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The Scavenger Receptor Serves as a Route for Internalization of Lysophosphatidylcholine in Oxidized Low Density Lipoprotein-induced Macrophage Proliferation*

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We have recently demonstrated that the growth of murine macrophages is induced by oxidized low density lipoprotein (Ox-LDL) and that lysophosphatidylcholine (lyso-PC), a major phospholipid component of Ox-LDL, plays an essential role in its mitogenic effect. The present study was undertaken to further characterize the role of the macrophage scavenger receptor (MSR) in Ox-LDL-induced macrophage growth. The growth-stimulating effect of Ox-LDL on murine resident peritoneal macrophages was inhibited by maleylated bovine serum albumin (maleyl-BSA), a non-lipoprotein ligand for MSR but a poor carrier of lyso-PC, while maleyl-BSA itself failed to induce macrophage growth even in the presence of lyso-PC. Moreover, it competitively inhibited the endocytic uptake of ^{125}I-Ox-LDL and the specific uptake of lyso-PC by MSR, whereas nonspecific lyso-PC transfer to cells was not affected. Furthermore, the Ox-LDL-induced cell growth of peritoneal macrophages obtained from MSR knockout mice was significantly weaker than that of macrophages obtained from their wild-type littermates. Our results suggest that the MSR is an important and efficient internalization pathway for lyso-PC in Ox-LDL-induced macrophage growth.

Macrophage-derived foam cells, characterized by massive deposition of cytoplasmic cholesterol esters, are the key cellular elements in the early stage of atherosclerosis and play an essential role in the progression of this process (1). Macrophages are known to take up chemically modified low density lipoproteins (LDL),[1] such as oxidized LDL (Ox-LDL) and acetylated LDL (acetyl-LDL), through the scavenger receptor pathway and to become foam cells *in vitro* (2). Among the chemically modified LDL, Ox-LDL has been proposed as an atherogenic lipoprotein *in vivo* (3, 4). It is also known to have various atherogenic effects, *e.g.* acting as a chemoattractant for circulating monocytes (5), impairs endothelium-dependent arterial relaxation (6), and is cytotoxic to endothelial cells (7). We recently described a new property for Ox-LDL, the induction of macrophage growth (8), in which lysophosphatidylcholine (lyso-PC), a major phospholipid component of Ox-LDL, plays an important role in its mitogenic effect (9, 10). Based on several lines of evidence suggesting that macrophage-derived foam cells proliferated *in situ* in atherosclerotic lesions (11–13), it is possible that the Ox-LDL-induced macrophage growth may be linked to the enhanced progression of atherosclerosis.

In contrast to Ox-LDL, acetyl-LDL that binds to the macrophage scavenger receptor (MSR) but has a negligible amount of lyso-PC, does not have a mitogenic effect on mouse resident peritoneal macrophages (9) and on human monocyte-derived macrophages (10). Lyso-PC itself also had no mitogenic effect. However, incubation of macrophages with acetyl-LDL together with lyso-PC or phospholipase A_2-treated acetyl-LDL, result in a significant enhancement of macrophage growth (9, 10). Based on these results, we considered that lyso-PC and a modified LDL are two essential factors for the induction of macrophage growth.

There are two different mechanisms involved in the transfer of lyso-PC from Ox-LDL to macrophages. One is the direct transfer from Ox-LDL to plasma cell membranes by aqueous diffusion or lipid exchange reaction. The other pathway is the endocytic uptake of Ox-LDL particles by MSR(s) leading to internalization of lyso-PC. At this stage, the exact route essential for the induction of macrophage growth is not known. To investigate this process, we investigated the role of MSR in Ox-LDL-induced macrophage growth using maleylated bovine serum albumin (maleyl-BSA), a non-lipoprotein ligand for MSR (14) but a less efficient carrier of lyso-PC than lipoproteins. Our results demonstrated that maleyl-BSA itself does not possess mitogenic activity even in the presence of lyso-PC. However, Ox-LDL-induced macrophage growth was inhibited by maleyl-BSA, under which the endocytic uptake of both ^{125}I-Ox-LDL and lyso-PC-labeled Ox-LDL by these cells was also competitively inhibited. Involvement of MSR in Ox-LDL-induced macrophage growth was further examined with peritoneal macrophages obtained from the MSR gene knockout mice. Our results showed that the Ox-LDL-induced cell growth of these cells was significantly weaker than that of macrophages from their wild-type littermates. These findings taken together support the notion that the MSR serves as an efficient pathway for internalization of lyso-PC, ultimately leading to macrophage growth.

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[1] The abbreviations used are: LDL, low density lipoprotein; acetyl-LDL, acetylated LDL; Ox-LDL, oxidized LDL; MSR, macrophage scavenger receptor; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; GM-CSF, granulocyte/macrophage-colony stimulating factor; maleyl-BSA, maleylated bovine serum albumin; PLA_2, phospholipase A_2; CE, cholesteryl esters; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

EXPERIMENTAL PROCEDURES

Chemicals—Palmitoyl-lyso-PC and phospholipase A_2 (PLA_2) from *Naja naja* venom were purchased from Sigma. [*methyl*-^{3}H]Thymidine (80 Ci/mmol), Na[^{125}I] (17 Ci/mg), lyso-3-phosphatidylcholine, and 1-[1-^{14}C]palmitoyl ([^{14}C]lyso-PC) (60 mCi/mmol) were purchased from DuPont NEN. Other chemicals were the best grade available from other commercial sources.

