Original Contributions

Induction of Murine Macrophage Growth by Modified LDLS

Satoru Yui, Toshinori Sasaki, Akira Miyazaki, Seikoh Horiuchi, and Masatoshi Yamazaki

We previously reported that cell membrane components and lipoproteins were able to induce the growth of murine peritoneal macrophages. The aim of the present study was to examine whether macrophage growth could also be induced by chemically modified lipoproteins, such as acetylated low density lipoprotein (acetyl-LDL) or oxidized LDL, ligands known to be endocytosed by the macrophage scavenger receptors. When murine peritoneal exudate macrophages were cultured in vitro with 25–100 μg/mL acetyl-LDL or oxidized LDL, significant growth was induced. On comparing the dose–response curves of these LDLs, a more potent effect was seen with oxidized LDL than acetyl-LDL, especially on resident macrophages. On the other hand, growth of these cells was not stimulated by native (unmodified) LDL or high density lipoprotein. These in vitro data revealed a new function of chemically modified LDLS as effective inducers of macrophage cell growth. This aspect may be physiologically relevant to the growth of macrophage foam cells in situ in the development of atherosclerosis. (Arteriosclerosis and Thrombosis 1993;13:331–337)

Key Words • macrophage growth • acetylated LDL • oxidized LDL • scavenger receptors

Foam cells, the key cellular elements in atherosclerotic plaques in the arterial wall, are derived from monocytes/macrophages.1-3 The macrophage scavenger receptors are known to mediate endocytotic uptake of chemically modified low density lipoprotein (LDL) such as acetylated LDL (acetyl-LDL), or oxidatively modified LDL, leading to foam cell formation.4,5 Recent immunohistochemical studies using monoclonal antibodies specific for modified portions of malondialdehyde-modified and oxidized LDLS have demonstrated the presence of these modified LDLS in atherosclerotic lesions.6-9 The proliferation of foam cells in atherosclerotic lesions was also suggested.10-12 Recent studies using cell type-specific monoclonal antibodies and other techniques have confirmed that many proliferating foam cells, particularly in the early phase of atherosclerosis, have been derived from macrophages.13-15 It is quite reasonable, therefore, to expect that a proliferative capacity of macrophage foam cells could be important in the development of atherosclerotic lesions.

To date, protein factors known to regulate the growth of peripheral macrophages are macrophage colony-stimulating factor (M-CSF),16,17 granulocyte/macrophage colony-stimulating factor (GM-CSF),16,18 and interleukin-3.19 Our previous studies disclosed that lipid components such as cholesterol esters (CEs), triglycerides, and some negatively charged phospholipids were also able to induce growth of murine peritoneal macrophages in vitro.21-23 It was stated, however, that the macrophage growth-stimulating activity of these lipids did not appear to depend on any protein factors23-24; rather, since these macrophages exhibited a foam cell-like appearance when incubated with the lipids, the lipids may have a direct role in macrophage growth.24

These findings, together with the above-mentioned possibility that modified LDL might be involved in foam cell formation in vivo, prompted us to examine whether macrophage cell growth was affected by acetyl-LDL or oxidized LDL, ligands for the macrophage scavenger receptors.25,26 The results demonstrated the capacity of these modified LDLS to induce growth of murine peripheral macrophages in vitro, indicating that the growth-stimulating capacity of the modified LDLS might be important in the atherogenic process in vivo.

Methods

Lipoproteins and Their Modifications

LDL (d=1.019–1.063 g/mL) was isolated by ultracentrifugation from fresh human plasma of normolipidemic subjects after overnight fasting as described previously.27 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 anhydride27 according to the method of Basu et al.28 To prepare oxidized LDL, 0.2 mg/mL LDL was incubated for 20 hours at 37°C with 5 μM CuSO4 in EDTA-free phosphate-buffered saline (PBS), followed by addition of 0.1 mM EDTA,29 using the method of Steinbrecher et al.30 Thiobarbituric acid–reactive substances of native LDL, acetyl-LDL, and oxidized LDL were 1.78±0.26, 2.49±0.38, and 34.17±2.42 nmol malondialdehyde per mg protein, respectively (n=3, mean±SD). The agarose gel electrophoretic pattern of each LDL is shown in Figure 1.

From the Faculty of Pharmaceutical Sciences (S.Y., T.S., M.Y.), Teikyo University, Kanagawa, and the Department of Biochemistry (A.M., S.H.), Kumamoto University Medical School, Kumamoto, Japan.

Address for correspondence: Dr. Masatoshi Yamazaki, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-cho, Tsukui-gun, Kanagawa 229-01, Japan.

