Macrophage-mediated oxidation of extracellular low density lipoprotein requires an initial binding of the lipoprotein to its receptor

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Abstract Cells of the arterial wall including macrophages were shown to oxidize low density lipoprotein (LDL) in vitro. Upon incubation of LDL with J-774.A1 macrophage-like cell line for 18 h in the presence of 1 μM $CuSO_4$, extensive macrophage-mediated oxidation of the LDL fatty acids and cholesterol moieties was demonstrated. Similar results were found with mouse peritoneal macrophages or human monocyte-derived macrophages. Several lines of evidence suggest that LDL binding to the LDL receptor on macrophages is required for the cell-mediated oxidation of LDL. *1)* Incubation of the cells in the presence of monoclonal antibody to the LDL receptor (IgG-C7), substantially inhibited lipoprotein oxidation. *2)* Pretreatment of LDL with monoclonal antibodies to the LDL receptor binding domains on the LDL apoB-100 (mAbs B1B6 and B1B3) inhibited cell-mediated oxidation of LDL by 52–95%. *3)* Down-regulation of the macrophage LDL receptors (by preloading the cells with cholesterol) reduced LDL oxidation by 42%. *4)* Up-regulation of the LDL receptor (by macrophage incubation in serum-free medium) was associated with 80% elevation in LDL oxidation. *5)* Macrophage activation with lipopolysaccharide up-regulated the LDL receptors and was associated with up to twofold increased LDL oxidation. *6)* Human monocyte-derived macrophages from a patient with homozygous familial hypercholesterolemia, which lack the LDL receptor, failed to oxidize the LDL. *7)* On using acetylated LDL or methylated LDL, which do not bind to the LDL receptor, macrophage-mediated oxidation of the lipoprotein did not occur. The binding of LDL to the macrophage LDL receptor under oxidative stress induced the oxidation of extracellular unbound LDL as demonstrated by cell-mediated lipid peroxidation of mAb B1B6-treated LDL by cells that were preincubated with native LDL. Furthermore, macrophage conditioned medium (MCM) that was obtained after 5 h of cells preincubation with native LDL under oxidative stress (1 μM $CuSO_4$), followed by lipoprotein removal and a further 18 h of cell incubation (but not MCM that was similarly obtained without cell preincubation with LDL), was found to contain oxidized linoleic and arachidonic acids and was able to induce LDL lipids peroxidation. In conclusion, macrophage-mediated oxidation of LDL requires an initial binding of the lipoprotein to the LDL receptor on the cell surface under oxidative stress. This interaction leads to the formation and release of cellular oxidized polyunsaturated fatty acids that can oxidize the LDL molecule extracellularly.—**Aviram, M., and M. Rosenblat.** Macrophage-mediated oxidation of extracellular low density lipoprotein requires an initial binding of the lipoprotein to its receptor. *J. Lipid Res.* 1994. **35:** 385–398.

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Oxidative modification of LDL has been shown to be associated with increased atherogenicity (1–9) which includes cytotoxicity to arterial wall cells (10), stimulation of hemostatic and thrombotic processes (11–13), and secretion of cytokines and growth factors from cells of the arterial wall (14–16). An important feature of oxidized LDL is its enhanced uptake by macrophages, which results in cellular cholesterol accumulation and foam cell formation (1–9), the hallmark of the early atherosclerotic lesion. LDL oxidation can be achieved in vitro by lipoprotein incubation with cells of the arterial wall including endothelial cells, smooth muscle cells, and macrophages (17–19).

The mechanism(s) of cell-mediated oxidation of LDL by endothelial cells, smooth muscle cells, and macrophages is different and was shown to involve different oxygenases, oxygen reactive species, calcium ions, and nitric oxide (20–24). As monocyte-macrophages are involved in the formation of the early atherosclerotic lesion (25) and as these cells were shown to contain oxidized lipids in the lesion area (26), macrophage-mediated oxidation of LDL may occur in vivo. Reactive molecules such as free radicals and lipid peroxides, which can be generated by cells

Abbreviations: Ox-LDL, oxidized low density lipoprotein(s); MQ-LDL, macrophage oxidatively modified LDL; EC, endothelial cells; SMC, smooth muscle cells; CE, cholesteryl ester; UC, unesterified cholesterol; PL, phospholipids; TG, triglycerides; MDA, malondialdehyde; TBARS, thiobarbituric acid reacting substances; Met-LDL, methylated LDL; HPLC, high performance liquid chromatography; HMDM, human monocyte-derived macrophages; MCM, macrophage conditioned medium; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid.

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of the arterial wall, can potentially oxidize LDL, either after its binding to the cell surface or as a result of the action of cellular oxidants that are released to the extracellular environment (27). Thus, we questioned the requirement of specific LDL binding to macrophages for its subsequent oxidation.

The results of the present study demonstrate that macrophage-mediated oxidation of extracellular LDL requires an initial binding of the lipoprotein to the cellular LDL receptor under oxidative stress.

METHODS

Materials

Ficoll-Paque and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, fetal calf serum (FCS), penicillin, streptomycin, and glutamine were obtained from Gibco Laboratories. Heparin, fucoidin, chloroquine, polyinosinic acid, arachidonic and linoleic acids, and phospholipids were obtained from Sigma Co. Ltd. (St. Louis, MO). Cu/Zn-superoxide dismutase (SOD) was a generous gift from General Biotechnology (Rehuvot, Israel). Monoclonal antibodies (mAbs B1B6 and B1B3) towards the apolipoprotein B-100 epitopes, which are located at the LDL receptor binding domains (mapped to amino acid residues 3214–3506 and 3506–3635, respectively), were a generous gift from Drs. G. Schonfeld and E. Krul (Washington University, St. Louis, MO). The mouse C-7 hybridoma cells producing monoclonal antibody (of the type IgG 2b) were obtained from American Type Culture Collection (ATCC, Rockville, MD).

Cells

J-774 A.1 murine macrophage-like cell line was purchased from ATCC (Rockville, MD). J-774 A.1 macrophages were plated at 1×10^6 cells/35 mm dish in DMEM supplemented with 10% fetal calf serum (FCS). The cells were fed every 3 days and were used for experiments within 7 days of plating.

Human monocytes were isolated by density gradient centrifugation (28) from blood derived from fasting normolipidemic subjects. Twenty ml of blood (anticoagulated with 10 U/ml of heparin) was layered over 15 ml of Ficoll-Paque and centrifuged at 500 *g* for 30 min at 23°C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice in DMEM culture medium containing 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine. The cells were plated at 2×10^6 monocytes/35 mm dish (Primaria brand, Falcon Labware, Becton Dickinson, Oxnard, CA) in the same medium, and further incubated in the presence of 20% autologous serum. Human monocyte-derived macrophages (HMDM) were used within 7–10 days of plating. After 7 days in culture, the cell population consisted of over 98% pure macrophages as analyzed by α naphthyl acetate esterase staining in the absence or presence of sodium fluoride (which blocks staining of macrophages).

Mouse peritoneal macrophages (MPM) were harvested from the peritoneal fluid of female BALB/c mice (15–25 g) 4 days after intraperitoneal injection into each mouse of 3 ml of thioglycolate (24 g/l) in saline (29). The cells ($10–20 \times 10^6$/mouse) were washed and centrifuged three times with phosphate-buffered saline (PBS) at 1000 *g* for 10 min, then resuspended to 10^9/l in DMEM containing 15% horse serum (heat-inactivated at 56°C for 30 min), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cell suspension was dispensed into 35 mm plastic petri dishes and incubated in a humidified incubator (5% CO_2, 95% air) for 2 h. The dishes were washed once with 5 ml DMEM to remove nonadherent cells, and the monolayer was then incubated under similar conditions for 18 h, prior to the beginning of the experiment.

