Induction of Murine Macrophage Growth by Oxidized Low Density Lipoprotein Is Mediated by Granulocyte Macrophage Colony-stimulating Factor*

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We have examined whether certain secreted factor(s) is involved in oxidized low density lipoprotein (Ox-LDL)-induced murine macrophage growth. An antibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) effectively inhibited Ox-LDL-induced macrophage growth by >80%. Ox-LDL as well as phospholipase A2-treated acetylated LDL enhanced mRNA levels and protein release of GM-CSF from macrophages, while neither acetylated LDL nor lysophosphatidylcholine (lyso-PC) showed such effects. The maximal induction of GM-CSF by Ox-LDL was noted at 4 h, followed by a time-dependent decrease to a basal level within 24 h. Ox-LDL-induced macrophage growth was inhibited by 75% by replacement of the culture medium at 24 h by a fresh medium containing the same concentration of Ox-LDL, when GM-CSF had already returned to the basal level. Thus, a cytokine(s) other than GM-CSF is also expected to participate in Ox-LDL-induced macrophage growth in a later phase. The Ox-LDL-induced GM-CSF release was inhibited by calphostin C, a protein kinase C inhibitor, and was significantly reduced in macrophages from the knockout mice lacking class A, type I and type II macrophage scavenger receptors (MSR-AI/AII). These results taken together indicate that effective endocytosis of lyso-PC of Ox-LDL by macrophages through MSR-AI/AII and subsequent protein kinase C activation have led to GM-CSF release into the medium which may play a priming role in conjunction with other cytokines in Ox-LDL-induced macrophage growth.

Macrophage-derived foam cells, a characteristic feature of the early stages of atherosclerosis, play a crucial role in the development of atherosclerotic lesions (1). Macrophages are known to take up chemically modified low density lipoproteins (modified LDLs) such as acetylated LDL (acetyl-LDL) and oxidized LDL (Ox-LDL) through the scavenger receptor pathway (2, 3) and become foam cells. It is generally accepted that LDL is oxidatively modified by cells of arterial walls such as smooth muscle cells, endothelial cells, and macrophages, and is converted to a ligand for the macrophage scavenger receptors (MSR) (3). Among chemically modified LDLs, Ox-LDL is thought to be the most likely candidate as an atherogenic lipoprotein in vivo because of its presence in human and rabbit atherosclerotic plaques (4, 5). In addition, in vitro experiments using cultured monocytes/macrophages have demonstrated the potential atherogenic effects of Ox-LDL such as chemotactic activity for monocytes (6), enhancement of monocyte adhesion to endothelial cells (7), initiation of monocyte differentiation into macrophages (8), inhibition of migration of tissue macrophages (9), and induction of macrophage cell death (10).

We have previously demonstrated a growth-stimulating effect of Ox-LDL in vitro on several types of macrophages, such as murine peritoneal macrophages (11, 12), rat peritoneal macrophages (13), and human blood monocyte-derived macrophages (14). Since previous morphological reports demonstrated that macrophage-derived foam cells proliferated in situ in atherosclerotic lesions (15–17), it seems reasonable to expect that the Ox-LDL-induced macrophage growth is linked to the development of atherosclerotic lesions. Therefore, it is important to characterize the molecular cascade(s) involved in the induction of macrophage growth by Ox-LDL. Our previous studies demonstrated that the specific uptake of lysophosphatidylcholine (lyso-PC) of Ox-LDL by class A, type I and type II MSR (MSR-AI/AII) was essential for Ox-LDL-induced macrophage growth (12, 14, 18), in which activation of protein kinase C (PKC) is involved (19). Recently, Martens et al. (20) reported that activation of phosphatidylinositol-3-OH kinase is involved in the induction of macrophage growth by Ox-LDL. However, the molecular cascade(s) leading to Ox-LDL-induced macrophage proliferation is still not fully understood.

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 growth. Three types of cytokines are known to exhibit growth stimulating activity for macrophages. These include the macrophage colony-stimulating factor (M-CSF) (21, 22), granulocyte-macrophage colony-stimulating factor (GM-CSF) (23, 24), and interleukin-3 (IL-3) (25). In the present study, we determined whether a soluble factor(s) is involved in the Ox-LDL-induced macrophage growth.

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¶The abbreviations used are: LDL, low density lipoprotein; acetyl-LDL, acetylated LDL; Ox-LDL, oxidized LDL; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IL, interleukin; lyso-PC, lysophosphatidylcholine; MSR, macrophage scavenger receptor; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; PKC, protein kinase C.
phage growth. The results indicated that Ox-LDL-induced GM-CSF release from macrophages may play a priming role in macrophage growth.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents for cell culture were obtained from Life Technologies, Inc. [methyl-³²H]Thymidine (80 Ci/mmol), [α-²⁵P]dCTP (370 MBq/ml), and Hyb-N⁺ nylon membrane were purchased from Amersham. Recombinant murine IL-3 and recombinant murine IL-5 were purchased from Research and Diagnostics Systems. Recombinant murine M-CSF was purchased from Upstate Biotechnology Inc. Recombinant murine GM-CSF was purchased from Genzyme. Goat polyclonal neutralizing antibodies against cytokines such as murine IL-3, murine IL-5, murine M-CSF, and murine GM-CSF were purchased from Research and Diagnostics Systems. ELISA kit for determination of GM-CSF level was purchased from Amersham. Phospholipase A₂ (PLA₂) from Crotalus atrox venom, calphostin C, and palmityl-hyophosphatidylcholine were purchased from Sigma. Calphostin C was stored at −20 °C as stock solutions of 500 μM in dimethyl sulfoxide and used within 1 week of dissolving. The final concentrations of dimethyl sulfoxide were less than 0.1% in the culture medium, which did not affect cell viability and macrophage growth. Calphostin C at <0.5 mM did not show any cytotoxic effect when assessed by lactate dehydrogenase release. Other chemicals were the best grade available from commercial sources.

