Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages

Hanfang Zhang, Harkamal J. K. Basra, and Urs P. Steinbrecher

Department of Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, B.C., Canada V6T 1W5

Abstract Oxidative modification of low density lipoproteins (LDL) has been shown to cause accelerated degradation of LDL via the scavenger receptor pathway in cultured macrophages, and it has been proposed that this process might lead to cholesterol accumulation in macrophages in the arterial wall in vivo. However, oxidation of LDL is accompanied by a substantial reduction in LDL total cholesterol content and hence the amount of cholesterol delivered by oxidatively modified LDL may be less than that delivered by scavenger receptor ligands such as acetyl LDL which results in massive cholesterol accumulation in cultured macrophages. The present studies were done to determine whether the decrease in total cholesterol content during LDL oxidation was due to oxidation of cholesterol and cholesteryl ester, and to determine whether the resulting oxidized sterols could affect cholesterol esterification in cultured macrophages. It was found that when LDL prelabeled with [^{3}H]cholesteryl linoleate was oxidized, there was a decrease in cholesterol mass but no change in radioactivity. The radioactive substances derived from cholesteryl linoleate appeared more polar than the parent compound when analyzed by reverse-phase liquid chromatography, but were not identical with free cholesterol. Thin-layer chromatography of oxidized LDL lipids confirmed the loss of esterified cholesterol, and revealed multiple new bands, some of which matched reference oxysterols including 7-ketocholesterol, 5,6-epoxycholesterol, and 7-hydroxy-cholesterol. In addition to oxysterols, oxidized cholesteryl esters were also present. Quantitation by gas chromatography indicated that 7-ketocholesterol was the major oxysterol present. The retention times of 25-hydroxycholesterol and 7-hydroxy-cholesterol were very similar under the conditions used for gas chromatography, but mass spectrometry showed that only 7-hydroxycholesterol was detectable in oxidized LDL. Incubation of mouse peritoneal macrophages with 5 µg/ml 7-ketocholesterol resulted in stimulation of cholesterol esterification, while 7-hydroxycholesterol had a much smaller effect. When oxysterols were added to macrophages together with 5 µg/ml acetyl LDL, 7-ketocholesterol resulted in further enhancement of cholesterol esterification while 7-hydroxycholesterol produced 55% inhibition of the cholesterol esterification caused by acetyl LDL. Oxysterols extracted from oxidized LDL resulted in up to 72% inhibition of acetyl LDL-induced cholesterol esterification. Oxidized LDL resulted in much less cholesterol esterification than the same concentration of acetyl LDL. Even after correction for the amount of intact cholesterol delivered to the cells, acetyl LDL was twofold more efficient at stimulating cholesterol esterification than oxidized LDL. It was noted that almost 50% of the internalized oxidized LDL was not degraded and hence might not be accessible to acyl-CoA:cholesterol acyltransferase (ACAT), whereas the proportion of acetyl LDL that was degraded exceeded 90%. When the stimulation of cholesterol esterification was corrected for the amount of cholesterol delivered via degraded LDL, the effects of oxidized LDL and acetyl LDL were very similar. **■** These results indicate that apparently inefficient stimulation of cholesterol esterification by oxidized LDL can be explained in large part by a lower content of cholesterol per LDL particle and altered intracellular processing, resulting in reduced delivery of cholesterol to the ACAT substrate pool. The different oxysterols present in oxidized LDL appear to have opposing effects on cholesterol esterification and, depending on the extent of oxidation of LDL and the relative proportions of oxysterols as well as the availability of cholesterol for esterification, the net result of the oxysterols could be either a modest inhibition or stimulation. —Zhang, H., H. J. K. Basra, and U. P. Steinbrecher. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J. Lipid Res.* 1990. **31:** 1361–1369.

One of the hallmarks of the atherosclerotic lesion is the presence of lipid-laden foam cells in the arterial intima. Several lines of evidence indicate that in early lesions foam cells are derived from macrophages, although in more advanced lesions, smooth muscle cells apparently can also undergo foam cell transformation (1–8). The mechanism by which these cells accumulate excess lipid in the artery wall in vivo has not been determined. However, studies using cultured macrophages have suggested potential mechanisms by which foam cell formation might occur. When macrophages are exposed to physiologic or even to high concentrations of normal lipoproteins in vitro, the rate at which they accumulate cholesterol is usually insufficient to cause foam cell formation (9). An

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exception to this is that J774 cells, a mouse macrophage-like cell line that exhibits unusually high acyl-CoA:cholesterol acyltransferase (ACAT) activity, can accumulate cholesteryl ester when incubated with normal LDL (10). This phenomenon apparently does not occur with primary cultured macrophages, and hence its applicability to foam cell formation in vivo remains speculative. Cultured macrophages can also accumulate lipid when exposed to beta-migrating very low density lipoproteins (β-VLDL) isolated from cholesterol-fed animals. The reason for this is not entirely clear, as the receptor responsible for the internalization of β-VLDL appears to be identical to the classical LDL receptor and its expression would therefore be expected to be regulated by cellular cholesterol content (11-13). In any case, uptake of β-VLDL and related triglyceride-rich lipoproteins remains a potentially important mechanism for lipid accumulation.