Lipoproteins

LDL was prepared from human plasma derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (30). The lipoprotein was washed at d 1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4). LDL was then sterilized by filtration (0.45 μm), kept at 3–6 mg of protein/ml under nitrogen in the dark at 4°C, and used within 2 weeks. Prior to oxidation, the LDL was dialyzed against 150 mM NaCl, EDTA-free solution pH 7.4. LDL was iodinated by the method of McFarlane as modified for lipoproteins (31). LDL (10 mg protein/ml) was methylated by adding sodium borohydride to the lipoprotein followed by the addition of 37% formaldehyde, and left to stand 30 min at 20°C. Methylated-LDL (Met-LDL) was then dialyzed against saline-EDTA prior to its use (32). LDL was acetylated by repeated additions of acetic anhydride to 4 mg/ml LDL diluted (1:1, v/v) with saturated sodium acetate at 4°C (33). Acetic anhydride was added at 40-fold molar excess with regard to total amino acid lysine residues in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital buffer (34). Lipoproteins were analyzed for their composition by determination of protein (35), unesterified and esterified cholesterol (36), triglyceride (37) and phospholipid (38) content. Free lysine amino groups in LDL were estimated with trinitrobenzenesulfonic acid (TNBS). LDL (50 μg of protein) was mixed with 1 ml of 4% $NaHCO_3$, pH 8.4, and 50 μl of 0.1% TNBS and heated for 1 h at 37°C, after which the absorbance at 340 nm was measured (39). For analysis of cholesterol oxides, 2 ml of LDL (0.2 mg protein/ml) was mixed with 2 ml 10% KOH

(in ethanol) and saponified for 1 h at 60°C. After cooling, 2 ml hexane was added, and the hexane phase was separated by centrifugation at 500 *g* for 10 min. The absorbance of the LDL lipid extract between 200–700 nm (versus hexane) was measured using a flow-through spectrophotometer (model 8452A Diode Array, Hewlett-Packard, Rockville, MD). LDL cholesterol oxides were measured in the hexane phase that was evaporated under nitrogen to complete dryness. The residue was redissolved in a minimal volume (200 μl) of hexane and injected into a high performance liquid chromatography (HPLC) analyzer. A stainless steel column of 25 × 4.6 mm (i.d.) packed with C18 reversed phase material was used. Elution was performed with methanol–acetonitrile 9:1 (v/v), at 1 ml/min.

A gradual increase of retention time after about 20 injections of samples was reversed by washing the column with methanol–acetonitrile–methylene chloride 8:1:1 (v/v/v). The content of the lipoprotein-associated 7-keto cholesterol was confirmed and determined using the appropriate standards. LDL carotenoid concentration and LDL vitamin E content were measured by HPLC (40). LDL fatty acid composition was analyzed, after methylation, by gas chromatography (41).

LDL oxidation by macrophages (MQ-LDL)

Cells (1 × 10^6/35 mm dish) were incubated in the incubator with LDL (0.2 mg protein/ml) in DMEM supplemented with 1 μM $CuSO_4$ for 18 h at 37°C. This incubation system was used because Ham's F-10 (which contains about 0.01 μM $CuSO_4$ and 2.5 μM $FeSO_4$) that is commonly used in LDL oxidation studies was found to be cytotoxic to human monocyte-derived macrophages (HMDM). LDL oxidation by HMDM in Ham's F-10 medium resulted in a 15–25% increase in cell-mediated LDL oxidation in comparison to LDL oxidation in DMEM+1 μM $CuSO_4$. On using Ham's F-10 medium, however, 20–33% of the cells died, whereas no cell cytotoxicity could be found on using DMEM+1 μM $CuSO_4$. Oxidation was terminated by the addition of 1 mM EDTA and refrigeration at 4°C. The extent of LDL oxidation was measured directly in the medium. In some experiments, lipid peroxidation was analyzed after re-separation of the lipoproteins by ultracentrifugation at d 1.210 g/ml, followed by a subsequent dialysis against 150 mM NaCl, 1 mM EDTA, pH 7.4.

LDL oxidation was assayed by measuring thiobarbituric acid-reactive substances (TBARS), which were quantified in terms of LDL malondialdehyde (MDA) equivalents (42), by the lipid peroxidation test that analyzes lipid peroxides by their capacity to convert iodide to iodine, which can then be measured photometrically at 365 nm (43); and also by determination of conjugated dienes in the LDL lipid extracts at 234 nm (44). Control LDL was always incubated under the same conditions in the absence of cells. Macrophage-mediated oxidation of LDL was calculated by subtraction of the oxidation rate in the absence of cells (control) from that obtained in the presence of macrophages.

LDL oxidation by copper ions

Copper ion-induced oxidized LDL (Cu-Ox-LDL) was produced by incubation of LDL (0.2 mg protein/ml) in EDTA-free PBS with 10 μM copper sulfate for 24 h at 37°C (45).

Immunoreactivity of MQ-LDL

Solid phase competitive binding radioimmunoassay of MQ-LDL, control LDL, Cu-Ox-LDL, and native LDL was performed in microtiter plates (46). The plates were coated with 150 μl of purified mAb B1B6 (10 μg/ml) overnight and then wells were blocked with 3% bovine serum albumin–phosphate-buffered saline (BSA–PBS). Serial dilutions of the lipoproteins in 1% BSA–PBS were added followed by the addition of a constant amount of ^{125}I-labeled LDL (500 ng). After incubation for 4 h at room temperature, the wells were washed 3 times with PBS and the binding (B) was determined. The maximal binding (Bo) was determined in wells where competing lipoprotein was not added. The results were expressed as B/Bo ratio.

Electrophoresis of MQ-LDL

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 3–10% gradient gel using mercaptoethanol as the reducing agent (47). Electrophoresis was performed at constant current (5 mA) for 16 h. The gels were stained with 0.1% Coomassie Brilliant Blue R and destained with 10% acetic acid. Nondenaturing polyacrylamide gradient gel electrophoresis (GGE) of the lipoprotein preparations was performed on 3–10% gels to compare their relative sizes (48).

Cellular binding and degradation of LDL by macrophages

Macrophage binding of ^{125}I-labeled LDL was determined at 4°C as previously described (49). Lipoprotein degradation by macrophages was analyzed as the TCA-soluble noniodide radioactivity after 5 h of cell incubation with ^{125}I-labeled lipoproteins (50).

Macrophage activation

The procoagulant activity (PCA) in activated macrophages was measured using the prothrombin time (PTT) assay. The supernatant of lipopolysaccharide (LPS)-treated cells or control cells (50 μl of 5 × 10^5 sonicated cells) was mixed with 50 μl standard human plasma. The mixture was incubated for 2 min at 37°C; 50 μl 25 mM $CaCl_2$ was then added and the time required for clot formation was determined (51). Superoxide release by the

cells was measured by analysis of the reduction in the presence of cytochrome C (18).

TLC separation of oxygenated lipids in macrophage conditioned medium (MCM)

MCM (1 ml) was acidified to pH 3.0 using 2 M citric acid and then the MCM lipids were extracted with chloroform–methanol 1:2 (v/v) followed by the addition of 0.6 ml chloroform. The chloroform phase was evaporated under N_2 and the residue was redissolved in a minimal volume of chloroform. TLC was performed on silica gel plates (F_{254}, 0.25 mm, Merck Co). The solvent system used for fatty acid analysis consisted of diethyl ether–hexane–acetic acid 60:40:1 (v/v/v). The solvent system for phospholipid analysis consisted of chloroform–methanol–ammonium hydroxide 60:35:8 (v/v/v). The plates were exposed to iodine vapor and the lipid spots were identified, scraped off, and extracted with hexane–isopropanol 3:2 (v/v) and analyzed for their content of conjugated dienes (44). Oxidized polyunsaturated fatty acids used as standards were purchased from Sigma Co. Ltd. Standards of oxidized fatty acids were also prepared by rotating linoleic acid, arachidonic acid, or the various phospholipids on the inner surface of a 20 ml round-bottomed flask at 37°C for 48 h.

Analysis of macrophage lipoxygenase activity

J-774 A.1 macrophages (10^6 cells/35 mm well) were incubated for 15 min at 37°C with 5 μM [^{14}C]arachidonic acid (55.9 Ci/mol, Amersham, U.K.) followed by the addition of 0.2 mg of LDL protein/ml and a further cell incubation for 1 h at 37°C, in the presence of 1 μM $CuSO_4$. The medium was acidified to pH 3.0 with 2 M citric acid and kept frozen at −20°C for up to 1 week. TLC analysis of the oxygenated labeled arachidonate was performed as described above. Standards of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) were identified by exposure of the plates to iodine vapor. The TLC spots related to the oxygenated fatty acid were scraped off and the radioactivity content of each spot was counted in a β-scintillation counter.