**Lipoproteins and Their Modifications**—Human LDL (d = 1.019 to 1.063 g/ml) was isolated by sequential ultracentrifugation from the plasma of normolipidemic subjects after overnight fasting (26). LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA (pH 7.4). Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride (26). Acetyl-LDL was dialyzed against phosphate-buffered saline and treated with PLA₂, as described by Quinn et al. (6) with minor modifications (12). Seventy-five percent of phosphatidylcholine in PLA₂-treated acetyl-LDL was converted to lysophosphatidic acid. Protein concentrations were determined by BCA protein kit reagent assay (Pierce) using bovine serum albumin as a standard, and were expressed as milligrams of protein/ml (27). The levels of endotoxin associated with these lipoproteins were <1 pg/μg of protein; these were measured by a commercially available kit (Toxicolor system, Seikagaku Corp.). Moreover, macrophage growth was not induced by endotoxin at a concentration of 1 ng/ml in our experimental system.

**Cell Culture**—Unless otherwise specified, cell culture experiments were performed at 37 °C in 5% CO₂. Peritoneal macrophages were collected from four different mice; those from non-stimulated male DDY mice (25–30 g), those from double-knockout mice lacking both the Fc receptor y chain, and the class II Fc receptor for immunoglobulin G (FcγR-FcRy) (Taconic Farms, Inc.) (28, 29), those from MSR-AI/AII-knockout mice (30), and those from their wild-type littermates (12). The peritoneal macrophages were collected with 8 ml of ice-cold phosphate-buffered saline and suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% heat-inactivated newborn calf serum (Life Technologies, Inc.), streptomycin (0.1 mg/ml), and penicillin (100 units/ml) (medium A). Cell suspensions were dispersed in each well of appropriate tissue culture plates and incubated for 90 min. Nonadherent cells were removed by washing three times with medium A. After washing, cell number was decreased to ~80%. More than 98% of adherent cells were judged to be macrophages by Giemsa staining. The macrophage monolayers thus formed were used for following cell experiments.

**RT-PCR and Northern Blot Analyses**—Standard molecular biology techniques were used (34). After incubation of murine peritoneal macrophage monolayers (2 × 10⁶ cells/well in 6-well plate, 3.5 cm in diameter, Nunc) with Ox-LDL (0 to 40 μg/ml) for different time intervals (0 to 7 h), total RNA was extracted with TRizol (Life Technologies, Inc.). Total RNA (5 μg) from SR-4987 cells (31) and WEHI-3 cells (35) was also extracted with TRizol. The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo(dT). Primers used for PCR amplification of GM-CSF, M-CSF, IL-3, IL-5, and β-actin were designed on the basis of murine GM-CSF cDNA (36), murine M-CSF cDNA (37), murine IL-3 cDNA (38), murine IL-5 cDNA (39), and murine β-actin cDNA (40) sequences as follows: for GM-CSF, forward primer, TGGTGCTCTTTGATGTCACGCACGATTTC (nucleotide 541 to 564 of murine GM-CSF coding sequence) and reverse primer, CAAAAGGGAATATCAGTCGAAAGGT (nucleotide 407 to 431 of murine GM-CSF coding sequence) (36); for M-CSF, forward primer, GTGTCTGGCTAGACCACGACCTCGAC (nucleotide 342 to 366 of murine M-CSF coding sequence) and reverse primer, TTAAACATTCCACCGTGTTCCAGGTTA (nucleotide 477 to 501 of murine IL-3 coding sequence) (38); for IL-3, forward primer, GACAGCAATGACAGATGATCAGG (nucleotide 129 to 152 of murine IL-5 coding sequence) and reverse primer, GACAGCAATGACAGATGATCAGG (nucleotide 342 to 366 of murine IL-5 coding sequence) (39); for β-actin, forward primer, GACAAGCACCGGCTTTCAGTATGTCG (nucleotide 45 to 65 of murine β-actin coding sequence) and reverse primer, CTCCTTGTATGGCCACCATTTTCGTTG (nucleotide 541 to 564 of murine β-actin coding sequence) (40); the sizes of RT-PCR products of GM-CSF, M-CSF, IL-3, IL-5, and β-actin were expected to be 368, 578, 423, 235, and 540 base pairs, respectively. The cycling conditions in the GeneAmp 9600 System consisted of a first step of 94 °C denaturation for 10 min, followed by 35 cycles of annealing at 54 °C for 60 s, extension at 75 °C for 90 s, and denaturation at 94 °C for 30 s, with a final elongation step at 75 °C for 10 min. Amplification products were analyzed by 1.5% agarose gel electrophoresis. To verify that the amplification products were consistent with the reported sequences of murine GM-CSF, M-CSF, IL-3, IL-5, and β-actin, they were ligated into pGEM-T (from Promega), and the resulting plasmids were transformed into Escherichia coli XL1-Blue and sequenced by using 373A DNA sequencing kit (version 2.2.1, PerkinElmer). For Northern blot analysis, GM-CSF cDNA inserted into pGEM-T was excised by restriction enzyme Apal and SacI, and labeled with [α-³²P]dCTP by random nanomer primer method using Megaprime™ DNA labeling system (Amersham) (41). After incubation of murine peritoneal macrophage monolayers (2 × 10⁶ cells/well in 6-well plate, 3.5 cm in diameter, Nunc) with 40 μg/ml Ox-LDL for 1 h, total RNA was
prepared from 5 dishes with TRIzol. Ten μg/lane of total RNA was fractionated by electrophoresis through a denaturing formaldehyde 1% agarose gel, transferred to Hybond-N+ nylon membrane as described (41) by capillary transfer with 10× SSC for 20 h, and then cross-linked by UV (FS 1500, Funakoshi). Hybridization of the membrane with 32P-labeled probe specific for GM-CSF was performed in a solution containing Hybridization buffer tablets (Amersham) with 50% formamide, 0.1 mg/ml salmon sperm DNA at 42 °C as described previously (41). Stringent washing of the membrane was performed with 0.2× SSC, 0.1% SDS at a higher temperature (from 42 to 65 °C) (41). The membrane was then exposed to a Fuji Imaging Plate BAS-III (Fuji Photo Film Co.) for 2 h at room temperature and analyzed using a BAS-2000 II (Fuji X).

