Defective catabolism of oxidized LDL by J774 murine macrophages

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Abstract In J774 murine macrophages, chemically oxidized LDL (OxLDL) and biologically oxidized LDL (BioOxLDL) have similar metabolic fates, characterized by a relatively poor degradation when compared with acetylated LDL (AcLDL), and a modest ability to activate acyl-CoA:cholesterol acyltransferase (ACAT) (850 and 754 pmol [^{14}C]oleate/mg cell protein in OxLDL- and BioOxLDL-incubated cells, versus 425 and 7070 pmol [^{14}C]cholesteryl oleate/mg cell protein in control and AcLDL-incubated cells) with a massive increase of cellular free cholesterol. Therefore, OxLDL were used to investigate the cellular processing of oxidatively modified LDL. Binding and fluorescence microscopy studies demonstrated that OxLDL are effectively bound and internalized by macrophages and accumulate in organelles with density properties similar to those of endo/lysosomes. Although the overall metabolism of OxLDL is modestly affected by 100 μM chloroquine, owing to the poor cellular degradation of the substrate, the drug can further depress OxLDL degradation, indicating that this process takes place in an acidic compartment. Failure to detect products of extensive degradation of OxLDL in the medium is due to their relative resistance to enzymatic hydrolysis, as demonstrated also by in vitro experiments with partially purified lysosomal enzymes, rather than to the intracellular accumulation of degradation products (degraded intracellular protein is, at most, 8.5% of total). This sluggish degradation process is not due to a cytotoxic effect since OxLDL do not affect the intracellular processing of other ligands like AcLDL or IgG. The accumulation of OxLDL-derived products within macrophages may elicit cellular responses, the relevance of which in the atherosclerotic process remains to be addressed.—**Roma, P., F. Bernini, R. Fogliatto, S. M. Bertulli, S. Negri, R. Fumagalli, and A. L. Catapano.** Defective catabolism of oxidized LDL by J774 murine macrophages. *J. Lipid Res.* 1992. **33:** 819–829.

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The fatty streak, the earliest atherosclerotic lesion, consists mostly of lipid-laden macrophages with some underlying smooth muscle cells (1). Therefore, studies on its genesis have focused on the mechanisms through which circulating monocytes are recruited from the blood stream and subsequently engorge lipoproteins from plasma.

A number of in vitro (2–4) and ex vivo (5–9) data support the hypothesis that a modified form of LDL, namely oxidized LDL (OxLDL), exists in vivo and, as the physiological ligand for the scavenger receptor (10, 11), may lead to the massive deposition of intracellular esterified cholesterol. However, recent findings indicate that OxLDL, as opposed to acetylated LDL (AcLDL), follow a cellular pathway that leads to a relatively minor hydrolysis (12, 13) of their protein moiety and poorly stimulate ACAT activity (13–15). Until now, few data are available to explain these observations. The purpose of the present work was to verify to what extent OxLDL are internalized and to localize them intracellularly. The possibility for an intracellular accumulation of degradation products of OxLDL and for the interference, by OxLDL, in the intracellular catabolism of other ligands was also considered.

METHODS

Cells

J774 murine macrophages, an established cell line (16), were from the Salk Institute Cell Repository. EAhy-926, a permanent human hybrid cell line that expresses factor VIII-related antigen (17), was kindly provided by Dr. C. J. S. Edgell and used as a model for human endothelial cells. Cells were maintained in standard conditions in MEM (Gibco, Madison, WI) with the addition of 10% FCS.

Abbreviations: LDL, low density lipoprotein; OxLDL, oxidatively modified low density lipoprotein; BioOxLDL, low density lipoprotein oxidatively modified by incubation with endothelial cells; LinOxLDL, low density lipoprotein oxidatively modified by the products of linolenic acid oxidation; AcLDL, acetylated low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; FAF-BSA, fatty acid-free bovine serum albumin; FCS, fetal calf serum; HAT, hypoxanthine-aminopterine-thymidine; LDH, lactate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; TBARS, thiobarbituric acid-reacting substances; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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HAT 1% was added to the culture medium of EAhy-926 cells to prevent the overgrowth of epithelial cells.

Cell viability

J774 were incubated in MEM + 0.2% FAF-BSA (medium A) alone or with OxLDL or AcLDL (50 μg lipoprotein cholesterol/ml). At different times, washed cells were gently scraped with a Teflon policeman, pelleted by centrifugation (80 *g*), and resuspended in Trypan Blue diluted 1:10 with saline. Dye-permeable versus -impermeable cells were counted under the microscope in a Neubauer chamber; a minimum of 100 cells per experiment were counted.

The release of LDH into the culture medium was quantitated by spectrophotometric measurement of NADH consumption over time, using a commercially available kit (Merck LDH, Merck, Darmstadt, Germany). The enzyme activity was expressed as U/l.

Lipoproteins

LDL (d 1.019–1.063 g/ml) were isolated from freshly isolated human plasma containing 0.01% EDTA (w/v) and 0.01% NaN_3 (w/v) by sequential ultracentrifugation (18) at 4°C and 40,000 rpm in a 60 Ti rotor, using a L5-50 ultracentrifuge (Beckman, Palo Alto, CA).

For acetylation, LDL were extensively dialyzed against 0.15 M NaCl, pH 7.4, diluted with an equal volume of saturated Na acetate and treated with acetic anhydride, according to Basu et al. (19).

For oxidation, LDL were desalted by gel filtration on Sephadex G-25 columns (PD-10, Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with PBS, pH 7.4. Chemical oxidation was performed under sterile conditions, by incubating LDL at 37°C for 24 h, at 0.2 mg protein/ml in PBS + 20 μM $CuSO_4$. Oxidation was blocked in ice, with the addition of BHT (final concentration 40 μM) dissolved in ethanol to the incubation mixture.

Biological oxidation was obtained, under sterile conditions, by incubating LDL in the presence of EAhy-926 cells at 37°C for 24 h, at a concentration of 0.07–0.1 mg protein/ml, in serum-free medium containing 12 μM $CuSO_4$ (20); since HAT acted as an antioxidant, lower concentrations of $CuSO_4$ could not be used. The ratio of lipoprotein protein/cell protein mass was 2. Oxidation was blocked as above. LDL incubated in the same conditions, but in the absence of cells, were used as control lipoproteins for BioOxLDL.

