Group-II phospholipase A$_2$ enhances oxidized low density lipoprotein-induced macrophage growth through enhancement of GM-CSF release

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Abstract

Inflammatory process plays an important role in the development and progression of atherosclerotic lesions. Recently, group-II phospholipase A$_2$ (PLA$_2$), an inflammatory mediator, was reported to exist in human atherosclerotic lesions and to enhance the development of murine atherosclerotic lesions. Oxidized low density lipoprotein (Ox-LDL) stimulates the growth of several types of macrophages in vitro. Since proliferation of macrophages occurs in atherosclerotic lesions, it is possible to assume that the Ox-LDL-induced macrophage proliferation might be involved in the progression of atherosclerosis. In this study, the role of group-II PLA$_2$ in the Ox-LDL-induced macrophage growth was investigated using thioglycollate-elicited mouse peritoneal macrophages. Thioglycollate-elicited macrophages significantly expressed group-II PLA$_2$ and released it into the culture medium. The Ox-LDL-induced thymidine incorporation into thioglycollate-elicited macrophages was three times higher than that into resident macrophages, whereas under the same conditions, granulocyte/macrophage colony-stimulating factor (GM-CSF) equally induced thymidine incorporation into both types of macrophages. Moreover, the Ox-LDL-induced GM-CSF release from thioglycollate-elicited macrophages was significantly higher than that from resident macrophages. In addition, the Ox-LDL-induced thymidine incorporation into macrophages obtained from human group-II PLA$_2$ transgenic mice and the GM-CSF release from these cells were significantly higher than those from their negative littermates, and the Ox-LDL-induced thymidine incorporation into human group-II PLA$_2$ transgenic macrophages was significantly inhibited by a polyclonal anti-human group-II PLA$_2$ antibody. These results suggest that the expression of group-II PLA$_2$ in thioglycollate-elicited macrophages may play an enhancing role in the Ox-LDL-induced macrophage growth through the enhancement of the GM-CSF release.

Keywords: Oxidized LDL; Macrophage growth; Atherosclerosis; Inflammation; Phospholipase A$_2$; GM-CSF

1. Introduction

The presence of a massive cluster of macrophage-derived foam cells in the subendothelial spaces of arter-
from the laboratory as well as by other investigators [8–17] have demonstrated that Ox-LDL could induce macrophage growth in vitro. Based on these findings, it was postulated that Ox-LDL might be involved in macrophage growth in vivo and linked to the progression of atherosclerotic process.

One of the pathological and immunohistochemical features of atherosclerotic lesions is accumulation of inflammatory cells and cytokines [1]. Moreover, dexamethasone, an anti-inflammatory agent, is known to exhibit an anti-atherogenic effect in animal models of experimental atherosclerosis, such as Watanabe heritable hyperlipidemic (WHHL) rabbit [18], cholesterol-fed rabbit [19] and rat balloon angioplasty model [20]. Based on these studies, it seems reasonable to consider atherosclerosis as a chronic inflammatory disease, or that the inflammatory process plays an important role in the development and progression of atherosclerosis. Moreover, recent reports using human group-II phospholipase A2 (PLA2) transgenic mouse demonstrated that group-II PLA2, one of the inflammatory mediators, played an enhancing role in the development of atherosclerotic lesions [21,22]. Thus, to further elucidate the pathophysiological significance of macrophage growth in atherosclerosis, it seems reasonable to investigate the growth promoting effect of Ox-LDL on inflammatory macrophages, and the effect of group-II PLA2 on the Ox-LDL-induced macrophage growth. Here, the growth stimulating effects of Ox-LDL on the thioglycollate-induced non-infectious inflammatory mouse peritoneal macrophages was examined. The results demonstrated that the responsiveness of thioglycollate-elicited macrophages to the growth-stimulating activity of Ox-LDL was significantly greater than that of resident macrophages, and the expression of group-II PLA2 might play, at least in part, an enhancing role in the growth of thioglycollate-elicited macrophage through the enhancement of granulocyte/macrophage colony-stimulating factor (GM-CSF) release.

