OXIDIZED LOW DENSITY LIPOPROTEIN CAUSES IMPAIRED EFFLUX OF CHOLESTEROL FROM MACROPHAGES:

IMPLICATIONS FOR ATHEROGENESIS

by

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ABSTRACT

Oxidation of low density lipoproteins (LDL) has been implicated as a causal factor in the pathogenesis of atherosclerosis. Oxidized LDL has been found to exhibit numerous potentially atherogenic properties, including receptor-mediated uptake by macrophages. The present study confirms that oxidized LDL is resistant to intracellular degradation and accumulates in a lysosomal compartment in macrophages. Continued accumulation of lipid by macrophages in the arterial intima results in transformation of these cells into lipid-laden "foam cells", which are one of the earliest signs of atherosclerosis. It is believed that high density lipoprotein (HDL) protects against atherosclerosis by removing excess cholesterol from cells of the artery wall, thereby retarding lipid accumulation by macrophages. We investigated the relative rates of HDL-mediated cholesterol efflux in murine resident peritoneal macrophages that had been loaded with acetylated LDL or oxidized LDL. It was found that acetylated LDL-loaded cells released approximately one-third of their total cholesterol to HDL-containing medium, whereas oxidized LDL-loaded cells released only one-tenth of their total cholesterol. To determine whether the impairment of cholesterol efflux was due to modification of the apolipoprotein B or the lipid component of LDL, cells were loaded with either acetylated LDL that had been oxidized for 5 hours or native LDL that had been modified with oxidized arachidonic acid. It was found that both the oxidized lipid component and the modified apolipoprotein B contributed to impaired efflux of cholesterol. To ascertain the proportion of cholesterol efflux mediated by the apolipoprotein component compared to the lipid component of HDL, we measured the rate of efflux when the cells were treated with trypsinized HDL. Very little efflux occurred when the

macrophages were treated with trypsinized HDL, underscoring the importance of intact apolipoprotein in HDL-mediated cholesterol efflux. We also investigated the subcellular distribution of cholesterol in oxidized LDL-loaded cells and acetylated LDL-loaded cells. Our findings suggest that in contrast to acetylated LDL-derived cholesterol, oxidized LDL-derived cholesterol accumulates within lysosomes. The observed impairment of cholesterol efflux in oxidized LDL-loaded macrophages may influence the generation of foam cells *in vivo,* and should, therefore, be considered one of the more important atherogenic effects of oxidized LDL.

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List of Abbreviations

ACAT	acyl CoA-cholesterol acyltransferase
acLDL	acetylated low density lipoprotein
aggreg. LDL	vortex-aggregated LDL
A-OP LDL	arachidonic acid-oxidation product low density lipoprotein
apo A-I	apolipoprotein A-I
apo A-II	apolipoprotein A-II
apo B	apolipoprotein B
BSA	bovine serum albumin
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
Ci	Curies
d	density
Da	daltons
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dpm's	disintegrations per minute
ε	epsilon
EDTA	ethylenediaminetetra-acetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
FC	free (unesterified) cholesterol

HDL	high density lipoprotein
IDL	intermediate density lipoprotein
kDa	kilodaltons
kPa	kilopascals
L	litre
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
lecithin	phosphatidylcholine
LPDS	lipoprotein-deficient serum (isolated from human plasma)
LPS	lipopolysaccharide
lyso-PC	lysophosphatidylcholine
mCi	milliCuries
μCi	microCuries
μl	microlitres
mmol	millimoles
M	molar
MARCO	macrophage receptor with collagenous structure
$NaBH_4$	sodium borohydride
NBD-	6-((N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)hexanoyl)-
nm	nanometers
oxLDL	oxidized low density lipoprotein
ox-acLDL	acetylated LDL that had subsequently been oxidized
PAF	platelet activating factor

PBS	phosphate-buffered saline
PC	phosphatidylcholine
PDGF	platelet-derived growth factor
R_f	relative electrophoretic mobility
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S.E.	standard error of the mean
SR-AI/II	scavenger receptor class A type I/II
SR-BI	scavenger receptor class B type I
SR-CI	scavenger receptor class C type I
TC	total cholesterol
VLDL	very low density lipoprotein
vol/vol	volume per volume
wt/vol	weight per volume

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Dedication

I dedicate this dissertation to my father, Jarnail Singh Dhaliwal, in appreciation of his support and wisdom throughout my life.

Chapter 1 Introduction

1.1 Plasma Lipoproteins and Cholesterol Homeostasis

Cholesterol is one of the essential lipid molecules for animal cells. In the membrane of animal cells, it modulates fluidity and maintains the barrier between cell and environment (1). Excessive levels of unesterified cholesterol in membranes have been shown to result in membrane dysfunction and toxicity (2). Cholesterol is also a precursor for the biosynthesis of various steroid hormones (3). Because unesterified cholesterol is insoluble in water, it is transported, in multicellular organisms, by esterifying the sterol with long-chain fatty acids and packaging these esters within the hydrophobic cores of plasma lipoproteins. With its polar hydroxyl group esterified, cholesterol remains sequestered within this core, which is essentially an oil droplet composed of cholesteryl esters and triglycerides, solubilized by a surface monolayer of phospholipid and unesterified cholesterol and stabilized by protein (Figure 1).

The major classes of lipoproteins are: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Table 1 (4) classifies these proteins based on their flotation density (g/ml) and composition (% mass).

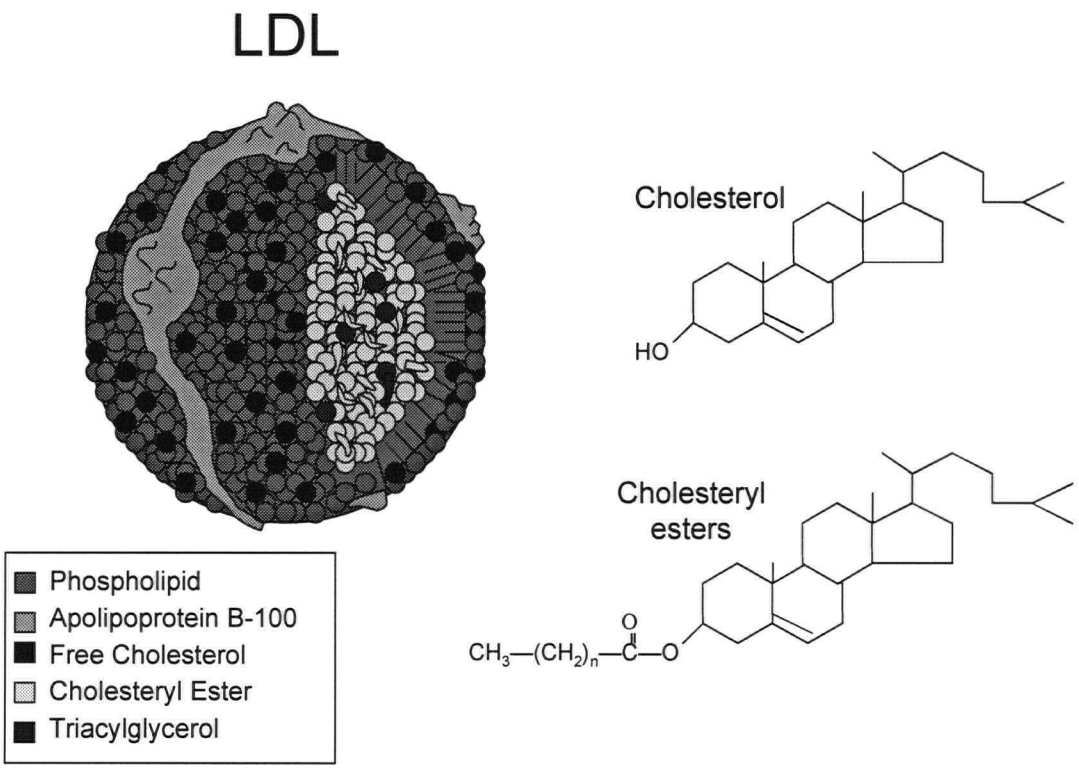


Figure 1 Structure of plasma low density lipoprotein

Low density lipoprotein is a spherical particle with a mass of 3 X 10^6 Daltons and a diameter of 22 nm. Each LDL particle contains about 1500 molecules of cholesteryl ester in an oily core that is shielded from the aqueous plasma by an amphipathic coat composed of 800 molecules of phospholipid, 500 molecules unesterified cholesterol, and one molecule of a 400,000 Dalton protein called apolipoprotein B-100. In addition, approximately 3.5% of the total mass of LDL is composed of triacylglycerols (1).

Table 1 Human plasma lipoproteins classified by density and composition

Density range (g/ml)	Lipoprotein fraction	Protein (%)	FC (%)	CE (%)	PL (%)	TG (%)
0.920<d<0.960	Chylomicron remnants	1.5	2.0	3.0	5.5	87.5
0.950<d<1.006	Very low density lipoprotein	10.4	5.8	13.9	15.2	53.4
1.006<d<1.019	Intermediate density lipoprotein	17.8	6.5	22.5	21.7	31.4
1.019<d<1.063	Low density lipoprotein	25.0	8.6	41.9	20.9	3.5
1.063<d<1.120	High density lipoprotein 2	42.6	5.2	20.3	30.1	2.2
1.120<d<1.210	High density lipoprotein 3	54.9	2.6	16.1	25.0	1.4
1.210<d<1.250	Very high density lipoprotein	62.4	0.3	3.2	28.0	4.6

Free cholesterol is abbreviated FC; cholesteryl ester is abbreviated CE; phospholipid is abbreviated PL; and triacylglycerol is abbreviated TG. The composition of each lipoprotein class is expressed as a percent of total mass. VLDL is also referred to as pre-β-lipoprotein, LDL as β-lipoprotein and HDL as α-lipoprotein, using an older system of nomenclature based on electrophoretic migration rate in agarose (4).