Lipoproteins and Their Modifications—Human LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of human plasma of normolipidemic subjects obtained after overnight fasting (15). 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 previously by our laboratory (16). To prepare Ox-LDL, LDL was dialyzed against phosphate-buffered saline to remove EDTA. LDL (0.1 mg/ml) was then incubated for 20 h at 37 °C with 5 μM $CuSO_4$, followed by the addition of 1 mM EDTA and cooling (9). Acetyl-LDL was dialyzed against phosphate-buffered saline and treated with PLA_2 as described by Quinn *et al.* (5). The concentrations of lyso-PC in lipoproteins were determined by the methods of Bartlett (17) as described previously (9). The concentration of proteins was determined by BCA protein assay reagent (Pierce) using bovine serum albumin (BSA) as a standard. Ox-LDL was iodinated with ^{125}I according to the method of McFarlane (18). Ox-LDL and LDL were labeled with [^{14}C]lyso-PC using the method of Albers *et al.* (19). Briefly, 20 μCi of [^{14}C]lyso-PC was dried under nitrogen and redissolved in 5 μl of ethanol. This solution was slowly added beneath the surface of 2 ml of Ox-LDL or LDL (1 mg/ml) with gentle stirring. The mixtures were incubated at 37 °C for 2 h to allow for equilibration of [^{14}C]lyso-PC with the lipoproteins and then dialyzed extensively against 0.15 M NaCl and 1 mM EDTA (pH 7.4). Specific radioactivities of [^{14}C]lyso-PC-Ox-LDL and LDL were 15,500 and 14,500 cpm/μg protein, respectively. The level of thiobarbituric acid-reactive substances in Ox-LDL was 80 nmol of MDA/mg protein, whereas those of LDL and acetyl-LDL were within 3.0 nmol of MDA/mg protein. The level of endotoxin associated with these lipoproteins was less than 1 pg/μg protein as measured by a commercially available kit (Toxicolor system; Seikagaku Corp., Tokyo, Japan). Moreover, macrophage growth was not induced by endotoxin at a concentration less than 1 ng/ml in our experimental system.

Preparation of Maleylated Bovine Serum Albumin (Maleyl-BSA)—Twenty milligrams of defatted BSA in 10 ml of 0.1 M sodium pyrophosphate buffer (pH 9) was added dropwise on ice to 1 M maleic anhydride in dioxane (total 750 μl). The pH was continuously adjusted to 9.0 using NaOH solution. After incubation on ice for 5 min, the mixture was dialyzed extensively at 4 °C against 0.15 M NaCl and 20 mM sodium phosphate buffer (pH 7.4) (20). To determine the capacity of maleyl-BSA to hold lyso-PC, 0.5 ml of 10 mM palmitoyl-lyso-PC was added to 100 ml of RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum in the presence of 10 μg/ml acetyl-LDL or 200 μg/ml maleyl-BSA. The mixtures were incubated for 2 h at 37 °C and separated into the LDL fraction ($d < 1.063$), the high density lipoprotein fraction ($1.063 < d < 1.21$) and the higher density fraction ($d > 1.21$) by sequential ultracentrifugation. The LDL fraction and the higher density fraction were run on agarose gel electrophoresis to separate acetyl-LDL from LDL, and maleyl-BSA from BSA, respectively. The bands corresponding to acetyl-LDL and maleyl-BSA were cut out and extracted, followed by determination of their lyso-PC contents by the methods described above (17).

Cell Culture—The experimental protocol was approved by the Ethics Committee for Animal Experimentation at our institution. Peritoneal macrophages were collected from non-stimulated male C3H/He mice (25–30 g) and then suspended in RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 0.1 mg/ml streptomycin, and 100 units/ml of penicillin (medium A). All cellular experiments were performed at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Tritiated Thymidine Incorporation Assay—The peritoneal cells were adjusted to 2×10^5 cells/ml and cell suspensions (0.1 ml) were dispersed in each well of a 96-well tissue culture plate (6.4 mm in diameter, Falcon) and incubated for 90 min at 37 °C. The non-adherent cells were removed by triplicate washing with 0.1 ml of prewarmed medium A. These macrophages were cultured at 37 °C with 0.1 ml of medium A in the presence or absence of the lipoproteins to be tested without a medium change. Eighteen hours before the termination of experiments, 10 μl of 10 μCi/ml [^{3}H]thymidine was added to each well. The medium was discarded and cells were dissolved in 0.1 ml of 0.5% sodium dodecyl sulfate and subsequently precipitated with 0.1 ml of ice-cold 10% trichloroacetic acid. The resultant trichloroacetic acid-insoluble material was collected on filters with Labomash LM-101 (Labo Science, Tokyo). The filters were dried and the radioactivity of the material was counted using a liquid scintillation spectrophotometer (8).

Cell Counting Assay—The peritoneal cells were adjusted to 4×10^4 cells/ml and 1 ml of cell suspension was dispersed in each well of a 24-well tissue culture plate (16 mm in diameter, Falcon) and incubated for 90 min at 37 °C. The non-adherent cells were removed by washing three times with 1 ml of prewarmed medium A. More than 98% of adherent cells were judged to be macrophages by both Giemsa staining and carbon-particle uptake (8). These macrophages were cultured at 37 °C in 1 ml of medium A with or without the lipoproteins to be tested. After incubation for 7 days without a medium change, the adherent cells in triplicate wells were lysed in 1% (w/v) Triton X-100, and all naphthol blue-black-stained nuclei were counted in a hemocytometer as described previously (8).

