Cholesterol deposition in macrophages: foam cell formation mediated by cholesterol-enriched oxidized low density lipoprotein

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Abstract Oxidized low density lipoprotein (LDL) is thought to mediate the transformation of macrophages to cholesterol-rich foam cells. Yet convincing evidence for this process is lacking in vitro. We suggest that oxidized LDL-mediated foam cell formation is not seen in vitro because the cholesteryl ester content of LDL particles (oxidized in the presence of transition metals) is dramatically reduced. Thus, if oxidized LDL could be cholesterol-enriched prior to its addition to macrophages, this lipoprotein would be made more capable of inducing the cellular deposition of cholesteryl esters. When we enriched cupric sulfate-oxidized LDL with cholesterol by incubation of this lipoprotein with unesterified cholesterol/phosphatidylcholine liposomes and added it to mouse peritoneal macrophage cultures, we found that: *a*) the enrichment of oxidized LDL with cholesterol did not alter the extent of oxidized LDL degradation; *b*) the cells accumulated massive amounts of cholesteryl ester (148 μg/mg cell protein) and unesterified cholesterol (260 μg/mg cell protein) after 24 h of incubation; and *c*) Sephacryl S-1000 chromatography of the cholesterol-enriched oxidized LDL verified the formation of large oxidized LDL-unesterified cholesterol/phosphatidylcholine complexes. These results demonstrate that oxidized LDL, when cholesterol-enriched, can mediate the formation of macrophage foam cells in culture.—**Greenspan, P., H. Yu, F. Mao, and R. L. Gutman.** Cholesterol deposition in macrophages: foam cell formation mediated by cholesterol-enriched oxidized low density lipoprotein. *J. Lipid Res.* 1997. **38:** 101–109.

Supplementary key words cholesterol liposomes • oxidized LDL • macrophages • foam cells

Foam cells are prominent features of the atherosclerotic plaque and their transformation from arterial wall macrophages is a key process in the development of this disease (1). The foam cell represents the endstage of a process by which the macrophage accumulates both cholesterol and cholesteryl esters. Atherogenic lipoprotein particles are believed to be responsible for producing this massive accumulation, but the identity of these particles and the mechanism by which they transform arterial macrophages into foam cells are still not well understood.

Steinberg et al. (2) have proposed that oxidized low density lipoprotein (oxLDL) is the atherogenic lipoprotein particle responsible for cellular accumulation of cholesterol and cholesteryl esters in vivo. Indeed, an abundance of oxLDL can be found in the arterial macrophage's in vivo environment (3) and can be efficiently endocytosed by these cells via their scavenger receptor (2). Yet, macrophages have not been shown to accumulate large amounts of cholesteryl esters when incubated with oxLDL in vitro (4–8). Thus it is difficult to understand how foam cells are generated by the lone action of oxLDL in the extracellular plaque environment.

We hypothesized that in order for oxidized LDL to experimentally promote cholesteryl ester accumulation, the modified lipoprotein must first be enriched with cholesterol. In a similar manner, McCloskey, Rothblat, and Glick (9) incubated acetylated low density lipoprotein (AcLDL) with cholesterol-rich phospholipid (PL) liposomes and found that macrophages accumulated more cholesteryl esters in the presence of these particles than from AcLDL alone. As our model system, we enriched oxLDL by pre-incubating the lipoprotein with cholesterol-bearing phosphatidylcholine (PC) liposomes, incubated the mixture with mouse peritoneal macrophage cultures, and examined the cells for cholesterol accretion. The cholesterol-bearing PC liposomes

Abbreviations: LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein; AcLDL, acetylated low density lipoprotein; PC, phosphatidylcholine; TBARS, thiobarbituric acid-reactive substances; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LPDS, lipoprotein-deficient serum; DMEM, Dulbecco's modified Eagle's medium; UC, unesterified cholesterol; PL, phospholipid.

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were of similar composition to those of McCloskey et al. (9). In our study, we show that only the oxLDL-complexed liposomes (and neither oxLDL nor the unesterified cholesterol/phospholipid liposomes themselves) promote significant cellular accumulation of both cholesteryl esters and unesterified cholesterol after a 24-h incubation period.

METHODS AND MATERIALS

Materials

Phosphatidylcholine (hen's egg PC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, cholesteryl ester standards, EDTA, BHT, and the Sephacryl S-1000 chromatography matrix was obtained from Sigma (St. Louis, MO). HPLC grade solvents were purchased from Fisher (Pittsburgh, PA). Culture media and supplements were obtained from Mediatech (Washington, DC). The source of ICR female mice was Harlan-Sprague-Dawley (Indianapolis, IN).

Lipoproteins

Human LDL was prepared by sequential density ultracentrifugation and extensive dialysis against 0.9% NaCl, 0.003% EDTA, pH 7.4, at 4°C (10). LDL was iodinated using the iodide monochloride method (11).