Macrophages have also been found to possess a high affinity uptake mechanism for certain types of modified lipoproteins (9, 14). This has been termed the scavenger or acetyl-LDL receptor. The expression of this receptor is not subject to regulation by cellular cholesterol content, and therefore can lead to massive cholesterol accumulation. Several different chemical modifications of LDL can lead to uptake via this receptor, including acetylation, acetoacetylation, carbamylation, and modification by malondialdehyde (14-19). These modifications share as a common feature the ability to derivatize lysine residues and result in a neutralization of the positive charge of the lysine epsilon amino group, resulting in a net increase in electrophoretic mobility. In addition to these chemical modifications of LDL, a biological modification of LDL has also been described that leads to uptake by the scavenger receptor. This modification is the result of peroxidation of LDL, either initiated by incubation of LDL with cultured cells or by exposure of LDL to redox-active metal ions in the absence of cells (20-27). The recognition of oxidized LDL by the scavenger receptor can be explained by the modification of lysine residues (24), although many other changes in LDL structure and composition result from oxidation. These include fragmentation of apolipoprotein B, loss of polyunsaturated fatty acids, and hydrolysis of phosphatidylcholine to lysophosphatidylcholine (22, 23). In addition, a very substantial decrease in the content of cholesteryl ester has been consistently seen with both cell-modified or copper-oxidized LDL (21, 23). The studies described in the present report were done to determine whether this decrease in cholesteryl ester content during oxidative modification of LDL was due to oxidation of cholesterol, and if so, to determine how oxysterols in oxidatively modified LDL would subsequently affect cholesterol esterification as well as cholesterol accumulation in cultured macrophages.

MATERIALS AND METHODS

Materials

Carrier free Na[^{125}I],[9,10(n)-^{3}H]oleic acid (sp act 9 Ci/mmol), and Aquasol scintillation fluid were obtained from New England Nuclear, Lachine, PQ. [1,2,6,7(n)-^{3}H]cholesteryl linoleate was purchased from Amersham Corp., Oakville, Ont. Bovine serum albumin, cholesterol, 7-ketocholesterol, 5,6-epoxycholesterol, 7-hydroxycholesterol, 25-hydroxycholesterol, 22(R)-hydroxycholesterol, and cholesteryl oleate were all purchased from Sigma Chemical Co., St. Louis, MO. Dulbecco's modified Eagle's medium, α-minimal essential medium, fetal bovine serum, and gentamicin were from Gibco, Mississauga, Ont. All other chemicals and solvents were of reagent grade or better and were obtained from Fisher Scientific, Vancouver, BC, British Drug Houses, Vancouver, BC, or Sigma. Enzymatic kits for the determination of free and total cholesterol were obtained from Boehringer Mannheim, Dorval, PQ.

Lipoprotein isolation, labeling, and modification

Normal human LDL (d 1.019-1.063 g/ml) was isolated by sequential ultracentrifugation as previously described (28). LDL was radioiodinated with carrier free Na^{125}I to a specific activity of 40-150 cpm/ng using iodine monochloride (29). Cholesteryl esters in LDL were labeled by exchange with a microemulsion containing [^{3}H]cholesteryl linoleate. The labeled microemulsion was obtained by cosonicating 5μl 10% Intralipid (Pharmacia, Dorval PQ) with 10^6 dpm [^{3}H]cholesteryl linoleate in 350 μl PBS for 30 sec at 20°C using an Artek 150 sonicator with titanium microprobe set at 30% power. Native LDL (5 mg) was then incubated for 4 h at 37°C with the labeled Intralipid together with 30 μg/ml d > 1.21 g/ml plasma fraction as a source of lipid transfer activity. LDL was reisolated by sequential ultracentrifugation between d 1.020 and 1.063 g/ml, and then dialyzed against several changes of PBS containing 10 μM EDTA. LDL was acetylated by sequential additions of acetic anhydride (30). LDL (200 μg/ml) was oxidized by incubation with 5 μM CuSO$_4$ in EDTA-free PBS at 37°C as previously described (23). Except as indicated in Fig. 2, incubation time with CuSO$_4$ was 20 h, which resulted in extensive modification as assessed by agarose gel electrophoresis.