Modification of LDL with the products of fatty acid oxidation (LinOxLDL) was performed according to Steinbrecher et al. (21). Briefly, linolenic acid (85 μl, equivalent to 85 μg) was incubated at 100°C for 1 h and subsequently extracted with 100 volumes of $CHCl_3$–PBS 1:1 (v/v). The aqueous phase was collected and incubated at room temperature for 18–20 h with 1.0 mg LDL at a final concentration of 0.2 mg/ml. Antiproteolytic agents (PMSF 1 mM, aprotinin 0.5 μg/ml, and phenantrolin 1 mM) were added to the incubation.

Oxidized LDL and LinOxLDL were concentrated by ultrafiltration under N_2 pressure on Diaflo Ultrafiltration Membranes YM 100 (American Corporation, Lexington, MA), desalted on Sephadex G-25 columns eluted with PBS, and sterile-filtered. Modification of lipoproteins was tested by nondenaturing gel electrophoresis in 0.8% agarose (Agarose A, Pharmacia Fine Chemicals) in 0.1 M Tris, pH 8.6, at 200 V (22). Gels were fixed in 70% ethanol, dried, and stained with Sudan Black in 70% ethanol. Quantitation of TBARS (23) and evaluation of apoB fragmentation by SDS-PAGE (24) were initially used to assess the oxidation of lipoproteins. For our purpose, however, these methods were not more informative than agarose electrophoresis, therefore the latter was used.

Iodination

Lipoproteins were labeled with ^{125}I according to Bilheimer, Eisenberg, and Levy (25), desalted against PBS by gel filtration over Sephadex G-25 and sterile-filtered. For unknown reasons, TCA-nonprecipitable radioactivity, usually about 2% of total for lipoproteins, was consistently higher (~10%) in the case of OxLDL, and was reduced (~3%) by extensive dialysis. Specific activity was 200–300 cpm/ng lipoprotein protein. Goat anti-mouse IgG (100 μg) was labeled with ^{125}I in the presence of Iodogen (100 μg) (Pierce, Oud-Beijerland, The Netherlands), according to a published procedure (26). TCA-nonprecipitable radioactivity was 2% of total and specific activity was 4,850 cpm/ng.

Cholesterol loading of cells and cholesterol determination

J774 cells were incubated at 37°C in medium A alone or containing modified lipoproteins at increasing concentrations for 18 h. At the end of the incubation, lipids were extracted from washed cells by a 30-min incubation at room temperature in hexane–isopropyl alcohol 3:2 (v/v), followed by a brief wash in the same mixture. Cellular proteins were dissolved in 1 N NaOH and quantitated according to Lowry et al. (27), using BSA as a standard. The combined lipid extracts were dried under a N_2 stream and redissolved in 200 μl of hexane; 70 μl of the extract was used for the determination of total cholesterol by an enzymatic colorimetric assay (28). Thin-layer chromatography (TLC) (HPTLC silica gel plates, Merck, Darmstadt, Germany) was performed on the remaining portion of the extract in petroleum ether–diethyl ether–acetic acid 70:30:1 (v/v/v). The percentages of free and esterified cholesterol were determined by enzymatic colorimetric assay on extracts of the corresponding areas scraped from the plates.

ACAT assay

ACAT activity was determined as the incorporation of [^{14}C]oleate into cholesteryl esters. Cell monolayers were washed overnight in medium A, then incubated for 2, 6, or 9 h in medium A alone or containing OxLDL, BioOxLDL (40 μg cholesterol/ml), or AcLDL (30 μg cholesterol/ml). Cells were then incubated with [^{14}C]oleate-albumin complex (0.1 mM) for 2 h. Lipids were extracted from washed cells and the radioactivity of esterified cholesterol, separated by TLC, was determined by liquid scintillation (29).

Uptake and degradation of lipoproteins

J774 cells were incubated for 18 h in medium A with or without 100 μM chloroquine, washed, and incubated for 5 h at 37°C in medium A containing ^{125}I-labeled OxLDL or ^{125}I-labeled AcLDL (10 μg/ml). Total uptake and degradation were determined as described (30).

J774 macrophages were incubated at 37°C for 4 h in medium A containing one of the following lipoproteins: ^{125}I-labeled AcLDL, ^{125}I-labeled OxLDL, ^{125}I-labeled LDL, incubated in MEM + 12 μM $CuSO_4$ in the absence of cells, and ^{125}I-labeled BioOxLDL (7 and 14 μg/ml). Nonspecific uptake and degradation were evaluated in the presence of a 30-fold excess of the unlabeled ligand.

Intracellular degraded ligands were quantitated according to Brown, Dana, and Goldstein (30). At the end of the incubation, washed cells were gently scraped and centrifuged at 3000 *g* for 20 min at 4°C. Cell pellets were incubated overnight at 4°C in 100 μl Kyro EOB, after which 900 μl of water was added. Protein was quantitated on 50-μl aliquots. To 400 μl of the remaining suspension, 400 μl of MEM containing 10% FCS and 200 μl 50% TCA were added; after 5 min the samples were centrifuged at 3000 *g* for 10 min and pellets and supernatants were counted.

To evaluate the effect of OxLDL on the metabolism of AcLDL, J774 cells were incubated with or without OxLDL (25 μg/ml) in medium A for 5 h, washed, and incubated for 3 h in medium A containing ^{125}I-labeled AcLDL (5 and 10 μg/ml). Uptake and degradation were quantitated as above. J774 cells were also incubated with OxLDL (25 μg/ml) and ^{125}I-labeled anti-mouse IgG previously incubated for 30 min at room temperature with or without equimolar anti-human apoA-I monoclonal antibody. The purpose of this experiment was to establish whether OxLDL affected the uptake and degradation of molecules bound by receptors other than the scavenger receptor, such as IgG and IgG/anti-IgG complexes, recognized by macrophages through the Fc receptor. This approach required the use of a first antibody irrelevant to apoB, i.e., anti-apoA-I, which should not interfere with the binding of OxLDL. After a 4-h incubation at 37°C, uptake and degradation of ^{125}I-labeled IgG were quantitated as above.