Statistical analysis

Statistical analysis was performed using nonpaired Student's *t*-test. Results are given as mean ± SD.

RESULTS

Physicochemical characterization of macrophage-mediated oxidized LDL (MQ-LDL)

Upon incubation of LDL (0.2 mg of protein/ml) with J-774 A.1 macrophage-like cell line (10^6 cells/35 mm dish) in DMEM supplemented with transition metal ions (Cu^{2+} or Fe^{2+}) for 18 h, LDL lipid peroxidation was substantially increased over the oxidation obtained with control LDL (incubated in the absence of cells). The medium TBARS contents, after cell incubation, in the presence of copper ions (1 μM $CuSO_4$) or ferrous ions (1 μM $FeSO_4$) were

TABLE 1. Low density lipoprotein modification by J-774 A.1 macrophages

Variable	Native LDL	Control LDL	MQ-LDL
Oxidation products (nmol/mg LDL protein)			
Peroxides	23 ± 5	29 ± 7	87 ± 10[a]
PUFA (mole%)			
Palmitic acid (16:0)	24 ± 3	25 ± 4	30 ± 4
Stearic acid (18:0)	7 ± 1	10 ± 2	15 ± 3
Oleic acid (18:1)	22 ± 3	25 ± 3	31 ± 4
Linoleic acid (18:2)	39 ± 5	34 ± 4	22 ± 3[a]
Arachidonic acid (20:4)	8 ± 1	6 ± 1	2 ± 1[a]
Antioxidants (μg/mg LDL protein)			
Vitamin E	3.1 ± 0.4	2.6 ± 0.2	1.8 ± 0.2
Carotenoids	0.20 ± 0.03	0.15 ± 0.03	0.13 ± 0.03
Oxycholesterol (μg/mg LDL protein)			
7-Keto-cholesterol	11 ± 2	23 ± 3	78 ± 9[a]
TNBS (% of native LDL)	100	89 ± 5	76 ± 4[a]
Lipid composition (mg/mg protein)			
Cholesteryl ester	1.08 ± 0.04	1.00 ± 0.03	0.84 ± 0.02[a]
Unesterified cholesterol	0.45 ± 0.02	0.43 ± 0.03	0.31 ± 0.03[a]
Triglyceride	0.11 ± 0.02	0.08 ± 0.02	0.06 ± 0.01
Phospholipid	0.77 ± 0.10	0.70 ± 0.10	0.60 ± 0.07
Lysophosphatidylcholine	0.08 ± 0.02	0.12 ± 0.03	0.24 ± 0.06[a]

Cells (10^6/35 mm dish) were incubated with LDL (0.2 mg protein/ml) in DMEM supplemented with 1 μM $CuSO_4$ for 18 h at 37°C. Lipoproteins were then reseparated from the medium by ultracentrifugation at d 1.210 g/ml. Control LDL represent LDL incubated under similar conditions without cells, and native LDL represent untreated lipoprotein. Results are given as means ± SD (n = 4). PUFA, polyunsaturated fatty acids; TNBS, trinitrobenzenesulfonic acid; MQ-LDL, macrophage oxidatively modified LDL.

[a] $P < 0.01$ versus control LDL.

23 ± 5 and 16 ± 4 nmol MDA equivalents/mg LDL protein, respectively, whereas control LDL that was incubated in the absence of cells (in the presence of 1 μM $CuSO_4$ or 1 μM $FeSO_4$) demonstrated only 4 ± 2 nmol MDA equivalents/mg LDL protein (n = 3). Incubation of the cells under similar conditions without LDL, in the absence or presence of 1 μM $CuSO_4$, resulted in the release of only 3 ± 2 or 5 ± 2 nmol MDA equivalents/mg cell protein, respectively. Similar results were obtained using Ham's F-10 medium which contains both copper and ferrous ions (data not shown).

In addition to the oxidation of the LDL fatty acids, the cholesterol moiety of LDL was also oxidized; HPLC analysis demonstrated the presence of 7-keto cholesterol (11–17% of total LDL cholesterol) in copper/macrophage oxidatively modified LDL (MQ-LDL). Substantial changes occurred in the oxidation state of LDL as well as in its chemical composition after 18 h of incubation with J-774 A.1 macrophages in the presence of copper ions (1 μM $CuSO_4$). LDL peroxide content was increased 3-fold in MQ-LDL in comparison to control LDL (**Table 1**). In MQ-LDL, in comparison to control LDL, the polyunsaturated fatty acids linoleate and arachidonate (C-18:2 and C-20:4, respectively) were reduced by 35% and 66%, respectively, and the lipoprotein antioxidants vitamin E and carotenoids were reduced by 24% and 13%, respectively (Table 1). 7-Keto cholesterol, the main cholesterol oxide that was produced during macrophage oxidation of LDL, was increased in MQ-LDL by 3.4-fold in comparison to control LDL. TNBS reactively, which reflects free lysine amino groups, was reduced by 15% in MQ-LDL in comparison to control LDL. LDL lipid composition was also changed with 16%, 25%, 25%, and 14% reduction in the lipid/protein ratio of cholesteryl ester (CE), unesterified cholesterol (UC), triglycerides (TG), and phospholipids (PL), respectively, and with an almost 3-fold elevation in LDL lysolecithin content (Table 1). The immunoreactivity of MQ-LDL against monoclonal antibody B1B6 (directed against the LDL receptor binding domains on LDL apoB-100) was substantially lower than that of the control LDL with ED-50 (lipoprotein concentration required to reduce the radioactivity in the immune complex by 50%) of 30 ± 5 μg of protein/ml for MQ-LDL in comparison to a value of 9 ± 2 μg of protein/ml that was obtained for control LDL (n = 3). SDS-PAGE analysis of the LDL apoB-100 molecule revealed in MQ-LDL a more substantial protein fragmentation than in the control LDL, but less fragmentation than that found in copper ion (10 μM)-induced Ox-LDL (data not shown). The size of MQ-LDL was found to be somewhat smaller than that of control LDL when analyzed by nondenaturing gradient polyacrylamide gel electrophoresis, as it migrated 10 ± 2 mm from the top of the gel in comparison to migration of 7 ± 1 mm for the control and the native LDL ($P < 0.01$, n = 3).

Lipoprotein electrophoresis on cellulose acetate revealed a significant change in the net charge of LDL with migrations of 0.8 ± 0.1 cm, 1.2 ± 0.2 cm, and 1.9 ± 0.2 cm from the origin for native LDL, control LDL, and MQ-LDL, respectively.

The ability of other types of macrophages to oxidize LDL was compared to that of J-774A.1 cells (**Table 2**).

Human monocyte-derived macrophages (HMDM) and mouse peritoneal macrophages (MPM) were also able to oxidize the LDL fatty acids as well as the cholesterol moieties (Table 2). When these results were expressed per mg cell protein, HMDM were found to be the most potent cells (Table 2).

The addition of 100 μg/ml of copper-superoxide dismutase (SOD) to the macrophages during cell-mediated oxidation reduced the content of LDL-associated MDA by 23 ± 9%, 65 ± 12%, and 61 ± 11% in J-774A.1 cells, HMDM, and MPM, respectively. Similar results were found with manganese-SOD as well as with catalase (data not shown).

The formation of MQ-LDL was a time-dependent process and could not be related to the proliferation capacity of the cells. Both J-774A.1 and MPM demonstrated a similar pattern of LDL oxidation with a major increment in the oxidation during the first 9 h of incubation (in the

TABLE 2. LDL oxidation by various types of macrophages

Cells	TBARS	Peroxides	7-Keto-cholesterol	Electrophoretic Mobility
	nmol/mg protein		*μg/mg*	*cm*
None (control)	4.2	26	31	1.0
J-774 A.1	25.7 (32)	95 (119)	133 (166)	2.1
HMDM	12.8 (67)	54 (284)	111 (584)	1.8
MPM	18.6 (20)	81 (88)	107 (116)	1.7

J-774 A.1 macrophages, human monocyte-derived macrophages (HMDM), and mouse peritoneal macrophages (MPM) were plated at densities of 1 × 10^6, 2 × 10^6, and 2 × 10^6 per 35 mm dish, respectively, and incubated with LDL (0.2 mg protein/ml) for 18 h at 37°C in DMEM supplemented with 1 μM $CuSO_4$. LDL lipid peroxidation and electrophoretic mobility were then analyzed. Results are the mean of duplicate dishes and are expressed per mg LDL protein and (in parentheses) per mg cell protein to allow for comparison between the cells.

presence of 1 μM $CuSO_4$), followed by a modest increment up to 24 h of incubation (**Fig. 1A**). This effect was shown in the proliferative J-774A.1 cells and in the quiescent MPM in spite of the proliferation capacity of the J-774A.1 cell line in comparison to the resting MPM (Fig. 1B). In a preliminary study, the proliferative cells (J-774A.1 macrophages) were found to oxidize LDL proportionally to the cell density at 0–1.5 × 10^6 cells/35 mm dish, whereas at cell density higher than 2.5 × 10^6/dish, cell-mediated oxidation of the lipoprotein substantially dropped (data not shown).