Analysis of sterols in oxidized LDL

LDL labeled with [^{3}H]cholesteryl linoleate was oxidized and the radioactive lipid products were analyzed by high pressure liquid chromatography on a 0.4 × 15 cm μBondapak C$_{18}$ column eluted at 1 ml/min with acetonitrile-isopropanol 80:20 for 6 min and then a 50:50 solvent mix for 15 min. Absorbance at 203 nm was used to

locate cholesterol and cholesteryl linoleate standards, and radioactivity of individual fractions was determined by scintillation counting. Lipids extracted from native and oxidized LDL were analyzed by thin-layer chromatography on Whatman K6 250 μ silica gel plates developed in hexane-acetone-acetic acid 80:20:1. Spots were visualized by spraying with 5% aqueous sulfuric acid, 5% acetic acid, 0.05% (w/v) FeCl$_2$, and heating at 155°C for 10 min. Quantitation of cholesterol and oxysterol mass in oxidized LDL was done by gas chromatography on a Hewlett Packard 5880A chromatograph fitted with a 0.53 mm × 30 m HP-5 (crosslinked 5% phenyl methyl silicone) fused silica capillary column and a flame ionization detector. Column temperature was held at 230°C for 4 min, increasing to 242°C at 0.6°C/min, and then maintained at 242°C for 15 min. Helium was used as carrier gas at a flow rate of 6 ml/min. The recovery of cholesterol and 7-ketocholesterol standards from the silica gel was 96–103%, and hence no corrections were made for recovery of individual oxysterols in oxidized LDL. No oxysterols were detected in cholesterol eluted from silica gel after TLC. Gas chromatography–mass spectrometry (GC–MS) was performed on a Kratos MS-80 instrument in the Regional Mass Spectrometry Unit, UBC. Samples were analyzed after ionization by electron impact as well as after chemical ionization with isobutane.

Cell culture

Resident peritoneal macrophages were harvested from Swiss CD-1 mice by peritoneal lavage with ice-cold calcium-free Dulbecco's phosphate-buffered saline (PBS) as previously described (20–22). Macrophages were suspended in α-minimal essential medium (α-MEM) with 10% fetal bovine serum and 50 μg/ml gentamicin and plated in six-well plates at a density of 2 × 10^6 cells/well. The adherent macrophages were cultured overnight in a humidified 5% CO$_2$ incubator before use in experiments.

Cholesterol esterification assay

Varying concentrations of oxysterols, oxidized ^{125}I-labeled LDL, or acetylated ^{125}I-labeled LDL were added to macrophages in α-MEM containing 2.5 mg/ml lipoprotein-deficient serum and 1.5 mg/ml bovine serum albumin complexed with 50 μM [^{3}H]oleic acid (sp act 50 mCi/mmol) and incubated for 10 h in a 37°C 5% CO$_2$ incubator. Because [^{3}H]oleic acid was present throughout the incubations, incorporation of radioactivity into cholesteryl ester served as an index of the cumulative amount of cholesteryl ester formed. In preliminary studies, results for [^{3}H]oleate incorporation into cholesteryl ester were found to correlate well with determinations of cholesteryl ester mass by gas chromatography (Fig. 1), and the accumulation of cholesteryl [^{3}H]oleate was linear up to 10 h.
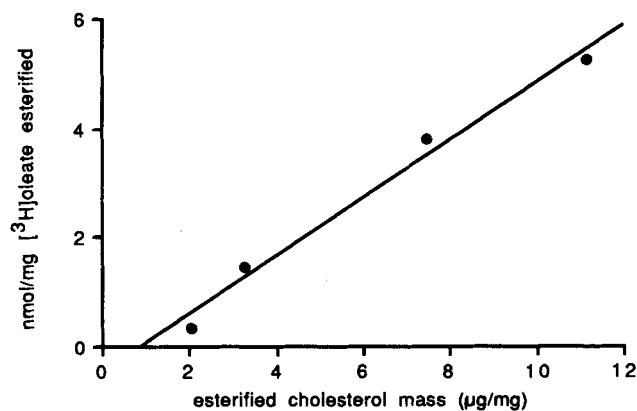
Fig. 1. Correlation between cholesteryl ester mass and cholesteryl [^{3}H]oleate formation in cultured macrophages. Mouse peritoneal macrophages were incubated for 10 h with 0, 5, 10, or 20 μg/ml acetyl LDL together with [^{3}H]oleate as described in Materials and Methods. Cells were then harvested and radioactivity in cholesteryl ester was measured. Cholesteryl ester mass was determined by gas chromatography. Each point is the mean of duplicate determinations. The data fit the regression line with a correlation coefficient of 0.98.

No attempt was made to compensate for possible hydrolysis of cholesteryl [^{3}H]oleate during the incubation because our intent was to measure net cholesteryl ester formation not ACAT activity per se. Because the amounts of cholesteryl ester mass present in macrophages after incubation with oxidized LDL were low, the analytical error in estimating cholesteryl ester mass was greater than with the measurement of [^{3}H]oleate incorporation. For this reason, in most experiments only [^{3}H]oleate incorporation was used to assess cholesteryl ester formation. It should be noted that these assays of cholesterol esterification were not performed under conditions of substrate excess, and hence the observed esterification rate was probably a reflection of both ACAT activity and the availability of cholesterol substrate. The medium was then removed and assayed for ^{125}I after precipitation with 1.5% AgNO$_3$ and 10% trichloroacetic acid (21). The content of degradation products in the medium served as a measure of the total amount of LDL degraded by the cells. Cells were washed three times with PBS with Ca^{2+}, solubilized in 1 ml 0.1 N NaOH, and harvested with a Teflon policeman. An aliquot was taken for measurement of cell-associated ^{125}I radioactivity. Cell lipids were then extracted with chloroform and methanol according to Bligh and Dyer (31). The organic phase was evaporated under N$_2$, redissolved in 60 μl chloroform-methanol 2:1 (vol/vol), and spotted on Whatman K6 250 μ silica gel thin-layer chromatography plates. The plates were developed in hexane-diethyl ether-acetic acid 80:20:1, and lipids were visualized with I$_2$ vapor. Cholesteryl ester zones were scraped into scintillation vials, mixed with Aquasol, and counted in a Beckman LS 7800 scintillation spectrometer. The recovery of cholesteryl ester by this