**Assay of PKC Activity**—PKC activity of macrophages was assayed by MESACUP Protein Kinase Assay Kit (Medical and Biological Laboratories) as described previously (19). The macrophage monolayers (5×106 cells/plate, 10 cm in diameter, Falcon) formed were cultured for the indicated times in 15 ml of serum-free RPMI 1640 with or without the lipoproteins to be tested. Cells were detached from the wells and homogenized by sonication for 30 s at 4 °C with Sonifier (Branson Sonic Power Co). The membrane fractions were collected by ultracentrifugation and were used in the PKC assay as described previously (19).

**Statistical Analysis**—Data were expressed as mean ± S.D. Differences between groups were evaluated by the Student’s t test. When the p value was less than 0.05, the difference was considered significant.

### RESULTS

**Effect of Medium Exchange on Ox-LDL-induced Macrophage Growth**—To determine whether the mitogenic activity of Ox-LDL is due to its direct effect on macrophage growth, or due to its indirect effect through induction of certain growth factors, we first tested the effect of medium exchange on Ox-LDL-induced cell growth of murine peritoneal macrophages. Incubation with 20 μg/ml Ox-LDL for 5 days without medium exchange induced a significant [3H]thymidine incorporation (Fig. 1B). However, when macrophages were incubated with 20 μg/ml Ox-LDL for 5 days replacing the medium at day 1 or 2 by medium A containing the same concentration of Ox-LDL, [3H]thymidine incorporation was markedly reduced by 75 or 60%, respectively (Fig. 1B). In contrast, replacement of the medium at day 3 or 4 by medium A containing the same concentration of Ox-LDL, did not change [3H]thymidine incorporation (Fig. 1B). Cell-counting assay also showed that medium exchange at day 1 or 2 significantly inhibited Ox-LDL-induced macrophage growth (Table I). These results suggest that a soluble factor(s) released from these cells into the medium during day 1 to 2 may be involved in the induction of macrophage growth by Ox-LDL.

### Table I

| Sample                      | Cell number (x 10⁶/well) | Mean ± S.D. of triplicate counts. |
|----------------------------|--------------------------|----------------------------------|
| Medium alone                | 3.52 ± 0.51 (100%)       |                                   |
| Ox-LDL No medium exchange   | 5.63 ± 0.32 (160%)       |                                   |
| Ox-LDL Medium exchange      |                          |                                   |
| Day 1                       | 4.77 ± 0.26 (135%)       |                                   |
| Day 2                       | 4.89 ± 0.21 (139%)       |                                   |
| Day 3                       | 5.84 ± 0.62 (165%)       |                                   |
| Day 4                       | 5.59 ± 0.29 (158%)       |                                   |

* p < 0.01, compared with no medium exchange (Student’s t test).

### Effect of Antibodies against GM-CSF on Ox-LDL-induced Macrophage Growth—Three types of cytokines are known to stimulate the growth of monocytes or macrophages, including M-CSF (21, 22), GM-CSF (23, 24), and IL-3 (25). Furthermore, a receptor for IL-5 is also known to share a β-subunit in common with those for GM-CSF and IL-3 (42, 43). To determine which factor is responsible for Ox-LDL-induced macrophage growth, we next examined neutralizing antibodies against these factors for their effect on Ox-LDL-induced macrophage growth. Our preliminary experiments showed that non-immune goat immunoglobulin G (IgG) had a minimal nonspecific effect on Ox-LDL-induced macrophage growth when compared with that of murine, rat, and rabbit (data not shown). We therefore used anti-cytokine antibodies raised in goats in the present study. Anti-M-CSF, anti-IL-3, and anti-IL-5 antibodies as well as goat non-immune IgG had no effect on Ox-LDL-induced [3H]thymidine incorporation, whereas anti-GM-CSF antibody significantly suppressed it by 80% in a dose-dependent manner (Fig. 2A). The cell-counting assay also showed that Ox-LDL-induced increase in the cell number was suppressed by 87% by 10 μg/ml neutralizing antibody against GM-CSF, while other neutralizing antibodies did not show inhibitory effects (Table II). Although detailed experiments will be shown later (Fig. 7), the neutralizing activity of each antibody was verified by assessing its inhibitory effect on the corresponding cytokine-induced [3H]thymidine incorporation. In fact, the capacity of recombinant murine GM-CSF to induce [3H]thymidine incorporation into macrophages under the present culture conditions was completely inhibited by the presence of 10 μg/ml anti-murine GM-CSF antibody (data not shown). Similarly, the capacities of recombinant murine M-CSF and recombinant murine IL-3 to induce [3H]thymidine incorporation were effectively neutralized by respective antibodies (data not shown).