Binding and internalization of lipoproteins

In this experiment, two sets of conditions were used: *a)* cells were incubated at 4°C for 2 h in HCO_3^--free medium A, 10 mM HEPES, containing ^{125}I-labeled OxLDL (10 μg/ml); or *b)* cells were incubated at 37°C for 5 h in medium A, containing the radioactive lipoproteins as above. Cells from *a)* and *b)* were washed and incubated in medium A at 37°C for 10 min with trypsin (0.05%) or pronase (0.25%), or at 4°C for 1 h in the presence of an excess of unlabeled ligand (1 mg/ml). Radioactivity released into the medium by these treatments was taken as the binding component of total uptake, whereas the remaining cell-associated radioactivity was taken as internalization.

Filipin stain

J774 cells were cultured on glass coverslips in 35-mm Petri dishes and incubated at 37°C for 5 or 18 h in medium A alone or with the addition of AcLDL or OxLDL (50 μg cholesterol/ml). At the end of the incubation the cells were washed and sequentially incubated in 4% paraformaldehyde at room temperature for 15 min, in 50 mM NH_4Cl at 4°C for 30 min, and in PBS containing 0.005% filipin (Filipin III, Sigma Chemical Company, St. Louis, MO) at room temperature for 30 min (31). Washed coverslips, mounted on microscopy slides, were viewed with a fluorescence microscope (Zeiss Octivert) at 630 × magnification and photographed using ASA 400 Ilford HP5 film.

Cell subfractionation

J774 cells were grown in 100-mm Petri dishes and incubated at 37°C for 18 h in medium A alone or containing OxLDL or AcLDL (50 μg cholesterol/ml). At the end of the incubation the dishes were put on ice and the cells were washed twice, for 1 min and 5 min, in 150 mM NaCl, 50 mM Tris, 2 mg/ml BSA, pH 7.4 (buffer B), and twice in 250 mM sucrose, 1 mM EDTA (solution C). Scraped cells were pelleted by centrifugation at 2000 *g* for 5 min, resuspended in solution C, homogenized in a Teflon/glass Potter homogenizer (10 strokes) and spun at 2000 *g* for 5 min. The pellet was resuspended, homogenized, and spun as above. Pooled supernatants (1 ml) were loaded on top of 11 ml of 7.8% Percoll (Pharmacia Fine Chemicals) in solution C and spun at 20000 *g* for 45 min at 4°C in a SW 41 rotor in an L5-50 ultracentrifuge. Protein content and the activity of two marker enzymes (N-acetyl β-glucosaminidase and 5'-nucleotidase) (32) were determined on eleven fractions (~1 ml) collected by aspiration from the top of the gradient. Cholesterol was quantitated on lipid extracts of the same fractions.

Degradation of lipoproteins by partially purified lysosomal enzymes

J774 cells were homogenized in 250 mM sucrose, 2 mM EDTA, 10 mM HEPES, pH 7.4, as described in Cell subfractionation. The lysate was centrifuged at 750 *g* for 10 min at 4°C and the supernatant was centrifuged at 10,000 *g* for 20 min at 4°C. The pellet was resuspended in water and freeze-thawed six times to disrupt the lysosomes (33); 25 μg of protein was then mixed with increasing amounts of ^{125}I-labeled LDL, ^{125}I-labeled AcLDL, or ^{125}I-labeled OxLDL (0.20 to 1.95 μg/ml) in a final volume of 0.1 ml of either citrate-buffered MEM, pH 4.5, or Tris-buffered MEM, pH 9, and incubated for 4 h at 37°C. The degradation of the radioactive substrate was measured as TCA-nonprecipitable, noniodide radioactivity, as above. The formation of lipoprotein aggregates during the incubation was tested in a blank incubation without membranes. Aggregated lipoproteins were pelleted by a 5-min centrifugation at 12,000 *g* and the radioactivity in pellets and supernatants was determined.

RESULTS

The observation that OxLDL induce the accumulation of free cholesterol in J774 cells prompted us to verify the validity of Cu^{2+}-oxidized LDL as a model for biologically oxidized LDL. This required the comparison of the metabolic behavior of OxLDL and BioOxLDL. Their electrophoretic mobilities were, respectively, 1.8 times and 1.5 times that of native LDL. **Fig. 1** illustrates the effect of increasing concentrations of modified lipoproteins on the cholesterol content of J774 macrophages. Both OxLDL and, although less efficiently, BioOxLDL caused a concentration-dependent accumulation of free cholesterol in J774 macrophages. Even at the highest loading, esterified cholesterol did not exceed 21% of total cholesterol (Fig. 1). Consistent with this observation, both lipoproteins activated ACAT very poorly (880 and 754 pmol [^{14}C]cholesteryl oleate/mg cell protein in OxLDL- and BioOxLDL-incubated cells, respectively, versus 425 and 7070 pmol [^{14}C]cholesteryl oleate/mg cell protein in control and AcLDL-incubated cells, respectively) (**Fig. 2**).

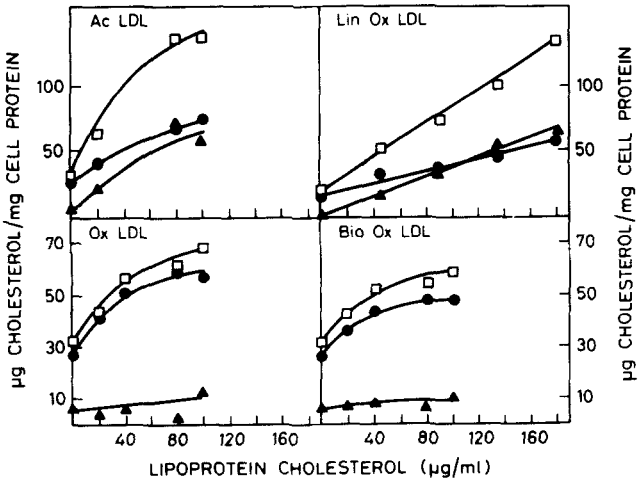

Fig. 1. Effect of modified LDL on the cholesterol content of J774 macrophages. Cells were washed and incubated at 37°C for 18 h in medium A alone or containing AcLDL, LinOxLDL, OxLDL, or BioOxLDL at the indicated concentrations of lipoprotein cholesterol. At the end of the incubation, lipids were extracted from washed cells and total (□), free (●), and esterified (▲) cholesterol were quantitated as described in Methods.

