The Effects of Interleukin (IL)-4 and IL-10 on Macrophage Growth-Stimulating Activities of Oxidized Low Density Lipoprotein (LDL), Acetylated LDL and Macrophage Colony-Stimulating Factor: The Activity of Oxidized LDL Is Refractory to the Inhibitory Cytokines

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We have previously reported that ligands of scavenger receptor such as acetylated low density lipoprotein (acetyl-LDL) and oxidized LDL induced growth of peripheral macrophages *in vitro*. This suggests the possibility that in addition to foam cell formation, modified or oxidized LDLs induce macrophage proliferation in atherosclerotic lesions. To learn further the physiological regulation of macrophage growth, we comparatively examined the effect of interleukin (IL)-4 and IL-10 which have been reported to be suppressive to various macrophage functions on macrophage growth-stimulating activities of the acetyl-LDL, oxidized LDL and macrophage colony-stimulating factor (M-CSF). An *in vitro* study showed that the activity of M-CSF-containing L-cell-conditioned medium was the most sensitive to the suppressive effects of these cytokines. The growth-inducing activity of acetyl-LDL was significantly inhibited by both IL-4 and IL-10. On the other hand, the activity of oxidized LDL was not attenuated by IL-4 or IL-10. These data indicate that macrophage growth-stimulating activity of oxidized LDL, in contrast to that of M-CSF or acetyl-LDL, is refractory to these suppressive cytokines. Oxidized LDL may act as a potent macrophage growth-stimulating factor in atherosclerotic or other inflammatory sites, even when these cytokines are produced by inflammatory and immunological reactions *in situ*.

Key words macrophage growth; M-CSF; oxidized LDL; acetylated LDL; interleukin 4; interleukin 10

It is well known that peripheral macrophages do not usually proliferate unless they are supplied with some growth-stimulating factor. However, growth of tissue macrophages was observed in inflammatory sites^{2–5)} or in tumors,^{6,7)} suggesting that in these circumstances, there is some factor which stimulates macrophage growth. It has also been reported that macrophages or macrophage-derived foam cells proliferate in *in vivo* atherosclerotic lesions of animal models,^{8,9)} or humans,^{10–13)} and that the local proliferation might be relevant to development of atherosclerosis.

Macrophage colony-stimulating factor (M-CSF) and granulocyte/macrophage CSF (GM-CSF) were known to be factors which induce peripheral macrophage growth *in vitro*.^{14–17)} On the other hand, we previously reported that liposomes including cholesterol esters or triglycerides,¹⁸⁾ phospholipids such as peroxidized phosphatidylserine and cardiolipin^{19,20)} induce macrophage growth *in vitro*. More recently, we also reported that acetylated low density lipoprotein (LDL) and oxidized LDL which are ligands of scavenger receptors^{21–23)} show growth-stimulating activity against murine peritoneal exudate macrophages.²⁴⁾ Oxidized LDL also stimulates proliferation of human monocyte-derived macrophages.²⁵⁾ Moreover, the growth was induced by advanced glycation end products (AGE) of proteins,²⁶⁾ the other type of ligands of scavenger receptor.²⁷⁾ These findings suggest that macrophage growth is induced by phagocytic stimuli through scavenger receptors. In atherosclerosis, it is thought that the modified or oxidized LDLs may be responsible for not only foam cell formation but also growth induction of macrophages.^{24,25,28)} On the other hand, M-CSF is a candidate for the factor which induces macrophage growth in atherosclerotic lesions, since M-CSF mRNA was reportedly detected in the lesions.^{29,30)}

It is considered that the atherosclerotic process involves inflammatory or immunological reactions.^{31–36)} Not only macrophages but also T lymphocytes are the cell components constituting atherosclerotic lesions, which possibly secrete a variety of cytokines. In fact, gene expression of cytokines including interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-4, IL-10 and IL-12 were observed in human atherosclerotic plaques.^{37,38)}