Another possibility taken into consideration is that, since macrophages express receptors for IgG (Fc receptors), the neutralizing antibody against GM-CSF could affect Fc receptors rather than neutralize the GM-CSF activity. To test this, we determined the effect of anti-GM-CSF antibody on Ox-LDL-induced growth of macrophages obtained from FeRγ/FeRRII double-knockout mice lacking all the receptors for IgG (28, 29). Ox-LDL-induced cell growth of macrophages from FeRγ/FeRRII...
double-knockout mice was significantly inhibited by anti-GM-CSF antibody but not by other antibodies when assayed both by \[^{3}H\]thymidine incorporation (Fig. 2B) and by the cell-counting assay (Table II). These results indicate that the inhibitory effect of anti-GM-CSF antibody on Ox-LDL-induced macrophage growth is not due to its nonspecific action on the Fc receptors of macrophages, but due largely to its neutralizing effect on GM-CSF released from macrophages into the culture medium.

**Table II**

| Macrophages | Sample                  | Cell number |
|-------------|-------------------------|-------------|
| DDY mice   | Medium alone            | 3.3 ± 0.3 (100%) |
|            | Ox-LDL                  | 5.7 ± 0.5 (172%) |
|            | + Anti-GM-CSF IgG       | 3.6 ± 0.3 (109%) |
|            | + Anti-M-CSF IgG        | 5.5 ± 0.5 (167%) |
|            | + Anti-IL-3 IgG         | 5.5 ± 0.2 (165%) |
|            | + Anti-IL-5 IgG         | 5.4 ± 0.4 (164%) |
|            | + Non-immune IgG        | 5.8 ± 0.2 (176%) |
| FcR/FcY RII double-knockout mice | Ox-LDL          | 9.1 ± 0.5 (260%) |
|            | + Anti-GM-CSF IgG       | 4.3 ± 0.3 (123%) |
|            | + Anti-M-CSF IgG        | 8.7 ± 0.2 (246%) |
|            | + Anti-IL-3 IgG         | 8.5 ± 0.5 (243%) |
|            | + Anti-IL-5 IgG         | 8.4 ± 0.5 (240%) |
|            | + Non-immune IgG        | 9.0 ± 0.6 (257%) |

\( p < 0.001, \) compared with Ox-LDL (Student’s t test).

