Isolation of macrophage-like cell mutants resistant to the cytotoxic effect of oxidized low density lipoprotein

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Abstract A high concentration of oxidized low density lipoprotein (Ox-LDL) showed a cytotoxic effect on mouse macrophage-derived J774 cells. Mutant cells were selected from these cells that were resistant to the cytotoxic effect of Ox-LDL. One mutant form, named JO21b cells, was characterized in the present study. In spite of a marked resistance to the cytotoxic effect of Ox-LDL, JO21b cells were apparently as sensitive as the parent cells not only to toxic moieties of Ox-LDL, such as 7-ketocholesterol and lysophosphatidylcholine, but also to *t*-butyl hydroperoxide, an artificial lipid hydroperoxide analog. However, the cellular association of ^{125}I-labeled Ox-LDL with, and subsequent endocytic degradation by JO21b cells was reduced by 70–80% compared with J774 cells. Similarly, accumulation of cholesteryl esters in JO21b cell by Ox-LDL was also reduced by 70%. Northern blot analyses of type I and type II macrophage scavenger receptors (type I and type II MSR) demonstrated that the mRNA levels of JO21b cells were lower than those of J774 cells. Moreover, peritoneal macrophages obtained from MSR-knockout mice showed a higher resistance to the cytotoxic effect of Ox-LDL than those from their wild-type littermates. Our results suggest, therefore, that macrophage scavenger receptor-mediated endocytic uptake of oxidized low density lipoproteins (Ox-LDL) may play an enhancing role in Ox-LDL cytotoxicity to macrophages or macrophage-derived cells.—**Hakamata, H., A. Miyazaki, M. Sakai, H. Matsuda, H. Suzuki, T. Kodama, and S. Horiuchi.** Isolation of macrophage-like cell mutants resistant to the cytotoxic effect of oxidized low density lipoproteins. *J. Lipid Res.* 1998. **39:** 482–494.

Supplementary key words oxidized low density lipoprotein • macrophage • somatic cell mutant • cytotoxicity • macrophage scavenger receptors • atherosclerosis

The early stages of atherosclerotic lesions are characterized by the presence of lipid-laden macrophage foam cells (1). Macrophages are known to take up chemically modified low density lipoproteins (modified LDLs), such as acetylated LDL (acetyl-LDL) and oxidized LDL (Ox-LDL), by the macrophage scavenger receptors (MSR), leading to foam cell formation and massive intracellular accumulation of cholesteryl esters (CE) (2). MSR specific for these modified LDLs are preferentially expressed by macrophages or macrophage-derived cells (3) and at least three classes (class A, class B, and class C) have been recognized (4, 5). MSR class A receptors were the first to be cloned and divided into type I and type II MSR, representing the products of alternative splicing of a single gene (6, 7). Expression experiments using the MSR cDNA showed that both type I and type II MSR could recognize acetyl-LDL as well as Ox-LDL as effective ligands (8).

Among the modified LDLs, Ox-LDL is regarded as a major atherogenic lipoprotein in vivo, due to its presence in human and rabbit atherosclerotic plaques (9, 10). Ox-LDL has several biological properties such as (*i*) enhancement of adhesion of circulating monocytes to endothelial cells and subsequent migration into subendothelial space; (*ii*) induction of monocyte–macrophage differentiation; (*iii*) macrophage foam cell generation; (*iv*) induction of smooth muscle cell migration, proliferation, and foam cell generation; (*v*) cytotoxicity to vascular cells; (*vi*) modulation of expression of cytokines and adhesion molecules; and (*vii*) effects on the coagulation pathways (for reviews see 2, 11). Of great importance is the cytotoxic effect of Ox-LDL on cells participating in the formation of atherosclerotic lesions, such as endothelial (12, 13) and smooth muscle cells (12) and macrophages (14, 15), as cellular

Abbreviations: LDL, low density lipoprotein; acetyl-LDL, acetylated LDL; Ox-LDL, oxidized LDL; CE, cholesteryl esters; MSR, macrophage scavenger receptors; MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; BSA, bovine serum albumin; TCA, trichloroacetic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehydephosphate dehydrogenase; ssDNA, single-strand DNA.

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components are lost from deep- to mid-intimal locations during the development of the lipid-rich necrotic core in atherosclerotic plaques (1). Among these cells, immunohistochemical observations suggest that those cells undergoing necrosis in human atherosclerotic plaque are mainly macrophages (16). In vitro experiments showed that Ox-LDL is able to induce the death of mouse peritoneal macrophages (14), $P388D_1$ cells, a macrophage-like cell line (15), and human monocytes-macrophages (17). However, the cellular mechanism(s) of Ox-LDL-induced macrophage cytotoxicity are not fully known.

To investigate the cytotoxic mechanisms of Ox-LDL, a molecular and cell genetic approach was used to isolate mutant cells resistant to the cytotoxic effect of Ox-LDL from J774 cells, a cell line derived from mouse macrophages. In particular, one mutant cell called JO21b was characterized in the present study. Our results suggest that the endocytic uptake of Ox-LDL via the MSR plays an enhancing role in Ox-LDL-induced cytotoxicity in macrophages and macrophage-derived cells.

MATERIALS AND METHODS

Materials

$Na^{125}I$ (3.7 GBq/ml) and [α-^{32}P]dCTP (111 TBq/mmol) were purchased from Amersham. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cell culture media and reagents were obtained from Life Technologies. Newborn calf serum (NCS) was purchased from Hyclone Laboratories. All other chemicals were of the best grade from commercial sources.

Lipoproteins and their modifications

LDL (d 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of fresh human plasma and dialyzed against 0.15 m NaCl and 1 mm ethylenediaminetetraacetic acid (EDTA) (pH 7.4). Acetyl-LDL was prepared as described previously (18). Ox-LDL was prepared as described previously (19, 20). Briefly, 0.1 mg/ml LDL was incubated for 18 h at 37°C with 5 μm $CuSO_4$ in EDTA-free phosphate-buffered saline (PBS). The reaction was stopped by the addition of 1 mm EDTA and cooling on ice, followed by 50-fold concentration using ultrafiltration cell (Amicon, Danvers, MA) equipped with a PM-10 membrane. To remove $CuSO_4$, Ox-LDL was subjected to extensive dialysis against 0.15 m NaCl and 1 mm EDTA (pH 7.4). Iodination of acetyl-LDL and Ox-LDL with ^{125}I was performed according to McFarlane (21). The protein concentration was determined by BCA protein assay reagent (Pierce Chemical Co.) using BSA as a standard (22).

Cell culture

Unless otherwise specified, cell cultures were performed at 37°C in 5% CO_2. J774 cells and their Ox-LDL-resistant cell lines were cultured in RPMI 1640 medium containing 10% newborn calf serum, 10 mm N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.4), 0.1 mg/ml streptomycin and 100 U/ml penicillin (medium A). Mouse peritoneal macrophages were cultured in RPMI 1640 medium containing 10 mm N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.4), 0.1 mg/ml streptomycin, and 100 U/ml penicillin (medium B).

Isolation of Ox-LDL-resistant mutants from J774 cells

J774 cells (1×10^6) were plated into a 75-cm^2 tissue culture flask (Falcon) in 20 ml of medium A and incubated at 37°C overnight. The medium was removed and the cells were treated with 400 μg/ml of ethylmethane sulfonate in 15 ml of medium A for 16 h to a survival rate of 10% of the initial cell number (23). The cells were washed three times with PBS and cultured for an additional 2 days in 20 ml of medium A without the mutagen. The harvested mutagenized cells (5×10^5) were seeded into 20 dishes (100 mm in diameter) and incubated for 2 weeks with 0.1 mg/ml of Ox-LDL in 10 ml of medium A. Each colony thus formed was transferred with the cloning cup to a culture dish containing 0.1 mg/ml of Ox-LDL in 10 ml of medium A, followed by incubation for 2 weeks (23). We obtained five colonies, subjected them to a further 3 months culture in the absence of Ox-LDL, and determined their Ox-LDL-resistant property by MTT assays described below. We finally obtained two Ox-LDL-resistant clones that were isolated independently from different dishes. One of them, JO21b clone, was investigated in the present study.