method was found to be greater than 95%, and hence no correction for recovery was made when results were analyzed. To determine whether changes in cholesterol esterification might have been due to changes in the size or specific activity of the acyl-CoA pool, in three experiments radioactivity in triacylglycerol and phospholipid was also measured, and it was found to remain constant under conditions where cholesteryl [³H]oleate formation increased more than 30-fold.

Analytical methods

Agarose gel electrophoresis was performed using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine serum albumin at a final concentration of 60 μg/ml was added to dilute lipoprotein samples to ensure reproducible migration distances. Protein was estimated by the method of Lowry et al. (32). Free and total cholesterol in LDL were determined by enzymatic kits according to the manufacturer's instructions except that the volumes of all reagents were reduced by one-half.

RESULTS AND DISCUSSION

Oxidation of LDL has previously been shown to cause a significant fall in the content of cholesteryl esters as determined with enzymatic or chemical methods (21, 23). The results shown in **Table 1** indicate that this decrease in cholesteryl ester mass is not accompanied by loss of cholesteryl ester radioactivity in LDL prelabeled with [³H]cholesteryl linoleate. There was no reciprocal increase in the mass of free cholesterol, indicating that the loss of cholesterol ester could not have been due simply to hydrolysis to free cholesterol. One possible explanation for this is that cholesterol and cholesteryl esters had undergone oxidation, and that the oxidized sterols failed to react with the cholesterol esterase or oxidase in the assay system, but remained physically associated with
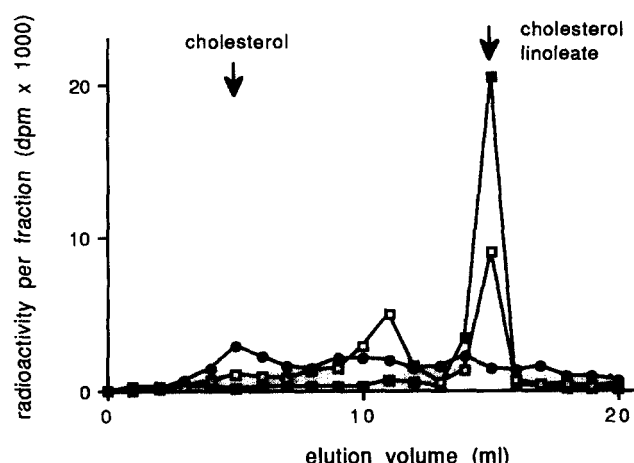

Fig. 2. Analysis of radioactive lipids in oxidized [³H]cholesteryl linoleate-labeled LDL. Lipids were extracted from the control and oxidized LDLs described in Table 1, dried under N₂, and redissolved in acetonitrile–isopropanol 50:50. An aliquot was injected into a Waters HPLC system equipped with a 0.4 cm × 15 cm μBondapak C18 column and eluted at 1 ml/min with acetonitrile–isopropanol 80:20 for 6 min and then a 50:50 mixture of the same solvents for 15 min. The column effluent was collected in 1-ml fractions and assayed for radioactivity. Recovery of radioactivity was 94% or greater. Elution positions of cholesterol and cholesteryl linoleate are indicated with arrows. Control LDL (■); LDL oxidized for 5 h (□); LDL oxidized for 20 h (●).

LDL. This conclusion was supported by the HPLC radiochromatogram of the products derived from [³H]cholesteryl linoleate shown in **Fig. 2**. Oxidation resulted in a progressive decrease in radioactivity from the cholesteryl ester region, and appearance across a broad region corresponding to more polar material. To further characterize the sterols in oxidized LDL, thin-layer chromatography of lipids from oxidized LDL was performed (**Fig. 3**). This confirmed the loss of cholesteryl ester, with the appearance of multiple new bands. The migration positions and color reactions of some of these oxysterol bands matched those of reference oxysterols, including 7-keto-cholesterol, 5,6-epoxycholesterol, and 7-hydroxycholesterol. The spots between cholesterol and triglycerides were

TABLE 1. Analysis of [³H]cholesteryl linoleate-labeled LDL after oxidation

LDL	Electrophoretic Mobility	Total Cholesterol	Free Cholesterol	Recovery of LDL Radioactivity
	cm	*mg/mg protein*	*mg/mg protein*	*%*
Control	0.6	1.50	0.48	79
Oxidized 5 h	1.7	0.84	0.32	87
Oxidized 20 h	2.3	0.48	0.23	77