FIG. 2. Effect of neutralizing antibodies against GM-CSF, M-CSF, IL-3, and IL-5 on Ox-LDL-induced growth of macrophages obtained from DDY mice (A) or FcR/FcY RII double-knockout mice (B). Peritoneal macrophage monolayers from DDY mice (5 × 10^5) were seeded in 3.5-cm dish and incubated for 90 min. A, the cell monolayers thus formed were incubated in 2 ml of medium A with (right panel) or without (left panel) 40 μg/ml Ox-LDL. After incubation for indicated time periods (0, 0.5, 1, 3, 5, and 7 h), total RNA was extracted from each dish with TRIzol. The expression of mRNA for GM-CSF (upper panel), M-CSF (middle upper panel), IL-3 (middle panel), IL-5 (middle lower panel), or β-actin (lower panel) was evaluated by RT-PCR as described under “Experimental Procedures.” M indicates molecular size marker. C indicates a positive control for each cytokine: for M-CSF, SR-4987 cell-derived total RNA; and for IL-3, WEHI-3 cell-derived total RNA, respectively. B, the cell monolayers were incubated in 2 ml of medium A with the indicated concentrations of Ox-LDL (0, 5, 10, 20, and 40 μg/ml) for 1 h and total RNA was prepared from each dish with TRIzol. Total RNA was also prepared from macrophage monolayers before incubation with Ox-LDL as a control (P). The expression of GM-CSF mRNA (upper panel) or β-actin (lower panel) was evaluated by RT-PCR as described under “Experimental Procedures.” M indicates molecular size marker. C, the macrophage monolayers (2 × 10^6) in 3.5-cm dish were incubated with or without 40 μg/ml Ox-LDL for 1 h and total RNA was prepared. Ten μg/lane of total RNA was fractionated by electrophoresis and transferred to Hybond-N+ nylon membrane. The membrane was hybridized with 32P-labeled GM-CSF specific probe at 42°C, and visualized as described under “Experimental Procedures.” To determine the amounts of total RNA per lane, 28S and 18S RNA were stained after electrophoresis with ethidium bromide and visualized by UV excitation (lower panel). bp, base pair(s); kb, kilobases.
as a control. When macrophages were incubated with medium A alone, GM-CSF mRNA was not detected during the 7-h incubation (Fig. 3A, left upper panel), whereas incubation with 40 μg/ml Ox-LDL for 30 min resulted in the appearance of the 368-base pair band of GM-CSF (Fig. 3A, right upper panel). The maximal level of GM-CSF mRNA was noted at 1 h after the addition of Ox-LDL, followed by a time-dependent decrease (Fig. 3A, right upper panel). In contrast to GM-CSF, neither M-CSF nor IL-3 was detected as clear bands even after incubation of macrophages with 40 μg/ml Ox-LDL (Fig. 3A, right panel). However, the primer pairs specific for M-CSF could detect a 578-base pair band of M-CSF in total RNA of SR-4987 cells which were known to express a high level of M-CSF mRNA (Fig. 3A, left side, middle upper panel). The primer pairs specific for IL-3 could also detect a 423-base pair band of IL-3 using total RNA of WEHI-3 cells, positive control cells for murine IL-3 mRNA expression (Fig. 3A, left side, middle panel). These results support the notion that each primer pair can detect mRNA of each cytokine if detectable amounts of M-CSF or IL-3 mRNA are transcribed. mRNA levels of β-actin as a control for each control cell line (SR-4987 cells and WEHI-3 cells) were indistinguishable from those of murine macrophages (data not shown). In contrast to M-CSF and IL-3, a significant expression of IL-5 mRNA was observed even when macrophages were incubated with medium alone, and its levels was gradually decreased with incubation time up to 7 h (Fig. 3A, right lower panel). However, levels of IL-5 mRNA were not affected by incubation with Ox-LDL (Fig. 3A, right side, middle lower panel), under which mRNA levels of β-actin did not change (Fig. 3A, right upper panel). Ox-LDL caused a dose-dependent increase of GM-CSF mRNA at 1 h after its addition (Fig. 3B, upper panel), while the level of β-actin mRNA was not affected by Ox-LDL (Fig. 3B, lower panel). Thus, from the studies with neutralizing antibodies (Fig. 2) as well as those of RT-PCR (Fig. 3), a cytokine involved in the Ox-LDL-induced macrophage proliferation is likely to be GM-CSF.

The result obtained by RT-PCR analysis for GM-CSF was confirmed by Northern blot analysis of total RNA of macrophages obtained after a 1-h incubation with Ox-LDL. To determine the amounts of total RNA per lane, 28 S and 18 S RNA were stained after electrophoresis by ethidium bromide and visualized by UV excitation (Fig. 3C, lower panel). As shown in the upper panel of Fig. 3C, 32P-labeled GM-CSF cDNA probe cross-hybridized with RNAs obtained from macrophages after a 1-h incubation with 40 μg/ml Ox-LDL, with strong signals at 3.9, 3.0, and 1.2 kilobases.

Ox-LDL Induces GM-CSF Secretion from Macrophages—In the next step, we determined whether Ox-LDL could induce GM-CSF secretion from macrophages. As shown in Fig. 4A, the addition of Ox-LDL at >10 μg/ml significantly induced the secretion of GM-CSF. The concentrations of GM-CSF in the medium reached a peak level at 4 h, followed by a time-dependent decrease to 48 h. The highest concentration of GM-CSF (2 pM) occurred at 4 h, produced by using 40 μg/ml Ox-LDL (Fig. 4A). In contrast, when macrophages were incubated with medium A alone, GM-CSF in medium showed a slight increase and reached basal level at 48 h (Fig. 4A). When macrophages were incubated with 40 μg/ml LDL or acetyl-LDL, the concentration of GM-CSF did not significantly change compared with medium alone (data not shown). These results suggested that induction of GM-CSF in these macrophages is specific for Ox-LDL. The inset of Fig. 4A indicated that Ox-LDL induced GM-CSF secretion from macrophages in a dose-dependent manner. Upon incubation of macrophages with Ox-LDL for more than 48 h (up to 120 h), we did not observe GM-CSF secretion in the medium during the extended incubation (data not shown). Therefore, these results when those of RT-PCR and Northern blot analyses (Fig. 3) are combined, indicate that the increase in GM-CSF mRNA by Ox-LDL is linked to the subsequent release of GM-CSF protein into the medium.

Our previous study demonstrated that PKC activation by Ox-LDL is important for Ox-LDL-induced macrophage growth (19). Consistent with this notion, incubation with 40 μg/ml Ox-LDL significantly increased membrane PKC activity with a peak time at 10 min, whereas LDL or acetyl-LDL did not show such an effect (Fig. 4B). In the next step, to evaluate a role of PKC in Ox-LDL-induced GM-CSF production, we tested the effect of calphostin C, a well known PKC inhibitor. As shown in
Fig. 5. Ox-LDL-induced GM-CSF secretion from macrophages obtained from MSR-AI/AII-knockout mice. Peritoneal macrophages obtained from MSR-AI/AII-knockout mice or their wild-type littersmates were incubated in 10 ml of medium A in the absence (open column) or presence (hatched column) of 40 μg/ml Ox-LDL. Aliquots 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 as described under “Experimental Procedures.” Data represent the mean ± S.D. of three experiments.