The relatively long time of LDL incubation with macrophages that was required for a substantial cell-mediated oxidation of LDL might be needed to deplete endogenous antioxidants in LDL in order to initiate peroxidation, or it may be related to the need for cellular protein synthesis. Indeed, the inhibitors of protein and RNA synthesis (cycloheximide and actinomycin D, respectively), substantially reduced macrophage-mediated oxidation of LDL, with no effect on LDL oxidation in a cell-free system (**Table 3, A and B**).

Requirement for lipoprotein binding to the LDL receptor for cell-mediated oxidation of LDL

The involvement of LDL binding to the macrophage LDL receptor, in cell-mediated oxidation of LDL, was analyzed in several ways. Upon incubation of ^{125}I-labeled LDL (10 μg protein/ml) with J-774A.1 macrophages in the presence of monoclonal antibody (mAb) to the cellular LDL receptor (mAb IgG C7, 10 μg/ml) or in the presence of mAbs directed against the LDL receptor binding domains on the LDL apoB-100 molecule [mAb B1B6 (10 μg/ml) and mAb B1B3 (10 μg/ml)], for 4 h at 4°C, cellular binding of LDL was reduced by 83%, 78%, and 73%, respectively (data not shown). Cellular cholesterol content in J-774A.1 macrophages that were incubated under similar conditions at 37°C with untreated LDL or with LDL that was preincubated with mAb IgG C7, mAb B1B6, or mAb B1B3 were: 73 ± 5, 40 ± 3, 44 ± 4, and 37 ± 3 μg, respectively, of cholesterol/mg cell protein (n = 3). Macrophage-mediated oxidation of LDL (0.2 mg of LDL protein/ml), was reduced by 92%, 95%, and 52% after cell incubation with LDL that was preincubated with 10 μg/ml of mAb IgGC-7, 100 μg/ml of mAb B1B6, or 100 μg/ml of mAb B1B3, respectively, in comparison to LDL that was incubated with cells in the absence of mAbs (Control, **Fig. 2**). J-774A.1 macrophage-mediated oxidation of LDL in Ham's F-10 medium was also reduced by preincubation of cells (1 h at 37°C, using 0.2 mg LDL protein/ml) with 10 μg/ml of mAb Ig G-C-7 (from 14 ± 3 to 3 ± 2 nmol MDA equivalents/mg LDL protein). An irrelevant IgG, mAb OKM1 (100 μg/ml), which recognizes a component of the cell surface complex (mediating neutrophil and monocyte adhesion), was used as a control for the mAb IgGC-7 (by its preincubation with the cells) as well as a control for mAb B1B6 and mAb B1B3 (by its preincubation with LDL). In both cases the mAb OKM1 had no effect on macrophage-mediated LDL oxidation (Fig. 2). Similar results were obtained when cell-mediated oxidation of LDL was performed in the presence of 1 μM

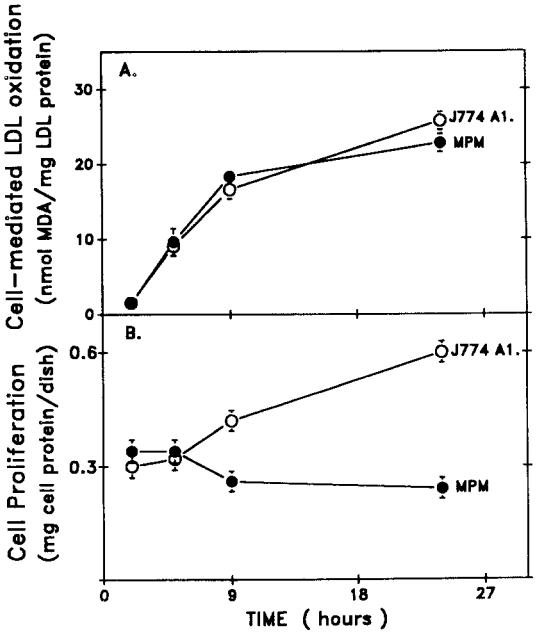

Fig. 1. Time course study of LDL oxidation by macrophages. LDL (0.2 mg protein/ml) was incubated with J-774 A.1 macrophages (○—○) or MPM (●—●) (10^6 cells/35 mm dish) in the presence of 1 μM $CuSO_4$ at 37°C for increasing periods of time. Cell-mediated lipoprotein oxidation was analyzed by determination of TBARS (A). Cell proliferation was assessed by determination of cellular protein content (B). Results are the mean ± SD of three different experiments.

TABLE 3. Effect of cycloheximide and actinomycin D on macrophage-mediated oxidation of LDL

Cells	LDL Oxidation
	nmol MDA/mg LDL protein
A. Macrophages	
Control	19 ± 3
+ Cycloheximide	8 ± 2[a]
+ Actinomycin D	10 ± 4[a]
B. No cells	
Control	8 ± 2
+ Cycloheximide	7 ± 3
+ Actinomycin D	9 ± 3

A: J-774 A.1 macrophages (10^6 cells/35 mm dish) were incubated for 12 h at 37°C with LDL (0.2 mg protein/ml) in the presence of 1 μM $CuSO_4$ with no additions (Control) or with the addition of 2.5 μM cycloheximide or 1.5 μM actinomycin D. Cell viability was 95%, 91%, and 92% for control, cycloheximide, or actinomycin D experiments, respectively. B: In the same experiment, a no-cell control was used under similar experimental conditions to rule out possible antioxidant properties of the drugs. Results are given as means ± SD (n = 3). MDA, malondialdehyde.

[a] $P < 0.01$ versus control.

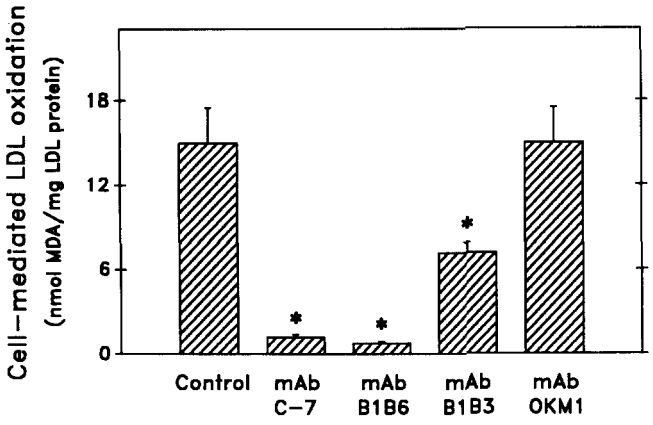

Fig. 2. Effect of monoclonal antibodies toward the LDL receptor on macrophage-mediated LDL oxidation. LDL (0.2 mg protein/ml) was incubated for 1 h at 37°C with no additions (Control), with mAb B1B3 (100 μg/ml), with mAb B1B6 (100 μg/nml), or with irrelevant mAb OKM1 (100 μg/ml). These LDL preparations were then incubated without or with J-774 A.1 macrophages, in the presence of 1 μM $CuSO_4$, for 18 h at 37°C. Similarly, J-774 A.1 macrophages were incubated for 1 h at 37°C with IgGC-7 (10 μg/ml), the monoclonal antibody to the cellular LDL receptor, or with mAb OKM1 (100 μg/ml) prior to the addition of native LDL and a further incubation for 18 h at 37°C. At the end of the incubation LDL oxidation was measured by the TBARS assay. Results are presented as mean ± SD (n = 3).

$FeSO_4$ instead of $CuSO_4$ (data not shown). All mAbs had no effect on LDL oxidation in a cell-free system (data not shown).