2. Methods

2.1. Chemicals

[methyl-3H]Thymidine and [1-14C]oleic acid were purchased from NEN Life Science (Boston, MA). A rabbit polyclonal anti-human group-II PLA2 antibody was purchased from Funakoshi (Tokyo, Japan). Other chemicals were of the highest grade available from commercial sources.

2.2. Cell culture

Peritoneal cells were collected from non-stimulated male C3H/He mice (25–30 g) (Japan SLC, Hamatsu, Japan) and commercially available human group-II PLA2 transgenic mice and their negative littermates, C57BL/6J strain [23] (Taconic Farms, NY). Serum PLA2 activity in transgenic mice was reported to be 8–10-fold greater than that in non-transgenic mice [23]. The preliminary experiment using RT-PCR showed the expression of human group-II PLA2 mRNA in PLA2-transgenic macrophages but not in their negative littermates. Kennedy et al. [24] demonstrated that some murine strain had natural null mutation for group-II PLA2, such as 129/Sv, B10.RIII and C57BL/6J, but C3H/He produced functional group-II PLA2. Thus, C3H/He mice were used in the present study for determination of the effect of group-II PLA2 on the macrophage growth. Thioglycollate-elicited macrophages were collected from C3H/He mice 5 days after intraperitoneal injection of 1.5 ml thioglycollate [25]. The collected peritoneal cells were suspended in RPMI 1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), streptomycin (0.1 mg/ml), and penicillin (100 U/ml) (medium A). After 90 min incubation at 37°C, non-adherent cells were removed by triplicate washing with prewarmed medium A. After washing, cell numbers of resident and elicited macrophages were decreased to ~70%. Macrophages were identified by the following features: (i) adherence to culture plates; (ii) morphological features resembling mononuclear cells after Giemsa staining; (iii) the capacity to take up carbon particles; and (iv) positive immunohistochemistry with antibody for CD68.

2.3. Tritiated thymidine incorporation assay

Peritoneal cells were adjusted to 4 × 10^4 cells/ml and 1 ml of cell suspensions were dispersed in each well of 24-well tissue culture plates (15.5 mm in diameter, Corning Glass works, Corning NY) and incubated for 90 min at 37°C. Nonadherent cells were removed by washing three times with 1 ml of medium A. After washing, cell numbers of resident and elicited macrophages were counted by haemocytometer, and they were decreased to ~70%. The macrophage monolayers thus formed were cultured at 37°C with 1 ml of medium A in the presence of the lipoproteins to be tested. Eighteen hours before the termination of the experiments, 20 μl of 50 μCi/ml [3H]thymidine was added to each well and incubated for 18 h at 37°C. After discarding the medium, each well was washed three times with 1 ml of PBS and the cells were lysed with 0.5 ml of 0.5 M NaOH by incubation on ice for 10 min. The cell lysates were neutralized with 0.25 ml of 1 M HCl, further precipitated with 0.25 ml of 40% trichloroacetic acid by incubation on ice for 20 min. The resulting trichloroacetic acid-insoluble material was collected on filters (Millipore PVDF filter; 0.45 μm in
pore size) and washed three times with 1 ml of 99.5% ethanol. The filters were dried under air and their radioactivity was counted in a liquid scintillation counter [13].

2.4. Cell-counting assay

Peritoneal cells were adjusted to $2 \times 10^4$ cells/ml, and 1 ml of cell suspension was dispersed in each well of 24-well tissue culture plates (16 mm in diameter, Falcon) and incubated for 90 min at 37°C. Non-adherent cells were removed by triplicate washing with 1 ml of prewarmed medium A. The macrophage monolayers thus formed were cultured at 37°C in 1 ml of medium A, with or without the test lipoproteins. After incubation for 7 days, adherent cells in triplicate wells were lysed in 1% (w/v) Triton X-100, and the number of naphthol blue–black-stained nuclei was counted in a hemocytometer, as described previously [8].