Cell viability

Cell viability was determined by the MTT method (20). Briefly, 50 μl of the suspension of JO21b cells or J774 cells (5×10^3 cells) in medium A was transferred to each well of 96-well tissue culture plates (7-mm diameter, Falcon). After incubation for 2 h at 37°C, 50 μl of the compound to be tested, such as lipoproteins (see Fig. 1) and toxic components of Ox-LDL (see Fig. 2), was added to each well, followed by further incubation for the indicated intervals (up to 7 days). At indicated times, 10 μl of 5 mg/ml of MTT was added to each well, followed by 4-h incubation at 37°C to reduce MTT

to a blue formazan product. To each well was added 150 μl of 10% SDS in 0.01 N HCl, and the mixture was further incubated for 16 h at 37°C to dissolve the blue formazan product. The absorbance at 570 nm was then measured with each well in a multiwell spectrophotometer. Because MTT assay simply reflects the mitochondrial dehydrogenase activity of cells, the absorbance at 570 nm was used as an index of cell viability.

Cellular assays

JO21b and J774 cells (2×10^5) in 1 ml of medium A were seeded into each well of 24-well plates (16-mm diameter, Falcon) and incubated overnight. For the binding experiments, the monolayers thus formed were washed once with 1 ml of ice-cold PBS containing 0.3% BSA and twice with 1 ml of ice-cold PBS. Each well was incubated for 90 min on ice in 0.5 ml of medium A with the indicated concentrations of ^{125}I-labeled Ox-LDL (83 cpm/ng protein) or ^{125}I-labeled acetyl-LDL (117 cpm/ng protein). The cells were washed once with 1 ml of ice-cold PBS containing 0.3% BSA and twice with 1 ml of ice-cold PBS. The cells were dissolved with 1 ml of 0.1 m NaOH and the cell-bound radioactivity was determined as described previously (22). Nonspecific binding was measured by parallel incubation with an excess amount of the same unlabeled ligand. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

To determine cellular association of ^{125}I–labeled Ox-LDL or ^{125}I-labeled acetyl-LDL and their endocytic degradation by macrophages, macrophage cell monolayers prepared as above were washed once with 1 ml of PBS containing 0.3% BSA and twice with 1 ml of PBS. Each well was incubated for 24 h at 37°C in 1 ml of medium A with various amounts of ^{125}I-labeled Ox-LDL or ^{125}I-labeled acetyl-LDL. After incubation, the culture medium was taken from each well and used for determination of endocytic degradation, while the cells in each well were washed three times with 1 ml of PBS, dissolved in 1 ml of 0.1 m NaOH, and the cell-associated radioactivity was determined as described previously (24). To determine endocytic degradation, to 0.75 ml of each culture medium was added 0.25 ml of ice-cold 40% TCA and 0.2 ml of 0.7 m $AgNO_3$ and the mixture was incubated at room temperature for 30 min to precipitate free iodine. After centrifugation at 700 *g* for 10 min, 0.6 ml of the resulting supernatant was used for the determination of radioactivity using a gamma-counter (25).

Mass determination of cellular cholesterol contents

Macrophage cell monolayers prepared as above were incubated with various concentrations of Ox-LDL or acetyl-LDL. After incubation for 24 h, cellular lipids were extracted and both the free cholesterol mass and CE mass were quantified by a modification (22) of the enzymatic fluorometric methods (26). Briefly, cellular lipids extracted were dried under nitrogen flush and dissolved in 180 μl of isopropanol. Aliquots (30 μl) of the lipid extract was added to 0.4 ml of enzyme mixtures and incubated at 37°C for 1 h (for free cholesterol) or for 2 h (for total cholesterol), followed by addition of 0.81 ml of 0.5 m NaOH to terminate the reaction. Enzyme mixtures were identical to those reported previously (26) except that Carbowax-6000 was replaced by 0.01% Triton X-100 and enzyme concentrations used were 2 times higher (cholesterol oxidase; 0.16 U/ml and cholesteryl ester hydrolase; 0.16 U/ml). Fluorescence intensity was measured with an excitation at 320 nm and an emission at 407 nm. CE was calculated by subtracting free cholesterol from total cholesterol. Cells were dissolved in 0.1 m of NaOH to determine cell proteins using a BCA protein assay reagent.

Proteolytic activity of cell extracts

The proteolytic activity of macrophage cell extracts was determined according to the method described by Hoppe, O'Neil, and Hoff (27). Briefly, JO21b cells or J774 cells (2×10^6) were suspended in 1.0 ml of 250 mm sucrose, 1 mm EDTA, and 0.1% ethanol (pH 6.8) and ruptured by passing 10 times through a 25-gauge needle, by 10 cycles of freezing and thawing, and finally by 15-sec bursts of sonication. The cell homogenate was centrifuged at 5,000 *g* for 60 min at 4°C to remove particulate fractions. The supernatant solution (10 μg cell protein/ml) was incubated for 1 h at 37°C in 1.0 ml of 100 mm sodium acetate buffer (pH 4.0) with various concentrations (up to 40 μg/ml) of ^{125}I-labeled acetyl-LDL as a protease substrate. After the reaction, the amount of degraded ^{125}I-labeled acetyl-LDL was determined as TCA-soluble radioactivity as described above.

Northern blot analysis

Standard molecular biology techniques were used in this analysis (28). Poly $(A)^+$ RNA prepared from J774 or JO21b cells using Fast Track® 2.0 Kit (Invitrogen) (2 μg/lane) was fractionated by electrophoresis through a denaturing formaldehyde 1% agarose gel and transferred to Hybond-N+ nylon membrane as described (29). Probes were generated according to Ashkenas et al. (30) from total RNA of J774 cells by RT-PCR (reverse transcriptase-polymerase chain reaction) (probes A through D; see Fig. 8). The first strand cDNA was synthesized from 1.0 μg of J774 total RNA primed with oligo-dT using Moloney murine leukemia virus reverse transcriptase (BRL/GIBCO). Sense and antisense primer pairs for PCR were as follows. For probe A, 5′-CGC CGAGCGGCCGCGCTGTCTTCTTTACCAGC-3′ and 5′-

CGCCGGTCTAGATTATACTGATCTTGATCCGC-3′; for probe B, 5′-CCCCCTTAAGACAGTTCGAC-3′ and 5′-GGTTTCATAATTGTAATTTC-3′; for probe C, 5′-ACT CCCTGAACATATTGGGG-3′ and 5′-TAATAGGATTCT GCACAGTTA-3′; for probe D, 5′-ATTGATTAAGTAT TAGTTCT-3′ and 5′-CGCCGGATCGATTTTAATAT ATTAGACTAC-3′. Specific primers for amplification of a 983 bp fragment of glyceraldehydephosphate dehydrogenase (GAPDH) (31) were included during the PCR process. The cycling conditions in the GeneAmp 9600 System consisted of 94°C denaturation for 2 min, followed by 30 cycles of annealing at 55°C for 60 sec, extension at 72°C for 90 sec, and denaturation at 93°C for 30 sec, with a final prolonged step of 75°C for 10 min. The amplification products were analyzed by 1% agarose gel electrophoresis. The amplification products extracted from the agarose gel were ligated into pGEM-T (Promega), and transfected into *E. coli* XL1-Blue. To obtain single-strand DNA (ssDNA), XL1-Blue cells containing this construct were infected with the VCSM13 helper phage. The purified ssDNAs were sequenced to verify that amplification products were consistent with the reported sequences of murine type I and type II MSR and GAPDH. For preparation of radioactive probes, the cDNA fragments were excised with *Apa* I and *Sac* I, and labeled with [α-^{32}P]dCTP (29). Hybridization was performed with 50% formamide at 42°C as described previously (29). The final wash stringency was 0.2 × SSC, 0.1% SDS at a higher temperature (from 42 to 65°C) (29). The membrane was then exposed to an X-ray film (RX Fuji, Tokyo) for 24 h at room temperature. The intensity of MSR mRNA relative to that of GAPDH was calculated by densitometry (Quantity One, version 2.7, PDI) of the radiograph on a Macintosh computer.

Cytotoxic effect of Ox-LDL on peritoneal macrophages from MSR-knockout mice

Cytotoxic effect of Ox-LDL on peritoneal macrophages obtained from MSR-knockout mice was examined by the MTT method (20). Peritoneal macrophages were collected with 8 ml of ice-cold PBS from MSR-knockout mice (32) and their wild-type littermates that were born to heterozygous mice (32). Cells were suspended in medium B. Fifty microliter of each cell suspension (5 × 10^5 cells) was transferred to each well of 96-well tissue culture plates (7-mm diameter, Falcon) and 50 μl of indicated concentrations of Ox-LDL in medium B was subsequently added to each well, followed by further incubation for 24 h or 48 h. Four hours before the termination of the experiment, 10 μl of 5 mg/ml of MTT was added to each well to reduce MTT to a blue formazan product. To each well was added 150 μl of 10% SDS in 0.01 N HCl, and the mixture was further incubated for 16 h at 37°C to dissolve the blue formazan product. The absorbance at 570 nm was then measured with each well in a multiwell spectrophotometer.