Human LDL was labeled with [1,2,6,7 (n)³H]cholesteryl linoleate as described in Materials and Methods, and then oxidized by exposing 200 μg/ml LDL to 5 μM CuSo₄ at 37°C for 5 h or 20 h. Further oxidation was arrested by refrigeration and addition of 50 μM butylated hydroxytoluene and 200 μM EDTA. The control LDL was left at 4°C without CuSO₄. Each sample was then washed and concentrated using Amicon CF25 membrane cones, and protein, radioactivity, and free as well as total cholesterol remaining with LDL were measured. Extent of oxidation was monitored by agarose gel electrophoresis. The radioactive lipids in LDL were further analyzed by HPLC as shown in Fig. 2.
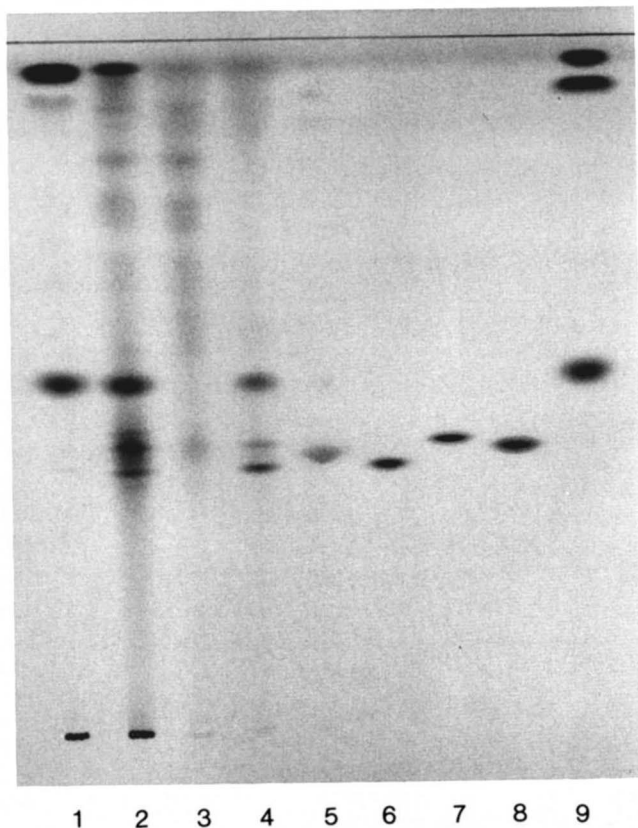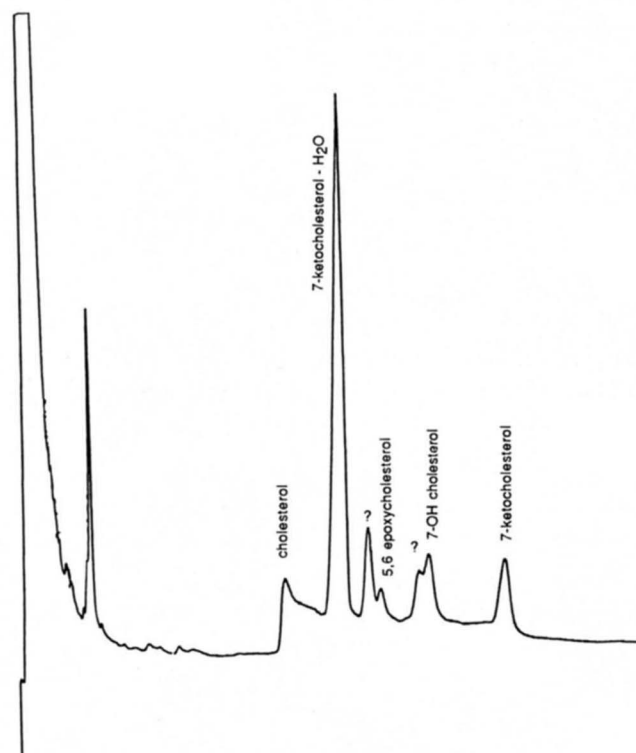
Fig. 3. Thin-layer chromatography of sterols in oxidized LDL. Lipids were extracted from 40 µg native LDL and from 40 µg oxidized LDL, and separated on silica gel G using hexane–acetone acetic acid 80:20:1 (v/v). Cholesterol, cholesteryl oleate, and oxysterols were run as standards. The plate was sprayed with sulfuric acid/FeCl$_2$ reagent and heated to 155°C to reveal the sterol spots. Lane 1: native LDL; lane 2: oxidized LDL; lane 3: rechromatography of oxidized LDL lipids from the zone between cholesterol and triacylglycerol (see lane 2); lane 4: same sample as lane 3 after hydrolysis; lane 5: 100 µg 7-ketocholesterol; lane 6: 4 µg 7-hydroxycholesterol; lane 7: 10 µg 5,6 epoxycholesterol; lane 8: 10 µg 25-hydroxycholesterol; and lane 9: cholesterol, trioleylglycerol, and cholesteryl oleate (in ascending order of R_f).