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Cells

Male C3H/He mice were purchased from breeding colonies at the Shizuoka Experimental Animal Farm, Shizuoka, Japan. Peritoneal cells were obtained 3 days after intraperitoneal injection of 30 mg starch into each animal. In some experiments, resident peritoneal macrophages were collected from untreated mice. These cells were suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% heat-inactivated fetal bovine serum (GIBCO, Grand Island, N.Y.), penicillin (100 units/mL), and kanamycin (60 μg/mL) (hereafter referred to as “medium A”). For the tritiated thymidine ([3H]Tdr) incorporation assay, the peritoneal cells were incubated in 96-well microplates (Corning, Corning, N.Y.) at 2x10⁴ cells/well. For cell counting, the peritoneal cells were incubated in 24-well microplates (Falcon, Lincoln Park, N.J.) at 4x10⁴ cells/well. The cells were incubated for 90 minutes at 37°C in a CO₂ incubator to allow them to adhere to the plates. The medium was then removed, and the nonadherent cells were removed by three vigorous washings with prewarmed PBS solution. More than 95% of the adherent cells were judged to be macrophages by both Giemsa staining and carbon-particle uptake. Unless otherwise specified, these macrophages were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air with 0.2 mL (for 96 wells) or 1.0 mL (for 24 wells) medium A in the presence or absence of the modified LDLs. After 3 or 7 days of culture, the cells were washed two times with 1.0 mL PBS containing 0.2% bovine serum albumin and then suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% heat-inactivated fetal bovine serum (GIBCO, Grand Island, N.Y.) with 0.2% bovine serum albumin and three times the culture medium. The cells were then counted by inverted, phase-contrast microscopy. The number of macrophages was determined in two standardized areas (0.25 mm²) in each of two wells. The number of macrophages was determined in two ways. To determine the number of cells per well during culture, the number of adherent cells within five standard-sized areas (0.25 mm²) in each of two wells was counted by inverted, phase-contrast microscopy. To determine the number of cells in the stationary phase of the culture, the medium was discarded, the adherent cells in triplicate wells were lysed in 1% (wt/vol) Triton X-100, and the number of naphthol blue-black-stained nuclei were counted in a hemocytometer as described by Nakagawara and Nathan.³¹ As judged from the uptake of carbon particles and the morphological features after staining with Giemsa, more than 99% of the cells cultured with either acetyl-LDL or oxidized LDL were macrophages.

Tritiated Thymidine Incorporation

Macrophage growth was assayed by the incorporation of [3H]Tdr into the acid-insoluble fraction.²¹ Briefly, [methyl-3H]thymidine (80 Ci/mmol, New England Nuclear, Boston, Mass.) was added to each plate at 1 μCi/mL and incubated for 18 hours. Then the medium was discarded, and the cells were dissolved in 100 μL cold 10% trichloroacetic acid. The resulting trichloroacetic acid-insoluble material was collected on filters with Labo mash LM-101 (Labo Science, Tokyo). The filters were dried, and their radioactivity was counted in a liquid scintillation spectrophotometer. All experiments were performed in triplicate.

Morphological Observations

Morphological observations of cultured macrophages were made by inverted, phase-contrast microscopy.

Uptake of Carbon Particles

One microliter of black drawing ink (Rotring, Art. 591 017, Hamburg, FRG) was added to the culture wells in 24-well plates, and the cells were incubated at 37°C for 2 hours in a CO₂ incubator. The cells were washed with PBS and subjected to microscopic observations.

Mass Determination of Macrophage Cholesterol Content

To determine the mass of cholesterol content in macrophages, peritoneal cells were incubated in six-well plates (Corning) at 2.5x10⁶ cells/well. After removal of nonadherent cells, macrophages were cultured at 37°C in a CO₂ incubator with 2.0 mL medium A in the presence or absence of the modified LDLs. After 3 or 7 days of culture, the cells were washed twice with a 1.0 mL PBS containing 0.2% bovine serum albumin and three times with 1.5 mL PBS, and then the cellular lipids were extracted and both free cholesterol and CE mass quantified by a modification of the enzymic fluorometric methods of Heider and Boyett³² as previously described.³³

Results

To examine whether acetyl-LDL could induce growth (i.e., increase in cell number) of mouse peripheral macrophages in vitro, we first measured the growth of peritoneal exudate macrophages by cell counting. As shown in Figure 2, the cell number of macrophages increased twofold after an 8-day incubation with 50 μg/mL acetyl-LDL and about threefold after a 10-day incubation. A parallel incubation with the same concentration of unmodified LDL or medium alone had almost no effect on the cell number. Acetyl-LDL was also found to increase cell number when the growth was assessed by counting solubilized nuclei (Table 1). These results suggest that acetyl-LDL has the capability to induce growth of macrophages.