Statistical Analysis

Data were evaluated by Student's *t*-test. Differences were considered significant when the *P* value was less than 0.05.

RESULTS

Cytotoxic effects of Ox-LDL on JO21b cells

JO21b cells, a mutant cell line resistant to the cytotoxic effect of Ox-LDL, were selected as described under Materials and Methods. In the first step, we compared the effects of Ox-LDL on the cell viability of JO21b cells with those on parent cells (J774 cells) using the MTT assay. As shown in **Fig. 1B**, 0.1 mg/ml of Ox-LDL had a negligible effect on cell viability until day 3, but became cytotoxic to J774 cells after day 4, indicating that J774 cells were sensitive to the cytotoxic effect of Ox-LDL. At the same concentration, however, Ox-LDL had no effect at all on the cell viability of JO21b cells, indicating resistance of JO21b cells to the cytotoxic effect of Ox-LDL (Fig. 1A). A similar concentration of acetyl-LDL exhibited no cytotoxic effect on JO21b cells as well as J774 cells (Fig. 1A and B), rather, it had a weak stimulating effect on the viability of J774 cells. Similarly, the cell viability of these cells was not influenced by unmodified LDL, suggesting that the cytotoxic effect of Ox-LDL on J774 cells was highly selective.

Cytotoxic effects of 7-ketocholesterol, *t*-butyl hydroperoxide and lysophosphatidylcholine on JO21b cells

Results of previous studies have suggested that the toxicity of Ox-LDL does not reside in its protein moiety, but rather in its lipid moiety (12). The potential cytotoxic compounds suggested so far include oxysterol (33–36), lipid hydroperoxide (37–39), and lysophosphatidylcholine (40). To examine the involvement of these compounds in Ox-LDL-induced cytotoxicity, we determined the cytotoxic effects of 7-ketocholesterol, tertiary-butyl hydroperoxide (a lipid hydroperoxide analog), and palmitoyl-lysophosphatidylcholine, on JO21b and J774 cells (**Fig. 2**). At 10 μg/ml, 7-ketocholesterol reduced the viability of JO21b cells at day 2 and that of J774 cells at day 1. The effect was more marked, causing death of all cells, at a concentration of 50 μg/ml

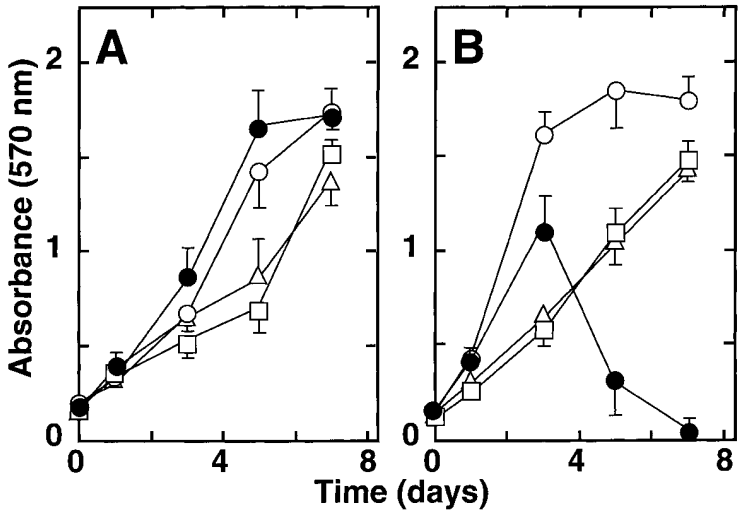

Fig. 1. Effects of Ox-LDL on the cell viability of JO21b cells (A) and J774 cells (B). The cells (5×10^3) were plated in microplates and incubated in 0.1 ml of medium A with 0.1 mg/ml Ox-LDL (●), acetyl-LDL (○), LDL (△), or medium alone (□) for the indicated times. Four hours before the termination of the experiment, 10 μl of 5 mg/ml of MTT solution was added to each well, followed by the addition of 150 μl of 10% SDS in 0.01 m HCl. The mixture was further incubated for 16 h to dissolve the blue formazan product and absorbance was read with a multiwell spectrophotometer at 570 nm. Data are representative of three separate experiments with duplicate wells.

(Fig. 2A and B), indicating that JO21b cells were as sensitive as J774 cells to the cytotoxic effect of 7-ketocholesterol. Similar experiments using 10 μm of *t*-butyl hydroperoxide, an artificial lipid hydroperoxide analog, did not influence the viability of J774 or JO21b cells, whereas a higher amount of the compound (30 μm) severely retarded an increase in the viability of J774 cells (Fig. 2D) and induced death of JO21b cells (Fig. 2C). These results suggest that the parent cells were slightly more resistant to *t*-butyl hydroperoxide. Lysophosphatidylcholine at 400 μm caused death of all JO21b and J774 cells (Fig. 2E and F), indicating that both cells were equally sensitive to the cytotoxic effects of lysophosphatidylcholine. Considered together, these results indicate that the sensitivity of JO21b cells to these cytotoxic compounds is essentially indistinguishable from that of the parent cells. Thus, these experiments failed to explain the mechanisms of resistance of JO21b cells to the cytotoxic effect of Ox-LDL. Accordingly, in the next series of experiments we examined the combined effects of these compounds on JO21b and J774 cells. Compared with the parent cells, however, JO21b cells were not specifically resistant to two or more compounds when combined together (data not shown), indicating again that the resistance of JO21b cells to Ox-LDL was not explained by these compounds.

Interaction of JO21b cells with Ox-LDL

The interaction of Ox-LDL with JO21b cells was determined by cellular binding, cell-association, and intracellular degradation. **Figure 3** shows the binding of ^{125}I-labeled Ox-LDL to JO21b and J774 cells at 0°C. Total binding was effectively replaced by >60% by an excess unlabeled ligand. The specific binding, calculated by subtracting nonspecific binding from the total bind-

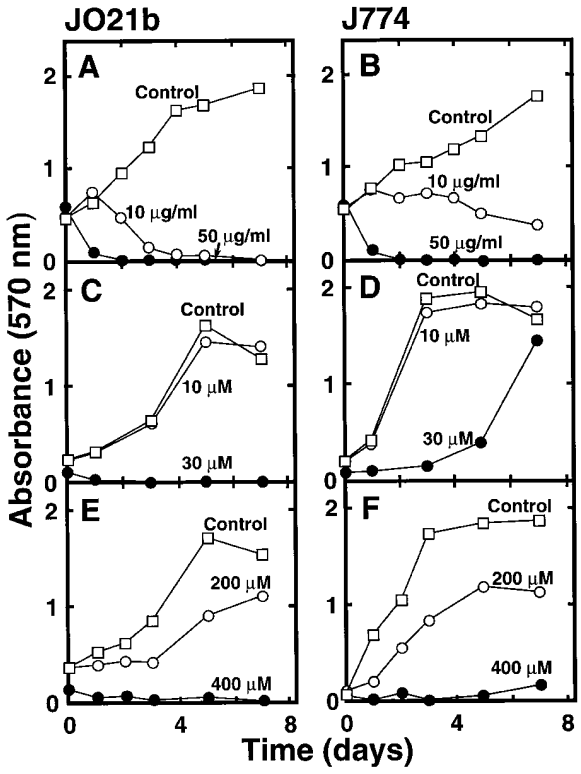

Fig. 2. Cytotoxic effects of 7-ketocholesterol, *t*-butyl hydroperoxide, and lysophosphatidylcholine on JO21b cells. JO21b cells (5×10^3) were incubated in 0.1 ml of medium A with 10 and 50 μg/ml of 7-ketocholesterol (A), 10 and 30 μm of *t*-butyl hydroperoxide (C) and 200 and 400 μm lysophosphatidylcholine (E) at the indicated time intervals. Under identical conditions, J774 cells were incubated with the same concentrations of 7-ketocholesterol (B), *t*-butyl hydroperoxide (D) and lysophosphatidylcholine (F). The cell viability was determined by the MTT assay as described in the legend of Fig. 1. Data are representative of three separate experiments with duplicate wells.