Down-regulation of the macrophage LDL receptors was achieved by cholesterol loading of the cells (macrophages were preincubated with acetylated LDL for 18 h at 37°C, followed by a cell wash). Macrophage-mediated oxidation of LDL (measured as LDL-associated conjugated dienes), was found to be inversely related to the cellular cholesterol content (**Fig. 3**). The cellular cholesterol content in control nonloaded cells and in cells that were preloaded with Ac-LDL (50 μg/ml and 100 μg/ml) were 57 ± 4, 89 ± 5, and 96 ± 5 μg of cholesterol/mg cell protein, respectively (Fig. 3, n = 3). On using 50 and 100 μg of Ac-LDL protein/ml, the binding of 10 μg/ml of ^{125}I-labeled LDL to the cells was reduced by 45% and 63%, respectively (data not shown), and macrophage-mediated oxidation of LDL was reduced under these conditions by 21% and 42%, respectively, in comparison to control cells that were not incubated with Ac-LDL (Fig. 3). Up-regulation of the LDL receptors by preincubation of the macrophages in serum-free medium resulted in increased LDL degradation by J-774A.1 macrophages that was paralleled by increased macrophage-mediated oxidation of the LDL. J-774 A.1 macrophages were incubated with 10 μg protein/ml of ^{125}I-labeled LDL in serum-free medium containing 0.2% bovine serum albumin (in the absence of fetal calf serum (FCS) in order to up-regulate the LDL receptor). This resulted in cellular LDL degradation of 938 ± 45 ng of LDL/mg cell protein in comparison to a degradation of 672 ± 37 ng LDL protein/mg cell protein that was obtained in the presence of 10% FCS ($P < 0.01$, n = 3). These changes were associated with elevation in cell-mediated oxidation of LDL (0.2 mg of protein/ml) from 16 ± 5 (in the presence of FCS) to 29 ± 5 nmol MDA/mg LDL protein under up-regulated conditions ($P < 0.01$, n = 3). Activation of macrophages with lipopolysaccharide (LPS) was shown to up-regulate the macrophage LDL receptors with no production of reactive oxygen species (51).

Macrophage activation was assessed by analysis of the cellular procoagulant activity (PCA). Increased PCA was found after 18 h of J-774A.1 macrophage incubation with 200 μg/ml of lipopolysaccharide (LPS) as evidenced by a reduction in the time required for clot formation from 168 ± 21 in nontreated cells to 42 ± 5 sec/mg cell protein in LPS-treated cells (n = 3). The effect of macrophage activation on the ability of J-774A.1 macrophages (**Fig. 4A**) and mouse peritoneal macrophages (Fig. 4B) to oxidize LDL was then studied. Cells that were preincubated with 200 μg/ml of LPS for 18 h at 37°C (followed by a cell wash), demonstrated increased capability to oxidize LDL as evidenced by 104% and 83% elevation in the TBARS content of LDL that was incubated with activated J-774 A.1 macrophages or with activated MPM, respectively, in comparison to the nonactivated cells (Fig. 4). Opsonized zymosan also resulted in a similar enhancement of LDL oxidation (data not shown). We next studied HMDM from a patient with homozygous familial hypercholesterolemia (HFH), in which there was a complete lack of LDL receptors on monocyte-macrophages (and on skin fibroblasts) in comparison to HMDM from control healthy subjects.

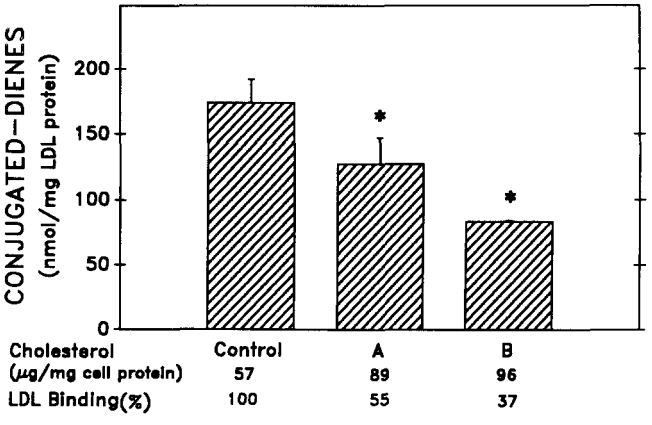

Fig. 3. Effect of macrophage cholesterol content on the ability of the cells to oxidize LDL. J-774 A.1 macrophages were incubated for 18 h with acetyl-LDL at a concentration of 50 μg protein/ml (A) or 100 μg protein/ml (B). The cells were then washed and incubated with LDL (0.2 mg protein/ml) in the presence of 1 μM $CuSO_4$ for 18 h at 37°C. Oxidation of LDL by cells that were not loaded with cholesterol serve as the control. At the end of the incubation, LDL oxidation was measured in the medium by analysis of its conjugated diene content. The cells were washed and extracted for analysis of the cellular cholesterol content. Results represent mean ± SD (n = 3).

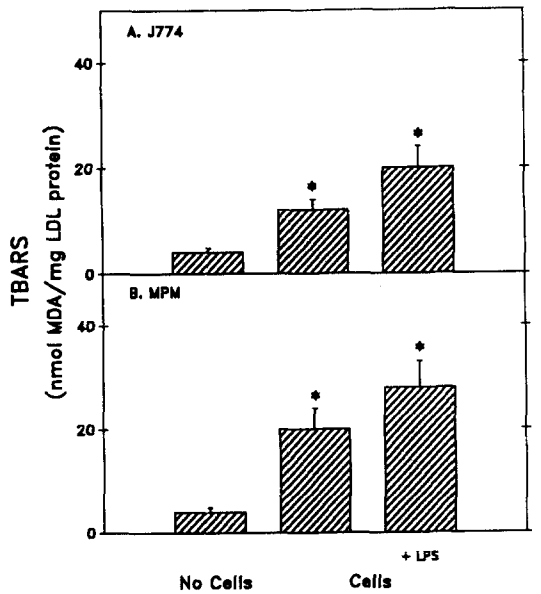

Fig. 4. Effect of macrophage stimulation with lipopolysaccharide (LPS) on their ability to oxidize LDL. J-774 A.1 macrophages (A) or mouse peritoneal macrophages (MPM, B) at a concentration of 10^6 cells/35 mm dish were incubated for 18 h at 37°C in the absence or presence of 200 μg/ml of LPS. The cells were then washed and further incubated for 18 h with LDL (0.2 mg protein/ml) in the presence of 1 μM $CuSO_4$, prior to the analysis of LDL-associated MDA. Results are mean ± SD of six determinations.

Upon incubation of control HMDM with 0.2 mg of LDL protein/ml for 18 h at 37°C, cellular cholesterol content was increased in control cells from 25 ± 5 to 77 ± 8 μg/mg cell protein, whereas in HMDM from the HFH patient cellular cholesterol content increased from 50 ± 9 only to 56 ± 3 μg/mg cell protein. Macrophage-mediated oxidation of LDL by control HMDM resulted in the formation of 132 ± 25 nmol of conjugated dienes/mg LDL protein whereas the oxidation of LDL by HFH-derived HMDM resulted in the formation of only 33 ± 6 nmol LDL conjugated dienes/mg LDL protein (means ± SD of three separate determinations).

Scavenger receptor and macrophage-mediated oxidation of LDL

The involvement of the macrophage scavenger receptor in cell-mediated oxidation of LDL was also studied by macrophage (J-774A.1) incubation with LDL in the presence of compounds (such as fucoidin or polyinosinic acid) that bind to the scavenger receptor. On using 100 μg/ml of polyinosinic acid or 50 μg/ml of fucoidin, the oxidation of LDL by the macrophages (10^6 cells/35 mm dish incubated for 18 h at 37°C in the presence of 1 μM $CuSO_4$) was increased by 32% and 37%, respectively, in comparison to the cell-mediated oxidation of LDL in the absence of these compounds (**Fig. 5**). Similar results were obtained with MPM (data not shown). As LPS contamination of poly I or fucoidin can stimulate macrophage-induced LDL oxidation, we have measured LPS concentration in poly I and in fucoidin with the Limulus Amebocyte Lysate (LAL) assay from Associates of Cape Cod Inc., Woods Hole, MA. No measurable LPS contamination could be found.