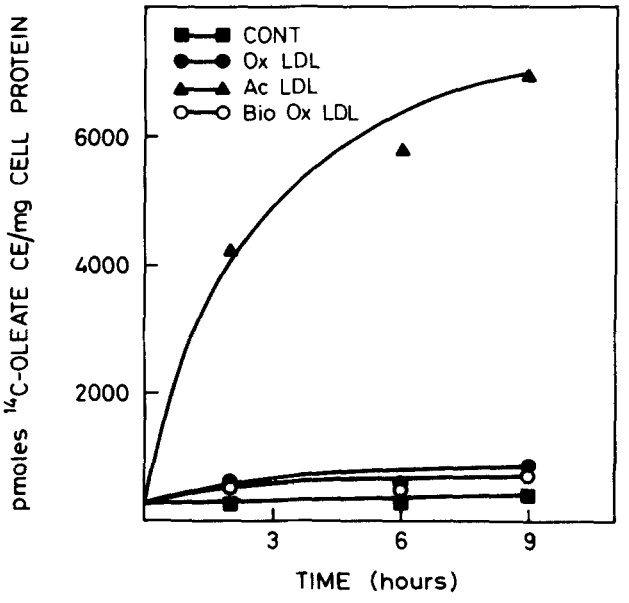

Fig. 2. Effect of modified LDL on ACAT activity in J774 macrophages. Cells were washed in medium A, then incubated at 37°C for the indicated times in medium A alone or containing OxLDL, BioOxLDL (50 μg lipoprotein cholesterol/ml), or AcLDL (30 μg lipoprotein cholesterol/ml). Cells were then incubated in medium A containing [^{14}C]oleate-albumin complex (0.1 mM) for 2 h. Lipids were extracted from washed cells and the radioactivity in esterified cholesterol was determined as described in Methods.

Fig. 3 illustrates the specific uptake and degradation of ^{125}I-labeled AcLDL, OxLDL, BioOxLDL, and sham-incubated LDL by J774 macrophages. LDL incubated in MEM without cells were neither taken up by macrophages nor degraded, indicating that cells are required to induce LDL modification. Specific uptakes of iodinated OxLDL, BioOxLDL, and AcLDL were comparable. Although ^{125}I-labeled AcLDL were efficiently degraded (4,231 and 7,426 ng/mg cell protein at 7 and 14 μg/ml) both ^{125}I-labeled OxLDL and ^{125}I-labeled BioOxLDL underwent relatively poor degradation (respectively, 840 and 1,250 ng/mg cell protein, at 7 and 14 μg/ml, 539 and 883 ng/mg cell protein, at the same concentrations) (Fig. 3). All together these data indicate that the behavior of OxLDL and BioOxLDL is very similar, if not identical. Moreover, these data stress the observation that EAhy-926

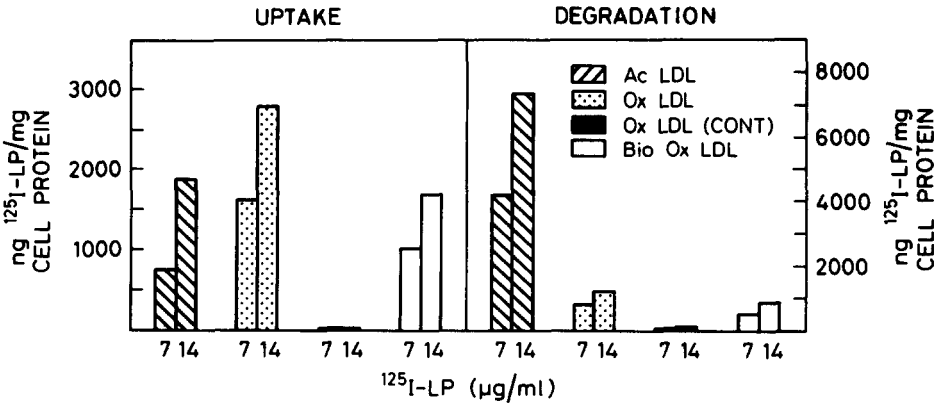

Fig. 3. Uptake and degradation of modified LDL by J774 macrophages. J774 macrophages were incubated at 37°C for 4 h in medium A containing one of the following lipoproteins: ^{125}I-labeled AcLDL, ^{125}I-labeled OxLDL, ^{125}I-labeled LDL incubated in MEM + 12 μm $CuSO_4$ in the absence of cells, and ^{125}I-labeled BioOxLDL (7 and 14 μg/ml). Nonspecific uptake and degradation were evaluated in the presence of a 30-fold excess of the unlabeled ligand. Specific uptake and degradation were quantitated as described in Methods.

cells, as differentiated endothelium, are capable of oxidizing lipoproteins.

An alternative model for oxidatively modified LDL, namely LDL modified by incubation with products of linolenic acid oxidation (LinOxLDL), was also studied. The effect of increasing concentrations of LinOxLDL on the content of cellular cholesterol was evaluated. The purpose of this experiment was to establish whether oxidatively modified fatty acids are responsible for altering the cellular fate of otherwise intact LDL. In fact, no oxidized sterol was present in these lipoproteins, with the exception of the trace amounts detectable in native LDL (B. Malavasi and G. Galli, personal communication). Only intact apoB, visible as a single band at the top of a 10% SDS-PAGE gel, was present, and the cholesterol/protein ratio was the same as in native LDL (data not shown). These modified lipoproteins apparently behaved like AcLDL in causing cellular cholesterol accumulation (Fig. 1): in two separate experiments, after 18 h of incubation with 124 and 180 μg lipoprotein cholesterol/ml, cellular cholesterol increased from 29.0 and 20.4 μg/mg cell protein to 90 and 125 μg/mg, respectively. Esterified cholesterol was 62% and 53%, in the two instances. Fig. 1 illustrates the results obtained in the second experiment.