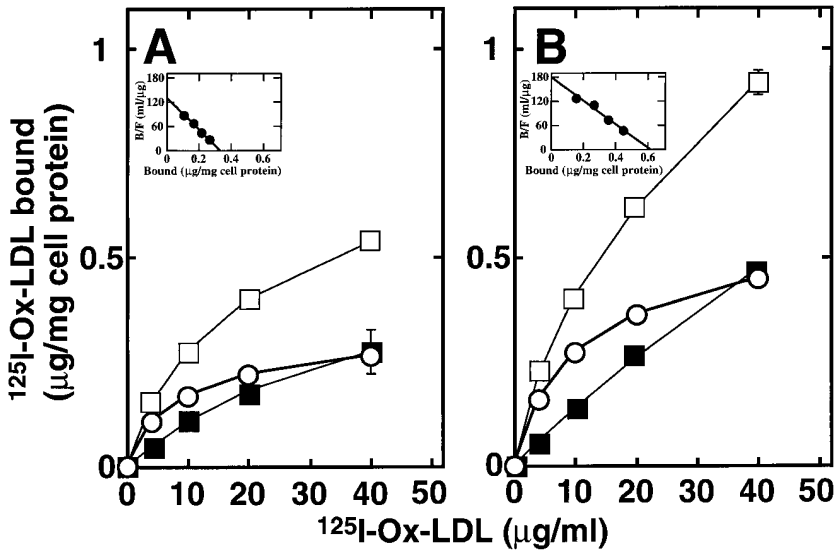

Fig. 3. The binding of ^{125}I-labeled Ox-LDL to JO21b cells. JO21b cells (A) or J774 cells (B) as a control (2×10^5) were incubated for 90 min on ice with increasing amounts of ^{125}I-labeled Ox-LDL with or without a 20-fold excess of unlabeled Ox-LDL. The cells were washed and the cell-bound ^{125}I-labeled Ox-LDL was determined as described under Materials and Methods. The specific binding (○) was calculated by subtracting the non-specific binding (■) from the total binding (□). The Scatchard plot of the specific binding is shown in the inset. Data are representative of three separate experiments with triplicate wells. Bars represent SD. SD values are small and are within the symbols.

ing, exhibited a saturation pattern in both JO21b and J774 cells. However, the total and specific bindings of ^{125}I-labeled Ox-LDL to JO21b cells (Fig. 3A) were reduced by 50% compared with those to J774 cells (Fig. 3B). The Scatchard analysis of this specific binding of Ox-LDL to JO21b cells disclosed a binding site with an apparent dissociation constant (K_d) of 2.6 μg/ml and maximal surface binding (B_{max}) of 335 ng/mg cell protein, whereas the same analysis of the specific binding to J774 cells showed a binding site with an apparent K_d of 3.4 μg/ml and B_{max} of 616 ng/mg cell protein. The nonspecific binding to JO21b cells was also reduced by 50% as compared with that to J774 cells. **Figure 4** shows the results of cellular association and degradation experiments. The total degradation of ^{125}I–labeled Ox-LDL by J774 cells in 24 h increased in a dose-dependent manner (Fig. 4B). The amount of Ox-LDL degraded by the mutant cells also increased dose-dependently (0.7 μg/mg cell protein/24 h at 100 μg/ml of Ox-LDL). However, the latter was only 10% of that degraded by J774 cells (6.3 μg/mg cell protein/24 h at 100 μg/ml of Ox-LDL) (Fig. 4B). Similarly, the total cellular association of ^{125}I-labeled Ox-LDL with JO21b cells was less than 20% of that with J774 cells (Fig. 4A). These results strongly suggested that the cellular binding of Ox-LDL and its subsequent endocytic degradation by JO21b cells were significantly less than those by parent cells.

Interaction of JO21b cells with acetyl-LDL

Although Ox-LDL is a ligand for type I and type II MSR, acetyl-LDL is known as a representative ligand for these MSR. We therefore determined the cellular binding of ^{125}I-labeled acetyl-LDL to JO21b cells at 0°C. As was the case with Ox-LDL, the specific binding of acetyl-LDL to both J774 and JO21b cells, calculated by subtracting nonspecific binding from the total binding, exhibited a saturation pattern. However, the amount of ^{125}I-labeled acetyl-LDL specifically bound to JO21b cells at each ligand concentration (**Fig. 5A**) was less than 30% of that obtained with the parent cells (Fig. 5B). The Scatchard analysis of this specific binding of acetyl-LDL to JO21b cells disclosed a binding site with an apparent K_d of 2.1 μg/ml and B_{max} of 480 ng/mg cell protein, whereas the same analysis of the specific binding to J774 cells showed a binding site with an apparent K_d of 4.2 μg/ml and B_{max} of 2,035 ng/mg cell protein. The endocytic activity of JO21b cells for acetyl-LDL was determined under conditions identical to those of Ox-LDL (Fig. 4). The cellular association of ^{125}I-labeled acetyl-LDL in JO21b cells was 60% of that in parent cells (**Fig. 6A**). Similarly, the subsequent endocytic degradation of ^{125}I-labeled acetyl-LDL by these cells was only 70% of that with J774 cells (Fig. 6B). Thus, the endocytic capacity of these mutant cells for acetyl-LDL is also reduced in the same way as that for

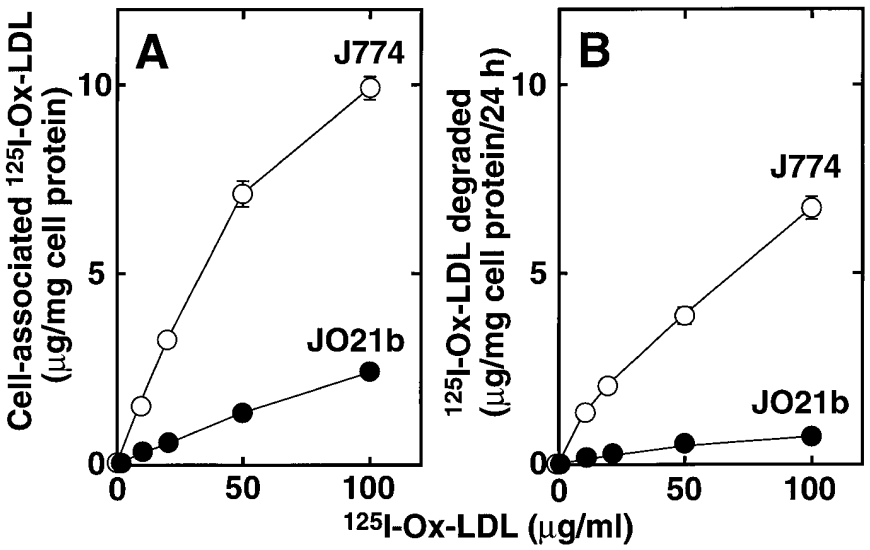

Fig. 4. Cell-association of ^{125}I-labeled Ox-LDL with and subsequent endocytic degradation by JO21b cells. JO21b cells or control J774 cells (2×10^5) were incubated for 24 h at 37°C with the indicated concentrations of ^{125}I-labeled Ox-LDL. The cell-associated ^{125}I-labeled Ox-LDL (A) and that degraded by the cells (B) were determined as described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. SD values are small and within the symbols.

Ox-LDL, suggesting a significantly reduced MSR function in JO21b cells.

CE-accumulation capacity of JO21b cells upon incubation with Ox-LDL or acetyl-LDL

Results of the above experiments (Figs. 3, 4, 5, and 6) suggested that the processes of Ox-LDL and acetyl-LDL binding to, and subsequent endocytic degradation by, JO21b cells were significantly weaker than J774 cells. As the endocytic degradation of these modified LDLs by macrophages is associated with intracellular accumulation of cholesteryl esters and foam cell formation, the mass of cholesteryl esters accumulating in JO21b cells was measured and compared with that in parent cells. Upon incubation with Ox-LDL, CE-accumulation increased dose-dependently in JO21b cells in a manner similar to J774 cells. However, the amount of accumulated CE in mutant cells was less than 20% of that in J774 cells (**Fig. 7A**). Furthermore, acetyl-LDL-induced CE-accumulation in JO21b cells was also less than one-third of that in J774 cells (Fig. 7B). As the loading of these cells with Ox-LDL and acetyl-LDL did not in-

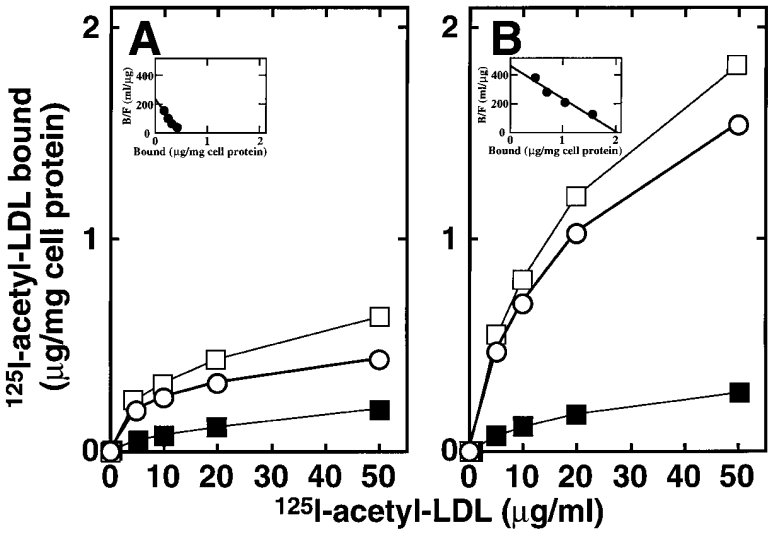

Fig. 5. The binding of ^{125}I-labeled acetyl-LDL to JO21b cells (A) and control J774 cells (B) were incubated for 90 min on ice with increasing amounts of ^{125}I-labeled acetyl-LDL with or without a 20-fold excess unlabeled acetyl-LDL. The cells were washed and the cell-bound radioactivity was determined as described under Materials and Methods. The specific binding (○) was calculated by subtracting the non-specific binding (■) from the total binding (□). The Scatchard plot of the specific binding is shown in the inset. Data are representative of three separate experiments with triplicate wells. SD values are small and within the symbols.