2.5. Lipoproteins

Human LDL ($d = 1.019–1.063$ g/ml) was isolated by sequential ultracentrifugation of plasma samples obtained from consented normolipidemic subjects after overnight fasting [26]. LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA, pH 7.4. Ox-LDL was prepared by incubation of 0.1 mg/ml of LDL in PBS with 5 μM CuSO₄ for 20 h at 37°C followed by the addition of 1 mM EDTA and cooling [27]. Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride as described previously [28]. The level of endotoxin associated with these lipoproteins was < 1 pg/μg protein as measured by a commercially available kit (Toxicolor system, Seikagaku, Tokyo). Moreover, macrophage growth and viability were not affected by endotoxin at a concentration below 1 ng/ml in the experimental system.

2.6. RT-PCR analysis for mouse group-II PLA₂

Standard molecular biological techniques were used [29]. Macrophages ($2 \times 10^6$ cells) were dispersed into each well (3.5 cm in diameter, Nunc) and incubated for 90 min. After washing with medium A, macrophage monolayers thus formed were cultured in 2 ml of medium A for 6 h, and then total RNA was extracted with TRIzol (Life Technologies). The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo dT. Primers used for PCR amplification of group-II PLA₂ and β-actin were designed on the basis of murine group-II PLA₂ cDNA [30] and murine β-actin cDNA [31] sequences as follows: for group-II PLA₂, forward primer; TTC TGG CAG TTC CAG AGG ATG G (nucleotide 241–262 of murine group-II PLA₂ coding sequence); reverse primer; AAG ACA CTC CCT AGA CAG CAA (nucleotide 678–709 of murine group-II PLA₂ coding sequence) [30]; for β-actin, forward primer; GTG GGC CGC TCT AGG CAC CAA (nucleotide 25–45 of murine β-actin coding sequence); reverse primer; CTC TTT GAT TTC CAC GAT TTC (nucleotide 541–564 of murine β-actin coding sequence) [31]. The cycling conditions in the GeneAmp 9600 System consisted of a first step of 94°C denaturation for 10 min, followed by 30 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 amplification products were consistent with the reported sequences of murine group-II PLA₂ and β-actin, they were ligated into pGEM-T (Promega, Madison, WI), transfected into Escherichia coli XL1-Blue and sequenced by using 373A DNA sequencer (Applied Biosystems, Foster City, CA).

2.7. Enzyme-linked immunosorbent assay (ELISA) for GM-CSF

Macrophages ($5 \times 10^6$ cells) were dispersed into each well (10 cm in diameter, Falcon) and incubated for 90 min. After washing with medium A, macrophage monolayers thus formed were cultured in 15 ml of medium A with or without the lipoproteins to be tested for the indicated times, and then 300 μl of the medium were collected. The concentration of GM-CSF protein was determined according to the instructions provided by the manufacturer of mouse GM-CSF-specific ELISA system (Amersham) using recombinant murine GM-CSF as a standard [13].

2.8. PLA₂ activity

PLA₂ activity was determined using [1-¹⁴C]oleate-labeled E. coli phospholipid as a substrate, as described previously [32]. Assay mixtures contained 100 mM Tris–HCl (pH 7.4), 1 mM CaCl₂, $2 \times 10^6$ cells of [1-¹⁴C]oleate-labeled E. coli and enzyme or conditioned serum-free medium. Reaction mixtures were incubated for 1 h at 37°C. The reaction was terminated by the addition of 5 ml propane-2-ol/n-heptane/1 M H₂SO₄ (40/10/1, by vol.), followed by 2 ml heptane and 3 ml water. After vortex and phase separation, an aliquot (2.5 ml) of the upper phase was passed over a column of silic acid. Free [1-¹⁴C]oleate was quantitatively elute with 1 ml ethyl acetate. Radioactivity was determined in a liquid scintillation counter. PLA₂ activity was expressed as [1-¹⁴C]oleate radioactivity released by indicated cell culture supernatant.
2.9. Miscellaneous

Data were expressed as mean ± S.D. Differences between groups were compared for statistical significance using the Student’s t-test. A probability value less than 5% was considered significant. The experimental protocol was approved by the Human Ethics Review Committee and the Ethics Review Committee for Animal Experimentation of the institution.