To test this notion, the effects of acetyl-LDL on the incorporation of [3H]Tdr into macrophages were determined (Figure 3). When incubated with unmodified LDL at concentrations ranging from 25 μg/mL to 200 μg/mL, the [3H]Tdr incorporation increased slightly but not significantly (the value obtained after incubation with 200 μg/mL unmodified LDL was less than twofold that obtained by parallel incubation with the medium alone). In sharp contrast, incubation with acetyl-LDL resulted in a marked increase in [3H]Tdr incorporation. The augmenting effect of acetyl-LDL became significant at 25 μg/mL (about threefold above the basal level) and increased linearly in a dose-dependent manner up

FIGURE 1. Agarose gel electrophoresis of native low density lipoprotein (LDL), oxidized LDL, and acetyl-LDL. Each LDL (5 μg as protein) was loaded onto agarose gel. After electrophoresis, the gel was stained with fat-red 7B. Lane A, native LDL; lane B, oxidized LDL; lane C, acetyl-LDL.
FIGURE 2. Line plot showing effect of acetylated low density lipoprotein (acetyl-LDL) on macrophage growth in vitro. Starch-induced murine macrophages were cultured with 50 µg/mL native LDL (○), 50 µg/mL acetyl-LDL (●), or with medium alone (■). After the indicated periods, the numbers of adherent macrophages per well were counted as described in "Methods." Bars represent the higher and lower counts of duplicate estimations. Data are representative of two separate experiments.

to sevenfold at 100 µg/mL, followed by a rapid decline to the basal level at >200 µg/mL. The dose-dependent effect of acetyl-LDL was maximal at 100 µg/mL, with which the incorporation was about 3.6-fold higher than that obtained with the same concentration of unmodified LDL. In some experiments, the dose-response curve shifted to a lower range.

It is well known that acetyl-LDL is taken up by macrophage scavenger receptors and that the uptake is effectively inhibited by several polyanions.4 To ascertain whether the binding of acetyl-LDL to the scavenger receptor was required for induction of macrophage growth, the effect of polyanions on growth induction by acetyl-LDL was examined. As shown in Figure 4, acetyl-LDL-induced growth of macrophages was obliterated in the presence of polyinosinic acid, an effective inhibitor. In contrast, polycytidylic acid, an abortive ligand for the scavenger receptor, did not affect the growth induction.

Oxidized LDL is one of the physiologically relevant ligand candidates for the scavenger receptors.26,34 Examination of its effect showed that the cellular incorporation of [³H]TdR became significant at day 6 when macrophages were incubated with 25–100 µg/mL oxidized LDL, whereas macrophage growth was not

TABLE 1. Estimations of Numbers of Starch-Induced Macrophages Cultured With Acetylated LDL by Counting of Solubilized Nuclei

<table>
<thead>
<tr>
<th>Sample (µg/mL)</th>
<th>Macrophage number (×10⁴/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>50</td>
<td>2.2±0.6</td>
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Starch-induced murine peritoneal macrophages were cultured with the indicated concentrations of acetylated low density lipoprotein (LDL) for 10 days. Counting of solubilized nuclei was performed as described in "Methods." Data are represented as mean±SD of triplicate counts.

FIGURE 3. Line plot showing tritiated thymidine ([³H]TdR) incorporation into macrophages by acetylated low density lipoprotein (acetyl-LDL). Starch-induced murine macrophages were cultured without (●) or with the indicated concentrations of native LDL (○) or acetyl-LDL (●). On day 6, the cultures were pulsed with [³H]TdR for an additional 18 hours and harvested as described in "Methods." [³H]TdR incorporation was assayed on day 6. Bars represent SD. Data are representative of five separate experiments. *p<0.05 compared with the medium-alone control (by Student's t test).

duced by native LDL or high density lipoprotein (Figure 5A). When compared at 25 µg/mL, oxidized LDL was much more potent than acetyl-LDL in its growth-stimulating activity (Figure 5A). This difference in the capacity between acetyl-LDL and oxidized LDL was more prominent with resident macrophages when compared with [³H]TdR incorporation (Figure 5B). This was also the case when macrophage growth was assayed by counting solubilized nuclei (Table 2).