shown to represent oxidized cholesteryl esters in that after hydrolysis these yielded cholesterol and oxysterols (Fig. 3). When assayed by gas chromatography, the sum of free cholesterol and cholesterol derived from oxidized cholesteryl ester gave a value for total cholesterol that was in good agreement with the result obtained with the enzymatic assay. Because color development with the TLC spray reagent was much less for 7-ketocholesterol than for most other oxysterols, gas chromatography for the oxysterols eluted from the TLC plate was performed to obtain a better estimate of relative amounts present. As shown in **Fig. 4**, this indicated that the predominant oxysterol in oxidized LDL was 7-ketocholesterol. The conditions that were used for gas chromatography did not permit satisfactory separation of 25-hydroxycholesterol from 7-hydroxycholesterol. It was noted that authentic 7-ketocholesterol consistently gave two peaks, even after

repurification by TLC. However, when 7-ketocholesterol was silylated prior to analysis, only a single peak was detected. No oxysterols were detected in cholesterol eluted from the silica gel, indicating that no artefactual oxidation of cholesterol had occurred during chromatography or extraction. To conclusively identify the oxysterols present in oxidized LDL, gas chromatography coupled with mass spectrometry was performed. Spectra obtained after chemical ionization with isobutane showed that the second peak seen with 7-ketocholesterol had the expected m/z of 401 (M + 1) while the first peak had m/z of 383 (M – 17), and hence was apparently a dehydration product generated during gas chromatographic analysis. Although both 25-hydroxycholesterol and 7-hydroxycholesterol had identical retention times, GC–MS showed that the relevant peak in Fig. 4 yielded a fragmentation pattern identical to 7-hydroxycholesterol and did not exhibit the fragment at m/z 367 (M – 35, chemical ionization) seen with 25-hydroxycholesterol.

It has been established that 25-hydroxycholesterol and 7-ketocholesterol stimulate cholesterol esterification in several cell types and that others, such as 22-hydroxycholesterol, are potent ACAT inhibitors (33–35). To deter-


Fig. 4. Analysis of oxysterols in oxidized LDL by gas chromatography. After separation of lipids in oxidized LDL by thin-layer chromatography as shown in Fig. 3, the oxysterol region was scraped and extracted with chloroform–methanol 2:1 (vol/vol). Aliquots were analyzed by gas chromatography as described in Materials and Methods. The identity of the peaks was established by mass spectrometry.
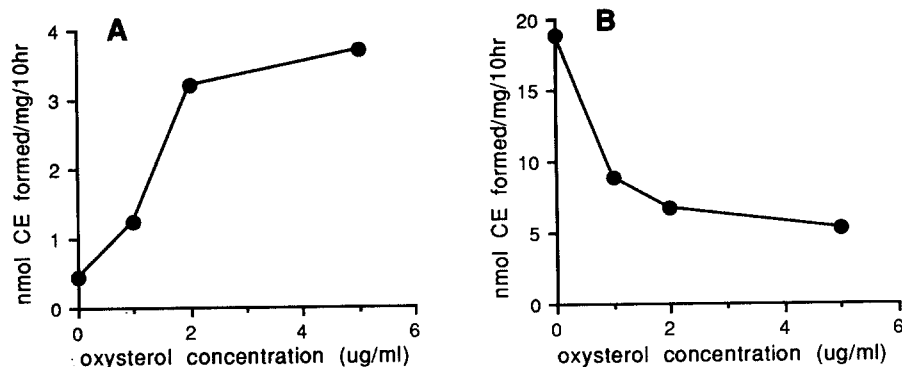
Fig. 5. Effect of oxysterols from oxidized LDL on cholesterol esterification in macrophages. Lipids were extracted from oxidized LDL and separated by thin-layer chromatography as described in Fig. 3. Cultured mouse peritoneal macrophages were incubated for 10 h with [³H]oleic acid together with the indicated concentration of oxysterols (based on 7-ketocholesterol content) from oxidized LDL alone (A) or in the presence of 5 μg/ml acetyl LDL (B). Cell lipids were then extracted and analyzed for content of radioactivity in cholesteryl ester. Each value is the mean of triplicate dishes that varied by less than 12%. All values differ significantly from the control values without oxysterol ($P<0.05$, two-tailed t-test).

mine what the combined effect of the oxysterols present in oxidized LDL would be on cholesterol esterification in macrophages, varying amounts of oxysterols isolated from oxidized LDL were incubated with macrophages together with [³H]oleic acid and the amount of radioactivity in cholesteryl ester was measured in the presence or absence of 5 μg/ml acetyl LDL. As shown in **Fig. 5**, addition of oxysterols from oxidized LDL alone resulted in a modest stimulation of cholesterol esterification, but when the same oxysterols were added in the presence of 5 μg/ml acetyl LDL, cholesterol esterification was inhibited. Possible explanations for this rather paradoxical finding are that the effect of some oxysterols on cholesterol esterification might differ depending on the availability of cholesterol and/or ACAT activity, that some oxysterols might exhibit a biphasic dose–response relationship, and that the amount of oxysterol internalized might be altered by the presence of acetyl LDL. This experiment demon-