Chylomicrons and VLDL are specialized for transport of triacylglycerols while the other lipoproteins carry cholesterol and cholesterol esters as their predominant neutral lipids. Chylomicrons carry absorbed dietary fat from the small intestine to other organs, whereas VLDL mainly carries triacylglycerol from the liver to other organs. VLDL is eventually converted to IDL and then to LDL, after the triacylglycerol is removed. IDL particles are at an important branchpoint in lipoprotein metabolism because they may be cleared from the circulation by the liver or they may be further processed to become LDL, which is the major transporter of cholesterol to human organs. HDL, on the other hand, is involved in

cholesterol efflux from tissue sites, as well as the transport of cholesterol from peripheral tissue to the liver for ultimate catabolism and excretion. HDL's role in "reverse cholesterol transport" is discussed in section 1.2. Since many of the biochemical properties of lipoproteins are a function of their apolipoprotein composition, a list of the apolipoproteins is provided in Table 2 (4).

Table 2 Major human plasma apolipoprotein

Apolipoprotein	**Protein molecular weight (kDa)**	**Lipoprotein distribution**
Apo A-I	28.1	HDL, Chylomicrons
Apo A-II	18.6	HDL
Apo A-IV	43.3	HDL, Chylomicrons
Apo B-48	240.8	Chylomicrons
Apo B-100	512.7	VLDL, LDL
Apo C-I	6.6	VLDL, HDL
Apo C-II	8.2	Chylomicrons, VLDL, HDL
Apo C-III	8.8	Chylomicrons, VLDL, HDL
Apo D	19.3	HDL
Apo E	34.2	Chylomicrons, VLDL, HDL

In general, cells outside the liver and intestine obtain cholesterol from plasma LDL rather than by synthesizing it *de novo*. As depicted in Figure 2 (5), the lipoprotein metabolism pathways in humans can be divided into exogenous and endogenous pathways, dealing with the transport of dietary and hepatically derived lipids, respectively (4).

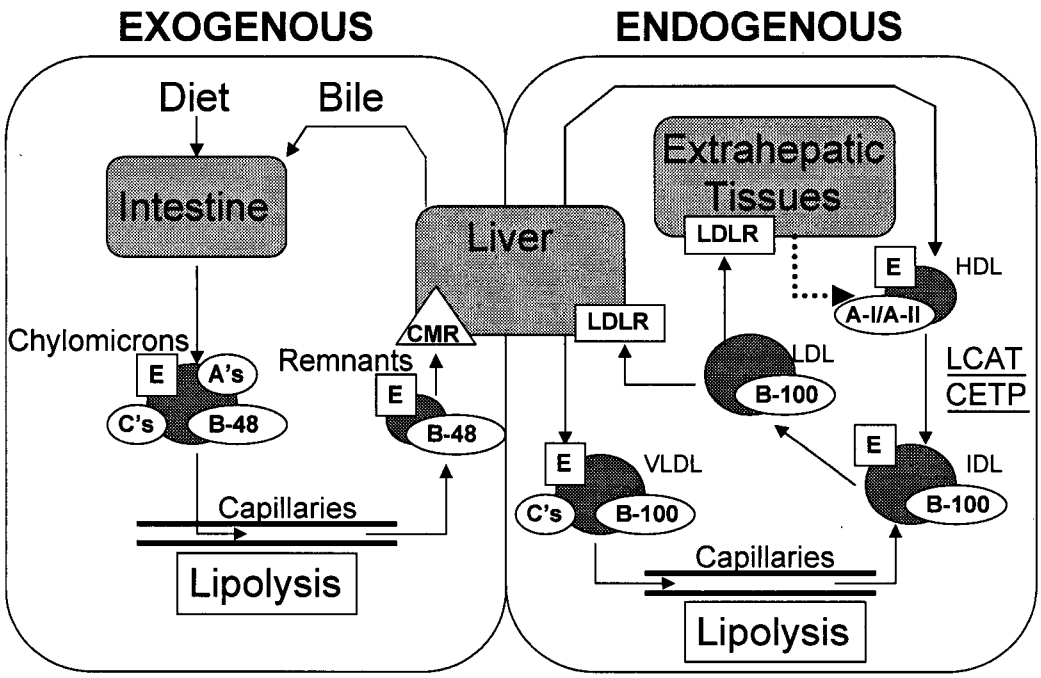


Figure 2 Pathways for lipoprotein metabolism in humans

The liver is the crossing point of the exogenous pathway dealing with dietary lipids and the endogenous pathway that starts with hepatically synthesized lipoproteins. The exogenous branch begins with production of chylomicrons, whereas the liver synthesizes VLDL. Apolipoproteins are denoted by E, C, B-48, B-100, A-I and A-II. Other abbreviations are as follows: Remnants, chylomicron remnants; LDL, low density lipoprotein; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; CMR, chylomicron remnant receptor; and LDLR, LDL receptor. (5)

Regulatory mechanisms have evolved to maintain the concentration of free (unesterified) cholesterol in cell membranes within narrow limits. Receptors for LDL, which are located on the surface of cells, bind to LDL and carry it into the cell by receptor-mediated endocytosis. The internalized lipoproteins are delivered to lysosomes where their cholesteryl

esters are hydrolyzed by acid cholesterol ester hydrolase, thereby liberating free cholesterol (6-11). The free cholesterol crosses the lysosomal membrane and enters a cytosolic pool accessible to the endoplasmic reticulum enzyme acyl CoA-cholesterol acyltransferase (ACAT), as depicted in Figure 3 (1, 12). Some of the free cholesterol is transported to the plasma membrane, and the remainder is esterified by ACAT and deposited in lipid droplets (13). A dynamic equilibrium is maintained by a continually active neutral cholesterol ester hydrolase, that releases free cholesterol from the droplets. In addition to increasing ACAT activity, the free cholesterol content of cells having an active LDL pathway is regulated in two ways. First, the released free cholesterol (or an oxygenated derivative of it) suppresses the transcription of the gene for 3-hydroxy-3-methylglutaryl CoA reductase, thereby blocking *de novo* synthesis of cholesterol (14, 15). Second, the native LDL receptor itself is subject to feedback regulation. When cholesterol is abundant inside the cell, new LDL receptors are not synthesized, and so the uptake of additional cholesterol from native LDL is blocked (16, 17). This allows cells to adjust the number of LDL receptors to provide sufficient cholesterol for metabolic needs without causing cholesterol overaccumulation (18). Under normal conditions, these regulatory mechanisms allow cells to keep their level of unesterified cholesterol remarkably constant despite wide fluctuations in cholesterol requirements and exogenous supply.

There are many important species differences in the activity of the cholesterol ester cycle, and it has been shown to be very rapid in murine cells, intermediate in the rat, and very slow in the rabbit (19). Comparisons of cholesterol ester turnover with human macrophages have not been reported, but it has been demonstrated that both THP-1 cells and human

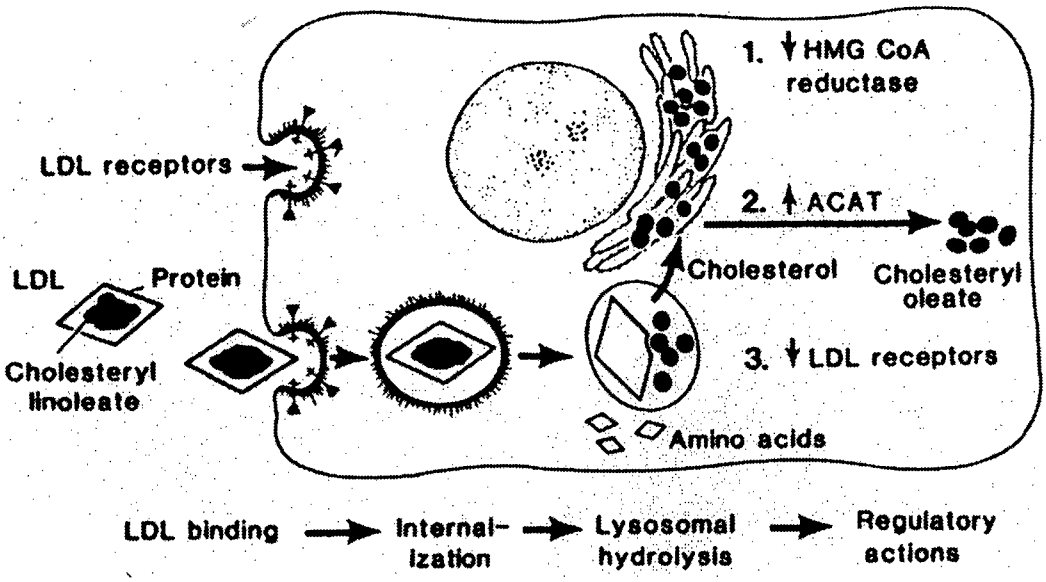


Figure 3 Sequential steps in the LDL receptor pathway of mammalian cells

Acyl-CoA:cholesterol acyltransferase is abbreviated as ACAT. 3-hydroxy-3-methylglutaryl CoA reductase is abbreviated as HMG CoA reductase. Vertical arrows indicate the directions of regulatory effects. (12)

monocyte-derived macrophages express hormone-sensitive lipase (HSL) mRNA (20). HSL is capable of hydrolyzing cholesteryl esters at approximately the same rate as triacylglycerol.

Isolated macrophages have few native LDL receptors and as a consequence, take up very little native LDL. As discussed in Section 1.3, some cells, including macrophages, express scavenger receptors on their cell surface, which recognize and take up modified LDL. Scavenger receptors, unlike the native LDL receptor, do not appear to be regulated by cellular sterol levels. Consequently, cholesterol delivery to these cells via scavenger receptors may depend solely on the presence and concentration of modified lipoproteins in the extracellular space. Studying the unregulated uptake of oxidized LDL by scavenger receptors on macrophages is of particular importance to atherosclerosis because such uptake can lead to cholesterol overaccumulation within these cells.

1.2 HDL-mediated cholesterol efflux

Animal cells other than hepatocytes and steroidogenic cells are incapable of catabolizing cholesterol, with the exception of the partial contribution made by the enzyme sterol 27-hydroxylase (21, 22). Hence, most of the body's excess cholesterol must be removed from the cells in tissues and transported to the liver for biological degradation to bile acids. Moreover, cholesterol efflux from macrophages within the arterial intima is essential to inhibit progression and cause regression of atherosclerosis (23-25). HDL is thought to play a central role in "reverse cholesterol transport", the pathway by which cholesterol is transported from extrahepatic cells to the liver from which it may be recycled to extrahepatic cells or excreted into the intestine in bile (3, 26). Furthermore, numerous population studies have shown an inverse correlation between risk for cardiovascular disease and plasma HDL levels (27-29), leading to the widely held view that HDL protects against atherosclerosis.