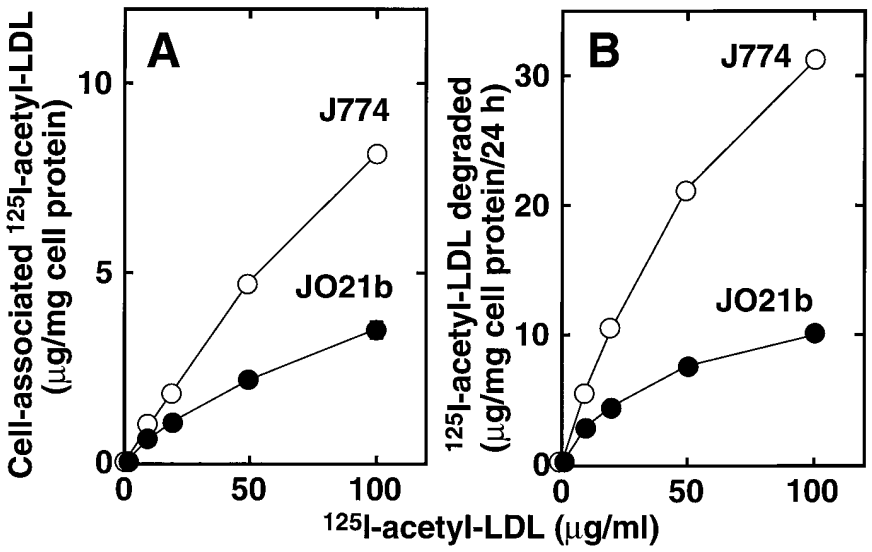

Fig. 6. Cell-association of ^{125}I-labeled acetyl-LDL with and subsequent endocytic degradation by JO21b cells. JO21b cells and control J774 cells (2×10^5) were incubated separately for 24 h at 37°C with the indicated concentrations of ^{125}I-labeled acetyl-LDL. The amount of cell-associated ^{125}I-labeled acetyl-LDL (A) and that degraded by these cells (B) was described under Materials and Methods. Data are representative of three separate experiments with triplicate wells. Bars represent SD. SD values are small and are within the symbols.

crease the cellular free cholesterol, the increase in total cholesterol was exclusively explained by intracellular accumulation of CE (data not shown).

These results are consistent with reduced endocytic activity of mutant cells for the ligands of MSR such as Ox-LDL and acetyl-LDL (Figs. 4 and 6). One cannot exclude, however, the possibility that the resistance of JO21b cells to Ox-LDL might be in part due to altered levels of lysosomal protease activity. However, determination of the lysosomal protease activity indicated that the activity in JO21b cells was not different from that in parent cells (data not shown), indicating that lysosomal

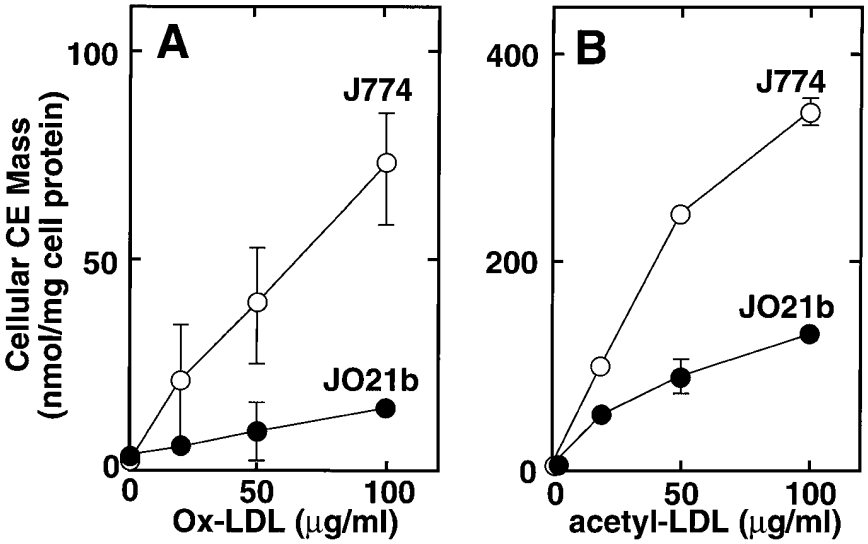

Fig. 7. The accumulation capacity of JO21b cells for cholesterol esters after exposure to Ox-LDL (A) and acetyl-LDL (B). JO21b cells or J774 cells (2×10^5) were plated and incubated in 1 ml of medium A with the indicated concentrations of Ox-LDL (A) and acetyl-LDL (B). The cells were harvested after 24 h incubation at 37°C, and the lipids were extracted and the mass of cellular cholesteryl esters (CE) were determined as described under Materials and Methods. Each value is the mean ± SD of quadruplicate experiments.

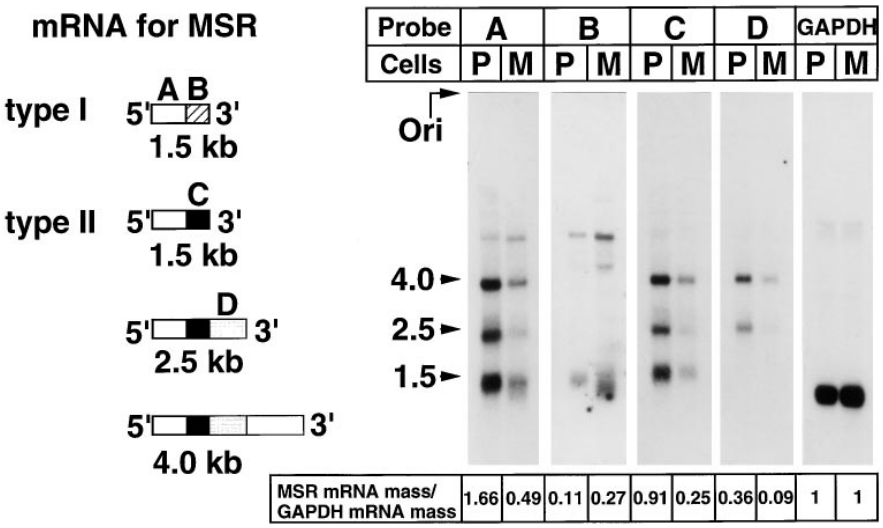

Fig. 8. Northern blot analysis of type I and type II MSR of JO21b cells. Schematic representation of mouse MSR mRNA (left panel). Open boxes: the common region of type I and type II forms (probe A), hatched box: the specific region for type I form (probe B), solid boxes: the specific region for all three type II forms (probe C), shaded boxes: the specific region for longer two type II forms (probe D). Complementary DNA probes were designated on the basis of the reported cDNA sequence and amplified by RT-PCR (probes A–D). Poly(A)$^+$ RNA from J774 (P, parent) or JO21b (M, mutant) cells (2 μg/lane) was subjected to agarose electrophoresis, transferred to Hybond-N+ nylon membrane and hybridized with ^{32}P-labeled probe (A–D, and GAPDH) as described under Materials and Methods (right panel). The mass of MSR mRNA compared with that of GAPDH was calculated by densitometry of the radiograph.

protease activity did not contribute to the resistance of JO21b cells to Ox-LDL.