Upon incubation of mouse peritoneal macrophages (MPM) or J-774A.1 cell line with 0.2 mg of protein/ml of methylated-LDL (Met-LDL) or acetylated LDL (Ac-LDL), which cannot bind to the LDL receptor, macrophage-mediated oxidation of these lipoproteins was minimal (**Table 4**). In the absence of cells, Met-LDL and Ac-LDL, like native LDL (0.2 mg of protein/ml), were oxidized by copper ions though only to 66 ± 8% and 70 ± 7% of the oxidation obtained with the native LDL (**Fig. 6**). However, in the presence of J-774 A.1 macrophages, only native LDL but not Met-LDL or Ac-LDL were oxidized by the cells over the oxidation rate of the lipoprotein that was obtained in the cell-free systems (Fig. 6).

As the effect of Ac-LDL can be secondary to cellular cholesterol loading after the cellular uptake of Ac-LDL (see Fig. 3), we have studied the effect of fucoidin (50 μg/ml) which blocks the cellular uptake of Ac-LDL (via the scavenger receptor) on cell-mediated oxidation.

In a separate experiment, fucoidin was found to reduce cellular degradation of ^{125}I-labeled Ac-LDL by 93 ± 3% and 87 ± 5% in MPM and in J-774A.1 macrophages, respectively (n = 3, data not shown). After incubation of J-774 A.1 macrophages or MPM with Ac-LDL in the presence of 50 μg/ml of fucoidin (Table 4), macrophage-mediated oxidation of Ac-LDL was still completely blocked as evidenced by analysis of LDL-associated MDA and conjugated dienes (Table 4).

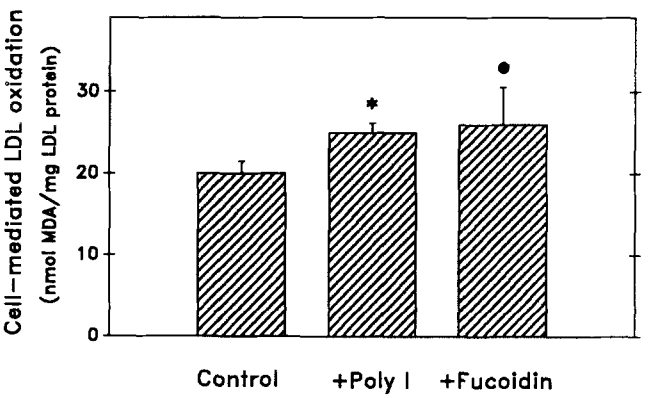

Fig. 5. Effect of polyinosinic acid (Poly I) and fucoidin on macrophage-mediated oxidation of LDL. J-774 A.1 macrophages were incubated with LDL (0.2 mg protein/ml) in the absence or presence of 100 μg/ml poly I or 50 μg/ml fucoidin. Lipid peroxidation was measured by analysis of lipoprotein-associated MDA. Results represent mean ± SD (n = 3).

TABLE 4. Macrophage oxidation of LDL, methylated LDL, and acetylated LDL

	Cell-Mediated Lipoprotein Oxidation			
	MPM	J-774 A.1	MPM	J-774 A.1
	nmol MDA/mg LDL protein		*nmol conjugated dienes/mg LDL protein*	
LDL	18 ± 3	19 ± 4	168 ± 11	189 ± 13
Met-LDL	6 ± 3	2 ± 1	37 ± 7	33 ± 9
Ac-LDL	3 ± 2	3 ± 1	29 ± 8	21 ± 5
Ac-LDL + fucoidin	4 ± 2	2 ± 1	20 ± 6	23 ± 7

Macrophages (2×10^6/35 mm dish) were incubated with 0.2 mg protein/ml LDL, methylated LDL (Met-LDL), or acetylated LDL (Ac-LDL) or Ac-LDL + 50 μg/ml fucoidin for 18 h at 37°C in DMEM containing 1 μM $CuSO_4$. Cell-mediated oxidation of the lipoprotein (LDL oxidation in the presence of cells − LDL oxidation in the absence of the cells) was then determined. Results are given as means ± SD (n = 3). In the absence of cells, the TBARS content ranged between 2 and 5 nmol of MDA equivalents/mg LDL protein and the conjugated diene content ranged between 9 and 17 nmol/mg LDL protein.

Oxidation of the unbound LDL

Although binding of the LDL to the macrophage LDL receptor was shown to be necessary for LDL oxidation, not only the bound LDL was oxidized but also the unbound LDL. Bound LDL (after 5 h of cell incubation with LDL) that was released (after cell wash) by treatment with 10 mg/ml of heparin (for 1 h at 4°C) was oxidized and contained 369 ± 55 nmol conjugated dienes/mg LDL protein. The oxidation rate of the unbound LDL in this study was only 146 ± 18 nmol of conjugated dienes/mg LDL protein. To analyze possible oxidation of the unbound LDL, MPM (2×10^6 cells/35 mm dish) were incubated with 0.2 mg of LDL protein/ml for 1 h at 37°C in the presence of 1 μM $CuSO_4$ (to induce oxidation signal), followed by cell wash and a further incubation of the cells for 18 h (in the presence of 1 μM $CuSO_4$) with 0.2 mg protein/ml of native LDL or of LDL that was pretreated with mAb B1B6 (to inhibit its ability to bind to the cells). Under these conditions, both LDL preparations (the native LDL and the mAb B1B6-treated LDL) were similarly oxidized (**Fig. 7B**). In a control experiment, mAb B1B6 (100 μg/ml) was shown to inhibit macrophage-mediated oxidation of LDL by 70% (Fig. 7A).

Upon incubation of LDL (0.2 mg of protein/ml), with macrophage conditioned medium (MCM, obtained after 18 h of cell incubation at 37°C, in DMEM supplemented with 1 μM $CuSO_4$), no increment in LDL oxidation could be shown in comparison to LDL that was incubated under similar conditions without MCM (Control LDL, **Fig. 8**). The medium was always centrifuged (10,000 *g* for 15 min) to assure a complete removal of any cells that might have detached during the incubation. The content of LDL-associated MDA, after 18 h of lipoprotein incubation with this MCM, was 7.1 ± 0.7 in comparison to a value of 6.4 ± 1.3 nmol MDA/mg LDL protein in the control LDL (Fig. 8). Similar results were obtained for LDL peroxides and conjugated diene content (Fig. 8, MCM vs. Control). Different results were found, however, when MCM was obtained from cells that were preincubated for 5 h with LDL (0.2 mg of protein/ml) in the presence of 1 μM $CuSO_4$, followed by cell wash and a further cell incubation for 18 h in the presence of 1 μM $CuSO_4$ (in the absence of LDL). Incubation of this MCM (MCM-LDL, Fig. 8) with LDL (0.2 mg protein/ml) resulted in its lipid peroxidation as determined by the significant ($P < 0.01$) elevation in the LDL content of MDA (24%, Fig. 8A), peroxides (51%, Fig. 8B), and conjugated dienes (36%, Fig. 8C) in comparison to control LDL. In the control LDL (no MCM) 24 ± 3 nmol peroxide was formed per mg LDL protein whereas in the LDL that was treated with MCM-LDL, 38 ± 2 nmol peroxide was produced per mg LDL protein. When LDL was incubated under similar conditions with MCM-HDL (prepared by 5 h of cells preincubation with HDL in the presence of 1 μM $CuSO_4$, followed by cell wash and a further cell incubation for 18 h in the presence of 1 μM $CuSO_4$) there was no enhancement of LDL oxidation with the forma-

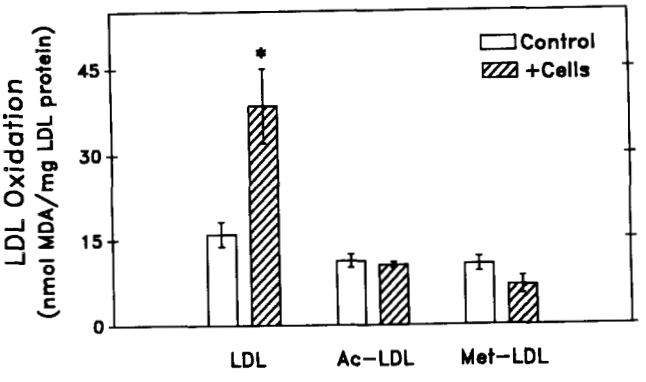

Fig. 6. Effect of macrophages on the oxidation of native LDL, methylated LDL, and acetylated LDL. Lipoproteins (0.2 mg protein/ml) were incubated without (control) or with (+cells) J-774 A.1 macrophages (10^6/35 mm dish), in the presence of 1 μM $CuSO_4$ for 18 h at 37°C prior to TBARS analysis of the lipoproteins. Results represent mean ± SD (n = 3); $^*P < 0.01$ (vs. no-cell Control).