Endocytic Degradation and Cell Association of ^{125}I-Ox-LDL—Mouse macrophages (2×10^6 cells) in 1.0 ml of medium A were seeded to each plastic culture dish (22 mm in diameter, Falcon) and incubated for 60 min at 37 °C. The monolayers thus formed were washed three times with 1.0 ml of medium A. Each well was incubated with ^{125}I-Ox-LDL for 6 h at 37 °C in the absence or presence of the unlabeled ligands to be tested. Endocytic degradation was determined by trichloroacetic acid soluble radioactivity in the medium after precipitating free iodine with $AgNO_3$ as described previously (21). Cells were solubilized with 1.0 ml of 0.1 N NaOH and the cell-associated radioactivity was determined as described previously (22).

Experiments with Peritoneal Macrophages from the MSR Gene Knockout Mice—Mice lacking both type I and type II MSR were established by targeted disruption of exon 4 of the MSR gene in A3-1 ES cells according to the method described previously by Kurihara *et al.* (23). Immunostaining using 2F8 anti-MSR monoclonal antibody indicated that the homozygote mice for MSR deficiency completely lacked both type I and type II receptor proteins and that peritoneal macrophages obtained from mice indicated very low (18%) acetyl-LDL degradation compared with their wild-type littermates.[2] These animals grow normal and fertile. Their establishment and phenotype will be reported in detail elsewhere.

We used the MSR knockout mice and their wild-type littermates that were born to heterozygous mice. Peritoneal macrophages (1×10^6 to 2×10^6) were obtained from each mouse. The cells obtained from 2 mice were used for one set of growth experiments (totally 7 sets) and those obtained from 3 mice were used for the cell-association and degradation of ^{125}I-Ox-LDL, and finally 17 knockout mice and 17 mice of their wild-type littermates were used in this study. Experimental protocols for the cell growth assay by [^{3}H]thymidine incorporation and the cell counting were essentially identical to those for C3H/He mice as described above. The cell association of ^{125}I-Ox-LDL with these cells was also determined in the same way, except the cell numbers were reduced from 2×10^6/well (C3H/He mice) to 0.5×10^6/well (the MSR knockout mice), because the MSR knockout mice and their wild-type littermates were limited in number.

Statistical Analysis—Data were expressed as mean ± S.D. Differences were examined by the paired Student's t test. A p value less than 0.05 denoted the presence of a significant difference.

RESULTS

Negligible Capacity of Maleyl-BSA to Hold Lyso-PC—Prior to experiments using maleyl-BSA, the capacity of maleyl-BSA to hold lyso-PC was compared with that of acetyl-LDL under the present culture conditions. When 10 μg/ml acetyl-LDL was incubated at 37 °C for 2 h with 50 μM palmitoyl-lyso-PC in 100 ml of RPMI 1640 medium containing 5% heat-inactivated fetal bovine serum, 10.2 nmol of lyso-PC was associated with 10 μg of acetyl-LDL (Table I). This value was higher than that of Ox-LDL (6.4 nmol of lyso-PC associated with 10 μg of Ox-LDL). In contrast, when 50 μM lyso-PC was incubated with 200 μg/ml maleyl-BSA under the same experimental conditions, 0.2 nmol of lyso-PC was associated with 200 μg of maleyl-BSA (Table I). From these results, it is evident that the capacity of maleyl-BSA to hold lyso-PC is negligibly weaker than that of acetyl-LDL.

Failure of Maleyl-BSA to Induce Macrophage Growth—We

[2] H. Suzuki, Y. Kurihara, and T. Kodama, unpublished observation.

TABLE I
Lyso-PC contents of lipoproteins and maleyl-BSA

The lyso-PC contents were determined as described under "Experimental Procedures." Values are mean ± SD (n = 3). PLA_2-treated acetyl-LDL; acetyl-LDL (2 mg/ml) was incubated with 5 units/ml PLA_2 for 2 h at 37 °C, and re-isolated from PLA_2 by ultracentrifugation ($d <$ 1.21). Acetyl-LDL incubated with Lyso-PC; acetyl-LDL was incubated with 50 μM lyso-PC in serum containing medium, and re-isolated by both ultracentrifugation and agarose electrophoresis. Maleyl-BSA incubated with lyso-PC; maleyl-BSA was incubated with 50 μM lyso-PC in serum containing medium, and re-isolated by both ultracentrifugation and agarose electrophoresis.

	Lyso-PC
	nmol/mg protein
LDL	22 ± 10
Ox-LDL	644 ± 53
Acetyl-LDL	25 ± 12
PLA_2-treated acetyl-LDL	923 ± 76
Acetyl-LDL incubated with lyso-PC	1020 ± 83
Maleyl-BSA incubated with lyso-PC	1 ± 1

have recently demonstrated that two essential factors for induction of macrophage growth are lyso-PC and a ligand for MSR such as acetyl-LDL and Ox-LDL (9, 10). In a strict sense, however, it is not clear whether a direct transfer of lyso-PC from these modified LDL to the cell surface membranes is involved in growth stimulation or whether endocytic internalization of lyso-PC by MSR is required for growth induction. To solve this issue, the capacity of maleyl-BSA to induce macrophage growth was compared with that of acetyl-LDL in the absence or presence of palmitoyl-lyso-PC. As shown in Table II, maleyl-BSA itself had no effect on macrophage growth in the absence or presence of lyso-PC. Similarly acetyl-LDL alone showed negligible growth stimulating activity. In the presence of lyso-PC, however, it showed a significant mitogenic effect on macrophages (Table II). Moreover, PLA_2-treated acetyl-LDL also showed a significant growth-stimulating activity for these macrophages (Table II). Since acetyl-LDL is an efficient carrier of lyso-PC compared with maleyl-BSA (Table I), lyso-PC may act as a growth inducer when it occurs with modified LDL. Thus, it seems reasonable to expect that internalization of lyso-PC, as a component of a modified LDL, by MSR pathway may be an essential step in macrophage growth. The likelihood of such process was further examined in the following series of experiments.