Northern blot analysis of type I and type II MSR

Our results so far suggested a possible reduction of MSR activity in JO21b cells. Northern blot analysis was performed to determine whether type I and type II MSR expression was reduced at the mRNA level using J774 cells as a control. Previous studies indicated that two isoforms (type I and type II) of MSR are formed by alternative splicing of transcripts derived from a single gene (41). Because type I has a single transcript, whereas type II has three transcripts (30), we designated four probes (A, B, C, and D) to detect these different species of MSR mRNA (**Fig. 8**); probe A can recognize all four species of transcripts, probe B can detect only type I transcript, probe C recognizes all three species of type II transcript, and probe D recognizes longer two type II transcripts. As shown in Fig. 8, [^{32}P]-labeled probe A cross-hybridized with poly(A)$^+$ RNAs of JO21b and J774 cells, with strong three signals at 4.0, 2.5, and 1.5 kb. Probe B cross-hybridized with poly(A)$^+$ RNAs, with a signal at 1.5 kb. Three signals at 4.0, 2.5, and 1.5 kb were detected by probe C, whereas two signals at 4.0 and 2.5 kb were detected by probe D. Parallel experiments showed that [^{32}P]-labeled GAPDH probe cross-hybridized with poly(A)$^+$ RNAs of JO21b and J774 cells, with a single signal at 1.0 kb. The densitometry units for MSR bands were divided by the corresponding units of GAPDH. The ratio of type I MSR mRNA level detected by probe B to the GAPDH level in JB23 cells (type I MSR mRNA mass/GAPDH mRNA mass = 0.27) was 2.5-fold larger than the corresponding ratio in J774 cells (type I MSR mRNA mass/GAPDH mRNA mass = 0.11). However, from the result using probe A which hydridized both type I and type II MSR mRNA, total MSR mRNA (type I and type II) was reduced by 70% in JO21b cells (MSR mRNA mass/GAPDH mRNA mass = 0.49) compared to J774 cells (MSR mRNA mass/GAPDH mRNA mass = 1.66). These results suggested the presence of a reduced activity of MSR in JO21b cells at the mRNA level.

Cytotoxic effect of Ox-LDL on peritoneal macrophages from MSR-knockout mice

In order to directly demonstrate the correlation of reduced MSR activity to reduced sensitivity to the cytotoxic effect of Ox-LDL, we attempted to transfect type II MSR cDNA or type I MSR cDNA into JO21b cells. However, several trials eventually failed. Thus, we compared the sensitivity of peritoneal macrophages obtained from MSR-knockout mice with that of their wild-type littermates by MTT methods (20). As shown in **Table 1**, incubation with 0.2 mg/ml of Ox-LDL for 48 h caused total cell death of both MSR-wild-type macro-

TABLE 1. Cytotoxic effect of Ox-LDL on peritoneal macrophages obtained from MSR-knockout mice

Ox-LDL	MSR-Knockout	Wild-Type
(mg/ml)		
0	0.152 ± 0.036 (100)	0.155 ± 0.019 (100)
0.1	0.150 ± 0.014 (98.7)[a]	0.098 ± 0.005 (63.1)[a]
0.12	0.030 ± 0.010 (19.8)[b]	0.019 ± 0.001 (12.3)[b]
0.2	0.001 ± 0.001 (0.7)	0.001 ± 0.001 (0.6)

Fifty microliter cell suspensions (5×10^5 cells) of peritoneal macrophages from MSR-knockout mice and their wild-type littermates were dispersed in each well of 96-well tissue culture plates. To each well was added 50 μl of 2-fold indicated concentrations of Ox-LDL in medium B, followed by further incubation for 48 h. The cell viability was determined by the MTT assay as described under Materials and Methods. Data are representative of three separate experiments with quadruplicate wells. Values are the mean optical density ± SD. Percentages of the control values are expressed in parentheses. Statistical significance by Student's *t*-test: [a]$P < 0.01$; [b]$P < 0.05$.

phages and MSR-knockout macrophages (Table 1). However, at 0.1 mg/ml of Ox-LDL, a decrease in cell viability of wild-type macrophages (36.9%) was significantly larger than that of knockout macrophages (1.3%) (Table 1). Similar results were obtained upon 24-h incubation with Ox-LDL (data not shown). These results clearly indicate that peritoneal macrophages from MSR-knockout mice are more resistant to the cytotoxic effect of Ox-LDL than those from MSR-wild-type mice, suggesting that MSR plays an enhancing role in the cytotoxic effect of Ox-LDL toward macrophages.

DISCUSSION

The present study provides several lines of experimental evidence that MSR plays an important role in Ox-LDL-induced macrophage cell death. First, we have isolated mutant cells resistant to the cytotoxic effect of Ox-LDL from J774 cells (a murine macrophage-like cell line), and one mutant form, JO21b cells, showed a marked reduction in MSR activity and a concomitant reduction in their ability to take up Ox-LDL. Second, macrophages from MSR-knockout mice (32), whose activity for endocytic uptake of Ox-LDL was less than 50% of that of wild-type macrophages (32), showed significantly higher resistance to the cytotoxic effect of 0.1 or 0.12 mg/ml of Ox-LDL than those from their wild-type littermates (Table 1). However, 0.2 mg/ml of Ox-LDL became cytotoxic to macrophages from both MSR-knockout mice and their littermates (Table 1), suggesting that MSR-mediated uptake of Ox-LDL enhances macrophage cell death, rather than being essential for cell death. It is therefore highly likely that MSR plays an enhancing role in the cytotoxic effect of Ox-LDL toward macrophages or macrophage-derived cells.

In the current study, JO21b cells were characterized by their reduced expression of MSR. However, it is not clear whether single or multiple gene alternations occur in JO21b cells. Subsequent observations seem to support the latter possibility. First, the specific binding of Ox-LDL to JO21b cells was reduced by 50% compared to that to J774 cells (Fig. 3A), while the endocytic degradation of Ox-LDL by JO21b cells was reduced by >80% compared to that by J774 cells (Fig. 4B). These results suggest that an additional gene alteration(s) in JO21b cells may independently affect the endocytic process of Ox-LDL. Alternatively, it is also possible that there are cell surface binding sites for Ox-LDL that do not mediate endocytosis. Second, as the nonspecific binding of Ox-LDL to JO21b cells was also reduced by 50% compared with that to J774 cells (Fig. 3), another gene alteration(s) could reduce the expression of cellular machinery involved in the nonspecific binding of Ox-LDL. This reduction may cause a decrease in the nonspecific transfer of toxic components of Ox-LDL and cause JO21b cells to be resistant to Ox-LDL. However, all these possibilities are speculative and have no molecular basis at the present. Further studies are therefore needed to identify a cDNA(s) that corrects the mutant phenotype of JO21b cells.

Ox-LDL is reported to be toxic not only to macrophages (14, 15, 17), but also to other cells, such as endothelial, smooth muscle, and fibroblast cells (for a review, see 42). Previous studies showed that the cytotoxic effects of Ox-LDL were not cell specific (43), suggesting that the essential toxic component(s) of Ox-LDL is delivered to the cell interior via a non-specific exchange reaction between lipoproteins and plasma membranes. However, Schmitt et al. (44) and Nègre-Salvayre et al. (45) showed that the cytotoxic effects of ultraviolet-oxidized LDL on cultural bovine aortic endothelial cells and EB virus-transformed B lymphocytes were related to the amount of oxidized neutral lipids taken up specifically by these cells through the apoB/E receptor pathway (the LDL receptor pathway), suggesting that receptor-mediated endocytosis of Ox-LDL is involved in the cytotoxic effect of Ox-LDL. This notion was greatly supported by the present conclusion that MSR plays an enhancing role in Ox-LDL-induced cell death of macrophages. As extensive oxidative modification of LDL by Cu^{2+} results in a loss of its ligand activity for the apoB/E receptor (2) and Ox-LDL thus formed is specifically taken up by macrophages via MSR which leads to foam cell formation (46), our experimental system using macrophages and Cu^{2+}-oxidized LDL likely reflects the events in the early stage of atherosclerotic lesions. Thus, the present study may explain that MSR-mediated uptake of Ox-LDL enhances the Ox-LDL-induced cell death of macrophages in a macrophage-specific manner during atherosclerotic lesion formation (16).

Recent cDNA expression and protein purification experiments have identified other Ox-LDL receptors, including FcγRII-B2 (47), SR-BI (4), macrosialin (48), CD36 (49) and LOX-1 (50). Interestingly, Nozaki et al. (51) showed that the specific endocytic degradation of 40 μg/ml of ^{125}I-labeled Ox-LDL by monocyte-derived macrophages from CD36-deficient subjects was 75% of that by the corresponding cells from normal subjects, suggesting an important role for CD36 in endocytosis of Ox-LDL by macrophages or macrophage-derived cells. However, our previous experiments showed that the endocytic degradation of ^{125}I-labeled Ox-LDL by peritoneal macrophages obtained from the MSR-knockout mice was reduced by more than 50% compared with those of their wild-type littermates (32, 52). Although the involvement of other Ox-LDL receptors in the endocytic uptake of Ox-LDL in J774 cells is not well known, from all these results it can be expected that type I and type II MSR play a major role in the endocytic uptake of Ox-LDL in J774 cells as well as mouse peritoneal macrophages.