3. Results

3.1. Thioglycollate-elicited macrophages express group-II \( \text{PLA}_2 \)

Various inflammatory bioactive molecules are secreted from cells in atherosclerotic lesions [1]. Among them, group-II \( \text{PLA}_2 \) as a candidate for enhancing the Ox-LDL-induced macrophage growth was focused on for the following reasons: (i) previous studies reported the presence of group-II \( \text{PLA}_2 \) in human atherosclerotic lesions [33,34], which played an enhancing role in mouse atherosclerotic lesions [21,22]; and (ii) the presence of group-II \( \text{PLA}_2 \) in peritoneal exudate induced by various stimuli [35–37]. RT-PCR analysis showed a faint band of mouse group-II \( \text{PLA}_2 \) mRNA in resident macrophages, whereas a clear band of mouse group-II \( \text{PLA}_2 \) mRNA was present in thioglycollate-elicited macrophages (Fig. 1A). Next \( \text{PLA}_2 \) activity in the macrophage conditioned medium was examined. Fig. 1B shows a significantly high \( \text{PLA}_2 \) activity in the conditioned medium containing thioglycollate-elicited macrophages compared to that of resident macrophages. These results suggested that thioglycollate-elicited macrophages produced a significantly higher amount of group-II \( \text{PLA}_2 \) into the medium than resident macrophages.

3.2. Ox-LDL stimulates growth of thioglycollate-elicited macrophages

The effects of Ox-LDL on the growth of mouse resident macrophages and thioglycollate-elicited macrophages were examined. Fig. 2A shows that thymidine incorporation into resident macrophages was induced by Ox-LDL in a dose-dependent manner, but neither by LDL nor acetyl-LDL. Fig. 2B shows that Ox-LDL also induced thymidine incorporation into thioglycollate-elicited macrophages, which was three times higher than that of resident macrophages (Fig. 2). As a control, thymidine incorporation induced by 1 nM of GM-CSF was almost equal in both types of macrophages (Fig. 2). Cell-counting assay also showed

![Fig. 1. Polymerase chain reaction (PCR) analysis of mouse group-II phospholipase A 2 (PLA 2 ) mRNA (A) and PLA 2 activity in medium (B). (A) Mouse peritoneal macrophages (2 × 10^6 cells) were seeded in 3.5 cm dish and incubated for 90 min. Non-adherent cells were removed by washing with medium A. The cell monolayers thus formed were incubated in 2 ml of medium A alone for 1 h. After incubation, total RNA was extracted from each dish with TRIzol. The expression of mRNA for mouse group-II \( \text{PLA}_2 \) (upper panel) or \( \beta \)-actin (lower panel) was evaluated by RT-PCR as described in Section 2. (B) Mouse peritoneal resident macrophages (○) or thioglycollate-elicited macrophages (●) (1 × 10^7 cells) were seeded in 10 cm dish and incubated for 90 min. After washing with medium A, the cell monolayers thus formed were incubated at 37°C for 18 h in 10 ml of RPMI 1640 with 3% bovine serum albumin (BSA). After incubation, the conditioned medium was collected and \( \text{PLA}_2 \) activity was determined as described under Section 2. After washing with PBS, cells were lysed with 0.1 N NaOH and protein contents were determined using BCA reagent (Pierce). Data represent the mean ± S.D. of three separate experiments. * \( P < 0.01 \) compared to medium alone by Student’s t-test.]
that the number of resident macrophages increased 1.8 times by Ox-LDL compared to non-loaded resident macrophages, whereas the number of thioglycollate-elicited macrophages was significantly increased 2.3 times by Ox-LDL (Table 1). These results demonstrated that the growth of thioglycollate-elicited macrophages was also induced by Ox-LDL and the responsiveness of thioglycollate-elicited macrophages to the growth-stimulating activity of Ox-LDL was significantly greater than that of resident macrophages.