Maleyl-BSA Inhibits Ox-LDL-induced Macrophage Growth—We examined the effect of maleyl-BSA on Ox-LDL-induced macrophage growth. When macrophages were incubated simultaneously with Ox-LDL and maleyl-BSA, Ox-LDL-induced macrophage growth determined by [^{3}H]thymidine incorporation was inhibited by 70% in a dose-dependent manner (Fig. 1). The cell counting assay also showed that maleyl-BSA significantly inhibited Ox-LDL-induced increase in cell number by 60% (Table III). When macrophages were incubated with 200 μg/ml maleyl-BSA, more than 98% of cells were viable as determined by both trypan blue staining and MTT method, which is based on the cellular reduction of MTT to MTT formazan. These results indicated a significant inhibitory effect of maleyl-BSA on Ox-LDL-induced macrophage growth.

Mechanism of the Inhibitory Effect of Maleyl-BSA on Ox-LDL-induced Macrophage Growth—To elucidate the exact mechanism of the inhibitory action of maleyl-BSA, we examined the effect of maleyl-BSA on endocytic uptake of Ox-LDL by macrophages. As shown in Fig. 2*B*, the cell association of ^{125}I-Ox-LDL was inhibited by maleyl-BSA as effectively as unlabeled Ox-LDL, whereas unlabeled LDL and BSA had no effect. A similar inhibitory pattern was observed in the endocytic degradation of ^{125}I-Ox-LDL (Fig. 2*A*), indicating that maleyl-BSA competitively inhibited cellular uptake of Ox-LDL by MSR.

TABLE II
Combined effect of maleyl-BSA and lyso-PC on macrophage growth determined by counting of solubilized nuclei

Resident mouse peritoneal macrophages (4×10^4) were incubated with 200 μg of protein/ml of maleyl-BSA or 10 μg of protein/ml of acetyl-LDL in the absence or the presence of 50 μM of palmitoyl-lyso-PC. Control incubations were performed with 10 μg of protein/ml of Ox-LDL or PLA_2-treated acetyl-LDL, or 23 ng/ml GM-CSF. On day 6, counting of solubilized nuclei was performed as described under "Experimental Procedures." Data are expressed as mean ± S.D. of triplicate counts.

Sample	Cell number (% of nonloaded)
	4×10^{-4}/well
Nonloaded	3.6 ± 0.3 (100%)
Palmitoyl-lyso-PC	3.6 ± 0.2 (100%)
Maleyl-BSA	3.5 ± 0.3 (97%)
Maleyl-BSA incubated with palmitoyl-lyso-PC	3.7 ± 0.3 (103%)
Acetyl-LDL	3.8 ± 0.4 (106%)
Acetyl-LDL incubated with palmitoyl-lyso-PC	6.8 ± 0.4[a] (189%)
PLA_2-treated acetyl-LDL	6.6 ± 0.4[a] (183%)
Ox-LDL	7.3 ± 0.5[b] (203%)
GM-CSF	7.5 ± 0.4[b] (208%)

[a] $p < 0.01$, compared with non-loaded control (Student's t test).
[b] $p < 0.001$, compared with non-loaded control.

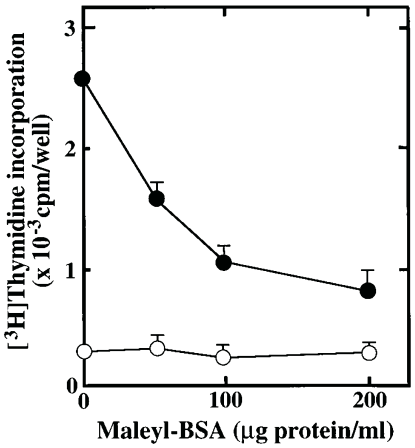

FIG. 1. **Effect of maleyl-BSA on Ox-LDL-induced macrophage growth determined by [^{3}H]thymidine incorporation assay.** Mouse resident peritoneal macrophages (2×10^4) were incubated with the indicated concentrations of maleyl-BSA in the absence (○) or presence (●) of 10 μg/ml Ox-LDL for 6 days. During the last 18 h of incubation, cells in each well were chased with [^{3}H]thymidine, harvested, and the radioactivity was determined as described under "Experimental Procedures." Data represent the mean ± S.D. of four separate experiments.