OxLDL was produced by incubating LDL (200 μg protein/ml) in PBS containing 5 μM $CuSO_4$ for 18 h at 37°C and then stopping the oxidation with 40 μM BHT and 20 μM EDTA. The average TBARS value of the oxLDL used in these experiments was found to be 55 nmol/mg protein (12). Preparations were then dialyzed against 0.9% NaCl, 0.003% EDTA, pH 7.4. The mean cholesteryl ester content of four preparations of oxLDL was 0.35 μg/μg LDL protein. This can be compared to the 1.68 μg cholesteryl ester/μg protein of the starting LDL and represents an 80% loss of cholesteryl esters from the particle. LDL oxidation did not produce a significant loss of unesterified cholesterol.

Liposomes

Unesterified cholesterol (UC)/phosphatidylcholine (PC) liposomes were made by first dissolving cholesterol and phosphatidylcholine in an approximate molar ratio of 4:1 in water washed, diethyl ether and then evaporating the solution to dryness under nitrogen followed by 1 h at 60°C in vacuo (13). A suspension was then prepared by adding 6 ml of a 0.9% NaCl, 0.003% EDTA, pH 7.4, solution to the dried lipids (24 mg cholesterol:12 mg PC) and probe sonicating according to the schedule of Glick et al. (13). The sonicate was then centrifuged at 3500 *g* for 30 min and the remaining suspension was 0.45 μm filtered (Gelman Sciences, Ann Arbor, MI). (The substitution of 3500 *g* for the 26,000 *g* spin of Glick et al. (13) did not alter cholesterol deposition in macrophages.) The phosphorus and cholesterol contents of this product were determined by the method of Bartlett (14) and by HPLC (15), respectively. The average final molar ratio of cholesterol:PC in the UC/PC liposomes was 1.6:1.

Macrophages

Macrophages were obtained from ICR female mice by peritoneal lavage. Cells were resuspended in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and plated in either 35-mm or 60-mm tissue culture dishes. The macrophages were allowed to adhere at 37°C in a moist, 5% CO_2/air incubator for 24 h.

Macrophage metabolism of oxLDL and liposomes

In a typical experiment, lipoproteins (oxLDL or native LDL) at a concentration of 200 μg protein/ml were combined with suspensions of UC/PC liposomes containing approximately 2000 μg cholesterol/ml. The volume pipetted of each constituent was dependent on the final ratio of lipoprotein to liposomal cholesterol to be used in the macrophage experiments. Prior to its addition to cells, this mixture was incubated at 37°C for 18 h. Preparations that went without preincubation or were preincubated for a period as short as 2 h, in agreement with McCloskey et al. (9), were less efficient in producing significant cholesteryl ester accumulation in macrophages. In control experiments, oxLDL or UC/PC liposomes were also preincubated for 18 h. Prior to the initiation of experiments, the appropriate concentrations of liposomes and lipoproteins were suspended in DMEM containing 5% lipoprotein-deficient serum (LPDS) and L-glutamine. In a typical experiment, each ml contained 25 μg oxLDL protein and 100 μg of liposomal unesterified cholesterol (a 1:4 protein to cholesterol mass ratio). Dishes were incubated at 37°C in a moist, 5% CO_2/air atmosphere for up to 48 h. The dishes were extensively washed at the end of incubation with PBS containing bovine serum albumin (BSA, 2 mg/ml) followed by PBS alone. The cells were then suspended in PBS by scraping each dish and the suspension was sonicated. An aliquot was removed for protein determination (16). The cellular lipids were extracted into chloroform (17) and analyzed by HPLC (15).

In other experiments, ^{125}I-labeled oxLDL preincubated with or without UC/PC liposomes (a 1:4 protein to cholesterol mass ratio) was added to macrophages

for 6 h at 37°C. Cellular degradation of the ^{125}I-labeled oxLDL was measured by quantitating TCA-soluble, non-iodide radioactivity (18).

HPLC of cholesterol and cholesteryl esters

Cholesterol and cholesteryl esters were quantitated by a modification of the method of Greenspan et al. (15). Briefly, the guard and analytical HPLC columns were composed of 7 μm cyanopropyl Zorbax packing material (Phenomenex, Torrance, CA). Cholesteryl esters were eluted from the column isocratically with heptane at a rate of 1 ml/min, while unesterified cholesterol was eluted with 0.1% isopropanol in heptane. The lipids were quantitated using a Sedex 45 light scattering detector (Sedere, Vitry Sur Seine, Cedex, France) attached to a HP 3394A Integrator (Hewlett-Packard, Avondale, PA). The standard used for macrophage cholesteryl ester analysis was a mix of cholesteryl oleate, cholesteryl linoleate, cholesteryl palmitate, cholesteryl palmitoleate, and cholesteryl myristate (ratio of 40:30:20:5:5) (19). Oxysterols that are present in both oxLDL (cupric sulfate oxidized) and macrophages incubated with oxLDL do not co-elute with either cholesteryl esters or unesterified cholesterol under these conditions.