It was reported that oxysterol (33–36), lipid hydroperoxide (37–39), and lysophosphatidylcholine (40) are cytotoxic components of Ox-LDL. In fact, all these compounds were cytotoxic to J774 cells as well as JO21b cells (Fig. 2). However, we could not identify a toxic component(s) of Ox-LDL specifically involved in Ox-LDL-induced death of J744 cells. A recent study identified 7β-hydroperoxycholesterol as a potent toxic component of Ox-LDL in human carotid atherosclerotic lesions (34). Moreover, Colles, Irwin, and Chisolm (35) reported that 7β-hydroperoxycholesterol was predominantly responsible for Ox-LDL-induced cytotoxicity to fibroblasts, smooth muscle cells, and endothelial cells. Further studies are necessary to compare the cytotoxic effects of this compound on J744 and JO21b cells.

Two types of cell death, apoptosis and necrosis, have been so far described (53, 54). However, the mechanisms of macrophage cell death induced by Ox-LDL are not fully understood. In a previous study, Reid, Hardwick, and Mitchinson (14) showed that exposure of murine macrophage-like cells ($P388D_1$ cells) to Ox-LDL led to DNA fragmentation, a process often used as a biochemical marker of apoptosis. We tested whether apoptosis might represent a mechanism of Ox-LDL-induced J774 cell death in accordance with Reid's procedure (14). However, repeated experiments failed to detect DNA fragmentation in J774 cells (data not shown). Several recent reports have demonstrated morphological apoptosis without DNA fragmentation (55–57). However, our electron microscopic observations failed to detect morphological features typical of apoptosis, such as condensation of nuclear chromatin, compact cytoplasmic organelles, and blebbing of the cell surface (data not shown). The exact reason for the apoptotic effect of Ox-LDL on $P388D_1$ cells (14) but not J774 cells is not completely understood. The different response may reflect differences in cell type or methodology, or both. When all these observations are combined it seems that J774 cells mainly undergo necrosis rather than apoptosis when they are exposed to a high concentration of Ox-LDL.

In contrast to Ox-LDL-induced macrophage cell death, we reported previously that Ox-LDL can paradoxically induce macrophage cell growth in mice (58), humans (59), and rats (60). In this process, endocytic uptake of Ox-LDL containing lysophosphatidylcholine by MSR plays an important and efficient role in the mitogenic effect of Ox-LDL (20, 52). Under these conditions, incubation of mouse peritoneal macrophages with Ox-LDL at concentrations less than 0.1 mg/ml led to the growth of these cells (20, 52, 58), whereas incubation with Ox-LDL at concentrations higher than 0.1 mg/ml resulted in cell death in a manner similar to that observed with J774 cells (Fig. 1A). Therefore, it seems likely that the mechanisms of Ox-LDL-induced macrophage cell growth are, in essence, related or similar to those of Ox-LDL-induced death of the same cells. This notion is supported by the fact that MSR-mediated endocytic uptake of Ox-LDL plays an enhancing role in both phenomena. Therefore, both the mitogenic and cytotoxic effects of Ox-LDL on macrophages should be pursued in parallel.

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REFERENCES

1. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for 1990s. *Nature*. **362:** 801–809.
2. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med*. **320:** 915–924.
3. Krieger, M., and J. Herz. 1994. Structures and functions of multiligand lipoprotein receptors: macrophage scaven-

ger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.* **63:** 601–637.
4. Acton, S. L., P. E. Scherer, H. F. Lodish, and M. Krieger. 1994. Expression cloning of SR-BI, a CD36-related class B scavenger receptor. *J. Biol. Chem.* **269:** 21003–21009.
5. Pearson, A., A. Lux, and M. Krieger. 1995. Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* **92:** 4056–4060.
6. Kodama, T., M. Freeman, L. Rohrer, J. Zabrecky, P. Matsudaira, and M. Krieger. 1990. Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. *Nature.* **343:** 531–535.
7. Rohrer, L., M. Freeman, T. Kodama, M. Penman, and M. Krieger. 1990. Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. *Nature.* **343:** 570–572.
8. Freeman, M., Y. Ekkel, L. Rohrer, M. Penman, N. J. Freedman, G. M. Chisolm, and M. Krieger. 1991. Expression of type I and type II bovine scavenger receptors in Chinese hamster ovary cells: lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **88:** 4931–4935.
9. Palinski, W., M. E. Rosenfeld, S. Ylä-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum. 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA.* **86:** 1372–1376.
10. Ylä-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S. Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
11. Holvoet, P., and D. Collen. 1994. Oxidized lipoproteins in atherosclerosis and thrombosis. *FASEB J.* **8:** 1279–1284.
12. Hessler, J. R., A. L. Robertson, Jr., and G. M. Chisolm. 1979. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis.* **32:** 213–229.
13. Berliner, J. A., M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamshad, M. Esterson, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85:** 1260–1266.
14. Reid, V. C., S. J. Hardwick, and M. J. Mitchinson. 1993. Fragmentation of DNA in $P388D_1$ macrophages exposed to oxidized low-density lipoprotein. *FEBS Lett.* **332:** 218–220.
15. Reid, V. C., and M. J. Mitchinson. 1993. Toxicity of oxidized low density lipoprotein towards mouse peritoneal macrophages in vitro. *Atherosclerosis.* **98:** 17–24.
16. Aqel, N. M., R. Y. Ball, H. Waldmann, and M. J. Mitchinson. 1985. Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies. *J. Pathol.* **146:** 197–204.
17. Marchant, C. E., N. S. Law, C. van der Veen, S. J. Hardwick, K. L. H. Carpenter, and M. J. Mitchinson. 1995. Oxidized low-density lipoprotein is cytotoxic to human monocyte-macrophages: protection with lipophilic antioxidants. *FEBS Lett.* **358:** 175–178.
18. Murakami, M., S. Horiuchi, K. Takata, and Y. Morino. 1987. Distinction in the mode of receptor-mediated endocytosis between high density lipoprotein and acetylated high density lipoprotein: evidence for high density lipoprotein receptor-mediated cholesterol transfer. *J. Biochem. (Tokyo)* **101:** 729–741.
19. Ohta, T., K. Takata, S. Horiuchi, Y. Morino, and I. Matsuda. 1989. Protective effect of lipoprotein containing apoprotein A-I on Cu^{2+}-catalyzed oxidation of human low density lipoprotein. *FEBS Lett.* **257:** 435–438.
20. Sakai, M., A. Miyazaki, H. Hakamata, T. Sasaki, S. Yui, M. Yamazaki, M. Shichiri, and S. Horiuchi. 1994. Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. *J. Biol. Chem.* **269:** 31430–31435.
21. McFarlane, A. S. 1958. Efficient trace-labelling of proteins with iodine. *Nature.* **182:** 53.
22. Miyazaki, A., M. Sakai, Y. Suginohara, H. Hakamata, Y. Sakamoto, W. Morikawa, and S. Horiuchi. 1994. Acetylated low density lipoprotein reduces its ligand activity for the scavenger receptor after interaction with reconstituted high density lipoprotein. *J. Biol. Chem.* **269:** 5264–5269.
23. Hara-Kuge, S., F. Amano, M. Nishijima, and Y. Akamatsu. 1990. Isolation of a lipopolysaccharide (LPS)-resistant mutant, with defective LPS binding, of cultured macrophage-like cells. *J. Biol. Chem.* **265:** 6606–6610.
24. Suginohara, Y., A. Miyazaki, H. Hakamata, Y. Sakamoto, T. Ohta, I. Matsuda, and S. Horiuchi. 1996. The heparin-bound fraction of human lipoprotein-deficient serum inhibits endocytic uptake of oxidized low density lipoprotein by macrophages. *Atherosclerosis.* **120:** 167–179.
25. Hakamata, H., A. Miyazaki, M. Sakai, Y. Sakamoto, H. Matsuda, K. Kihara, and S. Horiuchi. 1995. Differential effects of an acyl-coenzyme A:cholesterol acyltransferase inhibitor on HDL-induced cholesterol efflux from rat macrophage foam cells. *FEBS Lett.* **363:** 29–32.
26. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* **19:** 514–518.
27. Hoppe, G., J. O'Neil, and H. F. Hoff. 1994. Inactivation of lysosomal proteases by oxidized low density lipoprotein is partially responsible for its poor degradation by mouse peritoneal macrophages. *J. Clin. Invest.* **94:** 1506–1512.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Matsuda, H., H. Hakamata, A. Miyazaki, M. Sakai, C. C. Y. Chang, T-Y. Chang, S. Kobori, M. Shichiri, and S. Horiuchi. 1996. Activation of acyl-coenzyme A:cholesterol acyltransferase activity by cholesterol is not due to altered mRNA levels in HepG2 cells. *Biochim. Biophys. Acta.* **1301:** 76–84.
30. Ashkenas, J., M. Penman, E. Vasile, S. Acton, M. Freeman, and M. Krieger. 1993. Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. *J. Lipid Res.* **34:** 983–1000.
31. Tso, J. Y., X-H. Sun, T. Kao, K. S. Reece, and R. Wu. 1985. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* **13:** 2485–2502.
32. Suzuki, H., Y. Kurihara, M. Takeya, N. Kamada, M. Kataoka, K. Jishage, O. Ueda, H. Sakaguchi, T. Higashi, T. Suzuki, Y. Takashima, Y. Kawabe, O. Cynshi, Y. Wada, M. Honda, H. Kurihara, H. Aburatani, T. Doi, A. Matsumoto, S. Azuma, T. Noda, Y. Toyoda, H. Itakura, Y. Yazaki, S. Horiuchi, K. Takahashi, J. K. Kruijt, T. J. C van Berkel, U. P. Steinbrecher, S. Ishibashi, N. Maeda, S. Gordon, and T. Kodama. 1997. A