The removal of cholesterol from cells is the first and presumed rate-limiting step in reverse cholesterol transport. Reverse cholesterol transport is equally as important as forward cholesterol transport in maintaining intracellular cholesterol homeostasis in the animal cell. However, unlike the native LDL receptor pathway, the mechanism for the removal of cellular cholesterol has not been fully characterized (30). There are two proposed mechanisms of cholesterol efflux from cells (Figure 4) (3). The first is based on the fact that cholesterol is exchangeable between lipoproteins and cellular membranes by diffusion through the aqueous phase surrounding cells (31, 32). The net efflux of cholesterol can therefore be achieved by the gradient of cholesterol content between the cell's plasma

membrane and the extracellular HDL surface (33-35). This gradient is increased and maintained by the enzyme lecithin:cholesterol acyltransferase (LCAT) (Figure 5) (36-39). LCAT, as part of plasma HDL, converts cholesterol to nondiffusable cholesteryl esters that cannot recycle back to the cell (40). This process of cholesterol efflux is achieved without direct contact between the HDL particle and the plasma membrane (41). Other structural factors such as membrane lipid and protein compositions and shape of the acceptor lipoprotein particles may also influence the rate of cellular cholesterol efflux by this pathway (42-49). The specific function of HDL would therefore be attributed to its reactivity to LCAT, and also to its unique structure that may allow the particle to accommodate more cholesterol than other lipoproteins. This view, however, has been challenged by a second proposed mechanism of cholesterol efflux from cells, which operates when HDL interacts with a specific binding site on the surface of the cell (50-53). Many attempts have been made to identify a binding site for HDL (54-56); it is thought that the major function of the murine scavenger receptor class B type I (SR-BI) is to mediate the uptake of HDL cholesterol ester by the liver and steroidogenic tissues (57, 58). According to this hypothesis, it is thought to function as an HDL receptor, to allow the selective uptake of HDL cholesterol esters, without having to internalize the entire HDL molecule (59, 60). It has also been demonstrated that the SR-BI promotes cellular cholesterol efflux (61, 62). Although no single membrane protein has been conclusively identified as a mediator for cellular cholesterol efflux, specific cellular responses to the interaction with HDL have been implicated, supporting the idea for the presence of a specific cellular site of interaction. These cellular responses include translocation of intracellular cholesterol to the cell surface

(63-66), activation of protein kinase C (67-69), and other signal transduction pathways (70-72).

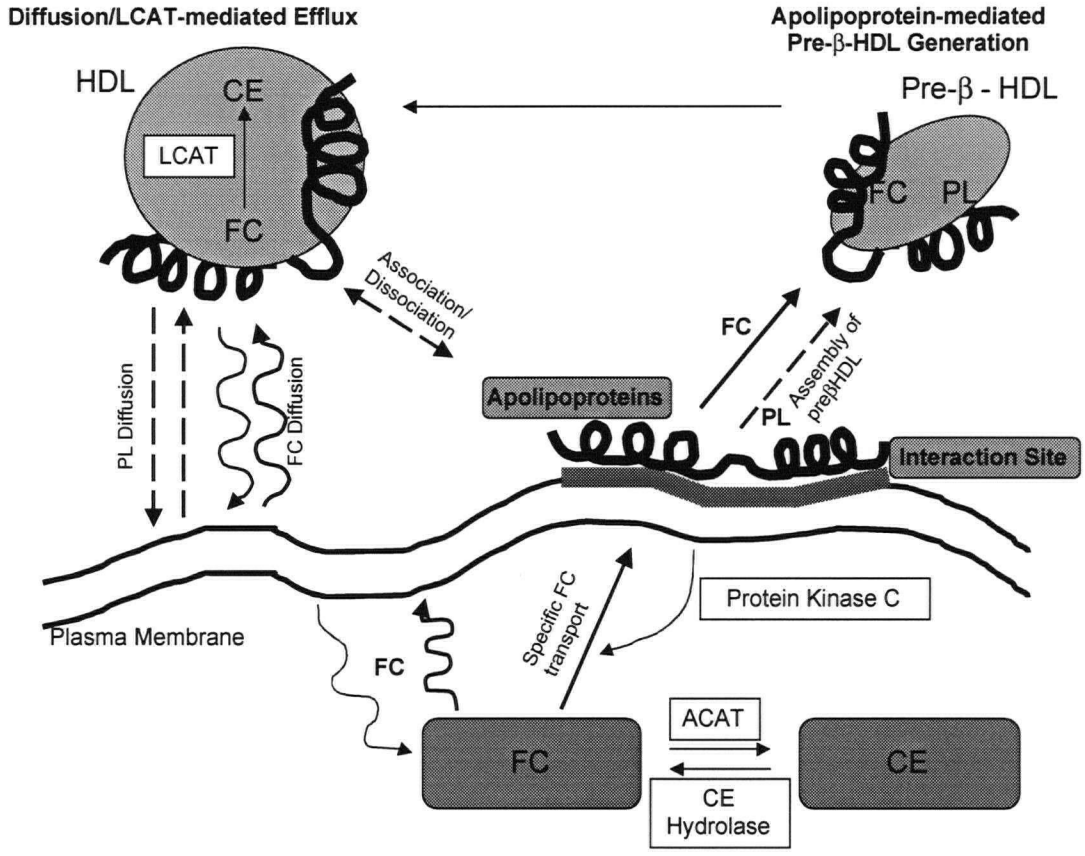


Figure 4 Overview of cellular cholesterol efflux

The left half represents diffusion-mediated efflux. Free cholesterol (FC) diffuses through the aqueous phase between plasma membrane and HDL. Phospholipid also diffuses through the aqueous phase between plasma membrane and HDL, but to a much lesser extent than FC. Lecithin:cholesterol acyltransferase (LCAT) reduces free cholesterol (FC) on the HDL surface to cholesteryl ester (CE), in order to maintain the gradient of free cholesterol. The right half of the figure represents generation of pre-β-HDL with extracellular apolipoproteins and cellular cholesterol and phospholipid. Apolipoproteins are thought to be released from HDL to interact with a specific site on the cell surface and intracellular cholesterol subsequently mobilized is utilized to generate pre-β-HDL by this reaction. Pre-β-HDL generated can be converted to regular HDL by various reactions such as free cholesterol exchange with other lipoproteins, or the LCAT reaction followed by CETP-mediated lipid exchange. (3)

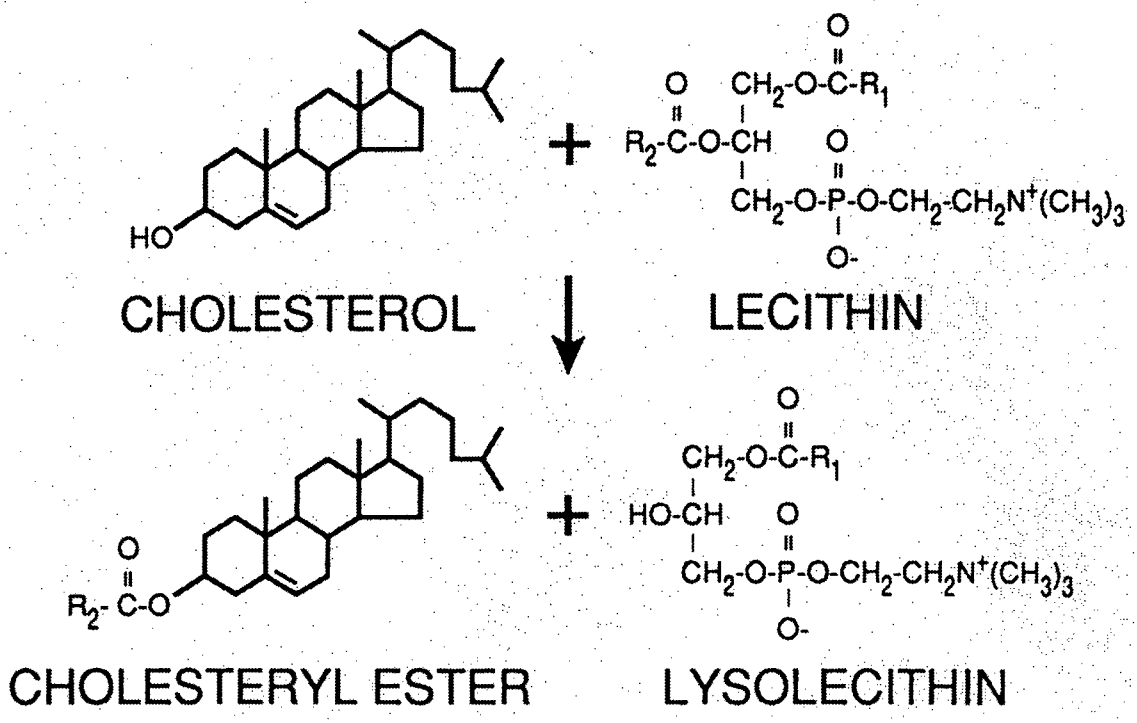


Figure 5 The LCAT reaction between free cholesterol and lecithin (phosphatidylcholine).

Cholesteryl ester is retained in the lipoprotein core while lysolecithin (lysophosphatidylcholine) is released and subsequently binds to albumin.

The relative contribution of the two mechanisms of HDL-mediated cholesterol efflux depends on the cholesterol content and growth state of cells. In rapidly proliferating cells that have been cholesterol-depleted, the number of apolipoprotein binding sites is at a minimum (51, 65, 73, 74) and apolipoprotein-mediated phospholipid and cholesterol efflux is relatively low (65, 75, 76). Conversely, arresting the growth of cells and loading them with cholesterol increases the number of apolipoprotein binding sites and enhances apolipoprotein-mediated cholesterol and phospholipid efflux (65, 73-75). Thus, with

proliferating cells grown in the presence of serum, the aqueous diffusion mechanism accounts for most of the cholesterol efflux that occurs in the presence of HDL, especially at high serum concentrations (77). However, with quiescent and differentiated cells, the apolipoprotein-mediated mechanism contributes significantly to the total HDL-mediated cholesterol efflux and may be the major process for clearing cholesteryl esters from cells (78).