strates that oxysterols extracted from oxidized LDL have the potential for inhibiting cholesterol esterification under some conditions, but the results are not equivalent to those with oxidized LDL itself because only oxysterols and not the cholesterol, cholesteryl ester, or oxidized cholesteryl ester components of oxidized LDL were present in the extract. A pattern similar to that seen with oxysterols from oxidized LDL was found with 7-hydroxy-cholesterol, although with most other oxysterols the effects on cholesterol esterification were in the same direction regardless of ACAT activity and substrate pool size (**Fig. 6**). No morphologic evidence of toxicity was observed with any of the oxysterols except 25-hydroxycholesterol and incorporation of [³H]oleate into phospholipid remained constant. Some rounding and detachment of macrophages was seen even with 5 μg/ml 25-hydroxycholesterol but cholesterol esterification was nevertheless increased.
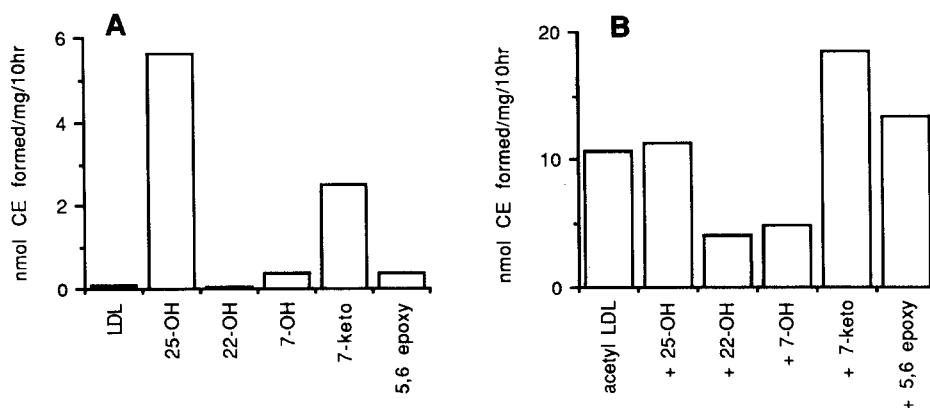

Fig. 6. Effect of individual oxysterols on cholesterol esterification. Cultured mouse peritoneal macrophages were incubated for 10 h with [³H]oleic acid and 5 μg/ml of the indicated oxysterol alone (A), or in the presence of 5 μg/ml acetyl LDL (B). Cell lipids were then extracted and analyzed for content of radioactivity in cholesteryl ester. Each value is the mean of triplicate determinations that varied by less than 15%.
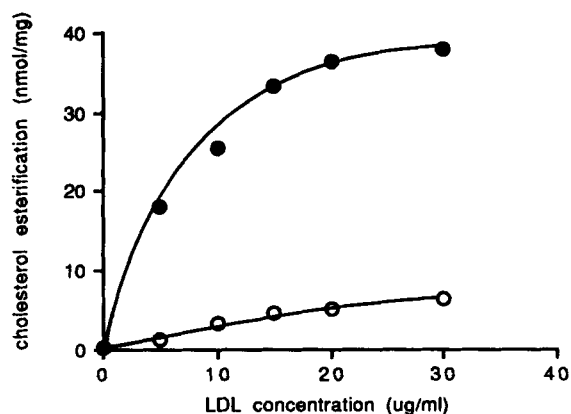
Fig. 7. Effect of oxidized LDL and acetyl LDL on cholesterol esterification. Varying concentrations of oxidized LDL and acetyl LDL were incubated for 10 h with macrophages together with [^{3}H]oleic acid, and then radioactivity in cholesteryl ester was determined.

Previous studies have shown that oxidized LDL can lead to a degree of cholesterol accumulation by phagocytic cells such as macrophages (21), but comparisons of the actual quantity of cholesteryl ester formed with oxidized LDL in comparison to other scavenger receptor ligands such as acetyl LDL have not been reported. **Fig. 7** shows that oxidized LDL resulted in much less cholesterol esterification than the same concentration of acetyl LDL. Even after correction for the amount of unoxidized cholesterol delivered to the cells (by multiplying the cholesterol:protein ratio of each modified LDL by the amount of cell-associated and degraded LDL protein), acetyl LDL was twofold more efficient at stimulating