Fig. 6. Effect of PLA2-treated acetyl-LDL on GM-CSF production by macrophages. A, peritoneal macrophage monolayers from DDY mice (5 × 10^6 cells/well in 24-well tissue culture plates) were incubated for 5 days in 1 ml of medium A with or without 40 μg/ml PLA2-treated acetyl-LDL, non-treated acetyl-LDL, or Ox-LDL in 1 ml of medium A. During the last 18 h of incubation, cells in each well were chased with [3H]thymidine, harvested, and radioactivity was determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of three experiments. B, peritoneal macrophage monolayers from DDY mice (2 × 10^6) in a 3.5-cm dish were incubated in 2 ml of medium A with or without 40 μg/ml PLA2-treated acetyl-LDL. After incubation for the indicated time periods (0, 0.5, 1, 3, 5, and 7 h), total RNA was extracted from each dish with Trizol. The expression of mRNA for GM-CSF (upper panel) or β-actin (lower panel) was evaluated by RT-PCR as described under “Experimental Procedures.” C, peritoneal macrophage monolayers from DDY mice (5 × 10^5) in a 10-cm dish were incubated in 15 ml of medium A in the absence (○) or presence of 40 μg/ml non-treated acetyl-LDL (△), PLA2-treated acetyl-LDL (○), and Ox-LDL (●). The level of GM-CSF was determined by ELISA as described in the legend to Fig. 4. Data represent the mean ± S.D. of three experiments. The concentrations of GM-CSF secreted at 4 h by 100 μM palmitoyl lyso-PC or 40 μg/ml acetyl-LDL was shown in the inset.

Effect of PLA2-treated Acetyl-LDL on GM-CSF Production—Our previous studies showed that GM-CSF-mediated specific uptake of lyso-PC of modified LDLs is a major pathway to induce macrophage growth (12, 14, 18, 44). To know whether this was also the case with Ox-LDL-induced GM-CSF production, we determined PLA2-treated acetyl-LDL for its effect on GM-CSF production. Our PLA2-treated acetyl-LDL preparation indeed showed the mitogenic activity for macrophages when assessed both by [3H]thymidine incorporation (8.8-fold increase above control, Fig. 6A) and by the cell-counting (Table III). Incubation with 40 μg/ml PLA2-treated acetyl-LDL for 30 min resulted in the appearance of the 388-base pair band of GM-CSF (Fig. 6B, upper panel). The maximal expression of GM-CSF mRNA was noted at 1 h after the addition of PLA2-treated acetyl-LDL, followed by a time-dependent decrease (Fig. 6B, upper panel). Under the conditions, levels of β-actin mRNA were not affected by PLA2-treated acetyl-LDL (Fig. 6B, lower panel). The addition of PLA2-treated acetyl-LDL at 40 μg/ml significantly induced the secretion of GM-CSF; its concentrations in the medium reached a peak level at 4 h, followed by a time-dependent decrease to 48 h (Fig. 6C). These patterns for increases in GM-CSF mRNA (Fig. 6B, upper panel) and protein release (Fig. 6C) by PLA2-treated acetyl-LDL were closely similar to those by Ox-LDL (Figs. 3A, right panel, and 4A). Incubation of these macrophages with 100 μM palmitoyl-lysophosphatidylcholine alone or with 40 μg/ml acetyl-LDL for 4 h, instead of PLA2-treated acetyl-LDL, did not lead to a significant GM-CSF release into the medium (inset of Fig. 6C). These results suggest that an effective uptake of lyso-PC of Ox-LDL is also important for induction of GM-CSF.

Effect of Recombinant Murine GM-CSF on Macrophage Growth—The above results strongly suggested that an increase in GM-CSF at mRNA level plays a crucial role in Ox-LDL-induced murine macrophage growth. To confirm these findings, we examined the effect of recombinant murine GM-CSF on murine macrophage growth. Incubation of macrophages with 1 pm recombinant GM-CSF led to a significant increase in [3H]thymidine incorporation (2.058 cpm/well). In addition, [3H]thymidine incorporation induced by >10 pm recombinant GM-CSF reached a plateau level (12,000 cpm/well at 10 pm)
Peritoneal macrophage monolayers from DDY mice (5 × 10^4 cells/well in 24-well tissue culture plates) were incubated for 5 days in 1 ml of medium A at indicated concentrations of recombinant GM-CSF (●). Parallel incubations were performed with identical concentrations of M-CSF (▲), IL-3 (■), and IL-5 (○). During the last 18 h of incubation, cells in each well were chased with [3H]thymidine, harvested, and the radioactivity was determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of three experiments.

Under our experimental conditions, incubation of these macrophages with 1 pM recombinant murine GM-CSF did not lead to a significant increase in cell number when determined by the cell-counting assay. Parallel incubation at concentrations higher than 1 nM showed a significant increase in cell number (Table IV).