Since the ability to eliminate cholesterol from extrahepatic cells is fundamental to life, it would be surprising if the process depended on a single extracellular acceptor. It has been hypothesized that all subpopulations of HDL, and most HDL apolipoproteins, are able to function as acceptors of cell cholesterol. The HDL fraction in human plasma is heterogeneous, comprising several discrete subpopulations of particles of varying size, density, electrophoretic mobility and lipid and apolipoprotein composition. Nondenaturing gradient gel electrophoresis separates HDL on the basis of size into at least five distinct subpopulations. HDL may also be subdivided into three subpopulations of apolipoprotein-specific particles: one with particles containing apo A-I but no apo A-II (A-I HDL), another with particles containing both apo A-I and apo A-II (A-I/A-II HDL), and a minor subpopulation of particles containing only apo A-II (A-II HDL). In terms of electrophoretic mobility, most HDL are α-migrating but there are also minor subpopulations of particles with pre-β or γ mobility. There is evidence that, in terms of effectiveness as acceptors of cell cholesterol, A-I HDL are superior to both A-I/A-II HDL and A-II HDL, and that pre-β-HDL (and possibly γ-HDL) are superior to α-HDL (38, 46, 77). Although apolipoproteins completely free of lipid have not been identified *in vivo,* those containing a relatively small amount of phospholipids (10-40% of the total weight) are present in plasma and lymph (77).

The best characterized of these is pre-β1-HDL a small particle containing only apo A-I and some phospholipid (24). The intracellular space of large arteries is known to contain a relatively high concentration of pre-β-HDL (79). Of the "classical" lipoprotein fractions, it has been proposed that HDL_3, which is predominantly composed of A-I HDL and A-I/A-II HDL, is the major promoter of cholesterol efflux (80-82). It is for this reason that all of the experiments presented in this thesis used HDL_3 as the cholesterol acceptor, unless otherwise stated.

1.3 The Pathogenesis of Atherosclerosis

Atherosclerosis is a chronic progressive disease that affects large and medium-sized elastic and muscular arteries. The name is derived from the Greek *athero* meaning paste and *sclerosis* meaning hardening. In the early stages of the disease, lipid (principally cholesterol) is deposited in the arterial intima. Lipoprotein transport into this subendothelial space is a concentration-dependent process and does not require receptor-mediated endocytosis (83, 84). Once low density lipoprotein (LDL) particles become trapped by proteoglycans within the arterial intima (85), they can undergo progressive oxidation since the intima lacks many of the antioxidant defense mechanisms that are present in the bloodstream (86-90). Initially, macrophages play a protective role by removing the modified LDL molecules via scavenger receptor-mediated endocytosis (91-93). Continued uptake of this modified LDL results in macrophage foam cell formation (94-96). As these cells die, they can release potent cytokines and oxidizing agents which can lead to smooth muscle cell proliferation, continued oxidation of incoming LDL, and recruitment of monocytes and T-lymphocytes into the

expanding intimal space (97, 98). There is a complex interplay of cellular signals (cytokines, growth factors, etc.) generated between the cells of the atherosclerotic lesion which influence each step of lesion progression, including differentiation of monocytes to macrophages (99). In addition, smooth muscle cells migrate from the media into the intima, where they begin to take up lipoproteins (100) and secrete large amounts of extracellular matrix (101-103). According to this model, these and other inflammatory events culminate to exacerbate lesion progression, resulting ultimately in the formation of atheromatous plaques or atheromas. Such plaques obstruct the blood flow and weaken the vessel wall, leading to thrombosis, internal blood coagulation, and aneurysms, where the internal wall is locally dilated. Also, plaques can rupture from the arterial wall and be washed downstream, where they lodge in smaller arteries. When an artery that feeds the heart, brain, or peripheral vasculature is occluded, it can result in myocardial infarction, stroke, or gangrene, respectively.

Atherosclerosis remains the principal cause of death in North America, Europe, and much of Asia (104, 105). Numerous risk factors that increase the likelihood of atherosclerosis have been identified. Four risk factors that can not be modified are age, estrogen deficiency (including male gender), family history of vascular disease, and personal history of symptomatic vascular disease. Some modifiable risk factors that are also causally related to atherosclerosis and its clinical sequelae include: smoking, hypertension, diabetes mellitus, obesity, sedentary lifestyle, psychosocial stress, and abnormal plasma lipoprotein levels (106). Investigating the mechanism of action of the risk factors and their interplay with a number of genes has increased our understanding of the complex interactions of circulating blood proteins, lipoproteins, growth factors, cytokines, vasoregulatory molecules, extracellular matrix proteins, and the cells of the artery wall (106).

Perhaps the most important and best studied of the major risk factors for atherosclerosis is hypercholesterolemia. In humans, LDL is the major transporter of cholesterol in blood, and it is the source of most of the cholesterol found in atherosclerotic lesions (1, 4, 11, 18, 107-113). In contrast, high density lipoprotein (HDL) acts as an extracellular cholesterol acceptor, thereby allowing extrahepatic cells to unload excess cholesterol, and this is consistent with the known protective effect of HDL cholesterol (3, 19, 30, 61, 65, 114, 115). In early lesions, much of the cholesterol is found as cytoplasmic lipid droplets within macrophages, and it is believed that cholesterol accumulation within these cells is an important initial step in the formation of more advanced lesions (116-122). The mechanism by which macrophages in the arterial intima accumulate massive amounts of cholesterol in patients with hypercholesterolemia is not readily apparent, as the activity of the LDL receptor is regulated by intracellular cholesterol content, and incubation of cultured macrophages with high concentrations of normal plasma LDL fails to bring about lipid accumulation (123, 124). However, subsequent studies showed that certain chemical modifications of LDL lysine residues, such as acetylation, converted it to a form that was recognized by a specific receptor on macrophages termed the acetyl LDL receptor or scavenger receptor class A type I/II (SR-AI/II) (123, 125). This scavenger receptor, unlike the native LDL receptor, is not down-regulated when the cholesterol content of the cell increases (123). Hence, continued uptake of modified LDL by this receptor allows massive cholesterol deposits to accumulate in macrophages *in vitro* (123, 126, 127).

Because there is no evidence that acetylation of LDL occurs *in vivo*, the search began for a biologically plausible modification of LDL that could account for foam cell formation and the initiation, or at least acceleration, of the atherosclerotic process (128). "Physiologic"

modifications of LDL that have been reported to facilitate foam cell formation include oxidation (87, 88, 128-130), glycation (131-133), aggregation (134, 135), association with proteoglycans (136-138), and incorporation into immune complexes (139-145). While there is evidence that all of these modifications may occur *in vivo,* oxidative modification has been the most extensively studied and there is increasing evidence that it plays a significant role in the pathogenesis of atherosclerosis (97, 128).

Arterial endothelial cells and smooth muscle cells are capable of inducing oxidative modification of LDL, and LDL modified in this fashion has been shown to bind with high affinity to the SR-AI/II on macrophages (86, 146-153). However, more recent studies have shown that uptake of oxidized LDL in macrophages is only partly attributable to the SR-AI/II. These studies have shown that as much as 70% of the uptake of oxidized LDL cannot be accounted for by the SR-AI/II alone (154). The search for additional receptors for oxidized LDL has lead to the identification of a number of cell membrane proteins that can bind oxidized LDL in both transfected cells and ligand blots (155). Table 3 lists some of these receptors along with the cell/tissue in which they are found, their putative functions, and their ligand specificity.

Table 3 Classification of scavenger receptors

CLASSIFICATION	CELL/TISSUE	PUTATIVE FUNCTION	LIGAND FEATURES	REFERENCES
Class A SR				
SR-AI/II	macrophages	innate immunity, cell adhesion, oxidized LDL uptake	polyanions, LPS	(10, 125, 156-171)
MARCO[§]	spleen macrophages	innate immunity?	acetyl LDL, bacterial components	(172-174)
Class B SR				
CD36	platelets, monocytes, endothelial cells, retinal pigment epithelium	multiple	native or modified lipoproteins	(175-187)
SR-BI/CLA-1	adrenal, gonads, liver	cholesterol transport		(57-60, 178, 181, 188-192)
others				
SR-CI	embryonic insect macrophages	innate immunity? phagocytosis of apoptotic cells?	polyanions	(193, 194)
macrosialin/CD68	macrophages/ Kupffer cells	abundant in lysosomes, function unknown	polyanions, phosphatidyl serine liposomes, apoptotic cells	(195-198)
Fcγ receptor	macrophages	immune complex binding	IgG	(142, 143, 199-201)
LOX-1[#]	endothelium	signaling?	polyanions, oxLDL	(202)
SREC*	endothelium	signaling?	acetyl LDL, oxLDL	(203)

[§]macrophage receptor with collagenous structure

#lectin-like oxidized LDL receptor
*scavenger receptor expressed by endothelial cells

Although all of these receptors can bind to and take up oxidized LDL, it is not known whether this is an important physiological function of any them *in vivo*. The efforts to isolate and characterize receptors for oxidized LDL have led us to a greater understanding of the potential role of such receptors in atherosclerosis. Identification of a receptor that functions primarily to take up oxidatively modified lipoproteins will afford the opportunity to further investigate the contribution of oxidized LDL to the development and progression of atherosclerosis.

1.4 Mechanisms of Oxidation and Structural Changes Associated with LDL

The physico-chemical changes that occur in the LDL particle that impart recognition by one or more scavenger receptors for oxidized LDL are very complex and incompletely understood. Oxidation of LDL by cells involves a transition metal-catalyzed, free radical-mediated lipid peroxidation process in which the polyunsaturated fatty acyl residues in the LDL lipids are degraded to a variety of peroxidation products (204). These products include hydroperoxy and hydroxy fatty acids (205-208), aldehydes and hydroxyaldehydes (169, 209), and more complex aldehydes (169). The aldehydic lipid peroxidation products that are formed are highly reactive and can derivatize the free amino groups of apolipoprotein B (86, 210). Such derivatization of the lysine ε-amino groups on apolipoprotein B results in an increased negative charge on the LDL particle, which is believed to be responsible for the

interaction of oxidized LDL with the scavenger receptor class A type I/II. Furthermore, derivatization of these amino groups not only prevents interaction with the native LDL receptor but it also permits binding to and unregulated uptake by scavenger receptors on phagocytic cells (86, 169, 170, 211). During metal-catalyzed oxidation, apolipoprotein B is also modified through radical-mediated scission of peptide bonds, and probably also by direct reaction with fatty acyl hydroperoxides (212). Oxidation of LDL also is accompanied by oxidation of cholesterol esters and free cholesterol (87), and by hydrolysis of phosphatidylcholine (PC) to lysophosphatidylcholine through the action of a lipoprotein-associated phospholipase A_2 known as platelet activating factor (PAF) acylhydrolase (213). PAF acylhydrolase can utilize the PC from oxidized LDL as a substrate but cannot convert PC from native LDL (213). Some lipid oxidation products found in oxidized LDL such as oxysterols, oxidized fatty acids, and oxidized phospholipids (for example, lyso-PC) have been shown to be important mediators or regulators of many biological effects associated with atherosclerosis, which will be discussed in Section 1.5.