Determination of [^{14}C]oleate incorporation into cholesteryl oleate

Macrophages were incubated for 24 h at 37°C in DMEM/5% LPDS containing either oxLDL (25 μg protein/ml), UC/PC liposomes (100 μg cholesterol/ml), or preincubates of oxLDL (25 μg/ml) and UC/PC liposomes (100 μg cholesterol/ml). Ten μl of 20 mM [^{14}C]oleate complexed to albumin (0.2 mM final concentration) was added to each dish and the incubation was continued for 2 h (20). The dishes were then extensively washed and [^{14}C]oleate incorporation into cellular cholesteryl oleate was then determined (21).

Sephacryl S-1000 chromatography

^{125}I-labeled oxLDL or ^{125}I-labeled oxLDL combined with UC/PC liposomes was incubated for 18 h at 37°C, suspended in 1 ml of saline-EDTA with 10% LPDS, and subjected to Sephacryl S-1000 chromatography. After elution from the (20 × 1.5 cm) column with 0.01 M Tris, 0.1 M NaCl, 0.1 mM EDTA, pH 7.4, the profile was determined by radioisotopic analysis.

Statistical analysis

Data are presented as the mean ± SE. Significance at the $P < 0.05$ level was determined, where applicable, by the Student's *t*-test.

RESULTS

UC/PC liposomes were preincubated with oxLDL for 18 h at 37°C prior to the initiation of experiments. To determine whether this preincubation altered the cellular degradation of oxLDL protein, macrophages were incubated 6 h with either ^{125}I-labeled oxLDL or ^{125}I-labeled oxLDL preincubated with UC/PC liposomes (at a 1:4 mass ratio). It was found that preincubation of oxLDL with UC/PC liposomes at this mass ratio did not significantly alter the cellular metabolism of oxLDL protein (**Fig. 1**).

The capability of the oxLDL-liposome mixture to promote cellular cholesterol accumulation was then examined. Cells were incubated with oxLDL or native LDL (25 μg protein/ml) in combination with various concentrations of UC/PC liposomes (0–200 μg cholesterol/ml) for 24 h at 37°C in the presence of DMEM containing 5% LPDS. No significant cholesteryl ester (**Fig. 2a**) or unesterified cholesterol (Fig. 2b) accumulation was seen in macrophages incubated in the presence of LDL and UC/PC liposomes, even in the presence of the highest concentration of UC/PC liposomes (200 μg cholesterol/ml). In contrast, incubation of macrophages with oxLDL and UC/PC liposomes resulted in significant cholesterol and cholesteryl ester accumulation, even when incubated in the presence of liposomes containing as little as 50 μg cholesterol/ml. When oxLDL was incubated with higher concentrations of liposomes (200 μg cholesterol/ml), the net choles-

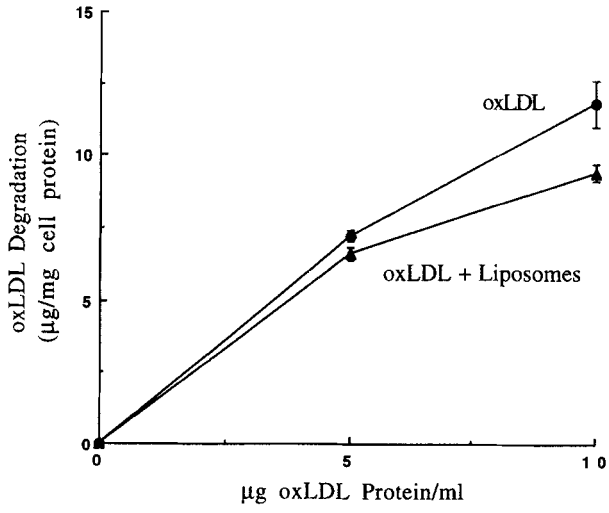

Fig. 1. Macrophage metabolism of oxLDL and oxLDL incubated with UC/PC liposomes. ^{125}I-labeled oxLDL or ^{125}I-labeled oxLDL preincubated for 18 h at 37°C with UC/PC liposomes (at a 1:4 protein to cholesterol mass ratio) was added to triplicate dishes of macrophages containing DMEM and 5% LPDS. The cellular degradation of ^{125}I-labeled oxLDL was determined after a 6-h incubation at 37°C. The results are expressed as the mean ± SE of two separate experiments.

