Granulocyte Macrophage Colony-Stimulating Factor Plays a Priming Role in Murine Macrophage Growth Induced by Oxidized Low Density Lipoprotein

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INTRODUCTION

One of the characteristic events in the atherosclerotic lesion is the proliferation of cellular components. It is generally accepted that smooth muscle cells migrated from media into intima proliferate in the atherosclerotic lesions. It was recently demonstrated, however, that macrophages also proliferate in the early stage of atherosclerotic lesions.1

Using an in vitro culture system, we have shown that macrophages obtained from mouse,2–5 rat, b and human7 are able to proliferate upon incubation with oxidized LDL (Ox-LDL). It then becomes clear that the specific uptake of lysophosphatidylcholine (lyso-PC) of Ox-LDL through the macrophage scavenger receptor type A-I/A-II (MSR-AI/AII) is essential for Ox-LDL–induced macrophage proliferation.3, 5, 7 The activation of protein kinase C (PKC) was also shown to be involved in this phenomenon.8 These in vitro observations strongly suggest that Ox-LDL acts as a growth factor for macrophage in vivo. The present study was undertaken to elucidate the molecular cascade(s) leading to Ox-LDL–induced macrophage proliferation. The results indicate that Ox-LDL–mediated release of granulocyte macrophage colony-stimulating factor (GM-CSF) from macrophages may play an important role in macrophage proliferation.

MATERIALS AND METHODS

Cell Culture

Murine peritoneal macrophages were collected from male DDY mice with 8 ml ice-cold PBS and suspended in RPMI 1640 supplemented with 10% heat-inactivated
newborn calf serum. Cell suspensions were dispersed in each well and incubated for 90 min for adherence.

**Determination of Macrophage Proliferation**

The macrophage monolayers (5 × 10⁴ cells/well in 24-well tissue culture plates) were cultured with 1 ml of RPMI 1640 medium in the presence of the lipoproteins to be tested. After a 5-day incubation, [³H]thymidine incorporation assay was performed as described previously. The number of adhered cells were counted at day 7.

**GM-CSF Analyses**

Cells were incubated with Ox-LDL for various periods followed by extraction of total RNA with TRIzol. The levels of GM-CSF mRNA expression were determined by Northern blotting as well as RT-PCR as described. The concentration of GM-CSF in the medium was determined by an ELISA kit purchased from Amersham.

**RESULTS AND DISCUSSION**

**Effect of Medium Exchange on Ox-LDL–Induced Macrophage Proliferation**

To test whether a soluble factor secreted from macrophages is involved in Ox-LDL–induced macrophage growth, we examined the effect of medium exchange on macrophage proliferation. Incubation of macrophages with 20 µg/ml of Ox-LDL for 5 days without medium exchange resulted in a significant [³H]thymidine incorporation. However, when macrophages 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, [³H]thymidine incorporation was markedly reduced by 75% or 60%, respectively. In contrast, replacement of the medium at day 3 or 4 did not affect [³H]thymidine incorporation. Cell-counting assay showed a consistent result, suggesting that a soluble factor(s) released from macrophages by Ox-LDL into the medium during day 1 to 2 may be involved in the induction of macrophage proliferation by Ox-LDL.

**An Anti–GM-CSF Antibody Suppresses Ox-LDL–Induced Macrophage Proliferation**

We next examined the effects of neutralizing antibodies against four cytokines, such as GM-CSF, M-CSF, IL-3, and IL-5, on Ox-LDL–induced macrophage proliferation. The anti–GM-CSF antibody significantly suppressed [³H]thymidine incorporation by 80%, whereas the other antibodies had no effect (Fig. 1). The cell-counting assay also showed a consistent result, suggesting the involvement of GM-CSF in Ox-LDL–induced macrophage proliferation.
Ox-LDL Induces GM-CSF Expression in Macrophages

