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OXIDIZED LDL INCREASE FREE CHOLESTEROL AND FAIL TO STIMULATE CHOLESTEROL ESTERIFICATION IN MURINE MACROPHAGES

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ABSTRACT: Oxidatively modified low density lipoproteins (Ox-LDL) may be involved in determining the formation of foam cells by inducing cellular cholesteryl ester accumulation. We studied the effect of copper oxidized LDL (Ox-LDL) on cholesterol accumulation and esterification in murine macrophages. Ox-LDL (44 ug/ml of lipoprotein cholesterol) increased the total cholesterol content of the cells from 29 to 69 ug/mg cell protein. Free cholesterol accounted for 85% of this increase. Acetyl LDL (Ac-LDL) (38 ug/ml of lipoprotein cholesterol), raised total cellular cholesterol content to a similar extent (76 ug/mg cell protein), however only 25% of the accumulated cholesterol was unesterified. When ACAT activity was determined after incubation of J774 cell with Ox- or Ac-LDL, Ox-LDL were 12 times less effective than Ac-LDL in stimulating cholesteryl ester formation. This was not due to an inhibition of ACAT by Ox-LDL since these lipoproteins failed to inhibit pre activated enzyme in cholesteryl ester-loaded macrophages. The uptake of ^{125}I-Ox-LDL was 175% that of ^{125}I-Ac-LDL, while degradation was only 20%. All together these data suggest an altered intracellular processing of Ox-LDL, which may be responsible for free cholesterol accumulation.

Various modifications (1-6) can convert native LDL into specific ligands for the scavenger receptor (1,7). Among these, oxidation is potentially occurring "in vivo" (8).

A current hypothesis (8) is that oxidized LDL (Ox-LDL) entering the cell via a poorly regulated receptor pathway cause massive accumulation of lipids, as cholesteryl ester droplets, and promote "foam cell" formation (1,9,10).

The major changes that LDL undergo upon oxidation are loss of esterified cholesterol (11), with a relative and absolute increase in free cholesterol content, and extensive breakage of lipid (12) and apoprotein (13) components, with derivatization of peptide chains by lipid decomposition products (14). These modifications result in denser particles (3), with increased

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electrophoretic mobility (3,14), which are cytotoxic (15), chemotactic for monocytes (16) and inhibit macrophage motility (17). The above changes are caused not only by the incubation of LDL with divalent cations (14,18), but also by incubating LDL in the presence of monocyte-derived macrophages (15), endothelial (12,19,20) and smooth muscle (20-22) cells.

Recent data support the hypothesis that oxidation occur "in vivo": Haberland et al. (23) demonstrated, by immunochemical analysis, the presence of malondialdehyde modified proteins that colocalize with extracellular apo B-100 in arterial lesions of WHHL rabbits. Yla-Herttuala et al. (24) isolated from atherosclerotic lesions of rabbits and man lipoproteins with many properties in common with "in vitro" oxidized LDL. Furthermore, oxidized lipids and lipoproteins have been observed in human (25) and experimental diabetes (26). Until recently it has been postulated that Ox-LDL are ligands for the same scavenger receptor responsible for acetyl-LDL uptake and degradation by macrophages (3,5). Recent data (27,28), however, suggest the existence of multiple receptors with different specificity for such modified lipoproteins. The cellular fate of Ac-LDL is well documented (1,2); on the contrary that of Ox-LDL is much less understood. Because of this and of the possible relevance of Ox-LDL in the atherosclerotic process (8) we investigated the impact of these lipoproteins on cellular cholesterol metabolism, as well as the fate of their protein moiety, in cultured macrophages.

MATERIALS AND METHODS

Cells. J774 murine macrophages, an established cell line (29), were obtained from Salk Institute Cell Repository. Cells were grown at 37°C in 5% CO_2 atmosphere in DMEM (Gibco, Madison, WI), with the addition of 10% FCS. Cells were used as confluent monolayers, in 35 mm tissue culture dishes (Corning, New York, NY). Thioglycolate-elicited mouse peritoneal macrophages (MPM) were harvested from CD1 female mice (Charles River, Italy) as described (30). Collected cells were washed once with DMEM, resuspended in the above medium with 10% FCS and seeded in 35 mm dishes (3 x 10^6 cells/dish).

Lipoproteins. LDL (1.019-1.063 g/ml) were isolated by sequential ultracentrifugation at 12°C, at 40.000 rpm in a 60 Ti rotor (Beckman, Palo Alto, CA), in a L5-50 ultracentrifuge (Beckman, Palo Alto, CA) and used within 10 days.