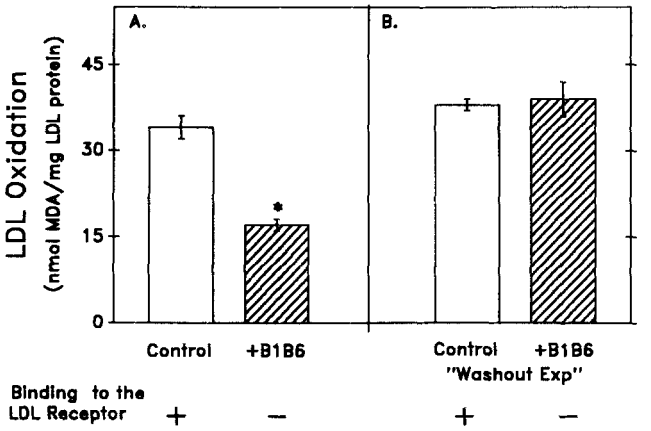

Fig. 7. Macrophage binding of LDL induces the oxidation of unbound LDL. A: Mouse peritoneal macrophages (2 × 10^6/35 mm dish) were incubated in the presence of 1 μM $CuSO_4$ for 18 h at 37°C with 0.2 mg protein/ml of native LDL (Control) or with LDL that was preincubated with 100 μg/ml of mAb B1B6 (for 1 h at 37°C). At the end of the incubation, LDL oxidation was measured by the TBARS assay. B: Mouse peritoneal macrophages (2 × 10^6/35 mm dish) were incubated in the presence of 1 μM $CuSO_4$ with LDL (0.2 mg protein/ml) for 1 h at 37°C. The cells were then washed and further incubated for 18 h at 37°C with native LDL (0.2 mg protein/ml) or with LDL that was preincubated with 100 μg/ml of mAb B1B6. At the end of the incubation, LDL oxidation was measured by the TBARS assay. Under these experimental conditions mAb B1B6 inhibited binding of 10 μg of ^{125}I-labeled LDL/ml to the macrophages by 78% (data not shown). Results represent mean ± SD (n = 3); * $P < 0.01$ (vs. Control).

tion of only 21 ± 4 nmol peroxides per mg LDL protein (n = 3).

Heat treatment (100°C, 15 min) of MCM-LDL did not affect the ability of MCM-LDL to oxidize native LDL (data not shown), suggesting that MCM-LDL does not contain cell-released oxygenases. Analysis of the MCM-LDL for a possible presence of cell-bound LDL that could have been released during the incubation periods was performed by using ^{125}I-labeled LDL. No radiolabeled LDL could be found in the medium, as analyzed by medium chromatography on Sephadex G-100 minicolumn (2 × 10 cm) that was followed by LDL radioactivity determination in the void volume.

Analysis of MCM-LDL, prior to its incubation with LDL, was performed by TLC separation of the oxidized fatty acids as well as of the oxidized phospholipids of the MCMs. Analysis of the TLC spots that migrated similarly to oxidized arachidonic acid (R_f = 0.81) revealed increased content of conjugated dienes in this oxidized PUFA that was obtained from MCM-LDL in comparison to MCM or control medium, respectively (63 ± 5 vs. 48 ± 5 or 42 ± 4 nmol conjugated dienes/ml, respectively). Similarly, the content of the oxidized linoleic acid in the TLC spots (R_f = 0.74) increased in MCM-LDL in comparison to MCM or control medium (37 ± 3 vs. 30 ± 3 or 25 ± 2 nmol conjugated dienes/ml respectively). HPLC analysis of the oxidized fatty acids revealed that the TLC spots that migrated like oxidized arachidonic and linoleic acids could be related to 15-hydroxyeicosatetraenoic acid (15-HETE) and to 13-hydroxyoctadecadienoic acid (13-HODE), respectively.

TLC analysis of the phospholipids in the MCMs revealed the presence of oxidized phosphatidyl serine (R_f = 0.73) in all three media (33 ± 3, 30 ± 2, and 25 ± 5 nmol conjugated dienes/ml, in MCM-LDL, MCM, and in control medium, respectively). The formation of the oxidized forms of arachidonic acid, 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) by J-774A.1 macrophages was analyzed by cell incubation with 0.1 μCi/ml of ^{14}C-radiolabeled arachidonic acid for 15 min followed by the addition of LDL (0.2 mg protein/ml) for 1 h at 37°C in the presence of 1 μM $CuSO_4$. The medium content of [^{14}C]-5-HETE and [^{14}C]-15-HETE was detected by TLC analysis. In the absence of

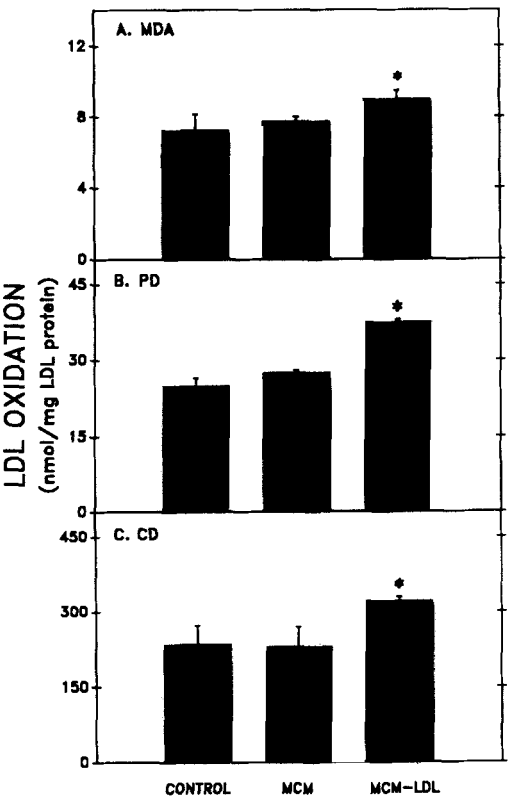

Fig. 8. Effect of macrophage conditioned medium (MCM) on LDL oxidation. J-774 A.1 macrophages (10^6/35 mm dish) were incubated with LDL (0.2 mg protein/ml) in the presence of 1 μM $CuSO_4$ for 5 h at 37°C, followed by a cell wash and a further cell incubation in LDL-free medium in the presence of 1 μM $CuSO_4$ for 18 h at 37°C. The medium (MCM-LDL) was then collected, centrifuged to remove any detached cells, and incubated with LDL (0.2 mg protein/ml) for 18 h at 37°C, at the end of which, LDL lipid peroxidation was analyzed. MCM represent LDL oxidation under similar conditions (as MCM-LDL) except that the first cell incubation with LDL was omitted. Control represent LDL incubation (without MCM) for 18 h with 1 μM $CuSO_4$. Results represent mean ± SD (n = 8); * $P < 0.01$ (vs. Control).

LDL, the TLC spots of 5-HETE and 15-HETE contained 677 ± 74 dpm/well and 374 ± 42 dpm/well, respectively. The addition of LDL to the incubation medium resulted in an increment in the production of these oxygenated arachidonic acid metabolites (5-HETE and 15-HETE) to 831 ± 78 dpm/well (23% increment) and to 721 ± 19 dpm/well (93% increment), respectively, suggesting that LDL probably activate the cell 15-lipoxygenase.

The source of the LDL-associated oxidized arachidonate was further studied. J-774A.1 macrophages were incubated with 0.1 μCi/ml of [^{14}C]arachidonate for 15 min, followed by cell wash and the addition of LDL (0.2 mg protein/ml) in the presence of 1 μM $CuSO_4$ for a further 18 h of incubation at 37°C. At the end of the incubation the medium was removed and the LDL was reseparated by ultracentrifugation (d 1.210 g/ml).