Endothelial cells, smooth muscle cells, T-lymphocytes, monocytes and macrophages have all been shown to be capable of enhancing the rate of oxidation of LDL, *in vitro* (86). The role of cells in the oxidative modification of LDL appears to be simply to accelerate the rate of peroxidation at low (nanomolar) free metal ion concentrations, either by providing thiols which can reduce transition metal ions to a catalytically active form (214, 215) or by "seeding" LDL in the medium with lipid hydroperoxides to initiate a peroxidation reaction (216). In fact, identical changes occur when LDL is oxidized in the absence of cells by incubation with micromolar concentrations of transition metal ions such as copper (86, 211). Typically, both cellular oxidation of LDL and transition metal ion oxidation of LDL result in

depletion of more than 70% of its linoleic acid and arachidonic acid content, complete depletion of its endogenous antioxidants, nearly half of its PC being converted to lyso-PC, and more than 30% of its lysine residues being derivatized by lipid peroxide decomposition products, resulting in an electrophoretic mobility greater than 2.5 times that of native LDL (210). In addition to these changes, oxidized LDL (as compared to native LDL) has an increased negative charge and an increased density (217). Lipoprotein oxidation by cells or transition metal *in vitro* is blocked by small concentrations of plasma or plasma proteins (218), such as albumin (219-224), and any oxidized lipoproteins that might appear in the plasma *in vivo* would be rapidly removed by the liver (225, 226), rather than be deposited into developing lesions within the arterial wall (227). As mentioned previously, oxidation of LDL is more likely to occur within the arterial wall, where increased levels of copper and iron ions are generally found and antioxidant levels are low (228).

1.5 The Biological Effects of Oxidized LDL

Oxidation of LDL has been implicated as a causal factor in the pathogenesis of atherosclerosis (88). This has been supported by several lines of evidence, including the demonstration that oxidatively modified LDL exists in atherosclerotic lesions *in vivo* in both experimental animals and humans (89, 90, 229, 230). In addition, inhibition of oxidation by several antioxidants (for example, probucol, butylated hydroxytoluene, diphenylphenylenediamine, and vitamin E) can slow the progression of the disease in several animal models, including the LDL-receptor-deficient rabbit, the cholesterol-fed New Zealand White rabbit, the cholesterol-fed hamster, the cholesterol-fed cynomolgus monkey, the LDL-

receptor-deficient mouse, and the apolipoprotein E-deficient mouse (231-239). Also, it was recently shown that the susceptibility of LDL to oxidation varied with the severity of coronary atherosclerosis, as evaluated by angiography (240). These observations prompted a number of investigators to explore the possible mechanisms by which oxidized LDL might contribute to atherogenesis.

Oxidized LDL has been found to exhibit a number of interesting biologic properties that are absent in native (unmodified) LDL (Figure 6). However, it should be emphasized here that a given biologic action of oxidized LDL can vary not only in magnitude but even in direction depending on the nature and extent of oxidative modification of LDL. In some studies, the extent of LDL oxidation was not controlled and/or inadequately characterized, thereby making it difficult to present anything more than a simple catalogue of actions that have been attributed to oxidized LDL. Most of the reports describing the biological effects of oxidized LDL have used extensively oxidized LDL, but some used mildly oxidized LDL.

Mildly oxidized or "minimally modified" LDL has an increased hydroperoxide content compared to native LDL, and unlike extensively oxidized LDL it retains at least some of its endogenous antioxidants. Furthermore, less than 5% of the unsaturated fatty acids are consumed in "minimally modified LDL", and it exhibits minimal derivatization of free amino groups on its apolipoprotein B component. The electrophoretic mobility of "minimally modified LDL" is not very different from that of native LDL. Biologic effects seen with mildly oxidized LDL include induction of monocyte chemotactic protein-1 expression by endothelial cells and smooth muscle cells (241), enhanced adhesiveness of cultured endothelial cells for monocytes (242), and expression of the inflammatory cytokines GM-CSF, M-CSF, G-CSF, GRO, and IL-1β (205, 243-245).

The remainder of this thesis will focus on the atherogenic properties of extensively oxidized LDL, and for simplicity, extensively oxidized LDL will be referred to as oxidized LDL. The physical characteristics and properties of oxidized LDL were discussed in Section 1.4. *In vitro* studies have revealed that it has many potentially atherogenic actions, which may account for the progression of atherosclerotic lesions (128). As already mentioned, one of the most important properties of oxidized LDL that makes it more atherogenic than native LDL is that it is recognized by scavenger receptors on the surface of macrophages and can therefore give rise to foam cells, which are one of the earliest hallmarks of atherosclerosis (147). Table 4 provides a catalog of some of the known biologic actions associated with oxidized LDL. The events listed in Table 4 would tend to increase foam cell formation and increase the number of macrophages in the arterial intima at sites of lesion formation. These findings suggest that oxidized LDL may play an important role in the development of early stage atherosclerotic lesions.

Table 4 Biologic actions associated with oxidized LDL

Biologic Action	*References*
accelerated uptake by macrophages via scavenger receptors	(210, 246, 247)
foam cell formation	(94, 217, 247)
cytotoxicity	(152, 248-253)
apoptosis	(254-261)
increased susceptibility to form aggregates	(262)
resistance to lysosomal degradation	(129)
enhanced binding to collagen	(221, 263)
monocyte chemotaxis and inhibition of migration of differentiated macrophages	(264-266)
stimulation of leukocyte adherence to the microvascular endothelium *in vivo*	(267, 268)
induction of macrophage proliferation	(269, 270)
activation of T lymphocytes	(271, 272)
increased monocytic cell expression of interleukin-8	(273)
increased monocytic cell release of interleukin-1β	(274)
induction of VCAM-1 and ICAM-1 expression in endothelial cells	(275, 276)
inhibition of PDGF and tumor necrosis factor-α expression	(277-279)
inhibition of lipopolysaccharide-induced interleukin-1β expression	(280)
increased growth factor gene expression in endothelial cells	(275, 281)
promotion of DNA synthesis, cell cycle entry and proliferation of vascular smooth muscle cells	(282-285)

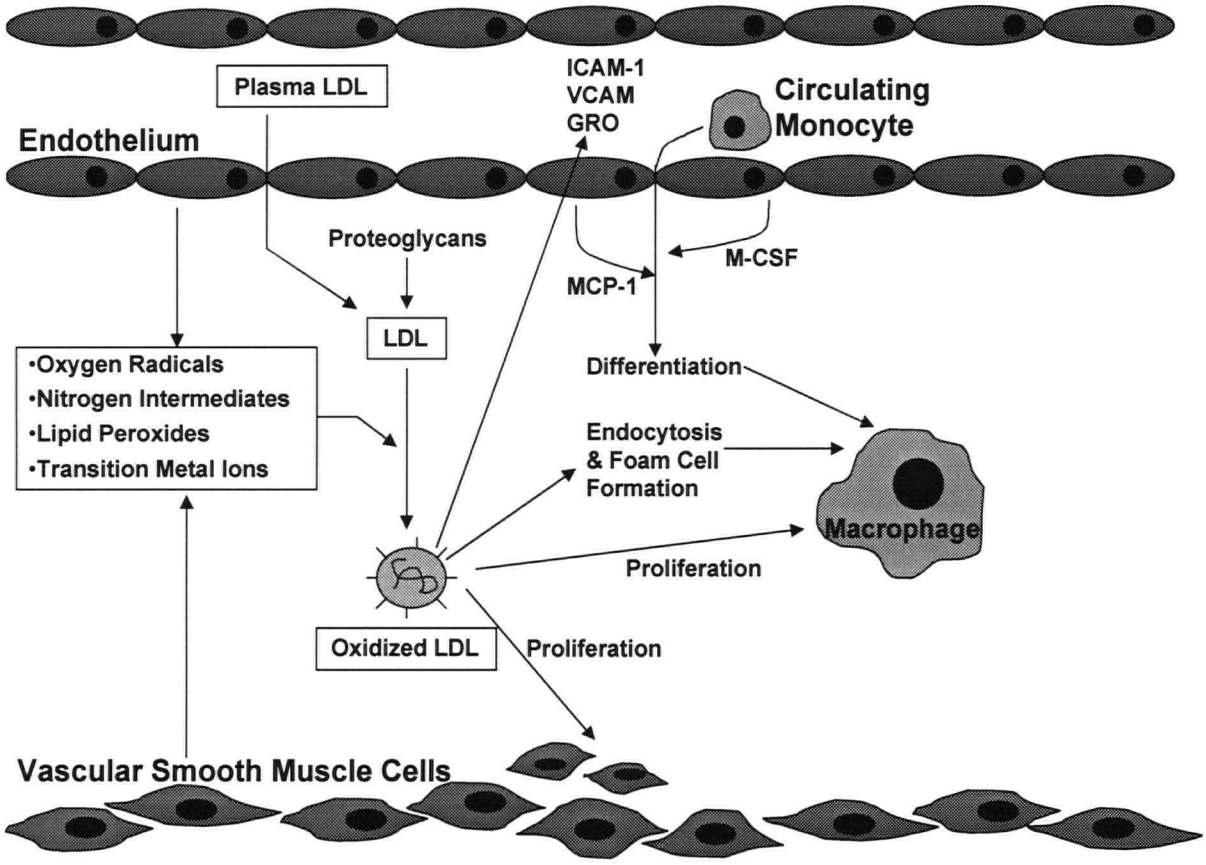


Figure 6 Oxidized LDL and atherosclerosis.