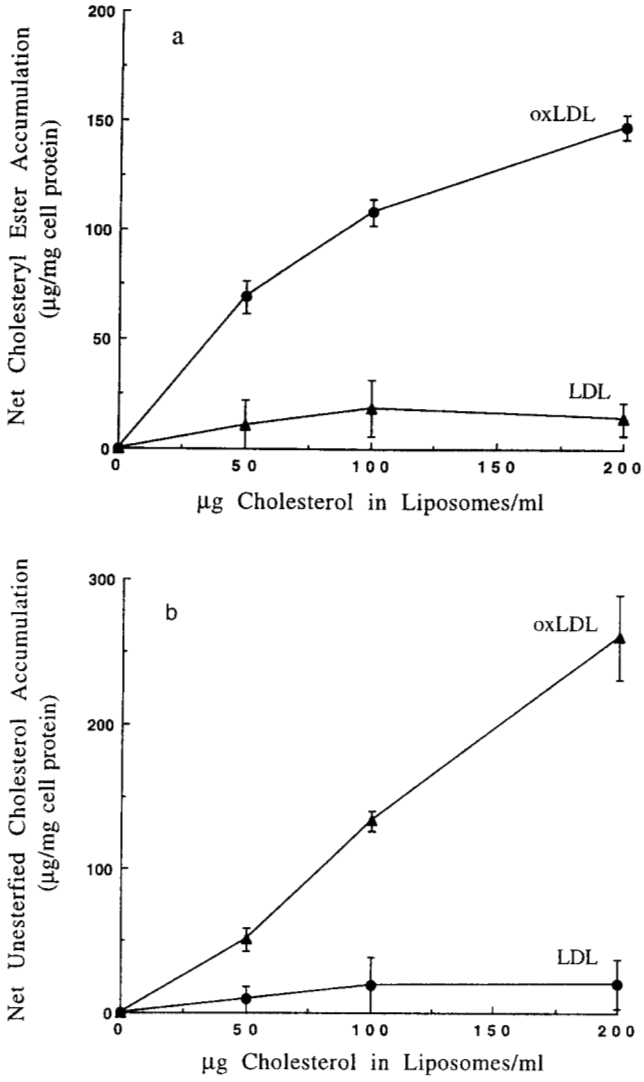

Fig. 2. Cholesteryl ester and unesterified cholesterol deposition in macrophages incubated with oxLDL or LDL in the presence of UC/PC liposomes. OxLDL or LDL was preincubated with UC/PC liposomes for 18 h at 37°C and then added to triplicate dishes of macrophages containing DMEM with 5% LPDS so that the final lipoprotein concentration was set at 25 μg protein/ml and the liposomal cholesterol ranged from 0–200 μg/ml. After 24 h of incubation, the cells were analyzed by HPLC for a) cholesteryl ester or b) unesterified cholesterol content. Baseline macrophage content of unesterified cholesterol was 20 μg/mg cell protein while cholesteryl esters were not detected. The results are expressed as the mean ± SE of three separate experiments. The cholesteryl ester and cholesterol contents of macrophages incubated with the oxLDL and liposomes are significantly different ($P < 0.05$) at all concentrations of liposomal cholesterol.

teryl ester accumulation rose to 148 μg/mg cell protein after a 24-h incubation period (Fig. 2a). In parallel experiments, [^{14}C]oleate:albumin complexes were added to dishes of macrophages after 24 h of incubation with either oxLDL, UC/PC liposomes, or the mixture of oxLDL and UC/PC liposomes. [^{14}C]oleate incorporation into cholesteryl oleate was determined after an additional 2 h of incubation. The incorporation of [^{14}C]oleate into cholesteryl oleate was found to be significantly greater in cultures incubated with both oxLDL and UC/PC liposomes than in cells incubated either with oxLDL or UC/PC liposomes (**Table 1**).

When macrophages were incubated with oxLDL and UC/PC liposomes, the cellular content of unesterified cholesterol was also found to be significantly increased (Fig. 2b). The net accumulation of unesterified cholesterol reached 260 μg/mg cell protein after 24 h of incubation with oxLDL (25 μg protein/ml) and liposomes containing 200 μg cholesterol/ml. This value, though seemingly high, approaches the cholesterol content of cells isolated from human atherosclerotic aortas (22). In control experiments, cells were incubated with oxLDL and UC/PC liposomes for 24 h and then treated with 0.2% trypsin for 10 min. Other cells were scraped from dishes, suspended in PBS, and layered over 10% sucrose (buoyant density 1.03 g/ml) or PBS. Neither trypsinization nor the pelleting of the cells by centrifugation through 10% sucrose (with the intent of stripping UC/PC liposomes from the cell surface) had any effect on the cellular content of cholesterol, strongly suggesting that the accumulation of cholesterol was not a result of the nonspecific binding of the oxLDL-UC/PC liposome mixture to the macrophage cell membrane.