**DISCUSSION**

Rajavashisth et al. (45) reported that minimally oxidized LDL induced expression of GM-CSF mRNA in vivo as well as endothelial cells in vitro. Although our present results obtained by macrophages may be related to their study, the interaction of Ox-LDL with macrophages seems to differ in nature from that of minimally oxidized LDL with endothelial cells. Minimally oxidized LDL is thought to be taken up by endothelial cells mainly through the scavenger receptor pathway (2, 3). In other words, minimally oxidized LDL is not recognized by the scavenger receptor (7), while Ox-LDL is not recognized by the LDL receptor (2, 3). Thus, one possible interpretation of our present result and their previous result would be that the receptor-mediated delivery of oxidatively modified LDL to endosomes and lysosomes may be important for induction of GM-CSF mRNA. This notion is supported by the recent finding by Nagy et al. (46), which shows that 9-hydroxyoctadecadienoic acid and 13-hydroxyoctadecadienoic acid generated by lysosomal processing of Ox-LDL are able to enhance expressions of several genes including CD36 by binding to peroxisome proliferator activated receptor γ in human monococytes and human monocytic THP-1 cells. Alternatively, the mechanism for GM-CSF induction by Ox-LDL in macrophages could be totally different from that induced by minimally oxidized LDL in endothelial cells.

GM-CSF is a well defined cytokine with a wide variety of potential effects on proliferation, maturation, and various functions of monocytes/macrophages (47). Wang et al. (48) reported that GM-CSF and M-CSF expression is associated with macrophage proliferation in progressing rabbit atheromatous lesions, suggesting an atherogenic role of GM-CSF endogenously expressed in atherosclerotic lesions. This notion may be consistent with the present finding that GM-CSF serves as a priming factor in Ox-LDL-induced macrophage proliferation.

In contrast to GM-CSF, the definite role of M-CSF in the progression of atherosclerosis is more generally accepted (1). In fact, Rosenfeld et al. (49) demonstrated the expression of M-CSF both at mRNA and protein levels in atherosclerotic lesions from humans, WIHLL, and cholesterol-fed rabbits. In particular, M-CSF mRNA and its protein were demonstrated in macrophage-derived foam cells freshly isolated from balloon injury-induced atherosclerotic lesions of cholesterol-fed rabbits. Since recombinant M-CSF in fact increased the [3H]thymidine incorporation into macrophages (Fig. 7), M-CSF produced by macrophage-derived foam cells may induce macrophage growth in a paracrine or autocrine fashion in atherosclerotic lesions. However, the present study showed that M-CSF gene expression was not increased by Ox-LDL in macrophages (Fig. 3A, right panel) and that anti-M-CSF antibody had no inhibitory effect on Ox-LDL-induced macrophage growth (Fig. 2 and Table II), indicating that M-CSF is unlikely to be involved in Ox-LDL-induced macrophage growth. This notion is supported by a report by Clinton et al. (50) in which M-CSF mRNA expression in both cultured human vascular endothelial cells and smooth muscle cells was significantly increased in vitro by endotoxin, human IL-1α, or tumor necrosis factor α, but not by Ox-LDL. Therefore, using the present data taken together, it is unlikely that M-CSF plays a crucial role in Ox-LDL-induced macrophage proliferation in vitro.

The size of murine GM-CSF mRNA reported by Gough et al. (51) was 1.2 kilobases. Two additional strong signals (3.9 and 3.0 kilobases) were detected in our system (Fig. 3C). It is not clear at present whether these signals really correspond to GM-CSF mRNA. Since we used 32P-labeled murine GM-CSF...
cDNA as a probe which was verified by dideoxy termination method, such signals probably correspond to GM-CSF mRNA or unknown RNAs with a high homology to GM-CSF. However, a possible contamination of the genomic DNA is unlikely because parallel preparation of total RNA from macrophages after incubation with the medium alone did not show such 3.9- and 3.0-kilobases signals.

In addition to MSR-AI/AII, several membrane proteins such as FcγRII-B2, SR-BI, macrosialin/CD68, CD36, and LOX-1 have been proposed as Ox-LDL receptors (52). Extents of contribution of MSR-AI/AII to endocytic uptake of Ox-LDL is somewhat deviated from one experiment to another, even though the results were obtained by using the same source of MSR-AI/AII-knockout mice. According to a recent report by Lougheed et al. (53), endocytic uptake of Ox-LDL by peritoneal macrophages from knockout mice was decreased only by 30%, indicating endocytic uptake of Ox-LDL mainly depends on receptors other than MSR-AI/AII. However, our previous study clearly showed that the cell association of Ox-LDL with MSR-AI/AII-knockout mice was decreased only by 30% as compared to that with their wild-type macrophages (18). Our subsequent study also showed that the capacity of peritoneal macrophages from MSR-AI/AII-knockout mice to degrade Ox-LDL was reduced to 50% of that of wild-type littermates (30). This difference in contribution of MSR-AI/AII might be derived from differences in ligand preparations and/or culture conditions. Based on our own experiments (18, 30), however, it seems reasonable to conclude that MSR-AI/AII serves as one of the major pathways for endocytic degradation of Ox-LDL. Therefore, a significant reduction in Ox-LDL-induced GM-CSF release from MSR-AI/AII-knockout macrophages (Fig. 5) is largely explained by the reduction in the uptake of Ox-LDL through MSR-AI/AII.