For oxidation LDL were dialyzed against phosphate buffer saline on a PD-10 Sephadex G-25 column (Pharmacia Fine Chemicals, Piscataway, NJ), sterile filtered, diluted to a concentration of 0.2 mg/ml with PBS and incubated for 24 h in the presence of $CuSO_4$ 20 uM. Oxidation was blocked with the addition of BHT 40

uM. Oxidized LDL were concentrated by ultrafiltration under N_2 pressure on Diaflo Ultrafiltration Membranes XM 100A (American Corporation, Lexington, MA) dialyzed against PBS and sterile filtered.

For acetylation LDL were extensively dialyzed in NaCl 0.15 M, diluted with an equal volume of saturated Na acetate and treated with acetic anhydride, according to Basu et al. (31). Acetylated LDL were dialyzed against PBS and sterile filtered.

Agarose electrophoresis. Non denaturing gel electrophoresis (32) of native and modified LDL was performed in 0.8% agarose A (Pharmacia Fine Chemicals, Piscataway, NJ), in 0.1% M TRIS, pH 8.6, at 200 V. Gels were fixed in 70% ethanol, dried and stained with Sudan Black 0.1% in 70% ethanol.

Cholesterol accumulation. Cells were washed with PBS and incubated with the appropriate dilutions of modified lipoproteins for 16-24 h. At the end of the incubation lipids were extracted from washed cells by a 30' incubation in hexane:isopropylic alchool 3:2, followed by a brief wash in the same mixture. Cellular proteins were dissolved in 1.0 M NaOH and quantitated according to Lowry et al. (33). Pooled lipid extracts were dried under a N_2 stream and redissolved in 300 ul of hexane. One half of the extract was used for the determination of total cholesterol by enzymatic colorimetric assay (34); from the second half, free and esterified cholesterol were separated by TLC (HPTLC silica gel plates, Merck, Darmstadt, FRG) and their relative proportion determined by a colorimetric assay on extracts of the respective areas scraped from the plate.

Uptake and degradation of lipoproteins. Lipoproteins were labelled with ^{125}I-NaI according to Bilheimer et al. (35), dialyzed against PBS on PD-10 columns and sterile filtered. TCA non precipitable radioactivity was 2-5% of total, specific activity was 200-300 cpm/ng of lipoprotein protein. Cells were washed with PBS and incubated for 5 h at 37°C in MEM, 0.2% BSA with the appropriate dilutions of the radioactive lipoproteins and with or without a 30 fold excess unlabelled lipoproteins. At the end of the incubation the media were collected for the determination of non iodide, TCA soluble, radioactivity (1) and washed cells dissolved in 0.1 M NaOH for the quantitation of cell-associated radioactivity (1) and protein determination (33).

ACAT assay. Cholesterol esterification activity was measured as the incorporation of $|^{14}C|$oleate into cholesteryl ester. Cell monolayers were incubated with $|^{14}C|$ oleic acid-albumin complex for 2 h. Cell monolayers were washed and extracted as above and esterified cholesterol was counted by liquid scintillation after TLC separation (36).

RESULTS AND DISCUSSION

Upon oxidation LDL underwent several physico-chemical changes: 1) loss of oxidized lipids, 2) changes in the cholesterol/protein ratio (1.1 vs 1.6), 3) breakage of apo B, 4) increased electrophoretical mobility (1.5-2 fold that of control LDL), 5) loss of the ability to interact with the apo B,E receptor on human skin fibroblasts, (data not shown). All such changes have been described as being typical for oxidized LDL (2,3,12-15).

Incubation of J774 macrophages with Ox-LDL induced a concentration dependent accumulation of cholesterol, with a 2.4 fold increase in cellular cholesterol/protein ratio at 44 ug/ml lipoprotein cholesterol (69 ug/mg versus 29 ug/mg for unloaded cells); higher concentrations of Ox-LDL (88 ug/ml) produced only a slight further increase of cellular cholesterol content. When free and esterified cholesterol were determined the bulk (85%) of accumulated sterol was found in the free form (Fig.1). In the same experiments Ac-LDL (38 ug/ml lipoprotein cholesterol) induced a comparable total cholesterol accumulation (76 ug/mg). However, free cholesterol represented only 25% of the net cholesterol accumulation. In a separate experiment we incubated MPM with amounts of modified lipoproteins (either Ox-LDL or Ac-LDL) capable of causing similar cholesterol accumulation. Again, we found that 82% of the cholesterol accumulated in the presence of Ox-LDL was unesterified, as compared to the 46% observed with the Ac-LDL (Table 1). Since cellular cholesteryl esters are the product of ACAT (2,36) we studied the effect of Ox-LDL on ACAT stimulation in J774 macrophages. Ac-LDL effectively stimulated in a time-dependent manner the enzyme activity, which increased up to 36 times the basal level. Ox-LDL, on the contrary, poorly induced