3.3. Group-II PLA2 enhances macrophage growth

To determine the role of group-II PLA2 in the Ox-LDL-induced macrophage growth, the effect of Ox-LDL on the growth of macrophages obtained from human group-II PLA2 transgenic mice was examined. Fig. 3 shows that the Ox-LDL-induced thymidine incorporation into macrophages derived from human group-II phospholipase A2 (PLA2) transgenic mice. Mouse resident peritoneal macrophages from human group-II PLA2 transgenic mice or their negative littermates (4 × 10^6 cells) were dispersed in 24 well plates, and incubated for 90 min. After washing, cell monolayers thus formed were incubated at 37°C for 6 days in 1 ml of medium A with the indicated concentrations of Ox-LDL (○), acetyl-LDL (△), LDL (□) or 1 nM of recombinant mouse granulocyte/macrophage colony-stimulating factor (GM-CSF) as a control. Thymidine incorporation was determined as described in Section 2. Data represent the mean ± SD of three separate experiments. * P<0.01 compared to medium alone by Student’s t-test.

### Table 1

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Lipoproteins</th>
<th>Cell number (×10^4/well)</th>
<th>% Medium alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resident macrophages</td>
<td>Medium alone</td>
<td>1.4 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>1.4 ± 0.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Acetyl-LDL</td>
<td>1.5 ± 0.3</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Ox-LDL</td>
<td>2.5 ± 0.2*</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>2.8 ± 0.4*</td>
<td>200</td>
</tr>
<tr>
<td>Elicited macrophages</td>
<td>Medium alone</td>
<td>1.5 ± 0.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>1.6 ± 0.4</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Acetyl-LDL</td>
<td>1.8 ± 0.3</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Ox-LDL</td>
<td>3.4 ± 0.2**</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>GM-CSF</td>
<td>3.0 ± 0.3**</td>
<td>200</td>
</tr>
</tbody>
</table>

* Peritoneal macrophages (2 × 10^6 cells) were dispersed into culture plates, and incubated for 90 min. After incubation, non-adherent cells were removed by triplicate washing with medium A. At the start of experiment, cell numbers of resident and elicited macrophages were 1.4 and 1.5 × 10^6 cells/well, respectively. Macrophage monolayers thus formed were incubated for 7 days in 1 ml of medium A with 20 µg/ml of lipoproteins or 1 nM of GM-CSF as a control. After incubation, counting of solubilized nuclei was performed as described under Section 2. Data are expressed as mean ± S.D. of quadruplicate counts. LDL, low density lipoprotein; Ox-LDL, oxidized low density lipoprotein; GM-CSF, granulocyte/macrophage colony-stimulating factor.

* P<0.01, compared to medium alone.

** P<0.01, compared to medium alone by Student’s t-test.
corporation into macrophages from human group-II PLA2 transgenic mice was twice that from their negative littermates. Moreover, the Ox-LDL-induced thymidine incorporation into macrophages from human group-II PLA2 transgenic mice was significantly but partially inhibited by 50% by a polyclonal anti-human group-II PLA2 antibody as compared to that by non-immune IgG (Fig. 4). These results suggested that group-II PLA2 might enhance, at least in part, the Ox-LDL-induced macrophage growth.