Once in the intimal space, LDL can become trapped by proteoglycans and subsequently oxidized by a transition metal catalyzed, free radical mediated reaction that converts unsaturated fatty acids to reactive lipid hydroperoxides and aldehydes. These reactive lipid substances then further accelerate oxidation of the LDL particle, leading to the depletion of polyunsaturated fatty acids and antioxidant activity, and the fragmentation of apolipoprotein B. Oxidized LDL stimulates the expression of cell adhesion molecules (VCAM, ICAM-1, and GRO) and the secretion of MCP-1 and M-CSF from endothelial cells, causing monocytes to enter the arterial intima and differentiate into macrophages. Oxidized LDL also becomes endocytosed via scavenger receptors and other receptors for oxidized LDL on the surface of macrophages, resulting in lipid accumulation and ultimately foam cell formation. Oxidized LDL has also been shown to promote chemotaxis of vascular smooth muscle cells from the tunica media, as well as stimulate cellular proliferation of both smooth muscle cells and macrophages.

1.6 Rationale

There is reason to believe that the simple model used to describe the native LDL receptor pathway may not adequately account for the distribution and metabolism of cholesterol in arterial foam cells. Ultrastructural studies of macrophage-derived foam cells within atherosclerotic lesions in animals suggest that a significant part of the lipid deposits in these cells remains in lysosomes rather than cytosolic lipid droplets (286-291). Similarly, in macrophage foam cells in human atherosclerotic lesions, a substantial proportion of lipid has been found within membrane-bound vacuoles, at least some of which appear to be of lysosomal origin (290-296). Several studies in J774 murine macrophages have revealed retention of free cholesterol in lysosomes after incubation of cells with cholesterol oleate droplets or oxidized LDL (92, 297-299). However, J774 cells also fail to efficiently esterify cholesterol delivered by acetylated LDL (300), and hence the results may not apply to primary macrophages. For instance, Kritharides et al. recently examined the subcellular distribution of sterols in murine macrophages incubated with oxidized LDL and they concluded that there was equilibration of cholesterol to all membrane pools (300). However, their data appear to show a significant increase in the proportion of cholesterol and oxysterol in the endosomal compartment, rather than the lysosomal compartment, and so this question remains unresolved. Moreover, the findings of Yancey et al. suggest that murine macrophages differ from both pigeon and THP-1 macrophages, in that murine macrophages stored most (71%) of their oxidized LDL-derived cholesterol within cytoplasmic inclusions, whereas pigeon and THP-1 macrophages stored most (60-90%) of it within lysosomes (301). In order to gain a more complete understanding of cholesterol metabolism in oxidized LDL-

loaded murine macrophages, the subcellular localization of oxidized LDL-derived lipid, both before and after HDL-mediated cholesterol efflux, must be ascertained.

Although much research has concerned possible mechanisms of reverse cholesterol transport, relatively little attention has been directed to the comparative release from lipid-loaded macrophages of free cholesterol derived from oxidized LDL or acetylated LDL. This may be of great significance since oxidized LDL has been shown to exhibit many potentially atherogenic actions, including accelerated uptake by macrophages (210, 246, 247) and macrophage foam cell formation (94, 217, 247). It has been demonstrated that extensively oxidized LDL is resistant to lysosomal enzymes (302), and also can inactivate lysosomal acid hydrolases (129, 303). Inactivation of lysosomal cathepsins by aldehydic adducts on oxidized LDL may be responsible for impaired efflux of cholesterol from oxidized LDL-loaded macrophages (302), but this hypothesis has never been tested directly. It has also been established that apolipoprotein B from oxidized LDL accumulates within the lysosomal compartment (129, 304, 305), and that oxidation-specific protein adducts (presumably representing undegraded oxidized apo B) are detectable within foam cells in atherosclerotic lesions (306). In contrast to acetylated LDL-derived *in vitro* foam cell models which accumulate free cholesterol and cholesteryl esters (307), oxidized LDL-derived foam cells contain much lower quantities of cholesteryl esters and contain a number of oxidized cholesterol compounds (308, 309). It has been demonstrated that there is a major difference in the ability of cells loaded with unoxidized cholesterol compounds (acetylated LDL) or oxidized cholesterol compounds (oxidized LDL) to release their respective lipid compound to an extracellular cholesterol acceptor (300). Further investigation into the nature of this phenomenon is valuable because impairment of cholesterol efflux from oxidized LDL-loaded

cells may influence the generation and persistence of the foam cell phenotype *in vivo* and may, therefore, contribute to the atherogenicity of oxidized LDL.

The purpose of this study was to further characterize the mechanism(s) by which oxidized LDL causes impaired efflux of cholesterol from macrophages. To accomplish this goal, we assessed the degree of HDL-mediated cholesterol efflux impairment in cells loaded with either oxidized LDL or acetylated LDL, we determined the subcellular location of oxidized LDL-derived lipid in macrophages before and after HDL-mediated cholesterol efflux, and finally, we investigated whether the lipid moiety or the protein moiety of the lipoproteins was predominantly involved in the process of cholesterol accumulation and efflux.

1.7 Objectives and Hypothesis

The main objective of this study was to investigate the role of oxidized LDL in lipid accumulation in macrophages, since this is pertinent to the formation of macrophage foam cells. In order to achieve this objective, the following specific hypotheses were addressed:

1. Macrophages loaded with oxidized LDL exhibit impaired efflux of cholesterol to HDL, compared to macrophages loaded with acetylated LDL.
2. Impairment of HDL-mediated cholesterol efflux in macrophages loaded with oxidized LDL involves both the oxidized lipid component and the oxidized apolipoprotein B moiety.

3. Cholesterol delivered by oxidized LDL is sequestered in lysosomes and therefore is inaccessible to HDL-mediated cholesterol efflux.

Chapter 2 Materials and Methods

2.1 Chemicals and Reagents

The Amplex Red reagent, NBD-C_6-sphingomyelin, and Texas Red sulfonyl chloride were purchased from Molecular Probes (Eugene, Oregon, USA). Butylated hydroxytoluene (BHT) was from J.T. Baker (Toronto, Ontario, Canada). Percoll, Folin and Ciocalteu's phenol reagent, fatty acid free bovine serum albumin, horseradish peroxidase, cholesterol esterase, cholesterol oxidase, arachidonic acid, 4-methylumbelliferyl α-D-glucoside, 4-methylumbelliferyl N-acetyl-β-D-glucosaminide, trypsin and trypsin inhibitor were purchased from Sigma. LPS-free sterile water was purchased from Baxter (Toronto, Ontario). Centriflo CF25 membrane cones were obtained from Amicon (Beverly, USA). Carrier-free $Na^{125}I$ was purchased from NEN/Mandel Scientific (Guelph, ON, Canada). [^{3}H]-cholesteryl linoleate was purchased from Amersham Corporation (Piscataway, NJ, USA). Dimethyl sulfoxide (DMSO), aquasol scintillation fluid, Scintisafe scintillation fluid, methanol, isopropyl alcohol, and chloroform were purchased from Fisher Scientific (Vancouver, BC, Canada). Dulbecco's modified Eagle's medium (DMEM) and gentamicin were purchased from GIBCO (Mississauga, Ontario). Hyclone defined fetal bovine serum was supplied by Professional Diagnostics (Edmonton, Alberta, Canada). Tissue culture plates were purchased from Canlab (Vancouver, B.C.). Female CD-1 Swiss mice were supplied by the Charles River animal breeding facility (Montreal, Quebec). Glass thin-layer chromatography plates were purchased from Mandel Scientific (Guelph, ON, Canada). Intralipid was purchased from Pharmacia (Dorval, PQ, Canada). The MPR 1 free cholesterol

assay kit, the MPR 1 total cholesterol assay kit, and the cholesterol standards were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were the highest grade available from Sigma Chemical (St. Louis, MO), BDH Chemical (Toronto, ON, Canada), Fisher Scientific (Vancouver, BC, Canada), or VWR Canlab (Vancouver, BC, Canada).

2.2 LDL Preparation

2.2.1 LDL isolation

Plasma from fasting, normolipidemic human volunteers was collected into EDTA (final concentration 3 mM). The density of the plasma was adjusted with sodium bromide solutions containing 10 mM EDTA, and LDL (d = 1.019-1.063 g/ml) was isolated by sequential ultracentrifugation in a Beckman Ti-60 fixed angle rotor by the method of Havel et al. (310). In order to reduce the high sodium bromide concentration, LDL preparations were then dialyzed against two changes of Dulbecco's PBS containing 10 μM EDTA at pH 7.4 at 4°C, and then stored at 4°C in the dark. This concentration of EDTA was sufficient to inhibit spontaneous oxidation on storage, but low enough to permit reproducible oxidation upon addition of 5 μM $CuSO_4$. Finally, the preparation was passed through a 0.22 μm filter to remove aggregates and ensure sterility.

2.2.2 LDL labelling

LDL was radio-iodinated using a modification of iodine monochloride method of McFarlane (311, 312). Typically, 5 mg of LDL was mixed with an equal volume of ice-cold 0.6 M glycine/0.4 M NaOH, pH 10. Lipid labelling is minimized at this pH. In a fume hood, 1 mCi carrier-free $Na^{125}I$ followed by 125 µl freshly diluted 1 mM ICl were then added with brief gentle vortexing. The mixture was transferred to a dialysis bag and dialyzed against four changes of Dulbecco's PBS with 10 µM EDTA. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility. Labelling efficiency was about 20% and specific radioactivities ranged from 80-140 cpm/ng. Over 99% of radioactivity in labelled LDL was precipitable with 10% trichloroacetic acid.

Cholesteryl esters in LDL were labelled by exchange with a microemulsion containing [^{3}H]-cholesteryl linoleate. The labelled microemulsion was obtained by cosonicating 5 µl of 10% Intralipid with 10^9 dpm [^{3}H]-cholesteryl linoleate in 350 µl PBS for 30 seconds at 20°C using an Artek 150 sonicator with titanium microprobe set at 30% power. Native LDL (5 mg) was then incubated for 4 hours at 37°C with the labelled Intralipid together with 30 mg/ml d > 1.21 g/ml plasma fraction as a source of lipid transfer activity. LDL was re-isolated by sequential ultracentrifugation between d 1.020 and 1.063 g/ml, and then dialyzed against several changes of Dulbecco's PBS containing 10 µM EDTA. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility. This method is nearly identical to that used by Zhang et al. (308), but this method promotes greater incorporation of [^{3}H]-cholesteryl linoleate into the LDL molecule. In

addition to labelling of native LDL, this procedure can be used to label modified forms of LDL either before or after the chemical modification of the LDL, depending on the purpose.