As OxLDL and BioOxLDL displayed similar metabolic behavior, the former were used as a model to investigate the cellular processing of oxidatively modified LDL. To address the question of whether these effects of OxLDL were due to cytotoxicity, permeability to Trypan Blue and release of LDH were evaluated in control, AcLDL- and OxLDL-incubated cells. Trypan Blue staining indicated appreciable cell death only after a 24-h incubation (**Table 1**); however, no difference could be detected between control and OxLDL-incubated cells. Also, quantitation of LDH activity in the medium revealed some enzyme loss only after 24 h. Furthermore, although the difference was modest, the loss was higher for control than for lipoprotein-treated cells (Table 1).

The hypothesis that the poor degradation of OxLDL may depend upon the blockade of a chloroquine-sensitive pathway was tested. In cells treated with 100 μM chloroquine, the degradation of both iodinated AcLDL and OxLDL dropped to 27–28% of the degradation in untreated cells (**Table 2**). Uptake of ^{125}I-labeled AcLDL was

TABLE 1. Effect of modified LDL on viability of J774 macrophages

	Trypan Blue-Impermeable Cells[a]				LDH Activity in Culture Medium[b]			
Lipoprotein	2 h	5 h	8 h	24 h	2 h	5 h	8 h	24 h
	%				U/l			
LDL	90	89 ± 4	90 ± 10	84 ± 4	52.6	52.6	68.8	252
OxLDL	85 ± 5	89 ± 4	89 ± 1	81 ± 5	72.8	60.7	64.8	119.4
AcLDL	84 ± 6	86 ± 2	92 ± 1	89 ± 5	64.8	52.6	68.8	175.4

Cells were incubated at 37°C in medium A alone or containing OxLDL or AcLDL (50 μg lipoprotein cholesterol/ml). At the end of the indicated times, cell viability was assessed as the percentage of cells impermeable to Trypan Blue or as LDH release into the culture medium.

[a]Mean ± SD.

[b]Mean of two determinations.

TABLE 2. Effect of chloroquine (CHL) on uptake and degradation of ^{125}I-labeled OxLDL and ^{125}I-labeled AcLDL in J774 macrophages

^{125}I-labeled Lipoprotein	CHL	Uptake	Degradation
		μg/mg cell protein	
OxLDL	–	2263 ± 28	1027 ± 75
OxLDL	+	3086 ± 509	282 ± 15
AcLDL	–	1289 ± 34	3069 ± 117
AcLDL	+	4380 ± 376	855 ± 140

Data are the means ± SD of three determinations. Cells were incubated for 18 h in medium A with (+) or without (–) 100 μM chloroquine, washed, and then incubated for 4 h at 37°C in medium A containing ^{125}I-labeled OxLDL or ^{125}I-labeled AcLDL (10 μg/ml). Total uptake and degradation were quantitated as described in Methods.

most sensitive to chloroquine, with a 3.4-fold increase over control values, versus 1.4-fold for OxLDL (Table 2). The observation that the degradation of OxLDL is somewhat sensitive to chloroquine (Table 2) suggests that this takes place within an acidic compartment; furthermore, these data indicate that the cellular processing of OxLDL is partially blocked or extremely slow. The possibility for an intracellular accumulation of products of apoB hydrolysis, which would lead to an underestimation of lipoprotein degradation, was also evaluated. With two different concentrations of ^{125}I-labeled OxLDL, the amount of intracellular degraded ligand did not exceed 8.5% of total intracellular lipoprotein. An explanation for these findings could be that, once bound to the cellular surface, OxLDL are poorly internalized. Binding and internalization of ^{125}I-labeled OxLDL at 4°C and 37°C were quantitated by means of three alternative methods. Binding of ^{125}I-labeled OxLDL to J774 cells, determined as the amount of lipoprotein displaced by a 100-fold excess of unlabeled ligand, was the same at 4°C and 37°C; similarly, only minor differences were observed after trypsin or pronase treatment of the cells. With all treatments, internalization was minimal at 4°C and increased 20- to 26-fold at 37°C (**Fig. 4**), indicating that OxLDL are bound and subsequently internalized. Internalization of bound OxLDL was confirmed by localization of cellular free cholesterol. In both control and AcLDL-incubated cells, probing with filipin revealed the diffuse presence of free cholesterol on the plasma membrane and within the cells. With OxLDL, besides what was seen in control cells, a punctuated intracellular fluorescence pattern was evident after 5 h and, more markedly, after 24 h of incubation (**Fig. 5**). To gain further insight into the distribution of cholesterol within cells exposed to OxLDL, cellular membranes and organelles were fractionated on density gradients and the cholesterol/protein ratio was calculated for each fraction (**Fig. 6**). In control cells this ratio peaked between light membranes and dense lysosomes. In AcLDL-loaded cells, in addition to this peak, a very high cholesterol/protein ratio was detected at the top of the gradient, presumably due to floating cholesteryl ester droplets. In cells incubated with OxLDL only a major peak was observed in the bottom fractions of the gradient, thus suggesting different localizations for OxLDL- and AcLDL-derived cholesterol.

The accumulation of OxLDL within the cell could result either from an inhibitory effect of these lipoproteins on proteolytic enzymes or from a lack of activity of these enzymes on OxLDL.