3.4. Ox-LDL augments GM-CSF release

It has recently been reported that GM-CSF played a priming role in the Ox-LDL-induced macrophage growth [13]. Thus, the production of GM-CSF from thioglycollate-elicited macrophages was next examined. When resident macrophages were incubated with medium alone for 6 h, GM-CSF was released at concentration of 8.2 fM/mg protein (Table 2). Ox-LDL significantly enhanced the GM-CSF release from resident macrophages into the medium in a dose-dependent manner with a maximal concentration of 30.2 fM/mg protein at 40 μg/ml of Ox-LDL (Table 2). On the other hand, when thioglycollate-elicited macrophages were incubated with medium alone, the concentration of GM-CSF in the medium was 7.8 fM/mg protein (Table 2). However, the GM-CSF release from thioglycollate-elicited macrophages was increased by Ox-LDL with maximal concentration of 40.5 fM/mg protein, which was significantly higher than that from resident macrophages induced by Ox-LDL (Table 2). In contrast, LDL or acetyl-LDL could not enhance the GM-CSF release from both types of macrophages (Table 2). Combined with the results of macrophage growth (Fig. 2) and PLA2 activity (Fig. 1), these results suggested that PLA2 might enhance the effect of Ox-LDL on the GM-CSF release as well as macrophage growth. To confirm this conclusion, the effect of Ox-LDL on the GM-CSF release from human group-II PLA2 transgenic macrophages was examined. As shown in Table 3, the Ox-LDL-induced GM-CSF release from macrophages obtained from wild type littermates was 2.1 times greater than that from wild cells which were incubated with medium alone. Consistent with macrophage growth (Fig. 2), the Ox-LDL-induced GM-CSF release from human group-II PLA2 transgenic macrophages was significantly greater than that from wild macrophages (Table 3). These results suggested that group-II PLA2 enhances GM-CSF release from thioglycollate-elicited macrophages, thereby enhancing the Ox-LDL-induced macrophage growth.

4. Discussion

Recent studies from the laboratory as well as by others have demonstrated that Ox-LDL could induce the growth of several types of macrophages in vitro [8–17], whereas the importance of in situ macrophage growth during the development of atherosclerotic lesions is still unknown. It is possible to assume that the Ox-LDL-induced macrophage growth is involved in the development and progression of atherosclerotic lesions because: (i) macrophages and macrophage-derived foam cells are believed to play an essential role in the development and progression of atherosclerosis via production of various active molecules [1]; and (ii) proliferation of macrophages has been observed in atherosclerotic lesions [2–4].

Recent immunohistochemical and pathological studies suggested that the inflammatory process was involved in the development and progression of atherosclerosis [1,18–20]. Moreover, recent reports using human group-II PLA2 transgenic mouse demonstrated that group-II PLA2, an inflammatory mediator, played an enhancing role in the development of atherosclerotic lesions [21,22]. Thus, it seems reasonable to investigate the growth promoting effects of Ox-LDL.
on inflammatory macrophages. Jutila and Banks [38] reported that the thymidine incorporation into non-stimulated thioglycollate-elicited inflammatory macrophage was greater than that into non-stimulated resident macrophages, whereas it was unclear whether Ox-LDL could induce the growth of thioglycollate-elicited macrophages. The present study demonstrated that Ox-LDL induced the growth of thioglycollate-elicited macrophages, which was significantly higher than the Ox-LDL-induced growth of resident macrophages (Fig. 2 and Table 1).

The results showed that the Ox-LDL-induced growth of thioglycollate-elicited macrophages was significantly greater than that of resident macrophages (Fig. 2 and Table 1), and the expression of group-II PLA₂ might play, at least in part, an enhancing role in this phenomenon (Fig. 1). This conclusion was supported by the following findings: (i) the Ox-LDL-induced thymidine incorporation into macrophages from human group-II PLA₂ transgenic mice was significantly higher than that from their negative littermates (Fig. 3); and (ii) such incorporation was significantly but partially inhibited by a polyclonal anti-human group-II PLA₂ antibody (Fig. 4). The presence of group-II PLA₂ in human atherosclerotic lesions is known to be associated with macrophages and lipid core lesions [44]. Interestingly, in addition to atherosclerotic lesions, macrophage proliferation has also been observed in tumors and inflamed lung [45–48], where the expression of group-II PLA₂ has been reported [40]. Thus, it is possible that group-II PLA₂ may enhance macrophage proliferation by certain stimuli in various tissues. In the present study, the enhancing effect of group-II PLA₂ on the Ox-LDL-induced macrophage growth has been focused on. It should be noted, however, that various active molecules are expressed in atherosclerotic lesions apart from group-II PLA₂ [1]. Thus, further studies are necessary to examine whether other active molecules enhance the Ox-LDL-induced growth of thioglycollate-elicited macrophages.