FIGURE 4. Line plot showing effect of polyinosinic acid (poly I) and polycytidylic acid (poly C) on stimulation of tritiated thymidine ([³H]TdR) incorporation into macrophages by acetylated low density lipoprotein (acetyl-LDL). Starch-induced murine macrophages were cultured without sample (●) or with 50 µg/mL acetyl-LDL in the presence of the indicated concentrations of poly I (●) or poly C (○) for 6 days. Data are representative of two separate experiments. [³H]TdR incorporation was assayed as described in the legend to Figure 3. Bars represent SD.
Figure 5. Tritiated thymidine ([3H]TdR) incorporation into macrophages cultured with various lipoproteins. Panel A: Starch-induced murine macrophages were cultured without (■) or with the indicated concentrations of native low density lipoprotein (LDL) (○), native high density lipoprotein (●), acetylated (acyetyl) LDL (◇), or oxidized LDL (▲) for 6 days. Data are representative of five separate experiments. Panel B: Resident macrophages were cultured without (■) or with the indicated concentrations of acetyl-LDL (◇) or oxidized LDL (▲) for 6 days. Data are representative of two separate experiments. [3H]TdR incorporation was assayed as described in the legend to Figure 3. Bars represent SD. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001 compared with the medium-alone control (by Student's t test).

Figure 6 shows the results of morphological observations of macrophages cultured for 10 days with acetyl-LDL and oxidized LDL. Many round cells with numerous cytoplasmic lipid droplets were seen in macrophages cultured with acetyl-LDL. In sharp contrast, the spreading of spindle-shaped cells was characteristic of the macrophages incubated with oxidized LDL; cytoplasmic lipid droplets, however, were less numerous in these macrophages. Macrophages cultured with native LDL or with the medium alone were small and round.

To assess the amount of lipid accumulation in these macrophages, CE and free cholesterol contents were measured. As shown in Figure 7, CE mass levels of macrophages cultured with acetyl-LDL for 3 and 7 days were about 360-fold and 480-fold higher, respectively, than those of control macrophages. With oxidized LDL, the CE contents were about 140-fold and 150-fold higher than controls, respectively. A CE mass level of macrophages cultured with oxidized LDL was less than half that of the macrophages cultured with acetyl-LDL.

Table 2. Estimations of Numbers of Resident Macrophages Cultured With Acetylated LDL and Oxidized LDL by Counting of Solubilized Nuclei

<table>
<thead>
<tr>
<th>Sample (μg/mL)</th>
<th>Macrophage number (×10⁴/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.4±0.4</td>
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<tr>
<td>Acetylated LDL</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>50</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>50</td>
<td>3.2±0.4</td>
</tr>
</tbody>
</table>

Resident murine peritoneal macrophages were cultured with the indicated concentrations of acetylated low density lipoprotein (LDL) or oxidized LDL for 10 days. Counting of solubilized nuclei was performed as described in “Methods.” Data are represented as mean±SD of triplicate counts.

Discussion

Recruitment of macrophage foam cells in atherosclerotic lesions is considered to be one of the important steps in the atherogenic process. Evidence has been mounting that macrophage-derived foam cells are indeed able to proliferate in situ in the early stages of lesion development. However, since peripheral macrophages do not usually possess the capacity to divide, it is expected that some growth factor(s) is generated in atherosclerotic plaques. The present study showed that modified LDLs, such as acetyl-LDL and oxidized LDL, were able to induce the growth of murine peritoneal macrophages in vitro. To our knowledge, this is the first report disclosing this new property of modified LDLs. Acetyl-LDL concentrations that were effective in inducing macrophage growth under the present in vitro conditions were closely similar to those of acetyl-LDL that induce intracellular CE accumulation in vitro. In fact, macrophages cultivated with the optimal concentration of acetyl-LDL or oxidized LDL were found to accumulate CE (Figure 7). Thus, it is possible that the in vitro growth-inducing capacity of these lipoproteins might account for the presence of proliferating foam cells derived from macrophages in atherosclerotic lesions in situ.