Phorbol 12-myristate 13-acetate and A23187 (a calcium ionophore) are reported to increase GM-CSF mRNA levels through activation of PKC in human Jurkat T cell line (54). The recent report from this laboratory demonstrated that Ox-LDL initiated an increase in intracellular Ca2+ and subsequent activation of PKC within 10 min after incubation with macrophages (19). This notion was also supported by the present study (Fig. 4B) and effective inhibition of calphostin C for Ox-LDL-induced GM-CSF release into the medium (Fig. 4C). It is therefore likely that PKC activation by Ox-LDL increases GM-CSF mRNA in macrophages. In addition to this pathway, the recent study by Martens et al. (20) showed the involvement of the phosphatidylinositol 3-OH kinase pathway in the Ox-LDL-induced macrophage growth. Our preliminary experiments showed that phosphatidylinositol 3-OH kinase inhibitors such as wortmannin and LY294002 had no appreciable effect on Ox-LDL-induced GM-CSF release from macrophages, suggesting that Ox-LDL-induced GM-CSF release is independent of phosphatidylinositol 3-OH kinase activation.

One important point indicated by the present study is the involvement of a cytokine(s) other than GM-CSF in murine macrophage growth. When macrophages were incubated with Ox-LDL, GM-CSF was promptly secreted into the medium with a maximal peak at 4–6 h but rapidly decreased to almost basal level at 24 h (Fig. 4). However, replacement of culture medium at 24 h after incubation with Ox-LDL by a fresh medium containing the same concentration of Ox-LDL resulted in a marked reduction of the growth-stimulating effect of Ox-LDL (Fig. 1 and Table I). If the macrophage growth was only explained by a direct cellular interaction of GM-CSF alone, such medium exchange should not influence Ox-LDL-induced macrophage growth (since the culture medium obtained 24 h after incubation with Ox-LDL contained only a basal level of GM-CSF). A simple interpretation of this finding would be that, in addition to GM-CSF, another factor(s) also participates in Ox-LDL-induced macrophage growth. This soluble factor can still be present in the medium even 24 h after incubation of macrophages with Ox-LDL, and is removed by the replacement of the original medium with a fresh medium, leading to a significant inhibition of macrophage growth. Since the increase in mRNA of several cytokines was determined within 7 h after Ox-LDL addition (Fig. 3A), it is possible to speculate that certain cytokines represented by GM-CSF induced initially by Ox-LDL may then interact with macrophages, leading to the induction of M-CSF, IL-3, IL-5, and other cytokines at the later stage, which could play some role in the macrophage growth. Further studies are needed to identify this factor.

Recombinant murine GM-CSF at 1 pM exhibited a significant increase in [3H]thymidine incorporation in macrophages (Fig. 7), but did not increase the cell number under identical conditions (Table IV). The concentration required for producing a significant increase in the cell number was 1 nM (Table IV), about 1,000 times higher than that required for a significant thymidine incorporation (Fig. 7). However, the concentration of GM-CSF released from macrophages upon incubation with Ox-LDL was 1–2 pM (Fig. 4A). Thus, GM-CSF induced by Ox-LDL would be sufficient for macrophage DNA synthesis but inadequate to increase the number of macrophages. It is generally accepted that cell growth is regulated by four phases of the cell cycle: G1, S, G2, and M phase (55). 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 (55). Based on the results of the present study, 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 growth of macrophages.

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REFERENCES

1. Ross, R. (1993) Nature 362, 801–809
2. Brown, M. S., and Goldstein, J. L. (1983) Annu. Rev. Biochem. 52, 223–261
3. Steinberg, D., Parthasarathy, S., Carew, T. E., Khour, J. C., and Witztum, J. L. (1989) N. Engl. J. Med. 320, 915–924
4. Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Scherer, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E., Steinberg, D., and Witztum, J. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1372–1376
5. Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., and Steinberg, D. (1989) J. Clin. Invest. 84, 1086–1095
6. Quinn, M. T., Parthasarathy, S., Feng, L. G., and Steinberg, D. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2995–2998
7. Berliner, J. A., Territo, M. C., Sevanian, A., Ramin, S., Kim, J. A., Ramsad, D., Akahoshi, E., and Foggelman, A. M. (1990) J. Clin. Invest. 85, 1260–1266
8. Frostegård, J., Nilsson, J., Hagestrand, A., Hamsten, A., Wiegell, H., and Gidlund, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 904–908
9. Martens, M., Fagerberg, B., and Steinberg, D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5949–5953
10. Hakamata, H., Miyazaki, A., Sakai, M., Matsuda, H., Suzuki, H., Kodama, T., and Horiiuchi, S. (1998) J. Lipid Res. 39, 482–494
11. Yui, S., Sasaki, T., Miyazaki, A., Horiiuchi, S., and Yamazaki, M. (1993) Arterioscler. Thromb. 13, 331–337
12. Sakai, M., Miyazaki, A., Hakamata, H., Sasaki, T., Yui, S., Yamazaki, M., Shichiri, M., and Horiiuchi, S. (1994) J. Biol. Chem. 269, 31430–31435
13. Sato, Y., Kohori, S., Sakai, M., Yano, T., Higashi, T., Matumura, T., Molikawa, W., Terano, T., Miyazaki, A., Horiiuchi, S., and Shichiri, M. (1996) Atherosclerosis 125, 15–26
14. Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matumura, T., Kohori, S., Shichiri, M., and Horiiuchi, S. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 600–605
15. Gordon, D., Reidy, M. A., Bennett, E. P., and Schwartz, S. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4690–4694
16. Rosenfeld, M. E., and Ross, R. (1990) Arterioscler. 10, 680–687
17. Sognoli, L. G., Orlandi, A., and Santeusanio, G. (1991) Atherosclerosis 88,