We also examined whether Ox-LDL could induce mRNA expression of GM-CSF. Time course studies by RT-PCR showed that a 368 bp band of GM-CSF appeared at 30 min and reached a peak at 1 h when cells were incubated with Ox-LDL. Northern blot analysis also showed induction of GM-CSF mRNA by Ox-LDL.9

In the next step, we determined whether Ox-LDL could induce GM-CSF secretion into the medium from macrophages. The concentrations of GM-CSF in the medium increased by addition of Ox-LDL and reached a peak level at 4 h, followed by a time-dependent decrease to basal level at 24 hours.9 Thus, the increase in GM-CSF mRNA by Ox-LDL is linked to the subsequent release of GM-CSF protein into the medium. Taken into consideration with the inhibitory effect of an anti-GM-CSF antibody on Ox-LDL–induced macrophage proliferation, Ox-LDL–induced macrophage growth was mediated by GM-CSF released from macrophages in an autocrine or paracrine manner.

Mechanism of GM-CSF Induction in Macrophages by Ox-LDL

To elucidate the involvement of lyso-PC in induction of GM-CSF by Ox-LDL, we examined the effect of phospholipase A2 (PLA2)–treated acetyl-LDL on GM-CSF secretion. Upon treatment with PLA2, 75% of the total PC in acetyl-LDL was converted to lyso-PC and a significant growth-promoting activity appeared. Al-

FIGURE 1. Anti–GM-CSF antibody inhibits Ox-LDL–induced macrophage growth. Mouse macrophages (5 × 10⁴ cells/well) were incubated with 20 µg/ml of Ox-LDL in the absence or presence of various antibodies against cytokines such as GM-CSF, M-CSF, IL-3, and IL-5. Macrophage proliferation was determined by [³H]thymidine incorporation (left) or by cell counting (right).
though untreated acetyl-LDL did not increase GM-CSF concentration in the medium. PLAr3-treated acetyl-LDL significantly induced GM-CSF secretion. Secretion of GM-CSF by PLAr3-treated acetyl-LDL was less than half of Ox-LDL, suggesting that some other components in Ox-LDL are also involved in GM-CSF production in macrophages.

To characterize the role of MSR-AI/AII, Ox-LDL–induced GM-CSF secretion from macrophages obtained from MSR-AI/AII-knockout mice was compared with those from their wild-type littermates. The level of GM-CSF release induced by Ox-LDL from MSR (−) macrophages was reduced by 75% as compared with that from MSR (+) macrophages. It is therefore likely that MSR-mediated endocytosis of lyso-PC (or some other components) also plays a crucial role in Ox-LDL–induced GM-CSF production.

To evaluate the role of PKC in GM-CSF production, we tested the effect of calphostin C, a PKC inhibitor, on Ox-LDL–induced GM-CSF release. Ox-LDL–induced GM-CSF release was effectively inhibited by calphostin C in a dose-dependent manner, suggesting the involvement of PKC activation in Ox-LDL–induced GM-CSF production.

**FIGURE 2.** The possible mechanism of macrophage growth by Ox-LDL. Endocytic uptake lyso-PC (or some other components) in Ox-LDL through macrophage scavenger receptors induces PKC activation. This PKC activation induces mRNA expression of GM-CSF and its secretion. Secreted GM-CSF acts on macrophages in an autocrine or paracrine fashion and induces macrophage proliferation. It is possible that another receptor for Ox-LDL and a cytokine(s) other than GM-CSF are also involved in Ox-LDL–induced macrophage growth.
CONCLUSIONS

The possible mechanism of macrophage growth by Ox-LDL is summarized in Figure 2. Endocytic uptake of mitogenic components in Ox-LDL including lyso-PC through MSR induces PKC activation followed by GM-CSF expression and secretion. Secreted GM-CSF acts on macrophages in an autocrine or paracrine fashion and induces macrophage growth. It is also suggested that a soluble factor(s) other than GM-CSF or another receptor for Ox-LDL is involved in Ox-LDL–induced macrophage proliferation.

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