There are several reports including ours dealing with cytokine regulation of macrophage growth: the growth was positively or negatively regulated by IFN- γ ,^{39–41)} tumor necrosis factor (TNF),^{41–44)} IL-4,^{44,45)} IL-6,⁴⁶⁾ IL-10,^{47,48)} transforming growth factor- β (TGF- β).^{49,50)} It is, therefore, considerable that macrophage growth in atherosclerotic lesions is influenced by macrophage- or T-cell-derived cytokines. We previously observed that the growth inducing activity of M-CSF was more sensitive to the inhibitory effects of IFN- γ and TNF than that of oxidized phosphatidylserine.⁴¹⁾

IL-4 and IL-10 are cytokines having suppressive effects on macrophage functions, including production of proinflammatory cytokines.^{51–53)} However, the effects of cytokines on growth-stimulating activity of acetyl-LDL or oxidized LDL have not been explored. Therefore, we examined the effect of the suppressive cytokines on the macrophage growth-stimulating activities of acetyl-LDL, oxidized LDL and M-CSF. We found that in sharp contrast with M-CSF or acetyl-LDL, the growth-stimulating activity of oxidized LDL is refractory to the suppressive effects of IL-4 or IL-10.

MATERIALS AND METHODS

Cytokines Recombinant mouse IL-4 (rmIL-4) was a

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Fig. 1. Effect of IL-4 on ³H-TdR Incorporation of Macrophages Cultured with Acetyl-LDL, Oxidized LDL or L-cell-Conditioned Medium

Starch-induced macrophages were cultured without (■) or with 100 μg/ml acetyl-LDL (○), 50 μg/ml oxidized LDL (●) or L-cell-conditioned medium (▽; 10%, ▼; 20%, v/v) in the presence of the indicated concentrations of rmIL-4 for 6 d. ³H-TdR incorporation was assayed as described in Materials and Methods. Bars represent S.D. Panels A and B are from different experiments. **p*<0.05, ***p*<0.01, ****p*<0.001 compared with each IL-4-free control (by Student's *t*-test).

generous gift of Ono Pharmaceutical Chemical Co. (Osaka). Recombinant mouse IL-10 (rmIL-10) was purchased from R and D Corp. (Minneapolis, MN, U.S.A.). L-cell-conditioned medium was used as crude M-CSF.⁵⁴

Lipoproteins and Their Modification LDL (d=1.019–1.063) was isolated by ultracentrifugation from fresh human plasma of normolipidemic subjects after overnight fasting as described previously.⁵⁵ 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 as described,⁵⁵ according to the method of Basu *et al.*⁵⁶ To prepare oxidized LDL, 0.2 mg/ml LDL was incubated for 20 h at 37 °C with 5 μM CuSO₄ in EDTA-free phosphate-buffered saline (PBS), followed by addition of 0.1 mM EDTA,⁵⁷ using the method of Steinbrecher *et al.*⁵⁸

Mice and Cells Male C3H/He mice were purchased from breeding colonies in Japan SLC, Inc. (Shizuoka). Peritoneal cells were obtained 3–5 d after i.p. injection of 30 mg of starch into each mouse. These cells were suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% heat-inactivated FCS (Summit, Ft. Collins, CO, U.S.A.), penicillin (100 U/ml) and kanamycin (60 μg/ml) (hereafter referred to as medium A). For [methyl ³H]-thymidine ([³H]-TdR) incorporation assay, the peritoneal cells were incubated in 96-well microplates (Corning, Corning, NY, U.S.A.) at 2 × 10⁴ cells/well. For cell counting, these cells were incubated in 24-well microplates (Nunc, OK-4000, Roskilde, Denmark) at 0.5 × 10⁵ cells/well. The cells were incubated for 90 min at 37 °C in a CO₂-incubator to allow them to adhere to the culture 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 macrophage both by Giemsa staining and carbon particle uptake. These macrophages were added with the sample to be tested and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air with 0.2 ml (for 96 well plates) or 1 ml (for 24 well plates) of medium A without changing the medium. For determination of the number of macrophages, the number of adherent cells within five standard-sized area (0.25 mm²) in

each duplicate well in 24 well plates was counted with an inverted microscope as described previously.²⁴

Counting the Solubilized Nuclei The number of cells in culture was also determined by counting the solubilized nuclei as described by Nakagawara and Nathan.⁵⁹ Briefly, the culture medium was discarded, and the adherent cells in triplicate well were lysed in 1% (w/v) Triton X-100 and the number of naphthol blue-black-stained nuclei were counted in a hemocytometer.