We then examined the effect of maleyl-BSA on the transfer of lyso-PC from Ox-LDL to cells. When macrophages were incubated with [^{14}C]lyso-PC-labeled Ox-LDL, the cell-associated radioactivity increased with time (Fig. 3). The presence of 20-fold excess of maleyl-BSA (on a protein basis) caused a significant 40% inhibition of cell association of [^{14}C]lyso-PC. Since the LDL receptor is negligibly expressed in mouse macrophages, we determined the transfer of [^{14}C]lyso-PC from LDL to cells as an index of the nonspecific transfer of [^{14}C]lyso-PC to cells by the lipid exchange reaction that also occurred to Ox-LDL. When macrophages were incubated with [^{14}C]lyso-PC-labeled LDL, the cell-associated [^{14}C]lyso-PC increased rapidly and reached a plateau level (5% of added radioactivity) after incubation for 2 h. In contrast to Ox-LDL, the transfer of [^{14}C]lyso-PC from LDL to cells was not affected by maleyl-BSA. The amount of [^{14}C]lyso-PC transferred from LDL to cells was very close to that transferred from [^{14}C]lyso-PC-labeled Ox-

TABLE III
Effect of maleyl-BSA on Ox-LDL-induced macrophage growth determined by counting of solubilized nuclei

Resident mouse peritoneal macrophages (4×10^4) were incubated with 10 μg of protein/ml of Ox-LDL and/or 200 μg of protein/ml of maleyl-BSA. On day 6, counting of solubilized nuclei was performed as described under "Experimental Procedures." Data are expressed as mean ± S.D. of triplicate counts.

Sample	Cell number (% of nonloaded)
	4×10^{-4}/well
Nonloaded	3.4 ± 0.2 (100%)
Ox-LDL	7.8 ± 0.5[a] (229%)
Maleyl-BSA	3.3 ± 0.3 (97%)
Ox-LDL and maleyl-BSA	5.2 ± 0.3[b] (157%)

[a] $p < 0.001$, compared with non-loaded control (Student's t test).
[b] $p < 0.001$, compared with Ox-LDL.

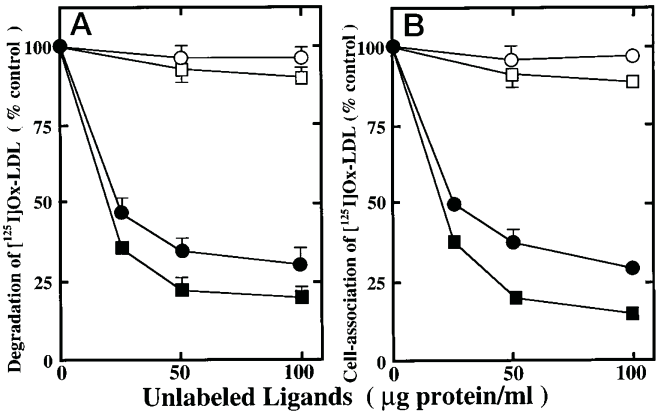

FIG. 2. **Effect of maleyl-BSA on endocytic uptake of ^{125}I-Ox-LDL by mouse macrophages.** Mouse resident peritoneal macrophages (2×10^6) were incubated at 37 °C for 6 h with 5 μg/ml ^{125}I-Ox-LDL in the absence or presence of the indicated protein concentrations of BSA (□), maleyl-BSA (■), LDL (○), or Ox-LDL (●). Trichloroacetic acid soluble radioactivity in the medium (*A*) and cell associated radioactivity (*B*) were determined as described under "Experimental Procedures." The raw values of endocytic degradation (*A*) and cell association (*B*) equivalent to 100% were 2.61 and 1.71 μg/6 h/mg of cell protein, respectively. Data represent the mean ± S.D. of four separate experiments.

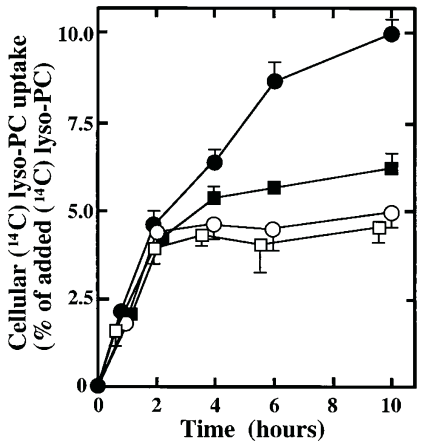

FIG. 3. **Effect of maleyl-BSA on the transfer of lyso-PC from Ox-LDL to macrophages.** Mouse resident peritoneal macrophages (2×10^6) were incubated for the indicated time periods with 10 μg/ml [^{14}C]lyso-PC-labeled Ox-LDL in the absence (●) or presence (■) of 200 μg/ml maleyl-BSA. A control incubation was performed with 10 μg/ml [^{14}C]lyso-PC-labeled LDL in the absence (○) or presence (□) of 200 μg/ml maleyl-BSA. After incubation, cellular radioactivity of [^{14}C]lyso-PC was measured as described under "Experimental Procedures." Specific radioactivities of [^{14}C]lyso-PC-Ox-LDL and LDL were 15,500 and 14,500 cpm/μg protein, respectively. [^{14}C]Lyso-PC contents of [^{14}C]lyso-PC-Ox-LDL and LDL were 108 and 104 nmol/mg protein, respectively. Data represent the mean ± S.D. of four separate experiments.