cholesterol esterification than oxidized LDL (**Fig. 8A**). It was noted that with oxidized LDL, cell-associated ^{125}I radioactivity was approximately equal to the amount degraded, whereas with acetyl LDL cell-associated radioactivity was less than 10% of the amount degraded. A similar finding has previously been reported by Sparrow, Parthasarathy, and Steinberg (36). Immunofluorescence microscopy using a polyclonal antibody to human LDL that has previously been shown to recognize oxidized LDL (23) revealed strong intracellular fluorescence in macrophages after incubation with oxidized LDL, suggesting that most of the cell-associated apoB had been internalized but remained immunologically intact (not shown). It has not yet been determined whether this incomplete degradation of oxidized LDL is due to the failure of some internalized oxidized LDL to be delivered to lysosomes, or to resistance of oxidized LDL to lysosomal proteases. However, it seemed likely that the cholesterol contained in the interalized but nondegraded LDL would not have been accessible to ACAT. When the stimulation of cholesterol esterification was plotted as a function of the amount of cholesterol delivered by LDL that underwent complete degradation, the effects of oxidized LDL and acetyl LDL were very similar (Fig. 8B). These results indicate that the apparently inefficient stimulation of cholesterol esterification by oxidized LDL can be explained in large part by a lower content of cholesterol per LDL particle and altered intracellular processing, resulting in reduced delivery of cholesterol to the ACAT substrate pool. The different oxysterols present in oxidized LDL appear to have opposing effects on choles-
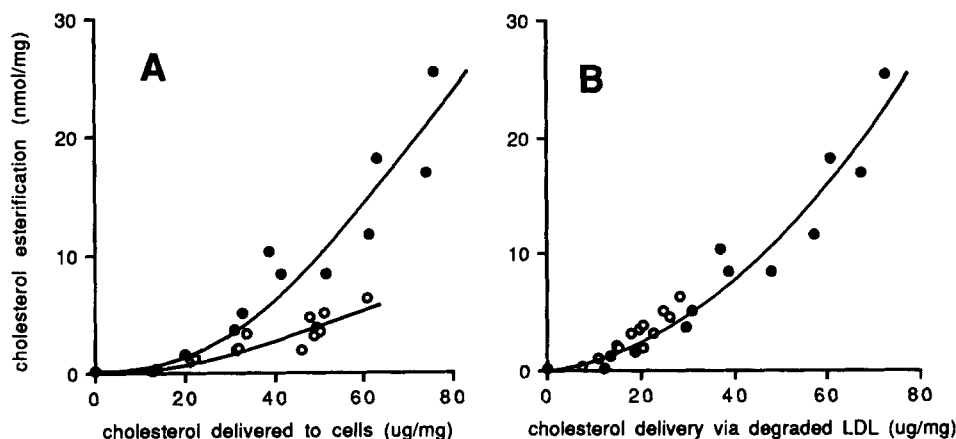
Fig. 8. Correlation of cholesterol delivery to cells with cholesterol esterification. Varying concentrations of ^{125}I-labeled oxidized LDL (O, cholesterol:protein ratio = 0.75 ± 0.11), and ^{125}I-labeled acetyl LDL (●, cholesterol: protein ratio = 1.56 ± 0.06) were incubated with macrophages for 10 h together with [^{3}H]oleic acid. Medium was then assayed for ^{125}I-labeled LDL degradation products and cells were assayed for content of radioiodine and cholesteryl [^{3}H]oleate. More than 95% of the cell-associated radioactivity with oxidized LDL was precipitable with 10% trichloroacetic acid. Cholesterol delivery to cells in panel A was calculated as (μg LDL degraded + internalized) × cholesterol:protein ratio. Only 50% of the total oxidized LDL delivered to cells was degraded, whereas more than 90% of the total acetyl LDL was degraded. Because the cholesterol in LDL that had not undergone degradation may have been unavailable for esterification, cholesterol delivery to the ACAT substrate pool (panel B) was calculated as μg LDL degraded × cholesterol:protein ratio.

terol esterification and, depending on the extent of oxidation of LDL and the relative proportions of oxysterols as well as the availability of cholesterol for esterification, the net result of the oxysterols could be either a modest inhibition or stimulation. Jialal and Chait (37) recently reported that oxidized LDL was associated with a reduction in cholesteryl ester accumulation and an actual inhibition of ACAT activity in cultured human umbilical vein endothelial cells. They concluded that the effect was due to a lipid component of oxidized LDL, and suggested that oxysterols might be implicated. In their experiments, ACAT activity was measured as a 2-h pulse at the end of a 24-h incubation of endothelial cells with modified LDL. Our initial studies with cultured macrophages were performed using a similar protocol wherein cholesterol esterification rate was assayed after a 24-h incubation with modified LDL, and the results also suggested inhibition of ACAT activity by oxidized LDL as well as by several oxysterols. However, the very low ACAT activity that was observed in these experiments did not seem consistent with the finding that a modest amount of cholesteryl ester did accumulate in macrophages incubated with oxidized LDL. This raised the possibility that the apparent suppression of ACAT when measured at the end of a 24-h incubation with oxidized LDL might have been due to toxicity rather than a specific effect on ACAT. No gross cytotoxicity was evident in this experiment or in the study of Jialal in endothelial cells, but only slightly higher concentrations of oxidized LDL or oxysterols induced obvious morphologic signs of toxicity in macrophages. Furthermore, when cumulative cholesteryl ester formation was measured throughout the incubation with modified LDL as described in the present report, the apparent inhibition of ACAT was no longer seen. We therefore concluded that the apparent inhibition of ACAT at the end of the incubation was probably due to a toxic effect on the cells rather than a specific inhibition of ACAT. It should be emphasized that such a cytotoxic effect, whether it be due to oxysterols or other lipid component of oxidized LDL, could have important pathogenetic consequences even in the absence of a specific effect on cholesterol esterification (38-40). ■■

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