TLC analysis of the LDL lipid extract revealed the presence of 1855 ± 145 dpm of [^{14}C]-15-HETE/dish (n = 3), whereas the medium that was obtained from cells that were incubated under similar conditions, but without LDL, contained only 155 ± 85 dpm/dish of [^{14}C]-15-HETE. In conclusion then, our results suggest that cellular oxidized arachidonate is released from the cells in the presence of LDL, under oxidative stress, and bind to extracellular LDL which can then be further oxidized leading to the formation of extensively modified atherogenic LDL.

DISCUSSION

The present study demonstrates that the LDL receptor on the surface of macrophages plays an important role in macrophage-mediated oxidation of LDL. Under oxidative stress, LDL binding to the macrophage LDL receptor was required in order to initiate cell-mediated oxidation of the lipoprotein.

Most types of mammalian cells including macrophages possess the LDL receptor on their surface membrane (52). The role of the LDL receptor is to regulate cellular cholesterol content, thereby preventing cells from cholesterol accumulation. In addition to the LDL receptor, macrophages possess the scavenger receptor that recognizes certain modified forms of LDL including Ox-LDL (1, 2). The scavenger receptor is not regulated by the macrophage cholesterol content and thus cellular uptake of Ox-LDL can lead to foam cell formation (1–9).

Although the unbound LDL was oxidized by macrophages, the initial binding of LDL to the macrophage LDL receptor was obligatory, under oxidative stress, to obtain cell-mediated oxidation of the lipoprotein. LDL binding to the cells may be necessary to activate cell membrane-associated oxidative enzyme(s) or other oxidant(s) that can then act upon the lipid constituents of LDL (20, 21, 53–55). Alternatively, the synthesis of cellular oxidative enzymes, secondary to the macrophage binding of LDL (under oxidative stress), can oxidize polyunsaturated fatty acids (PUFA) on the cell membrane phospholipids, which can then be released from the cell surface (by the action of phospholipase A_2) and be transferred from the cells to the extracellular LDL, producing oxidized lipoprotein (20, 21). The present demonstration of macrophage-released oxidized PUFA (oxidized arachidonate and linoleate) under experimental conditions that required the initial binding of LDL to the macrophage LDL receptor suggest that this latter mechanism is responsible for macrophage-mediated oxidation of LDL. Similarly, activation of 15-lipoxygenase by LDL in vascular endothelial cells was demonstrated and a linkage between cellular binding of LDL and signal transduction pathway to activate lipoxygenases was suggested (56).

The involvement of macrophage-derived free radicals in cell-mediated oxidation of LDL may also require an initial LDL binding to the cells. J-774A.1 macrophages do not secrete measurable amounts of superoxides (57) and their LDL oxidation capability was not substantially affected by superoxide dismutase (SOD). In contrast, MPM and HMDM were shown to secrete superoxides (58), and SOD inhibited cell-mediated oxidation by these cells. These cells, however, like the J-774 A.1 cell line, also required the binding of LDL to the LDL receptor in order to oxidize the lipoprotein, suggesting that superoxides can be involved in macrophage-mediated oxidation of LDL following LDL binding to its receptor.

A crucial role for LDL binding to the macrophage LDL receptor for its subsequent oxidation by these cells is supported by the following data. *1)* The blocking of LDL binding to the cells by using monoclonal antibodies to the LDL receptor or monoclonal antibodies to the receptor binding domains on the LDL apoB-100 molecule prevented cell-mediated oxidation of LDL. *2)* Down-regulation or up-regulation of the LDL receptor on macrophages inhibited or increased cell-mediated oxidation of LDL, respectively. *3)* Ac-LDL and Met-LDL, which do not bind to the LDL receptor, could not be oxidized by the macrophages. *4)* HMDM derived from an HFH patient (that lacks the LDL receptor) failed to oxidize LDL. This latter finding, however, did not rule out the presence of oxidized LDL in the arterial wall of HFH patients, since LDL oxidation by other cells of the arterial wall such as EC and SMC (by different mechanisms) can produce oxidized LDL. The inhibitory effect of increased macrophage cholesterol content on cell-mediated oxidation of LDL may be related to the down-regulation of the LDL receptors. However, an effect of the macrophage cholesterol per se, on cellular events such as activation of the antioxidative enzymes, can also be operative. Macrophage activation (which occurs during atherogenesis) was shown to increase cellular uptake of LDL (51) (resulting

in cellular cholesterol accumulation) as well as to stimulate cell-mediated oxidation of LDL. The stimulation of cell-mediated oxidation of LDL following macrophage activation can be the result of up-regulation of the LDL receptors (51) or it might be secondary to the activation of cellular oxidative systems. Macrophage-oxidized LDL resemble LDL that was oxidized in a cell-free system in the presence of copper ions (19, 53). In the present study a detailed analysis, not previously published, of the physicochemical characteristics of MQ-LDL was performed including the lipoprotein oxidants and anti-oxidants content, its immunoreactivity, and its electrophoretic properties.

It was demonstrated that macrophage-mediated oxidation of LDL required several hours and that inhibition of macrophage protein synthesis resulted in the inhibition of cell-mediated oxidation of LDL. It should be emphasized, however, that the long time required for macrophage-mediated oxidation of LDL made it necessary to include the protein synthesis inhibitors for the whole incubation period and thus cellular proteins with short half-lives could have been depleted. As cell-released oxidized fatty acid was shown to induce LDL oxidation, the time required for the formation and release of these cellular oxidized lipids may also be responsible for the several hours that were required for cell-mediated LDL oxidation. The time required for macrophage-mediated oxidation of LDL may be related to cellular synthesis of oxidative enzymes such as lipoxygenases (54–56), NADPH oxidases (58), or phospholipases (59), which may be involved in cell-mediated LDL oxidation. Although 15-lipoxygenase (15-LPO) was shown to be involved in LDL oxidation by macrophages or endothelial cells (54–56), a recent study suggests that LDL oxidation by either macrophages or endothelial cells may not require lipoxygenases (60).

A mechanism similar to macrophage-mediated oxidation of LDL may not be operative for other cells of the arterial wall such as endothelial cells and smooth muscle cells (17, 18). In EC-modified LDL, the main cholesterol oxide derivative was shown to be epoxide (61), whereas in macrophage-mediated oxidation of LDL, 7-keto cholesterol was found to be the main cholesterol oxide (62). In the early atherosclerotic plaque, the presence of both 7-keto cholesterol and epoxides was shown (63) and it is possible that different mechanisms are involved in LDL oxidation by EC, SMC, and macrophages. Different mechanisms may operate in vivo at different stages of LDL interaction with cells of the arterial wall during atherogenesis. In activated monocytes/macrophages the respiratory burst leads to NADPH oxidase-mediated superoxide production (58), whereas in EC and SMC superoxides are produced continuously (63–66). SMC-mediated oxidation of LDL involves superoxide production which is followed by the secretion of reduced thiol compounds (65). The initiation of LDL oxidation by endothelial cells, however, was shown to be superoxide-independent (67).

Whereas blockage of the LDL receptor inhibited macrophage-mediated oxidation of LDL, the blockage of the macrophage scavenger receptors (with fucoidin or polyinosinic acid) demonstrated significantly increased macrophage-mediated oxidation of LDL. This phenomenon was the result of the inability of the newly produced Ox-LDL to be removed from the medium via the macrophage scavenger receptor. At the same time, this blockage of the scavenger receptor also prevented cellular cholesterol accumulation, which was shown in the present study to inhibit macrophage-mediated oxidation of LDL.

LDL modifications by cells of the arterial wall involve not only lipid peroxidation but also LDL aggregation (68, 69) and enzymatic modifications of its various lipid moieties (54, 55, 70–78). Macrophage foam cell formation is the hallmark of the early atherosclerotic process and these cells accumulate in addition to cholesteryl ester and unesterified cholesterol and lipid peroxides in areas of the atherosclerotic lesion (1–9, 27, 55, 63). LDL oxidation by macrophages can trigger some early events in atherosclerosis such as the recruitment of monocytes to the injured arterial wall, the conversion of monocytes into macrophages, and the formation of foam cells. Oxidation of LDL at later stages by other cells, such as arterial wall smooth muscle cells, to a more extensively oxidized form of LDL act via different mechanisms and can have a more deleterious effect on the progression of atherosclerosis.

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