Acetylated LDL and oxidized LDL were fluorescently labelled with NBD-C_6-sphingomyelin. NBD-C_6-sphingomyelin is the common name for 6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl) sphingosyl phosphocholine. 2 mg of modified LDL was pipetted into a sterile conical tube, to which 2-4 ml of LPDS was added. NBD-C_6-sphingomyelin was dissolved in methanol to create a 1 mg/ml stock solution. 400 µl of this was removed and dried down in a glass test tube under a stream of nitrogen. This was redissolved in 100 µl DMSO and then transferred to the conical tube containing the modified LDL/LPDS mixture. The tube was then inverted a number of times and then incubated for 8 hours at 37°C. Throughout the labelling procedure, the NBD-C_6-sphingomyelin was protected from light as much as possible. Upon completion of the incubation, the density of the mixture was adjusted with sodium bromide solutions containing 10 mM EDTA, and the modified LDL was isolated by ultracentrifugation in a Beckman Ti-60 fixed angle rotor. Isolation of fluorescently labelled acetylated LDL required adjustment of the density of the mixture to 1.063 g/ml; isolation of fluorescently labelled oxidized LDL required adjustment of the density of the mixture to 1.100 g/ml. In order to reduce the high sodium bromide concentration, the fluorescently-labelled modified LDL preparations were then dialyzed against two changes of Dulbecco's PBS containing 10 µM EDTA at pH 7.4 at 4°C, and then stored at 4°C in the dark. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility.

2.2.3 LDL modification

Acetylation of LDL was performed by sequential addition of acetic anhydride (123). Seven aliquots each of 1 µl of acetic anhydride were added at 15 min intervals to 4 mg of LDL in ice-cold 50% saturated sodium acetate with gentle stirring, and then dialyzed against Dulbecco's PBS containing 10 µM EDTA. Before addition of acetic anhydride, BHT (20 µM) was added to prevent oxidation. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility.

LDL was oxidized by incubation of 200 µg/ml LDL in EDTA-free Dulbecco's PBS containing 5 µM $CuSO_4$ for 24 hours, at 37°C (86, 210). Oxidation was arrested by refrigeration and addition of 50 µM BHT and 200 µM EDTA. The oxidized LDL was then washed with EDTA-free Dulbecco's PBS and reconcentrated on CF25 membrane cones. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility.

To produce $NaBH_4$-treated oxidized LDL, 500 µg of oxidized LDL (prepared as above) was incubated with 150 µl of a freshly prepared solution of 1 M $NaBH_4$ in 0.1 M NaOH (302, 313). Following the incubation for 4 hours at room temperature, the oxidized LDL sample was dialyzed against Dulbecco's PBS containing 10 µM EDTA. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility.

To produce acetylated LDL that is subsequently oxidized, we simply prepared acetylated LDL as described above, and then we oxidized it for either 5 hours or 24 hours, using the method for oxidation of native LDL, given above.

In order to produce arachidonic acid-oxidation product LDL, we first generated reactive fatty acid oxidation products (OP) by thermal autoxidation of arachidonic acid (129, 170). Arachidonic acid (10 mg) was oxidized by exposure to air at 37 °C for 72 hr in a glass vial. The yellow-brown products were dissolved in Dulbecco's PBS with 300 μM EDTA at pH 7.4, and aliquots containing the residue from 0.8-1.5 mg of oxidized fatty acid were added to 1 mg LDL, in 1 ml Dulbecco's PBS containing 300 μM EDTA and 50 μM butylated hydroxytoluene. Irreversible derivatization of proteins occurred within minutes at 20°C and was substantially complete within a few hours. After overnight incubation at 20 °C, reaction mixtures were washed repeatedly with PBS on Amicon Centricon 30 microconcentrators to remove soluble non-bound materials. Omission of this wash step resulted in cytotoxicity at higher concentrations of modified LDL during subsequent incubation with macrophages.

Aggregated LDL was prepared by vortexing a 1 mg/ml native LDL solution for 15 seconds in a 15 ml conical tube with a benchtop vortex mixer at medium speed setting. The extent of aggregation (as reflected in turbidity) was monitored as absorbance increase at 680 nm and was typically 09-1.0 (314). BHT (20 μM) and EDTA (10 μM) were present throughout and there was no appreciable oxidation of these preparations.

2.2.4 Characterization of LDL

Lipoprotein electrophoresis was performed using a Ciba-Corning apparatus and 1% Universal agarose film in 50 mM barbital buffer (pH 8.6) according to the manufacturer's instructions. Bovine serum albumin was added to lipoprotein samples to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with Fat Red 7B. Native

(unmodified) LDL was used as a reference. The relative electrophoretic mobility of modified LDL was calculated by dividing the distance travelled during electrophoresis by the distance travelled by native LDL, and satisfactory acetylation or oxidation was confirmed if the relative electrophoretic mobility (R_f) was greater than or equal to 3.

Protein was assayed by the method of Lowry (315) in presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard.

2.3 HDL Preparations

2.3.1 HDL_3 isolation

Plasma from fasting, normolipidemic human volunteers was collected into EDTA (final concentration 3 mM). The density of the plasma was adjusted with sodium bromide solutions containing 10 mM EDTA, and HDL_3 (d = 1.125-1.210 g/ml) was isolated by sequential ultracentrifugation in a Beckman Ti-60 fixed angle rotor by the method of Havel et al. (310). In order to reduce the high sodium bromide concentration, HDL_3 preparations were then dialyzed against three changes of Dulbecco's PBS containing 10 μM EDTA at pH 7.4 at 4°C, and then stored at 4°C in the dark. This concentration of EDTA was sufficient to inhibit spontaneous oxidation on storage. Finally, the preparation was passed through a 0.22 μm filter to remove aggregates and ensure sterility.

2.3.2 HDL labelling

HDL_3 was radio-iodinated using a modification of iodine monochloride method of McFarlane (311, 312). Typically, 5 mg of HDL_3 was mixed with an equal volume of ice-cold 0.6 M glycine/0.4 M NaOH, pH 10. Lipid labelling is minimized at this pH. In a fume hood, 1 mCi carrier-free $Na^{125}I$ followed by 125 µl freshly diluted 1 mM ICl were then added with brief gentle vortexing. The mixture was transferred to a dialysis bag and dialyzed against four changes of Dulbecco's PBS with 10 µM EDTA. Finally, the preparation was passed through a 0.22 µm filter to remove aggregates and ensure sterility. Labelling efficiency was about 20% and specific radioactivities ranged from 80-140 cpm/ng. Over 99% of radioactivity in labelled HDL_3 was precipitable with 10% trichloroacetic acid.

Native HDL_3 was fluorescently labelled with Texas Red sulfonyl chloride. 1 ml of 0.5 M sodium phosphate buffer (pH 8) was added to 10 mg of native HDL_3 in a vial wrapped in aluminum foil (to protect from light). 1 mg of Texas Red sulfonyl chloride was dissolved in 50-100 µl of 100% ethanol, and then added directly to the vial containing the buffer and the HDL_3. A stir bar was added to the vial and it was stirred in the dark at the slowest speed (to prevent aggregation) for 7-8 hours at 4°C. Upon completion of the incubation, the density of the mixture was adjusted to 1.210 g/ml with sodium bromide solutions containing 10 mM EDTA, and the labelled HDL_3 was isolated by ultracentrifugation in a Beckman Ti-60 fixed angle rotor. In order to remove sodium bromide and unreacted Texas Red sulfonyl chloride, the fluorescently-labelled HDL_3 preparation was then dialyzed against three changes of Dulbecco's PBS containing 10 µM EDTA at pH 7.4 at 4°C, and then passed through a 0.22 µm filter to remove aggregates and ensure sterility. The labelled product was

stored at 4°C in the dark. Texas Red sulfonyl chloride reacts via a displacement reaction with primary amines such as the amino acid, lysine; it forms a stable sulfonamide linkage to the side chain of this amino acid.

2.3.3 Trypsinization of HDL_3

Trypsinized HDL_3 was prepared by isolating HDL_3 as described above and then treating 5 mg of HDL_3 with 125 μg of trypsin for 1 hour at 37°C (316, 317). The reaction was stopped with excess trypsin inhibitor. Trypsin and trypsin inhibitor were removed from HDL_3 by dialysis against Dulbecco's PBS containing 10 μM EDTA. Finally, the preparation was passed through a 0.22 μm filter to remove aggregates and ensure sterility.

2.3.4 Apolipoprotein A-I isolation

HDL_3 was obtained from human plasma as described above. After delipidating it with ethanol : diethyl ether (2 : 1) at -20°C (318), apolipoprotein A-I was isolated by chromatography at 4°C on a Superose 12 Column (Pharmacia) in 0.01 M Tris-HCl, 6 M Urea, pH 8.0 (319). Each fraction collected was analyzed for protein content and run on SDS-PAGE to identify the purest fraction. The purest fractions were pooled and dialyzed at 4°C against 0.1 M ammonium bicarbonate and then thoroughly dialyzed against distilled water. Lyophilized protein was stored at -20°C under nitrogen atmosphere. Before use, lyophilized apo A-I was dissolved in Dulbecco's PBS at 4°C by stirring overnight. The

dissolved apolipoprotein was then passed through a 0.22 μm filter to remove aggregates and ensure sterility.