To verify that OxLDL are not responsible for a nonspecific blockade of lysosomal functions, the intracellular degradation of other ligands, such as AcLDL and IgG, was evaluated. In order to avoid any competition between OxLDL and ^{125}I-labeled AcLDL for the scavenger receptor, as well as any transfer of small peptides from the former to the latter, the cells were sequentially incubated with the two lipoproteins, and the degradation of ^{125}I-labeled AcLDL was quantitated. A 5-h preincubation with OxLDL (25 μg/ml) did not affect the degradation of ^{125}I-labeled AcLDL at concentrations of 5 and 10 μg/ml (1.23 and 2.27 μg/mg versus 1.52 and 2.32 μg/mg cell protein in preincubated and control cells, respectively; **Table 3**). The possibility that a lysosomal blockade was a transient effect, and therefore could only be observed in the presence of the causative factor (i.e., OxLDL), was examined by incubating the cells with ligands that are not taken up via the scavenger receptor (i.e., IgG and IgG/anti-IgG complexes). Uptake and degradation of such ligands were unaffected by a simultaneous incubation with OxLDL (**Table 4**). This suggested that

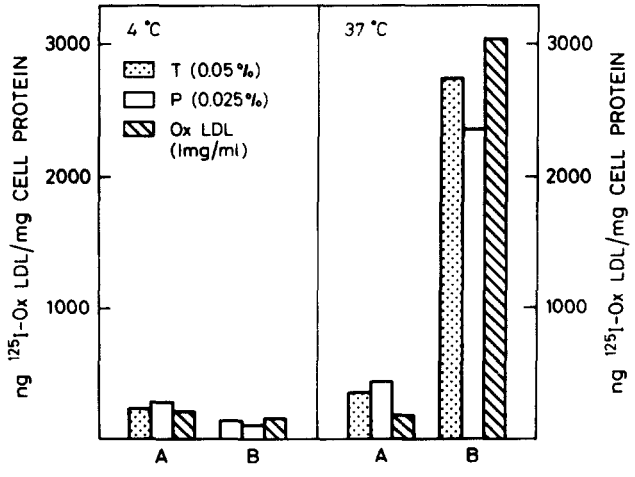

Fig. 4. Binding and internalization of ^{125}I-labeled OxLDL by J774 macrophages. Cells were incubated for 2 h at 4°C or for 5 h at 37°C in medium A containing ^{125}I-labeled OxLDL (10 μg/ml), washed, and incubated for 10 min at 37°C in medium A with the addition of 0.05% trypsin (T), 0.025% pronase (P), or 1 h at 4°C in medium A containing OxLDL (1.0 mg/ml). Binding (A) was quantitated as the radioactivity released into the medium by the above treatments; internalization (B) was the radioactivity that remained associated to the cells after extensive washing.

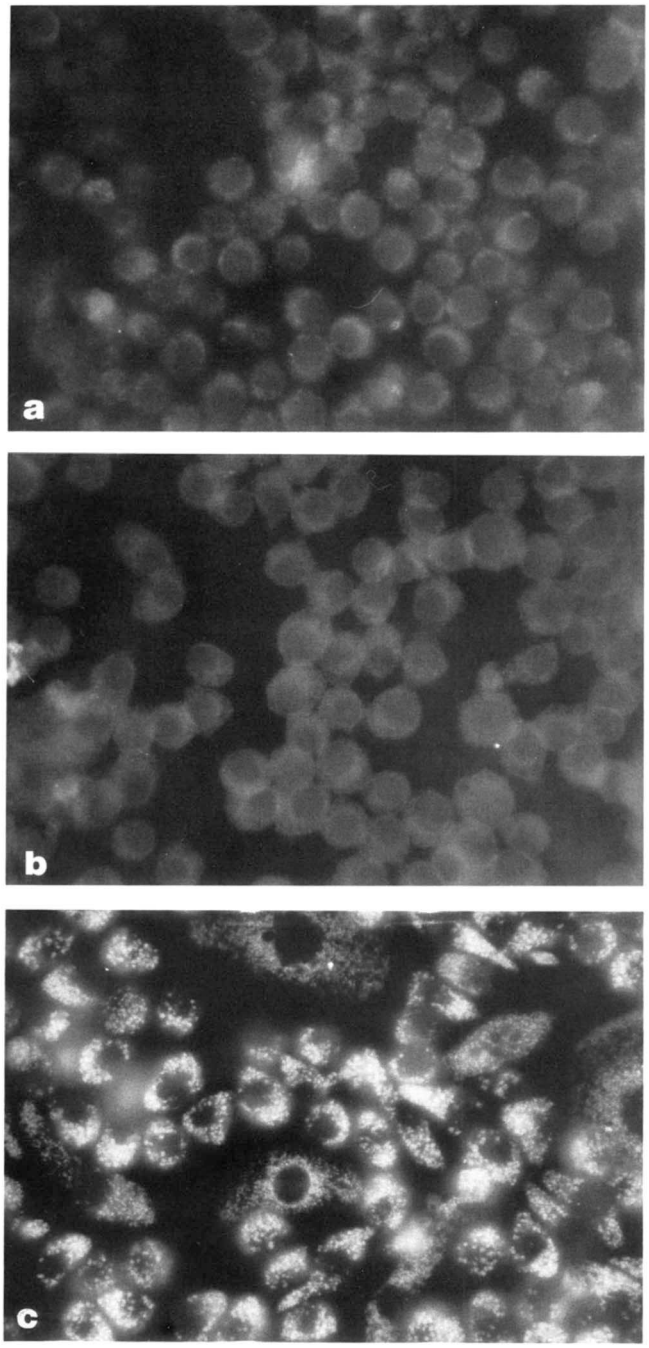

Fig. 5. Filipin staining of J774 macrophages. Cells, grown on coverslips, were incubated at 37°C for 18 h in medium A alone (a) or containing AcLDL (b) or OxLDL (c) (50 μg lipoprotein cholesterol/ml). Washed cells were fixed in 4% paraformaldehyde (room temperature, 15 min), incubated in 50 mM NH_4Cl (4°C, 30 min), and finally incubated in PBS containing 0.005% filipin (room temperature, 30 min). Washed coverslips were mounted on microscopy slides and viewed with a fluorescence microscope.

lysosomal function is not generally impaired by OxLDL, but rather that these lipoproteins themselves are relatively resistant to degradation. To investigate this aspect in further detail, the susceptibility of OxLDL, AcLDL, and native LDL to hydrolysis by lysosomal enzymes was evaluated by direct incubation of the iodinated lipoproteins with cellular fractions enriched in lysosomes. **Fig. 7** illustrates the results of this experiment. At pH 4.5 degradation was linear in the concentration range used for ^{125}I-labeled OxLDL, however, it was markedly lower than for the other substrates (0.166 ng/mg protein versus 1.13 ng/mg protein for ^{125}I-labeled AcLDL and 0.76 ng/mg protein for ^{125}I-labeled LDL at 2 μg lipoprotein protein/ml; Fig. 7). Degradation of any of the lipoprotein was not detectable either at pH 9 or in the absence of cellular protein (not shown).