[³H]-TdR Incorporation The incorporation of [³H]-TdR into cultured macrophages was measured as previously described.²⁴ Briefly, [³H]-TdR (2960 TBq/mmol, New England Nuclear, Boston, MA) was added to each plate, at 37 kBq/ml and incubated for 18 h. The medium was discarded, and the cells were dissolved in 100 μl of 0.5% sodium dodecyl sulfate, followed by addition of 100 μl of cold 10% trichloroacetic acid. The resulting trichloroacetic acid-insoluble material was collected on filters with Labo mash LM-101 (Futaba Medical, Tokyo). The filters were dried, and their radioactivity was counted in a liquid scintillation spectrophotometer. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Effect of IL-4 on Macrophage Growth-Inducing Activity of M-CSF, Acetyl-LDL or Oxidized LDL

We first examined the effect of IL-4 on macrophage growth-stimulating activity of M-CSF-containing L-cell-conditioned medium, oxidized LDL or acetyl-LDL. Figures 1A and B show that 10 or 20% of L-cell-conditioned medium, 100 μg/ml of acetyl-LDL or 50 μg/ml of oxidized LDL enhanced ³H-TdR incorporation into macrophages cultured for 6 days, respectively. When more than 0.1 ng/ml of rmIL-4 was added to macrophages simultaneously with L-cell-conditioned medium, the enhanced ³H-TdR incorporation was markedly attenuated. Similarly, the enhancing effect of acetyl-LDL was inhibited by 1 ng/ml IL-4, although acetyl-LDL seemed to be less sensitive to the negative effect of IL-4 (Fig. 1A). In contrast, the activity of oxidized LDL was completely refractory against IL-4: even 100 ng/ml IL-4 did not reduce the ³H-TdR incorpora-

tion that was enhanced by oxidized LDL (Fig. 1B).

It is necessary to examine whether the effect of IL-4 on macrophages cultured with L-cell-conditioned medium or acetyl-LDL is temporal, or if the cytokine inhibits the increase in macrophage cell number even in prolonged culture periods. Also, it is unclear whether oxidized LDL is refractory to IL-4 for longer culture periods. To address these problems, we performed the cell-counting assay from day 4 to day 9. Figure 2 shows that the macrophage numbers were

Fig. 2. Time-Course Study of the Suppressive Activity of IL-4 against Macrophage Growth

Starch-induced macrophages were cultured without (□) or with 10% L-cell-conditioned medium (panel A; ○, ●), 50 µg/ml acetyl-LDL (panel B; ▽, ▼) or 50 µg/ml oxidized LDL (panel C; △, ▲) in the absence (open symbols) or presence (closed symbols) of 100 ng/mL rmIL-4. The number of macrophages was successively estimated by counting adherent cells as described in Materials and Methods. Bars represent upper and lower values in duplicate determinations.

gradually elevated from day 4 to day 9 by the addition of L-cell-conditioned medium, acetyl-LDL or oxidized LDL, respectively. IL-4 markedly suppressed macrophage growth induction by L-cell-conditioned medium from day 4 to day 14, while the inhibitory effect on acetyl-LDL was potent on day 9 (Fig. 2A, B). On the other hand, the cytokine did not inhibit induction of macrophage growth by oxidized LDL on days 4 and 6 and only a very small reduction in macrophage cell number was observed on day 9 (Fig. 2C).

Effect of IL-10 on Macrophage Growth-Inducing Activity of M-CSF, Acetyl-LDL or Oxidized LDL We next examined the effect of IL-10 on macrophage growth-stimulating activity of M-CSF, acetyl-LDL or oxidized LDL. The effects of IL-10 were similar to IL-4: rmIL-10 effectively inhibited macrophage ³H-TdR incorporation stimulated by L-cell-conditioned medium (Fig. 3). It significantly inhibited the incorporation stimulated by acetyl-LDL (Fig. 3A). On the other hand, the enhancing effect of oxidized LDL was not attenuated by even 100 ng/ml IL-10 (Fig. 3B), suggesting that macrophage growth-inducing activity of oxidized LDL is refractory to the suppressive effects of both IL-4 and IL-10.