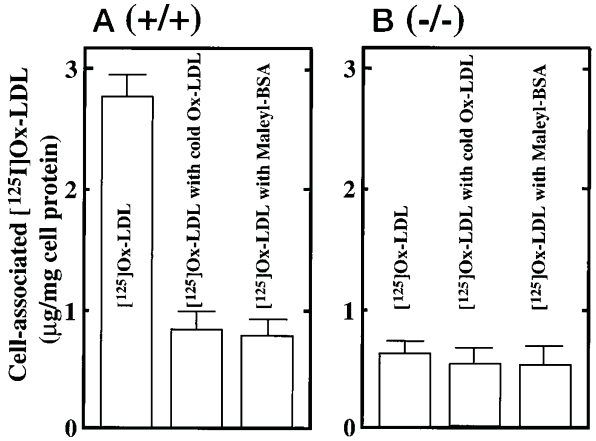

FIG. 4. **Effect of maleyl-BSA on cell association of ^{125}I-Ox-LDL to the MSR knockout macrophages.** Mouse resident peritoneal macrophages (0.5×10^6) obtained from wild mice (*A*) and the MSR knockout mice (*B*) were incubated at 37 °C for 18 h with 5 μg/ml ^{125}I-Ox-LDL in the absence or presence of 100 μg/ml cold Ox-LDL or maleyl-BSA. After incubation, the cell-associated radioactivity was determined as described under "Experimental Procedures." Data represent the mean ± S.D. of triplicate.

LDL in the presence of maleyl-BSA, suggesting that maleyl-BSA might suppress [^{14}C]lyso-PC transfer from Ox-LDL to cells to almost nonspecific level. Combined together, these results suggested that maleyl-BSA might inhibit "specific" cellular uptake of lyso-PC by MSR rather than "nonspecific" transfer to cell membrane.

Growth-stimulating Effect of Ox-LDL on Scavenger Receptor-deficient Macrophages—To confirm the involvement of MSR in Ox-LDL-induced macrophage growth, we compared the mitogenic effect of Ox-LDL on peritoneal macrophages from the MSR gene knockout mice with that on peritoneal macrophages from their wild-type littermates (24). These cells were incubated with 5 μg/ml ^{125}I-Ox-LDL at 37 °C for 18 h, washed with medium, and amounts of the radiolabeled ligand associated with these cells were determined. As shown in Fig. 4, the amount of ^{125}I-Ox-LDL associated with the MSR knockout macrophages was less than 23% of that of their wild-type littermate macrophages. Moreover, the cell association of ^{125}I-Ox-LDL with wild-type macrophages was inhibited by 70% by maleyl-BSA, whereas maleyl-BSA had no effect on the cell association of ^{125}I-Ox-LDL with the MSR knockout macrophages (Fig. 4). Fig. 5 and Table IV show the effect of Ox-LDL on cell growth of these macrophages. Upon [^{3}H]thymidine incorporation assay, Ox-LDL significantly induced the growth of wild-type macrophages (Fig. 5, *A* and *C*), whereas the growth-stimulating effect of Ox-LDL for the MSR-deficient macrophages was significantly weaker (Fig. 5, *B* and *D*). Upon the cell counting assay, 7 days incubation with 20 μg/ml Ox-LDL caused a 2-fold increase in the wild-type macrophages but only a 1.3-fold increase in the MSR knockout macrophages (Table IV). PLA_2-treated acetyl-LDL also induced cell growth of wild-type macrophages, whereas its effect on the MSR knockout macrophages was negligible (Table IV). Moreover, in wild-type macrophages, the inhibitory effect of maleyl-BSA on PLA_2-treated acetyl-LDL-induced cell growth was much more prominent as compared with that on the Ox-LDL-induced cell growth (Table IV). In contrast to the marked difference between these two macrophage populations in their response to the growth-stimulating effect of Ox-LDL and PLA_2-treated acetyl-LDL, their responses to GM-CSF were equally effective when determined under identical conditions (Fig. 5 and Table IV). It is likely from these results, therefore, that the MSR

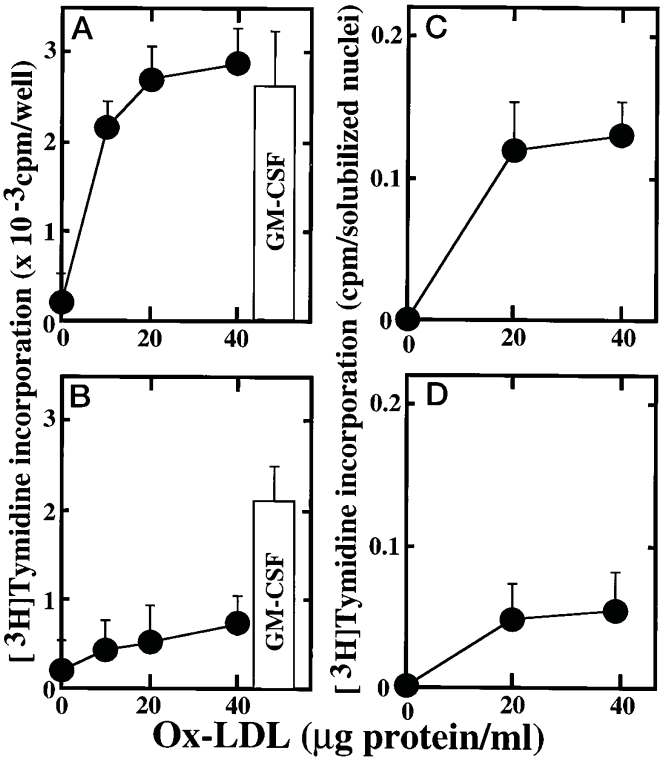

FIG. 5. **Growth stimulating effect of Ox-LDL on scavenger receptor-deficient macrophages determined by [^{3}H]thymidine incorporation assay.** Mouse resident peritoneal macrophages (2×10^4) obtained from wild C57/BL6 (*A* and *C*) or MSR knockout C57/BL6 (*B* and *D*) mice were incubated with the indicated concentrations of Ox-LDL or 23 ng/ml GM-CSF for 7 days. During the last 18 h of incubation, cells in each well were chased with [^{3}H]thymidine, harvested, and the radioactivity was determined as described under "Experimental Procedures." To normalize the [^{3}H]thymidine uptake into these two macrophage populations on the basis of solubilized nuclei, determination of solubilized nuclei was performed under the same conditions. Data were represented on the basis of counts/min/well (*A* and *B*) and on the basis of counts/min/solubilized nuclei (*C* and *D*). Data represent the mean ± S.D. of three separate experiments.