Macrophages were also incubated with a mixture of oxLDL and cholesterol-free PC for 24 h. No significant cholesteryl ester or cholesterol accumulation was seen in these cells. Moreover, when cells were first incubated with the UC/PC liposomes for 24 h and then incubated with oxLDL for another 24 h, no significant accumulation of cholesterol or cholesteryl esters was observed. In fact, when these liposomes and oxLDL were added without preincubation, cellular accumulation of choles-

TABLE 1. Incorporation of [^{14}C]oleate into cholesteryl oleate by macrophages incubated with oxLDL and unesterified UC/PC liposomes

Incubation	Incorporation of [^{14}C]oleate
	μg/mg cell protein
OxLDL	0.81 ± 0.12
UC/PC liposomes	1.38 ± 0.21
OxLDL + UC/PC liposomes	6.13 ± 0.28

Macrophages were incubated in DMEM/5% LPDS containing oxLDL (25 μg protein/ml), UC/PC liposomes (100 μg cholesterol/ml), or preincubates of oxLDL (25 μg protein/ml) and UC/PC liposomes (100 μg cholesterol/ml). After 24 h at 37°C, [^{14}C]oleate:albumin complex was added to each dish. The incubation continued for an additional 2 h and the [^{14}C]oleate incorporated into cholesteryl oleate was then determined. The results are expressed as the mean ± SE of three separate experiments.

terol and cholesteryl esters was found to be negligible. Thus, the mere presence of cholesterol-containing liposomes does not account for the increased cellular content of either the cholesterol or cholesteryl esters.

The time course of cholesterol accumulation in macrophages incubated with oxLDL (25 μg protein/ml) in the presence of UC/PC liposomes (100 μg cholesterol/ml) was examined. When the oxLDL-UC/PC liposomes mixture was added to dishes of macrophages, a significant cholesteryl ester accumulation was observed after 12 h of incubation (**Fig. 3**). The cholesteryl ester content continually increased during the incubation period; its net accumulation reaching 157 μg cholesteryl ester/mg cell protein after 48 h of incubation. The unesterified cholesterol also increased during the first 24 h of incubation, reaching a peak value of 140 μg/mg cell protein (Fig. 3). It is interesting to note that during the second 24-h period, the cholesteryl ester content increased in the presence of a declining unesterified cholesterol level, indicative of a continuing and efficient cholesterol esterification process. It should also be noted that cell number and total cell protein were found to increase during the experiment and to reach maximum by 48 h of incubation, in agreement with the previous findings of the mitogenic nature of oxLDL (23). Cells cultured for 24 h with the oxLDL, UC/PC liposomes were also found to remain more than 98% viable by the trypan blue exclusion test.

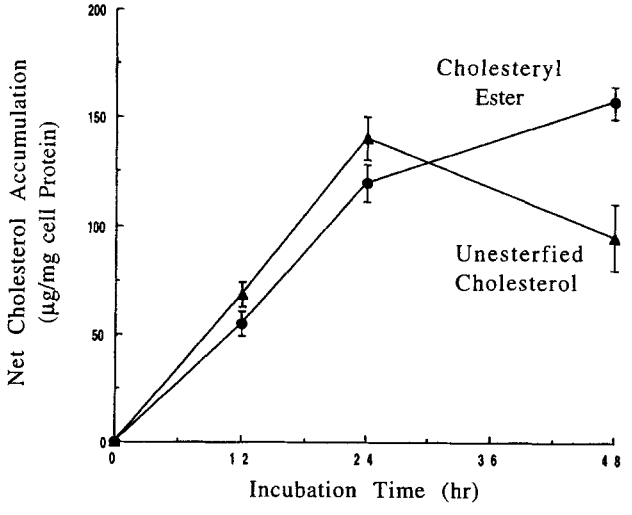

Fig. 3. Time course of cholesteryl ester and unesterified cholesterol deposition in macrophages incubated with oxLDL and UC/PC liposomes. OxLDL was preincubated with UC/PC liposomes for 18 h at 37°C and then added to triplicate dishes of macrophages containing DMEM and 5% LPDS so that final concentration of oxLDL was 25 μg protein/ml and the liposomal cholesterol was 100 μg/ml. After the designated incubation periods, the macrophage content of cholesteryl esters and unesterified cholesterol was determined. The results are expressed as the mean ± SE of three separate experiments.