The mechanism of growth induction by these modified LDLs is unknown. Our previous studies have shown that triglycerides, cholesterol palmitate, stearate, linoleate, and linolenate and negatively charged phospholipids (phosphatidylserine, phosphatidylglycerol, cardiolipin, etc.) are able to induce macrophage growth, so a lipid component(s) of these modified LDLs could be responsible for this phenomenon. Although these lipid components seem to stimulate macrophage growth in a direct manner, the involvement of M-CSF or GM-CSF as autocrine macrophage growth factors cannot be ruled out; the interaction of the modified LDLs with macrophages might stimulate the latter to release a factor(s) that might in turn act on macrophages themselves as a cell growth inducer. Sup-
porting this possibility is the finding that modified LDL stimulates endothelial cells to release CSFs. Alternatively, since acetyl-LDL and oxidized LDL are endocytosed via the scavenger receptor,
the macrophage growth-stimulating activity of acetyl-LDL (see Figure 4) and that of oxidized LDL (data not shown) were effectively inhibited by the presence of polynosinic acid, an effective inhibitor of the scavenger receptor. Thus, the endocytic uptake of modified LDL through the receptor might be necessary for growth induction, and the ligand binding to the receptor may initiate signal transduction, which leads to macrophage growth. Alternatively, intracellular accumulation of CEs may be linked to this activity, since our previous study showed that macrophages accumulated a large amount of lipid droplets during culture when growth was induced by CEs or acidic phospholipids. In the present study, we did observe CE accumulation in macrophages cultured with both acetyl-LDL and oxidized LDL (Figure 7). Therefore, lipid accumulation per se seems to play a key role in

![Image of photomicrographs of macrophages cultured with modified low density lipoproteins (LDLs).](https://example.com/figure6)

**FIGURE 6.** Photomicrographs of macrophages cultured with modified low density lipoproteins (LDLs). Starch-induced murine peritoneal cells (1×10⁵/mL) were cultured for 10 days with medium alone (panel a), 50 µg/mL native LDL (panel b), acetylated LDL (panel c), or oxidized LDL (panel d). Original magnification, ×200; final magnification, ×160.

![Image of bar graphs of cholesterol ester and free cholesterol contents in macrophages cultured with modified low density lipoproteins.](https://example.com/figure7)

**FIGURE 7.** Bar graphs of cholesterol ester and free cholesterol contents in macrophages cultured with modified low density lipoprotein (acetyl-LDL) or oxidized LDL. Starch-induced murine peritoneal macrophages were cultured with 50 µg/mL acetyl-LDL or 50 µg/mL oxidized LDL for 3 days (panel A) or 7 days (panel B). Cholesterol ester (▲) and free cholesterol (□) contents were measured as described in “Methods.” Bars represent SD of quadruplicate determinations.
the macrophage growth induced by modified LDLs. These issues should be pursued in future research. The growth-stimulating activity of negatively charged phospholipids such as phosphatidylserine was strongly enhanced on peroxidation. Here, the macrophage growth-stimulating activity of oxidized LDL was shown to be more potent than that of acetyl-LDL (Figure 4 and Table 1), although oxidized LDL induced about a threefold lower CE accumulation than acetyl-LDL (Figure 7). Since oxidized LDL has been demonstrated to occur in atherosclerosis plaques in vivo, the present finding appears to be pertinent to an understanding of the pathogenesis of atherosclerosis. Taking into account that macrophages cultured with oxidized LDL were clearly distinct in morphological appearance from those cultured with acetyl-LDL (Figure 3), the growth-stimulating mechanism of oxidized LDL could in part be different from acetyl-LDL. Cross-competitive assays between acetyl-LDL and oxidized LDL suggested that in addition to a receptor common to both ligands, other scavenger receptors specific to each ligand may exist, suggesting that several receptors for these modified LDLs constitute a family of scavenger receptors. Moreover, unlike acetylation of LDL, oxidation of LDL resulted in peroxidation of phospholipids, as well as production of oxysterols such as 7-ketocholesterol and 7-hydroxycholesterol. These factors might account for the difference in the macrophage growth activity between oxidized LDL and acetyl-LDL. Further studies are needed to elucidate this issue.

It was demonstrated that oxidized LDL has a cytotoxic effect on certain cell types in vitro. In the present study, however, oxidized LDL showed no appreciable cytotoxicity against murine peritoneal macrophages at concentrations as high as 100 μg/mL. The differences may reflect a difference in the sensitivity of each cell type toward oxidative stress. Several biological features of chemically or oxidatively modified LDLs have been disclosed with respect to the potential involvement of these LDLs in atherogenesis. They include cytotoxic activity, chemoattractant activity toward monocytes, modulation of monocyte–endothelial interaction, induction of cell differentiation, the capacity to enhance prostaglandin E₂ production, induction of enzyme release, modulation of cytokine production, and the induction of growth factors. The present results disclose a new role for chemically modified LDL as a cell growth inducer for macrophages, the precursors of foam cells in atherosclerotic lesions. This capacity of oxidized LDL in particular may be functionally linked to the proliferative property of foam cells observed in atherosclerotic plaques in situ. Further studies along this line will provide a new clue to the mechanism governing macrophage recruitment in atherosclerosis.

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