Group-I PLA₂ is another type of secretary PLA₂ derived mainly from pancreatic juice [40]. This enzyme is known to exhibit a growth stimulating activity for fibroblasts through its specific receptor [49]. However, it remains unclear whether group-II PLA₂ directly induces cell growth. Kurizaki et al. [50] has reported that group-II PLA₂ induced fibroblast growth, although the exact mechanism of this process remains unknown at present. In the present study, following incubation of macrophages with medium alone, macrophage growth did not occur in thioglycollate-elicited macrophages and human group-II PLA₂-transgenic macrophages which produced a significant amount of group-II PLA₂ (Figs. 2 and 3). Moreover, the preliminary experiments showed that PLA₂ alone did not induce resident macrophage growth. These findings suggested that
group-II PLA\(_2\) did not induce macrophage growth directly, and that it exhibited a growth enhancing activity for macrophages via phospholipid metabolites, such as lysophospholipids, arachidonate itself or its metabolites. Since it was previously reported that receptor-mediated endocytosis of lysophosphatidylcholine in Ox-LDL through the scavenger receptors played an important role in the Ox-LDL-induced macrophage growth [8], it was possible that PLA\(_2\) might increase in lysophosphatidylcholine content in Ox-LDL, thereby enhancing the Ox-LDL-induced macrophage growth. However, a significant change of lysophosphatidylcholine content in the conditioned medium could not be detected, in spite of an increase in PLA\(_2\) activity. The exact reason of these apparently inconsistent results remains unclear, but it is possible to assume that during incubation with macrophages, lysophosphatidylcholine hydrolyzed from phosphatidylcholine by PLA\(_2\) may be further reduced by other enzymes to glycerolphosphate or other metabolites, or may receive re-acylation to phosphatidylcholine in the cell culture system. Therefore, the change of lysophosphatidylcholine contents in the conditioned medium after incubation with macrophages might not be detected. On the other hand, Martens et al. [16] recently demonstrated that oxidized arachidonate might modify apoB and then induce macrophage growth. Moreover, it was reported that free fatty acids, another part of phosphatidylcholine metabolite by PLA\(_2\), in LDL were oxidized by cells and enhanced further oxidation of LDL [51]. These findings suggested that arachidonate or its oxidatively modified metabolite(s) might enhance the Ox-LDL-induced growth of thioglycollate-elicited macrophages. However, the precise mechanisms of action are still unknown at present and further studies are needed to elucidate PLA\(_2\)-mediated enhancement of the Ox-LDL-induced macrophage growth.

In the present study, the Ox-LDL-induced thioglycolate-elicited macrophage growth, PLA\(_2\) activity in conditioned medium and GM-CSF release from these cells were all significantly greater than those of resident macrophages (Figs. 1 and 2, and Table 2). Moreover, the Ox-LDL-induced growth of human group-II PLA\(_2\) transgenic macrophages and the GM-CSF release from these cells were both significantly greater than those from wild macrophages (Fig. 3 and Table 3). Furthermore, both elicited and resident macrophages were equal in their response to the growth-promoting activity of recombinant GM-CSF (Fig. 2). These results suggested that group-II PLA\(_2\) promotes the Ox-LDL-induced macrophage growth through enhancement of the GM-CSF release. A recent study demonstrated that dexamethasone inhibited the Ox-LDL-induced macrophage growth which was explained by the inhibition of the GM-CSF expression [52]. Moreover, dexamethasone was reported to inhibit the expression of group-II PLA\(_2\) in rat macrophages [53]. These findings also supported the conclusion that group-II PLA\(_2\) might enhance the expression of GM-CSF. However, the enhancing mechanism of group-II PLA\(_2\) for the GM-CSF release from macrophages remains unclear at present. It was previously demonstrated that a rise in intracellular calcium and a subsequent increase in PKC activity were involved in the Ox-LDL-induced GM-CSF release and the subsequent macrophage growth [14]. Moreover, Martens et al. [15] recently reported that phosphatidylinositol 3-kinase (PI3K) was involved in the Ox-LDL-induced macrophage growth. Further studies are necessary to elucidate the mechanism of the group-II PLA\(_2\)-mediated enhancement of GM-CSF release and the subsequent macrophage growth.