role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature.* **386:** 292–296.
33. Guyton, J. R., B. L. Black, and C. L. Seidel. 1990. Focal toxicity of oxysterols in vascular smooth muscle cell culture: a model of the atherosclerotic core region. *Am. J. Pathol.* **137:** 425–434.
34. Chisolm, G. M., G. Ma, K. C. Irwin, L. L. Martin, K. G. Gunderson, L. F. Linberg, D. W. Morel, and P. E. DiCorleto. 1994. 7β-Hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **91:** 11452–11456.
35. Colles, S. M., K. C. Irwin, and G. M. Chisolm. 1996. Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7β-hydroperoxycholesterol. *J. Lipid Res.* **37:** 2018–2028.
36. Hughes, H., B. Mathews, M. L. Lenz, and J. R. Guyton. 1994. Cytotoxicity of oxidized LDL to porcine aortic smooth muscle cells is associated with the oxysterols 7-ketocholesterol and 7-hydroxycholesterol. *Arterioscler. Thromb.* **14:** 1177–1185.
37. Thomas, J. P., P. G. Geiger, and A. W. Girotti. 1993. Lethal damage to endothelial cells by oxidized low density lipoprotein: role of selenoperoxide in cytoprotection against lipid hydroperoxide- and iron-induced reactions. *J. Lipid Res.* **34:** 479–490.
38. Kuzuya, M., M. Naito, C. Funai, T. Hayashi, K. Asai, and F. Kuzuya. 1991. Lipid peroxide and transition metals are required for the toxicity of oxidized low density lipoprotein to cultured endothelial cells. *Biochim. Biophys. Acta.* **1096:** 155–166.
39. Naito, M., K. Yamada, T. Hayashi, K. Asai, N. Yoshimine, and A. Iguchi. 1994. Comparative toxicity of oxidatively modified low-density lipoprotein and lysophosphatidylcholine in cultured vascular endothelial cells. *Heart Vessels.* **9:** 183–187.
40. Chen, Y., S. Morimoto, S. Kitano, E. Koh, K. Fukuo, B. Jiang, S. Chen, O. Yasuda, A. Hirotani, T. Ogihara. 1995. Lysophosphatidylcholine cause Ca^{2+} influx, enhanced DNA synthesis and cytotoxicity in cultured vascular smooth muscle cells. *Atherosclerosis.* **112:** 69–76.
41. Freeman, M., J. Ashkenas, D. J. G. Rees, D. M. Kingsley, N. G. Copeland, N. A. Jenkins, and M. Krieger. 1990. An ancient, highly conserved family of cysteine rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors. *Proc. Natl. Acad. Sci. USA.* **87:** 8810–8814.
42. Chisolm, G. M. 1991. Cytotoxicity of oxidized lipoproteins. *Curr. Opin. Lipidol.* **2:** 311–316.
43. Hessler, J. R., D. W. Morel, L. J. Lewis, and G. M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxity. *Arteriosclerosis.* **3:** 215–222.
44. Schmitt, A., A. Nègre-Salvayre, M. Troly, P. Valdiguié, and R. Salvayre. 1995. Phospholipid hydrolysis of mildly oxidized LDL reduces their cytotoxicity to cultured endothelial cells. Potential protective role against atherogenesis. *Biochim. Biophys. Acta.* **1256:** 284–292.
45. Nègre-Salvayre, A., M. Lopez, T. Levade, M-T. Pieraggi, N. Dousset. L. Douste-Blazy, and R. Salvayre. 1990. Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. II. Uptake and cytotoxicity of ultraviolet-treated LDL on lymphoid cell lines. *Biochim. Biophys. Acta.* **1045:** 224–232.
46. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
47. Stanton, L. W., R. Tyler White, C. M. Bryant, A. A. Protter, and G. Endemann. 1992. A macrophage Fc receptor for IgG is also a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **267:** 22446–22451.
48. Ramprasad, M. P., W. Fischer, J. L. Witztum, G. R. Sambrano, O. Quehenberger, and D. Steinberg. 1995. The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. *Proc. Natl. Acad. Sci. USA.* **92:** 9580–9584.
49. Endemann, G., L. W. Stanton, K. S. Madden, C. M. Bryant, R. Tyler White, and A. A. Protter. 1993. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **268:** 11811–11816.
50. Sawamura, T., N. Kume, T. Aoyama, H. Moriwaki, H. Hoshikawa, Y. Aiba, T. Tanaka, S. Miwa, Y. Katsura, T. Kita, and T. Masaski. 1997. An endothelial receptor for oxidized low-density lipoprotein. *Nature.* **386:** 73–77.
51. Nozaki, S., H. Kashiwagi, S. Yamashita, T. Nakagawa, B. Kostner, Y. Tomiyama, A. Nakata, M. Ishigami, J. Miyagawa, K. Kameda-Takemura, Y. Kurata, and Y. Matsuzawa. 1995. Reduced uptake of oxidized low density lipoproteins in monocyte-derived macrophages from CD36-deficient subjects. *J. Clin. Invest.* **96:** 1859–1865.
52. Sakai, M., A. Miyazaki, H. Hakamata, T. Kodama, H. Suzuki, S. Kobori, M. Shichiri, and S. Horiuchi. 1996. The scavenger receptor serves as a route for internalization of lysophosphatidylcholine in oxidized low density lipoprotein-induced macrophage proliferation. *J. Biol. Chem.* **271:** 27346–27352.
53. Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer.* **26:** 239–257.
54. Vaux, D. L., H. Georg, and A. Strasser. 1994. An evolutionary perspective on apoptosis. *Cell.* **76:** 777–779.
55. Cohen, G. M., X-M. Sun, R. T. Snowden, D. Dinsdale, and D. N. Skilleter. 1992. Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem. J.* **286:** 331–334.
56. Oberhammer, F., G. Fritsch, M. Schmied, M. Pavelka, D. Printz, T. Purchio, H. Lassmann, and R. Schulte-Hermann. 1993. Condensation of chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J. Cell Sci.* **104:** 317–326.
57. Tomei, L. D., J. P. Shapiro, and F. O. Cope. 1993. Apoptosis in C3H/10T1/2 mouse embryonic cells: evidence for internucleosomal DNA modification in the absence of double-strand cleavage. *Proc. Natl. Acad. Sci. USA.* **90:** 853–857.
58. Yui, S., T. Sasaki, A. Miyazaki, S. Horiuchi, and M. Yamazaki. 1993. Induction of murine macrophage growth by modified LDLs. *Arterioscler. Thromb.* **13:** 331–337.
59. Sakai, M., A. Miyazaki, H. Hakamata, Y. Sato, T. Matsumura, S. Kobori, M. Shichiri, and S. Horiuchi. 1996. Lysophosphatidylcholine potentiates the mitogenic activity of modified low density lipoproteins for human monocyte-derived macrophages. *Arterioscler. Thromb. Vasc. Biol.* **16:** 600–605.
60. Sato, Y., S. Kobori, M. Sakai, T. Yano, T. Higashi, T. Matsumura, W. Morikawa, T. Terano, A. Miyazaki, S. Horiuchi, and M. Shichiri. 1996. Lipoprotein[a] induces cell growth in rat peritoneal macrophages through inhibition of transforming growth factor-β activation. *Atherosclerosis.* **125:** 15–26.