DISCUSSION

Recently two relevant features of the in vitro metabolism of OxLDL by murine macrophages have been out-

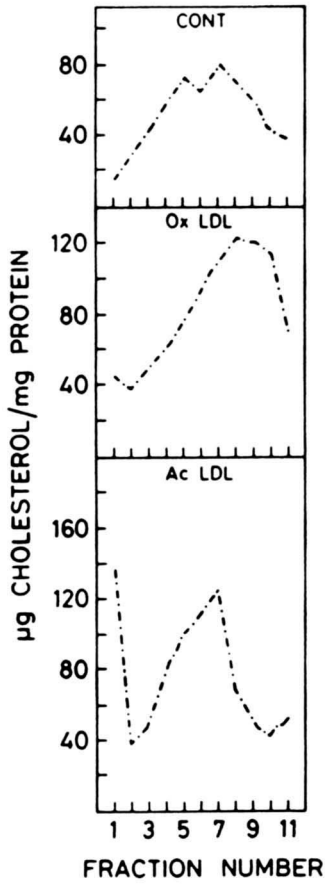

Fig. 6. Effect of modified LDL on the cholesterol/protein ratio in cellular subfractions of J774 macrophages. Cells were incubated at 37°C for 18 h in medium A alone or containing AcLDL or OxLDL (50 μg lipoprotein cholesterol/ml). Washed cells were homogenized in solution C and centrifuged at 2000 *g* twice. Pooled supernatants (1 ml) were loaded onto 11 ml of 7.8% Percoll in solution C and spun at 20,000 *g* for 40 min. Eleven fractions were collected from the gradient; protein and cholesterol were quantitated as described in Methods.

TABLE 3. Effect of OxLDL on uptake and degradation of ^{125}I-labeled AcLDL by J774 macrophages

Preincubation with OxLDL	^{125}I-labeled AcLDL	Uptake	Degradation
	μg/ml	*μg lipoprotein protein/mg cellular protein/3 h*	
–	5	0.5	1.5
+	5	0.6	1.2
–	10	1.0	2.3
+	10	1.2	2.3

Cells were incubated for 5 h at 37°C in medium A with (+) or without (–) OxLDL (25 μg/ml), washed, and then incubated for 3 h in the same medium with ^{125}I-labeled AcLDL (5 and 10 μg/ml). Total uptake and degradation were determined as described in Methods. Data are the average of duplicates that did not differ by more than 10%.

lined: *i)* OxLDL load the cells with high amounts of cholesterol, largely unesterified, and *ii)* the protein moiety of OxLDL is relatively poorly degraded, thus resulting in a disproportion between degradation and uptake by the cells.

The aim of our study was to elucidate the pathway that OxLDL follow after binding to the cell membrane. Several lines of evidence indicate that OxLDL are bound and subsequently internalized by macrophages. *i)* A high percentage of ^{125}I-labeled OxLDL associated to the cells during an incubation at 37°C was not displaced by treatments that selectively remove membrane-bound ligands. *ii)* When cells were incubated with OxLDL and then fractionated on density gradients, dense lysosome-containing membranes were enriched in cholesterol. *iii)* Fluorescence microscopy, using filipin as a probe to localize free cholesterol, indicated, as expected on the basis of parallel biochemical determinations, a much higher content of free cholesterol in cells preincubated with OxLDL. Furthermore, cholesterol was mainly intracellular with a perinuclear distribution, suggestive of a lysosomal localization.

Zhang, Basra, and Steinbrecher (34) reported that at least one intact apoB epitope can be detected by immunofluorescence microscopy in cells incubated with chemically oxidized LDL. We performed similar observations with a mixture of four monoclonal antibodies that recognized apoB on both AcLDL and OxLDL (data not shown). This is in agreement with the finding that only a minor percentage of cell-associated OxLDL radioactivity was not precipitable with trichloroacetic acid. That different lipoproteins may follow different pathways and may be degraded within lysosomes to various extents has recently been proposed. Ellsworth et al. (33) found that in murine macrophages the internalization rate of chylomicron remnants was lower than that of β-VLDL; their degradation rate, too, was lower, possibly reflecting a direct effect on lysosomal pH by chylomicron-associated fatty acids. Tabas et al. (35) studied the processing of endocytosed LDL and β-VLDL in mouse peritoneal macrophages. Along with the distinct localizations of the two lipoproteins, they observed differences in the rates of degradation, as well as in their ability to stimulate ACAT activity. Our data on OxLDL could be explained either by the presence of a cellular pathway specific for oxidized LDL, not leading to significant degradation of the internalized ligand, or by a defective action of lysosomal enzymes on OxLDL. The data on the degradation of OxLDL by partially purified lysosomal enzymes favor the latter possibility. We cannot exclude, however, that OxLDL may follow a specific intracellular pathway leading to their accumulation in organelles that contain little enzymatic activity.

Aggregation has been considered crucial for the uptake of OxLDL by macrophages (36) and Hoff et al. (37) suggested that the little degradation of oxidized LDL extracted from human atherosclerotic lesions depends on

TABLE 4. Effect of OxLDL on uptake and degradation of ^{125}I-labeled IgG and ^{125}I-labeled IgG/anti-IgG by J774 macrophages

Mab Anti-A-I	OxLDL	Uptake	Degradation
		μg lipoprotein protein/mg cellular protein/5 h	
–	–	4.3	11.1
–	+	4.3	10.0
+	–	4.6	11.2
+	+	4.4	10.2

^{125}I-labeled goat anti-mouse IgG (0.5 μg/ml) was incubated for 30 min at room temperature with (+) or without (–) anti-human apoA-I mouse monoclonal antibody (Mab) (0.5 μg/ml). Total uptake and degradation by J774 macrophages during an incubation of 5 h at 37°C in medium A with (+) or without (–) OxLDL were then determined as described in Methods. Data are the average of duplicates that did not differ by more than 10%.