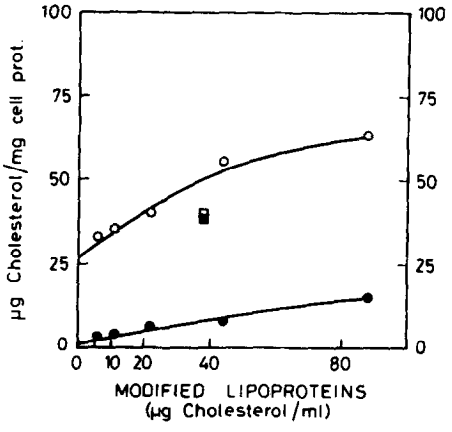

Fig.1. Effect of Ox-LDL (●) and Ac-LDL (■) on free (open) and esterified (filled) cholesterol content in J774 macrophages.
Cells were incubated for 16 h with increasing amounts of Ox-LDL or with 37.5 ug/ml of Ac-LDL cholesterol.
At the end of the incubation lipids were extracted from washed cells and free and esterified cholesterol determined as described in Methods. Data are the mean of triplicate determinations that did not differ by more than 7%.

TABLE 1

EFFECT OF MODIFIED LDL ON TOTAL (TC) ESTERIFIED (EC) AND FREE CHOLESTEROL (C) CONTENT IN J774 AND MOUSE PERITONEAL MACROPHAGES (MPM)

LIPOPROTEIN CHOLESTEROL ADDED (ug/ml)	TC	EC	C
	ug/mg cell protein		
J774			
NONE	29.7	1.4	28.3
Ox-LDL (88)	79.2	16.0	63.2
Ac-LDL (38)	76.5	38.0	38.5
MPM			
NONE	21.7	2.8	18.9
Ox-LDL (88)	81.8	13.7	68.1
Ac-LDL (38)	83.0	35.7	47.3

Cells were incubated with the indicated amount of lipoproteins for 16 h, washed and their protein and cholesterol content determined as described in Methods. Data are the average of duplicates that did not differ by more than 5%.

cholesterol esterification: at the same time point the activation was only 8% of that observed with Ac-LDL (Fig.2). Similar observations have been reported by Yokode et al. (37), who observed in MPM a 40 fold stimulation of ACAT activity over basal levels with both Ac-LDL and β-VLDL as compared to a 10 fold stimulation with Ox-LDL. Furthermore, Ox-LDL suppress ACAT activity in human endothelial cells, possibly via a direct inhibition of enzyme activity (38). To test this possibility we studied the effect of Ox-LDL on cholesterol esterification in Ac-LDL pre loaded cells. In those cells ACAT activity rose 30 fold over unloaded cells; Ox-LDL failed to suppress ACAT activity (Fig.2B).

All together those findings suggest that the cholesterol transported by Ox-LDL into the J774 macrophages is sequestered into a pool which is not readly available to the ACAT; a direct inhibitory effect on the enzyme therefore does not play a role in determining free cholesterol accumulation. Tabas et al. have shown that lipoprotein derived cholesterol, after lysosomal hydrolysis, rapidly reaches the plasma membrane, and from there moves to the ACAT substrate pool (39) of which it constitutes the bulk. This physiological mechanism may be modulated by using the pharmacological agent U18666A, which blocks the