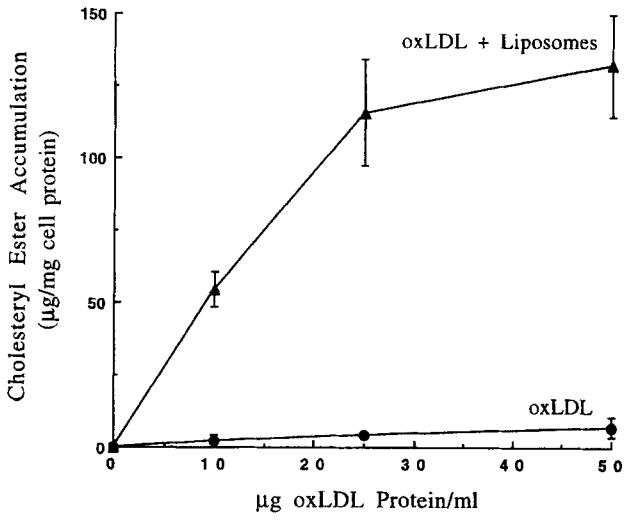

Fig. 4. Effect of oxLDL concentration on the cholesteryl ester deposition in macrophages incubated with oxLDL and UC/PC liposomes. OxLDL was preincubated with UC/PC liposomes for 18 h at 37°C and then added to triplicate dishes of macrophages containing DMEM and 5% LPDS so that the final concentration of oxLDL ranged from 0–50 μg protein/ml and the liposomal cholesterol was set at 100 μg/ml. After 24 h of incubation, the macrophage content of cholesteryl esters was determined. The results are expressed as the mean ± SE of three separate experiments. The cholesteryl ester content of macrophages incubated with oxLDL and liposomes is significantly different ($P < 0.05$) at all oxLDL concentrations.

A third experiment was performed to further define the optimum conditions of macrophage cholesteryl ester accumulation by oxLDL and UC/PC liposomes. In this experiment, different concentrations of oxLDL were preincubated with UC/PC liposomes so that the final liposomal cholesterol concentration was set at 100 μg cholesterol/ml. Macrophages were also incubated with the various concentrations of oxLDL in the absence of liposomes. After a 24-h incubation period, oxLDL, at all concentrations examined, did not produce significant cholesteryl ester accumulation (**Fig. 4**). In contrast, when oxLDL was pre-incubated with UC/PC liposomes, the amount of cholesteryl ester accumulation was found to depend on the concentration of oxLDL present in the culture medium; net cholesteryl ester accumulation was found to be 132 μg/mg cell protein after a 24-h incubation with oxLDL (50 μg protein/ml) and UC/PC liposomes. When these cells were stained with nile red and viewed by fluorescence microscopy (24), the cells were filled with brillantly fluorescent yellow-gold droplets, indicative of massive cholesteryl ester accumulation (data not shown).

Finally, we attempted to analyze the nature of the oxLDL-UC/PC liposome mixture. ^{125}I-labeled oxLDL was preincubated with UC/PC liposomes for 18 h at 37°C. The mixture was then diluted with saline/EDTA containing 10% LPDS so that the final concentration

of oxLDL and liposomes was 100 μg protein/ml and 400 μg cholesterol/ml, respectively. The mixture was then subjected to Sephacryl S-1000 chromatography and compared to the elution profile of ^{125}I-labeled oxLDL alone. As seen in **Fig. 5,** the elution profile of 100 μg oxLDL (measured by radiolabel analysis) was a single peak. Preincubation of oxLDL with UC/PC liposomes reduced the height of the oxLDL peak and caused a significant portion of oxLDL to elute from the column starting at the void volume. This shift in the elution profile of oxLDL indicates that oxLDL and the UC/PC liposomes have complexed.

In later experiments, larger amounts of preincubated oxLDL (500 μg) and UC/PC liposomes (2000 μg cholesterol) were chromatographed on Sephacryl S-1000 to obtain sufficient quantities of fractions I and II (see Fig. 5) for cellular studies. Peak tubes, representing complexes of oxLDL and UC/PC liposomes (fraction I) and oxLDL (fraction II), were collected and analyzed for lipid composition. Fraction I had an unesterified cholesterol to protein ratio of 2.16 (on a μg basis), representing an approximately 5.5-fold cholesterol-enrichment of the oxLDL in fraction II (**Table 2**). To confirm the importance of complex formation in cellular cholesteryl ester accretion, cells were incubated with the purified fractions I and II (at a concentration of 25 μg oxLDL protein/ml). The cholesteryl ester and unesterified cholesterol accumulation in macrophages was found to be much greater after 24 h incubation with fraction I than with fraction II (Table 2). It was found that the tubes intermediary between the oxLDL-liposome complex (fraction I) and oxLDL (fraction II) peaks, with approximately 50% less cholesterol enrichment than fraction I, also produced less cellular cholesterol accretion than fraction I, though more than that observed for fraction II (data not shown).