might play an important role in Ox-LDL-induced macrophage growth.

DISCUSSION

We have recently demonstrated that two factors, lyso-PC and modified LDL that are recognized by MSR, are required for the induction of macrophage growth (9, 10). The present study was undertaken to elucidate the route for lyso-PC transfer to cells in order to induce macrophage growth. Our results showed that (i) maleyl-BSA, a ligand for MSR but a poor carrier of lyso-PC, did not induce macrophage growth even in the presence of lyso-PC, whereas acetyl-LDL induced cell growth in the presence of lyso-PC; (ii) when macrophages were incubated with Ox-LDL and maleyl-BSA, Ox-LDL-induced macrophage growth was inhibited; (iii) maleyl-BSA also inhibited specific cellular uptake of Ox-LDL and that of lyso-PC by the MSR but not nonspecific lyso-PC transfer to cells; (iv) the growth of the MSR-deficient macrophages was induced by Ox-LDL but the response of these cells to Ox-LDL was significantly weaker than that of their wild-type littermate macrophages; (v) PLA_2-treated acetyl-LDL showed a significant growth stimulating activity for wild-type macrophages, whereas its mitogenic effect on the MSR knockout macrophages was negligible. Combined together, these observations strongly suggest that the specific cellular uptake of lyso-PC by the MSR (type I and type II macrophage scavenger receptor) (25) is functionally linked to the induction of macrophage growth (Fig. 6).

The results of the present study likely suggest a functional link between the MSR-mediated internalization of lyso-PC and subsequent macrophage growth. However, although lyso-PC contents of PLA_2-treated acetyl-LDL and acetyl-LDL incubated with lyso-PC were much higher than that of Ox-LDL (Table I), their mitogenic effects were almost the same or slightly weaker than that of Ox-LDL (Tables II and IV). The reason for this is not clear. There could be two possibilities. First, the receptors other than the MSR might be involved in the internalization of lyso-PC in Ox-LDL-induced macrophage growth. In fact, the results of the recent studies showed that Ox-LDL is also recognized by other receptors such as Fc receptor, CD 36, and SR-BI (26–36). Second, in addition to lyso-PC, oxidized compounds such as oxidized sterols and aldehydes or degraded apoB, which are generated during oxidation of LDL (2), could also serve as mitogens or enhancers for the macrophage growth. Recently, Heery *et al.* (36) reported that oxidized phospholipid other than lyso-PC could induce SMC proliferation via platelet-activating factor receptor. In this connection, the present study showed that the growth stimulating effect of Ox-LDL on macrophages obtained from the MSR knockout mice was low but significant, whereas PLA_2-treated acetyl-LDL did not have such an effect (Table IV). Furthermore, the inhibitory effect of maleyl-BSA on cell growth of the wild-type macrophages was almost complete for PLA_2-treated acetyl-LDL-induced cell growth, but partial (60%) for the Ox-LDL-induced macrophage growth (Table IV). Thus, these two possibilities must be taken into account to further elucidate the mechanism of the Ox-LDL-induced macrophage growth.

Since maleyl-BSA did not induce macrophage growth even in the presence of lyso-PC (Table II), the ligand binding to MSR is probably not involved in growth induction. In contrast, secretion of lipoprotein lipase from macrophages was stimulated by maleyl-BSA as well as acetyl-LDL (37). Therefore, lipoprotein lipase secreted from macrophages seems to be coupled with the ligand binding to MSR. The inability of β-very low density lipoprotein to stimulate secretion of lipoprotein lipase supports this notion (37). In Ox-LDL-induced macrophage growth, MSR may serve as a route for internalization of lyso-PC. It would be interesting to know whether another lipoprotein receptor could replace this function. To address this issue, we are currently investigating the effect of β-very low density lipoprotein on macrophage growth.

Maleyl-BSA inhibited Ox-LDL-induced macrophage growth probably by interfering with specific uptake of lyso-PC by MSR. However, in addition to inhibiting the specific uptake of Ox-LDL, maleyl-BSA is also expected to prevent the accumulation of cholesteryl esters (CE) in macrophages. Since phagocytic uptake and intracellular accumulation of CE led to proliferation of "starch-induced" murine macrophages (38), it is possible that CE accumulation itself may be a factor involved in Ox-LDL-induced macrophage growth. However, incubation of macrophages with 20 μg/ml Ox-LDL together with 80 μg/ml apoA-I, an efficient cholesterol acceptor from macrophage foam cells (39), inhibited CE accumulation by 60% whereas macrophage growth was not affected, as demonstrated in our previous study (9). These results suggest that intracellular accumulation of CE is not an essential event for Ox-LDL-induced cellular growth in mouse resident peritoneal macrophages.