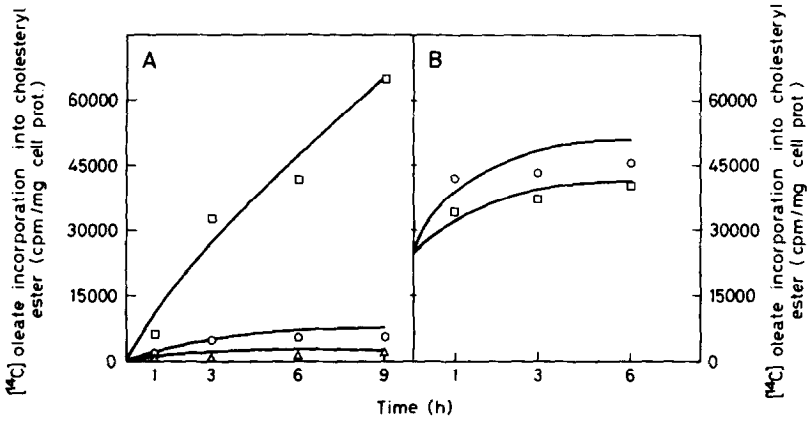

Fig.2. Panel A: time-dependent effect of Ox-LDL and Ac-LDL on cholesterol esterification in J774 macrophages. Cells were pre-incubated for the indicated time with medium alone (△) or containing Ox- (○) or Ac- (□) LDL (44 and 38 ug/ml lipoprotein cholesterol). Panel B: time-dependent effect of Ox-LDL on cholesterol esterification in cholesteryl ester loaded J774 macrophages. Cells were pre-incubated for 24 h with medium containing Ac-LDL (50 ug/ml lipoprotein cholesterol), washed for 18 h in medium alone and incubated for the indicated time with medium alone (□) or containing Ox-LDL (○) (40 ug/ml lipoprotein cholesterol).
The monolayers were washed and then incubated for two hours with ^{14}C oleate-albumin complex: cells were then extracted and the ^{14}C esterified cholesterol determined as described in Methods. Data are the average of duplicates that did not differ by more than 9%.

ability of the LDL derived cholesterol to stimulate cholesterol esterification (40).

A defect in the processing of LDL-derived cholesterol has been described in fibroblasts of patients with Niemann-Pick C disease (41), where the transport of the sterol from the lysosomes to cellular regulatory sites (or pools) is impaired. Trapping of Ox-LDL derived cholesterol within the lysosomes, its irreversible localization into the plasma membrane or, alternatively, a defective lipoprotein processing at an even earlier step of intracellular catabolism, could explain our observations.

To evaluate possible defects in the intracellular processing of Ox-LDL we performed studies on the uptake and degradation of ^{125}I-Ox-LDL and ^{125}I-Ac-LDL in J774 macrophages. The specific uptake, at the concentration of 30 ug/ml of lipoprotein protein, was higher for Ox-LDL as compared to Ac-LDL, 2065 ng and 1455 ng of lipoprotein protein/mg of cell protein respectively (Fig.3). However, the specific degradation of Ox-LDL was much lower than that of Ac-LDL (1,541 ng and 11,224 ng

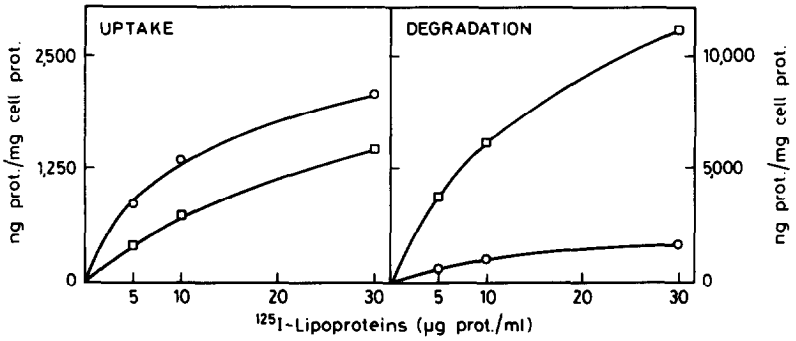

Fig.3. Specific uptake and degradation of ^{125}I-Ox-LDL (○) and ^{125}I-Ac-LDL (□) by J774 macrophages.
Cells were incubated for 5 h at 37°C with the indicated amounts of lipoprotein protein. Data are averages of duplicate determinations that did not differ by more than 8%.

lipoprotein protein/mg cell protein for Ox-LDL and Ac-LDL respectively). A similar discrepancy between cell association and degradation of endothelial cell oxidized LDL has been observed by Nagakerke et al. (42) in rat liver endothelial cells and Sparrow et al. (27) in MPM. Lack of degradation may depend either on a failure to reach the lysosomal compartment or on sequestration and defective hydrolysis of Ox-LDL at this level. Thus, the hypothesis of a peculiar intracellular processing of Ox-LDL we envisioned to explain their relative inability to activate ACAT is supported by the fate of their protein moiety.

Although the "in vivo" significance of such a pathway remains to be addressed, we speculate that Ox-LDL may cause massive accumulation of free cholesterol in the vessel wall. Changes in the free cholesterol content can alter membrane physico-chemical properties, the function of membrane bound enzymes, and ion transport (43). An increase in the permeability to Ca^{++}, observed in red blood cells with high membrane cholesterol/lecithin ratio (44), is only but one example of the disruptive effect of free cholesterol loading on cellular metabolism which could potentially lead to atherosclerotic degeneration.

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