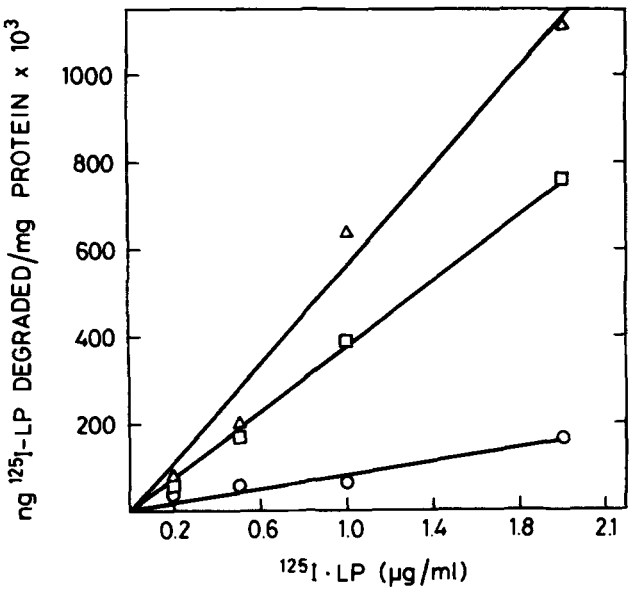

Fig. 7. Degradation of native and modified LDL by lysosomal enzymes. A lysosome-enriched cell subfraction was obtained from J774 macrophages as described in Methods. Lysosomes were lysed by repeated freeze-thawing and 25 μg of protein was incubated at 37°C for 4 h in citrate-buffered MEM, pH 4.5 (final volume 100 μl), containing the indicated concentrations of ^{125}I-labeled LDL (□), ^{125}I-labeled AcLDL (△), or ^{125}I-labeled OxLDL (○). Degradation of lipoproteins was quantitated as TCA-nonprecipitable, noniodide radioactivity present in the medium as described in Methods.

the formation of lipoprotein aggregates, which could be only partially processed. This explanation seems unlikely in our case since lipoproteins were sterile-filtered after concentration and desalting, as well as after iodination. Furthermore, although at the end of an incubation with a lysosome-enriched fraction similar percentages of ^{125}I-labeled OxLDL and ^{125}I-labeled AcLDL could be pelleted by low speed (12,000 *g*) centrifugation, only ^{125}I-labeled OxLDL were resistant to degradation.

From these observations one could speculate that, as far as OxLDL are concerned, cells are in a "chloroquine-like" state: lysosomal proteolysis is almost blocked, and chloroquine treatment of cells cannot cause further accumulation of undegraded ligand. On the other hand, because treatment with chloroquine abolishes the slight amount of degradation that OxLDL undergo, degradation is likely to take place in an acidic endo/lysosomal compartment. This is in agreement with the observations made with partially purified lysosomes.

After this paper was submitted, Lougheed, Zhang, and Steinbrecher (38) published evidence that the intracellular accumulation of oxidized LDL in macrophages may depend in part on their resistance to cathepsins. The present study confirms and extends this observation, suggesting that OxLDL are resistant not merely to cathepsin D but also to other proteolytic enzymes that are present in lysosomes. Lysosomal proteolytic digestion of other substrates is not affected by OxLDL, since cellular degradation of AcLDL and of immunoglobulins was not reduced by a preincubation (AcLDL) or by a co-incubation (immunoglobulins) with these lipoproteins; this speaks against a nonspecific toxic effect. On the other hand, we failed to detect any toxicity of OxLDL on J774 macrophages. It appears that at 24 h the control cells suffered more than cells exposed to lipoproteins. This could reflect the fact that control cells were incubated in a medium containing 0.2% albumin and no lipids. Thus, it can be speculated that the inhibition of lysosomal hydrolysis would not depend on a nonspecific blockade, but rather on an intrinsic resistance of OxLDL to enzymatic attack.

Consistent with the resistance of OxLDL to proteolysis is the observation that they modestly stimulate ACAT activity (13). An inhibitory effect of the lipid moiety of oxidized LDL on ACAT activity in human umbilical vein endothelial cells has been reported by Jialal and Chait (15). We failed to detect such an effect; different experimental methods and conditions, as well as the use of different cells (endothelial cells vs. J774) might contribute to explain these discrepancies.

It has been postulated that the only requirement for the binding of oxidized LDL to the scavenger receptor(s) is the presence of some oxygenated molecules, derived from the oxidation of fatty acids, that bind apoB (21). The effect on cellular cholesterol content of LDL that had been modified with water-soluble products derived from the oxidation of linolenic acid (LinOxLDL) was similar to that obtained with AcLDL. Such oxidized lipoproteins do interact with the scavenger receptor, but, at least with respect to cellular accumulation of cholesterol, are not an appropriate model for particles modified as a consequence of the interactions with cells present in the vessel wall (39).

Further investigation is required to assess the relevance of the above findings to the development of atherosclerotic lesions, provided that OxLDL are an adequate model for in vivo oxidized lipoproteins. However, some speculations are suggested by the present results. Cells may overload with free cholesterol as an acute response to high local concentrations of oxidized lipoproteins; only at later times may ACAT be activated to yield the massive amounts of esterified cholesterol generally observed in lesion areas. However, the observation of free cholesterol in lesions is not an uncommon finding (40, 41). Moreover, even a temporary accumulation of free cholesterol, by alteration of the cellular pools of lipid, may elicit regulatory as well as inflammatory responses. It would be of interest to know whether macrophages can respond to the overload of free cholesterol with an increased transcription of the apoE gene (42), in view of the observed association between apoE production and cholesterol loading (43). Similarly, the transcription of other genes could be modulated by OxLDL, as was already reported for the TNF gene (44). Of relevance would be an effect on the synthesis of various

growth factors (45) and modulators of immune functions, which may play a role in atherosclerosis.

In summary, the present study demonstrates that OxLDL are efficiently internalized by cultured murine macrophages. While a small percentage of OxLDL undergoes degradation in an acidic compartment, most is undegraded and accumulates in dense, perinuclear organelles, and is resistant to hydrolysis by lysosomal enzymes. The relevance of these findings to the formation of foam cells and to the initiation of atherosclerotic lesions remains to be addressed.

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