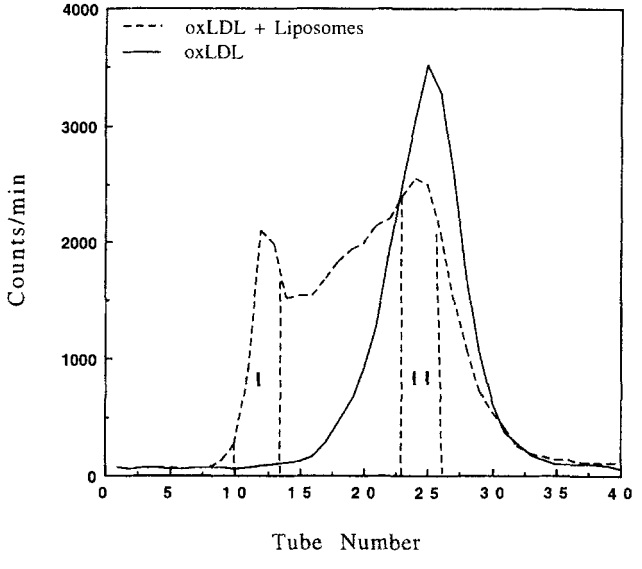

Fig. 5. Sephacryl S-1000 gel chromatography of ^{125}I-labeled oxLDL and ^{125}I-labeled oxLDL incubated with UC/PC liposomes. OxLDL (100 μg protein, with a trace of ^{125}I-labeled oxLDL) was preincubated with either UC/PC liposomes (400 μg cholesterol) or the equivalent volume of 0.9% NaCl, 0.003% EDTA, pH 7.4, for 18 h at 37°C. Then the preincubates were diluted up to 1 ml with saline-EDTA and 10% LPDS before loading the column. The elution profiles of ^{125}I-labeled oxLDL and ^{125}I-labeled oxLDL incubated with UC/PC liposomes were generated by monitoring radioactivity in the collected fractions. The location of the LPDS protein in the elution profile (the reference peak) was determined by absorbance at OD_{280}.

TABLE 2. Cholesteryl ester and unesterified cholesterol (UC) deposition in macrophages incubated with Sephacryl S-1000 fractionated oxLDL-UC/PC liposomes

Fraction Number[a]	Unesterified Cholesterol Content[b]	Net Cholesterol Accumulation[c]	
		Cholesteryl Ester	Unesterified Cholesterol
		μg/mg cell protein	
I	2.16	47 ± 4.7	85 ± 35
II	0.40	15 ± 6.1	—
Unfractionated	4.30	118 ± 7.7	130 ± 25

Macrophages were incubated in DMEM containing 5% LPDS and fractions I and II were collected from Sephacryl S-1000 gel chromatography or the unfractionated mixture consisting of oxLDL and UC/PC liposomes. For all three groups, macrophages were incubated with oxLDL at a concentration of 25 μg protein/ml for 24 h at 37°C. The content of cellular cholesteryl esters and unesterified cholesterol was then determined.

[a]OxLDL (500 μg) (with a trace of ^{125}I-labeled oxLDL) and UC/PC liposomes (2000 μg) were preincubated for 18 h at 37°C before being chromatographed on Sephacryl S-1000. The resulting fractions were eluted with 0.01 M Tris, 0.1 M NaCl, 0.1 mM EDTA, pH 7.4. Fractions I and II represent the two peaks of the elution profile seen in Fig. 5.

[b]OxLDL-liposome mixture expressed as μg cholesterol/μg protein.

[c]The results are expressed as the mean ± SE of three separate experiments.

DISCUSSION

Several previous studies have demonstrated that LDL oxidized by either cupric sulfate or by endothelial cells does not produce significant in vitro accumulation of cholesteryl esters in macrophages (4–8). These results, along with those presented in Fig. 4, demonstrate the inability of oxLDL to promote cholesteryl ester accretion in macrophages. A recent report of Brown, Dean, and Jessup (25) indicates that the majority of the cholesteryl esters in macrophages incubated with oxLDL contain oxidized fatty acyl chains. Previous studies have also demonstrated that UC/PC liposomes also cannot promote significant cholesterol and cholesteryl ester accumulation in macrophages (9, 26). Thus, it is

extremely interesting to note that these two cholesterol-bearing particles, which individually do not promote significant cholesterol accumulation in cells, can, when combined, produce macrophage foam cells. This amplification is not due to an increased or accelerated degradation of oxLDL protein by the cell, but rather is a direct consequence of cholesterol enrichment of the oxLDL (Table 2). It is important to note that the oxidation of LDL (using either a cell-based system or incubation with cupric sulfate) causes the oxidation of the lipid constituents of the particle. In fact, the cholesteryl ester component of LDL is responsible for the majority of aldehydes produced during LDL oxidation (27, 28). The content of cholesteryl esters in oxLDL is significantly decreased when compared to native LDL (28–30) and the density of this particle was found to be 1.07 g/ml, a density outside the classic LDL density range (1.019–1.063 g/ml) (29). While oxLDL is metabolized by macrophages, the total amount of cholesterol that can be delivered to cells by this oxidized lipoprotein is therefore diminished by oxidation.

