protein kinase C, GM-CSF; granulocyte/macrophage colony-stimulating factor, RC; receptor, PIP₂; phosphatidylinositol diphosphate, IP₃; inositoltriphosphate, DAG; diacylglycerol, PI3K; phosphatidylinositol-3 kinase.
Miyazaki for their collaborative endeavor throughout this study.

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