It was previously demonstrated that PLA\(_2\)-treated acetyl-LDL significantly induced macrophage growth [8]. However, when elicited-macrophages or human PLA\(_2\)-transgenic macrophages, which released a significant amount of group-II PLA\(_2\), were incubated with acetyl-LDL, macrophage growth did not observed (Figs. 2 and 3). These results were apparently inconsistent. When macrophages were incubated with acetyl-LDL, acetyl-LDL bound to the scavenger receptors and

<table>
<thead>
<tr>
<th>Macrophages</th>
<th>Ox-LDL (μg/ml)</th>
<th>GM-CSF (fmol/mg protein)</th>
<th>% Medium alone</th>
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<tr>
<td>Wild macrophages</td>
<td>0</td>
<td>7.8 ± 1.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>16.5 ± 2.8*</td>
<td>212</td>
</tr>
<tr>
<td>PLA(_2)-transgenic macrophages</td>
<td>0</td>
<td>5.6 ± 1.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>20.9 ± 2.2**</td>
<td>373</td>
</tr>
</tbody>
</table>

* Peritoneal macrophages (2 × 10\(^6\) cells) were dispersed into culture plates, and incubated for 90 min. After incubation non-adherent cells were removed by triplicate washing with medium A. Macrophage monolayers thus formed were incubated for 6 h in 1 ml of medium A with indicated concentrations of lipoproteins. After incubation, culture medium were taken and the levels of GM-CSF was determined by ELISA as described under Section 2. Contents of cellular proteins were determined using BCA reagent (Pierce). Data are expressed as mean ± S.D. of triplicate. Ox-LDL, oxidized low density lipoprotein; GM-CSF, granulocyte/macrophage colony-stimulating factor; PLA\(_2\), phospholipase A\(_2\).

* \(P < 0.01\), compared to non-loaded wild macrophages.

** \(P < 0.01\), compared to non-loaded PLA\(_2\) transgenic macrophages by Student’s \(t\)-test.
was endocytosed by macrophages immediately. Moreover, since the medium used in the present study contained 10% fetal calf serum (FCS), a large amount of phosphatidylcholine might prevent hydrolysis of phosphatidylcholine in acetyl-LDL. Thus, it is possible to assume that if a small amount of phosphatidylcholine would be hydrolyzed to lysophosphatidylcholine in acetyl-LDL by the action of PLA2, it might not be enough for the induction of macropahge growth.

Based on the finding that Ox-LDL significantly induced the growth of peritoneal cells obtained from M-CSF and GM-CSF double knockout mouse, Hamilton et al. [17] proposed that the GM-CSF release from macrophages was not required for the Ox-LDL-induced macropahge growth. Since both M-CSF and GM-CSF are well known growth factors regulating the differentiation and the proliferation of monocytes/macrophages, it is possible to assume that peritoneal cells from double knockout mice could represent immature macrophages or poorly differentiated monocytic cells. In fact, M-CSF knockout mice are known to develop osteopetrosis which is caused by immaturatation of osteoclast, a resident macrophage [54,55]. Moreover, it is reported that Ox-LDL is able to induce the differentiation of monocytes into macrophages [56]. These findings suggested that the effect of Ox-LDL on immature macrophages might differ in quality from that on mature macrophages.

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References