The results presented in this paper show that the ability of oxLDL to deliver cholesterol to macrophages can be augmented by UC/PL liposome supplementation. While the exact nature of the interaction between oxLDL and UC/PC liposomes is unknown, Sephacryl S-1000 chromatography confirmed the importance of complex formation in the extent of cellular cholesteryl ester deposition; the more the particles were enriched with cholesterol, the greater was the cellular accumulation of cholesterol and cholesteryl esters.

Aggregation of lipoproteins is an important process in the formation of cholesteryl ester-rich foam cells during atherogenesis (31). Tertov et al. (32) reported that certain LDL particles can aggregate in culture medium and these aggregated LDL can promote cholesteryl ester accumulation. In a similar manner, vortexing of LDL (33), phospholipase treatment of LDL (34), and interaction of LDL with negatively charged particles (proteoglycans and cardiolipin) (35, 36) causes the aggregation of this lipoprotein and cholesteryl ester deposition in macrophages. OxLDL has also been shown to undergo aggregation (37). By measuring the electrophoresis in agarose gels of ^{125}I-labeled oxLDL, it was determined that only 17% of the oxLDL preincubated with UC/PC liposomes is aggregated and cannot enter the gel. Liposome-induced aggregation of oxLDL, independent of the formation of the oxLDL-liposomal complex, may well be responsible for some of the cholesterol accumulation seen in these experiments.

The mechanism of the cholesterol and cholesteryl ester accumulation in macrophages incubated with oxLDL-UC/PC liposome mixture remains to be elucidated. In preliminary experiments, the metabolism of oxLDL and UC/PC was studied in the presence (100 μg/ml) or absence of fucoidin, a competitive inhibitor of macrophage metabolism of modified LDL (38). Fucoidin was found to inhibit the accumulation of cholesteryl ester by 56%, suggesting scavenger receptor-dependence of this cellular process (data not shown). Similar results were found when acetylated LDL was preincubated with UC/PC liposomes and then incubated with macrophages (9). It should be noted that surface transfer of cholesterol from the AcLDL-liposome mixture to cells was responsible for an estimated 30% of the total cellular cholesterol accretion (9).

The oxysterols present in oxLDL have been shown to alter the cellular metabolism of cholesterol (39); a relatively large proportion of the cholesterol delivered by oxidized LDL becomes trapped in the lysosome and unable to undergo esterification (40). However, in our studies, cholesterol esterification did not appear to be inhibited. A large accumulation of cholesteryl esters was observed in macrophages incubated with the oxLDL-UC/PC liposome mixture (25 μg oxLDL protein/ml and 100 μg liposomal cholesterol/ml). This mixture contains an unesterified cholesterol/cholesteryl ester ratio of approximately 12 to 1. The appearance of a dramatic increase of cellular cholesteryl esters must have been a result of the cellular esterification of the endocytosed unesterified cholesterol of the oxLDL-liposome complex. When the rate of radiolabeled oleate incorporation into cholesteryl oleate was determined (Table 1), a large increase in cholesterol esterification was observed when compared to cells incubated with oxLDL alone. These results suggest that the inhibition of oxysterols in the oxLDL particle on cholesterol esterification can be overcome by the delivery of a large load of unesterified cholesterol into the cell.

The in vivo relevance of the principal findings of this communication is supported by the existence of both oxLDL and UC/PL liposomes in atherosclerotic lesions. The presence of oxLDL in atherosclerotic aortas has been demonstrated immunochemically (3). Antibodies against oxLDL, such as anti-malondialdehyde-conjugated LDL, or against oxLDL itself (3, 41) show positive immunostaining in atherosclerotic lesions. OxLDL can be extracted from atherosclerotic lesions and is recognized by antibodies specific to malondialdehyde-conjugated LDL (3, 42). In addition to the presence of oxLDL, Chao et al. (43, 44) and Mora, Simionescu, and Simionescu (45) have isolated UC/PL particles from the artery wall. These particles have been visualized by a variety of techniques including filipin staining (46–48), electron microscopy (49), and freeze-fracture microscopy (49). The UC/PL particles were observed in cholesterol-fed rabbits, rats, and monkeys (46), and also in human atherosclerotic lesions (47). It

was also noted that deposits of these particles developed prior to the appearance of cholesterol-rich arterial foam cells in the subendothelial space (48). Recently, it has been shown that similar particles can be obtained when plasma LDL is treated with trypsin and cholesteryl esterase (50) or possibly from lipolysis of very low density lipoprotein remnants (51). These results suggest that the metabolism of lipoproteins is responsible for the formation of these aortic UC/PL liposomes. While the importance of the UC/PL particles in the atherosclerotic process has yet to be proven, the results of the present study suggest that "aortic liposomes", the in vivo analog of these particles, could well play a significant role in the formation of macrophage foam cells in the atherosclerotic lesion.

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