Lyso-PC is known to have various atherogenic functions, including (i) a chemotactic activity for circulating monocytes (40); (ii) induction in endothelial cells of a variety of cell adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (41); (iii) induction in endothelial cells of growth factors such as platelet-derived growth factor and heparin-binding epidermal growth factor-like pro-

TABLE IV

Growth stimulating effect of Ox-LDL on the scavenger receptor-deficient macrophages determined by cell counting assay

Resident mouse peritoneal macrophages (4×10^4) obtained from the scavenger receptor gene knockout mice (−/−) and its wild-type littermates (+/+) were incubated with 20 μg of protein/ml of Ox-LDL or PLA_2-treated acetyl-LDL in the presence or the absence of 200 μg of maleyl-BSA, or 23 ng/ml GM-CSF. On day 7, counting of solubilized nuclei was performed as described under "Experimental Procedures." Data are expressed as mean ± S.D. of triplicate counts.

Cell	Sample	Cell number (% of nonloaded)
C57/BL6 (+/+)	Nonloaded	3.5 ± 0.3 (100%)
	Ox-LDL	6.6 ± 0.4[a] (194%)
	Ox-LDL with maleyl-BSA	4.8 ± 0.3[b] (137%)
	PLA_2-treated acetyl-LDL	6.1 ± 0.4[c] (185%)
	PLA_2-treated acetyl-LDL with maleyl-BSA	3.9 ± 0.2[d] (110%)
	GM-CSF	7.3 ± 0.5[a] (209%)
C57/BL6 (−/−)	Nonloaded	3.3 ± 0.2 (100%)
	Ox-LDL	4.3 ± 0.2[e] (130%)
	Ox-LDL with maleyl-BSA	4.1 ± 0.4 (124%)
	PLA_2-treated acetyl-LDL	3.7 ± 0.3 (112%)
	PLA_2-treated acetyl-LDL with maleyl-BSA	3.6 ± 0.3 (109%)
	GM-CSF	6.0 ± 0.4[f] (182%)

[a] $p < 0.001$, compared with non-loaded control (Student's t test).
[b] $p < 0.05$, compared with Ox-LDL.
[c] $p < 0.01$, compared with non-loaded control.
[d] $p < 0.05$, compared with PLA_2-treated acetyl-LDL.
[e] $p < 0.05$, compared with non-loaded.
[f] $p < 0.01$, compared with non-loaded control.

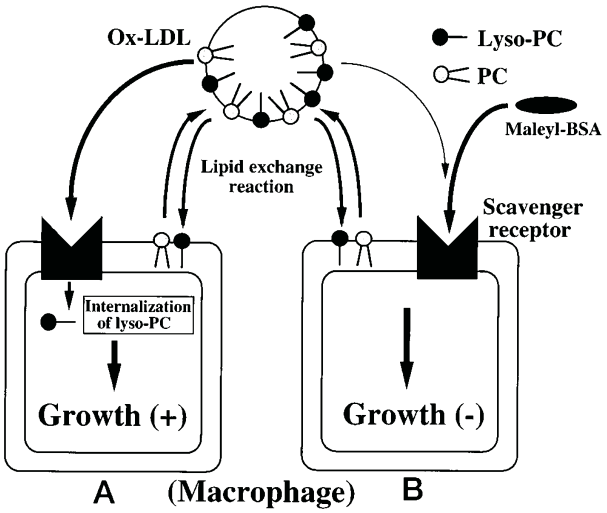

FIG. 6. **Schematic representation of lyso-PC internalization by the scavenger receptor and subsequent induction of macrophage growth.** *A*, when macrophage are incubated with Ox-LDL, lyso-PC, a major phospholipid component of Ox-LDL, is transferred to cells by two independent pathways. The first involves a nonspecific transfer of lyso-PC to plasma membrane by lipid exchange reaction while the second involves endocytic internalization of lyso-PC by MSR. *B*, when macrophages are incubated with Ox-LDL in the presence of excess amounts of maleyl-BSA, endocytic uptake of Ox-LDL as well as that of lyso-PC is inhibited whereas nonspecific lyso-PC transfer to plasma membrane is unaffected. Macrophage growth is initiated only when lyso-PC of Ox-LDL is effectively endocytosed by MSR. It is likely that internalized lyso-PC plays an essential role in generating intracellular signal for macrophage growth.

tein (42); (iv) impairment of endothelium-dependent arterial relaxation (6); (v) stimulation of plasminogen activator inhibitor-1 release and inhibition of tissue-type plasminogen activator release from endothelial cells thereby reducing overall fibrinolytic activity (43). In addition to these functions, we recently elucidated another essential function for lyso-PC in the mitogenic effect of Ox-LDL on macrophages (9, 10). It should be noted that macrophage growth is induced only when lyso-PC is presented by a modified LDL, while various atherogenic events described above are induced by lyso-PC alone.

Although the signal transduction in Ox-LDL-induced macrophage growth is not clear, it is possible that protein kinase C is involved in this process. Hamilton and Dientsman (44, 45) demonstrated that mouse macrophage growth was induced by phorbol esters. Consistent with this notion, Ox-LDL is known to induce accumulation of inositol triphosphate in human smooth muscle cells (46) and mouse macrophages (47) and increase the concentration of cytosolic calcium in mouse macrophages (47). However, further studies are required to define the functional link between protein kinase C and Ox-LDL-induced macrophage growth.

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