The results described in Fig. 3 were confirmed by counting solubilized nuclei from macrophages on day 11. As shown in Fig. 4, 100 ng/ml IL-10 significantly reduced the macrophage cell numbers that were enhanced by L-cell-conditioned medium or acetyl-LDL, respectively. On the other hand, the macrophage number cultured with oxidized LDL was not significantly reduced by the presence of IL-10.

The present data indicate that both IL-4 and IL-10 markedly inhibit the macrophage growth-stimulating activity of M-CSF and acetyl-LDL, but that oxidized LDL is refractory to the effects of the suppressive cytokines. The gene expression of various cytokines including IL-4 and IL-10 were observed in atherosclerotic lesions.^{36,37} Accordingly, oxidized LDL might be a potent growth stimulator against macrophages or macrophage-derived foam cells in environments in which activated T cells and macrophages secrete these cytokines.

IL-4 and IL-10 are cytokines produced by the T helper 2 (Th2) subset of helper T cells.⁶⁰ IL-10 is also produced by macrophages and it down-regulates the T helper 1 (Th1) cy-

Fig. 3. Effect of IL-10 on ³H-TdR Incorporation of Macrophages Cultured with Acetyl-LDL, Oxidized LDL or L-cell-Conditioned Medium

Starch-induced macrophages were cultured without (■) or with 100 µg/ml acetyl-LDL (○), 50 µg/ml oxidized LDL (●) or L-cell-conditioned medium (▽; 10%, ▼; 20%, v/v) in the presence of the indicated concentrations of rmIL-10 for 6 d. ³H-TdR incorporation was assayed as described in Materials and Methods. Bars represent S.D. Panels A and B are from different experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with respective IL-10-free controls (by Student's *t*-test).

Fig. 4. Estimation of the Effect of IL-10 on Macrophage Growth by Counting Solubilized Nuclei

Starch-induced macrophages were cultured without or with 50 μ g/ml acetyl-LDL, 50 μ g/ml oxidized LDL or L-cell-conditioned medium (10%, v/v) in the absence (white bars) or the presence (shaded bars) of 100 ng/ml rIL-10 for 11 d. Counting of solubilized macrophage nuclei was performed as described in Materials and Methods. Bars represent S.D. of triplicate determinations. * $p < 0.05$, ** $p < 0.01$, compared with respective IL-10-free controls (by Student's *t*-test).

tokine response to elicit a shift towards Th2-dominated immunity.⁶⁰⁾ Therefore, when such Th2-dominated immunity is dominant in the atherosclerotic process, oxidized LDL might govern macrophage growth in the lesions because its macrophage growth-stimulating activity is refractory to IL-4 and IL-10. However, we do not underestimate the role of M-CSF in induction of macrophage growth in atherosclerosis. When the balance of cytokines changes, it is possible that macrophage growth-stimulating activity of M-CSF may be more potent than that of oxidized LDL. For example, we observed that in accordance with the previous report,⁵⁰⁾ TGF- β augmented M-CSF-stimulated macrophage ³H-TdR incorporation, while the incorporation stimulated by either acetyl-LDL or oxidized LDL was not changed by TGF- β (T. Sasaki, unpublished observation). When TGF- β is the predominant cytokine in atherosclerotic lesions, M-CSF may be a potent growth factor for macrophages.

Why is oxidized LDL, but not acetyl-LDL, refractory to IL-4 and IL-10? We previously reported that the growth-inducing capacity of oxidized LDL is effective to not only peritoneal exudate macrophages but also resident macrophages, while the effect of acetyl-LDL on resident macrophages is very weak.²⁴⁾ Lysophosphatidylcholine (lysoPC) included in oxidized LDL is an essential element of macrophage growth.⁶¹⁾ Therefore, the lysoPC-mediated pathway in mitogenic signal transduction might be responsible for oxidized LDL being refractory to the suppressive cytokines. Alternatively, it was suggested that the scavenger receptors which bind oxidized LDL and acetyl-LDL are not totally equal.⁶²⁾ The signals transduced by a selective receptor for oxidized LDL might make oxidized LDL refractory against IL-4 and IL-10. To elucidate why and how oxidized LDL is refractory may open the way to control macrophage proliferation in atherosclerosis.

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