2.3.5 Characterization of HDL and apo A-I

Purity of HDL_3 and apolipoprotein A-I preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a Bio-Rad mini-gel apparatus (320). The separating or resolving gel that we used contained 10% acrylamide. 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.1% (wt/vol) bromophenol blue, and 2% (vol/vol) β-mercaptoethanol were mixed with samples of either HDL_3, trypsinized HDL_3, apolipoprotein A-I, or column fractions containing apolipoprotein A-I. This mixture was then heated to 100°C for 2 minutes. The amount of trypsinized HDL_3 applied to the well was equivalent to the amount of native HDL_3, as assessed by phosphorus content. As for the fractions collected from the Superose 12 column, an equal volume was applied in order to accurately compare the content of purified apolipoprotein A-I in each fraction. Nonradioactive low range molecular weight markers (range, 14,400 Da to 97,400 Da; Bio-Rad) were run concurrently for calibration. The bands were visualized by staining for protein with Coomassie brilliant blue R-250. Apo A-I migrated as a single band (321). Gels were run in 10 mmol/L Tris-glycine buffer (pH 8.3) for 50 minutes at 150 Volts and were fixed in ethanol/acetic acid/water (38:10:52, vol/vol/vol).

Protein was assayed by the method of Lowry (315) in presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard.

2.4 Cell Culture

Procedures were followed in accordance with ethical guidelines of the University of British Columbia and the Medical Research Council of Canada. Resident peritoneal macrophages were obtained from female CD-1 Swiss mice (after asphyxiation using carbon dioxide gas) by peritoneal lavage with ice-cold Dulbecco's PBS (86, 123). The isolated cells were pelleted, and then resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS) and gentamicin (50 μg/ml). Next, the cells were plated either in 35 mm diameter (Falcon) or 100 mm diameter (Corning) tissue culture plates at a density of either 6.0×10^6 cells/plate or 2.0×10^7 cells/plate, respectively. The larger plates were used for the subcellular fractionation experiments as well as all experiments in which the colorimetric cholesterol assay was used. The smaller plates were used in all experiments in which the fluorimetric cholesterol assay was used. The freshly plated cells were incubated at 37°C for 1 to 2 hours, and then washed four times with prewarmed Dulbecco's PBS to remove nonadherent cells. After washing, cells were cultured overnight in DMEM containing 10% FBS and gentamicin (50 μg/ml) prior to use in experiments. Cells were washed with serum-free DMEM and then, unless otherwise specified, 50 μg/ml modified LDL was added in DMEM containing gentamicin (50 μg/ml) and 0.5 mg/ml fatty acid free bovine serum albumin (tissue culture grade) to minimize toxicity. After 24 hours of incubation with modified LDL, cell were washed with warm Dulbecco's PBS three times to remove LDL-containing medium. At this point, representative cultures were then taken for analysis. The remaining cultures were then incubated for a further 12 hours in DMEM (with gentamicin and BSA as specified above)

containing 75 μg/ml HDL_3. Upon completion of this "efflux" incubation, these cultures were also taken for analysis. The cells that were removed for analysis both before and after the 12 hour "efflux" incubation were lysed by incubating in 1 ml of cold 0.1 M NaOH for 15 minutes at 4°C. The contents of the plate were then scraped off and collected in a glass test tube. Of this, 0.1 ml was removed and stored at -20°C for later protein assay, and the remainder was lipid extracted with chloroform and methanol according to the method of Bligh and Dyer (322). Lipid extracts were stored at -20°C until cholesterol analysis, which was usually performed within 24 hours.

In the experiments in which we measured the retention of radiolabelled lipid or apoproteins, cells were dissolved in NaOH, scraped, and assayed for protein content as described above. The radioactivity of these cells was counted on either an LKB 1282 gamma spectrometer or a Beckman LS5000CE scintillation spectrometer, depending on whether radioactivity was emitted by ^{125}I or ^{3}H, respectively. In some cases, medium was collected and counted on the beta counter to measure the counts of ^{3}H-cholesterol effluxed into the medium. Furthermore, degradation of ^{125}I-labelled lipoproteins was measured using the degradation assay, described in section 2.6.6.

2.5 Microscopy

Cells treated with fluorescently-labelled lipoproteins were cultured on sterile coverslips. Upon completion of the experiment, cells were washed and then fixed with PBS containing Ca^{2+} and 2% formaldehyde. A microscope slide was prepared by adding 7 μl of a Glycerol/PBS/DABCO solution to it before mounting the cover slip on it. The edges of the

coverslip were sealed with clear nail polish. These slides were then viewed and photographed using a Zeiss Axioskop fluorescence microscope for transmitted light and incident light fluorescence microscopy. For experiments related to retention of NBD-C_6-sphingomyelin, cells were examined with a fluorescein filter set. A rhodamine filter set was used to image Texas Red sulphonyl chloride-labelled HDL_3.

Morphological observations of cultured macrophages were made by inverted phase-contrast microscopy. Cell viability was established by trypan blue staining at the end of either the 24 hour "loading" incubation or the 12 hour "efflux" incubation. Counting was preformed twice in representative plates, and the result was averaged in each case. Both acetylated LDL-loaded cells and oxidized LDL-loaded cells showed a viability of 90%.

2.6 Experimental Assays

2.6.1 Protein determination

Protein was assayed by the method of Lowry (315) in presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard.

2.6.2 Colorimetric cholesterol assay

Lipid extracts were dissolved in isopropanol and 50 µl aliquots were assayed for total cholesterol and free cholesterol using MPR 1 colorimetric enzymatic kits (Boehringer Mannheim; catalog numbers 1 442 341 and 310 328) with a final reaction volume of 1 ml.

Absorbances of the colored product were read at 500 nm using an LKB 4054 Ultrospec Plus spectrophotometer. Cholesterol standards were purchased from Boehringer Mannheim. Both of these assays are based on the assay method originally described by Trinder (323, 324).

2.6.3 Fluorimetric cholesterol assay

Lipid extracts were dissolved in DMSO and 50 µl aliquots were assayed for total cholesterol and free cholesterol using the Amplex Red cholesterol assay (Molecular Probes; catalog number A-12216) with a final reaction volume of 2 ml. After excitation at 540 nm, the fluorescence emission of the product, resorufin, was measured at 585 nm using a Turner Model 450 fluorometer, as previously described (325). Cholesterol standards were purchased from Boehringer Mannheim.

2.6.4 Phosphorus assay

The phosphorus content of both HDL_3 and trypsinized HDL_3 was assayed according to the method described by Rouser et al. (326). Briefly, lipoprotein samples were lipid extracted with chloroform and methanol (322), and the organic phase was dried under nitrogen. The extracted lipid was dissolved in 0.45 ml perchloric acid by heating at 180°C for 30 minutes, and subsequently treated with 2.5 ml distilled water, 0.5 ml of 2.5% ammonium molybdate, and 0.5 ml of 10% ascorbate. Samples were then incubated in a 37°C

water bath for 15 minutes, and the absorbances read at 820 nm using an LKB 4054 Ultrospec Plus spectrophotometer. Na_2HPO_4 was used as the standard.

2.6.5 Degradation assay

The degradation of lipoproteins by cultured macrophages was measured. Resident peritoneal macrophages harvested from CD-1 Swiss mice were cultured as previously described (86, 123). To measure the degradation of various modified LDL's, the LDL-containing medium was harvested at the end of the 24 hour "loading" incubation. To measure the degradation of HDL_3, the HDL_3-containing medium was harvested at the end of the 12 hour "efflux" incubation. Lipoprotein degradation was estimated as the amount of trichloroacetic acid-soluble noniodide radioactivity in the medium. Undegraded lipoprotein was precipitated with 10% trichloroacetic acid in the presence of 3% BSA as a carrier. Free iodine was removed by precipitation with 2% $AgNO_3$. Radioactivity in the supernatant, representing iodotyrosine and peptide fragments, was counted in an LKB 1282 gamma spectrometer. The cells were washed and dissolved in 0.1 M NaOH. Aliquots were taken for determination of cell-associated radioactivity and protein content. Degradation rates were corrected by subtraction of values obtained in cell-free control dishes incubated in parallel.

2.6.6 ER and lysosome enzyme assays

In the subcellular fractionation experiments, endoplasmic reticulum and lysosomes were identified in density gradient fractions by fluorescent assay of neutral α-glucosidase

and N-acetylglucosaminidase activities, respectively, using the corresponding 4-methylumbelliferyl substrates (327, 328). Latency of N-acetyl β-glucosaminidase was determined as the ratio of activity in post-nuclear supernatant without Triton X-100 to that with 0.1% Triton X-100.

2.7 Subcellular Fractionation

Resident peritoneal macrophages harvested from CD-1 Swiss mice were cultured as previously described (86, 123). Upon completion of the 24 hour "loading" incubation in the presence of either radiolabelled acetylated LDL or radiolabelled oxidized LDL, the cells were washed three times with Dulbecco's PBS and representative cultures were removed and prepared for subcellular fractionation. The remaining plates were incubated for a further 12 hours in the presence of HDL_3, as previously described. Finally, these plates were also washed three times with Dulbecco's PBS and prepared for subcellular fractionation. In order to prepare cells for subcellular fractionation, they were washed twice with ice-cold Dulbecco's PBS and incubated with Dulbecco's PBS containing 0.02% EDTA for 10 minutes at 4°C. Macrophages were disrupted and fractionated essentially as described by Dean (329). Cells were gently removed from the plates using cell lifters and sedimented at 1100 × g for 10 minutes at 4°C. Between 18 and 20 million cells were resuspended in 2 ml cold 10 mM Tris, 0.25 M sucrose, 1 mM EDTA (pH 7.5) and disrupted by nitrogen cavitation at 445 kPa for 15 minutes in a precooled Kontes mini cell disruption chamber (Mandel Scientific, Edmonton, AB). Nuclei, mitochondria and intact cells were removed by centrifugation at 100 × g for 5 minutes at 4°C. An aliquot of the resultant post-nuclear supernatant was reserved for latency determination and the remainder was mixed with 10 mg of BSA and layered over 10 ml of 40% Percoll in 10 mM Tris, 0.25 M sucrose, 1 mM EDTA

(density 1.070 g/ml) on a cushion of 0.5 ml 2 M sucrose. Self-forming gradients were generated by centrifugation at 20,000 rpm for 3 hours at 4°C in a Beckman SW-40 Ti rotor. Fractions of 0.5 ml were collected from the bottom of the gradient and assayed for radioactivity using a Beckman LS5000CE scintillation spectrometer. Individual fractions were frozen at -20°C and assayed the following day for neutral α-glucosidase and N-acetylglucosaminidase. Density profiles of blank gradients spun in parallel were measured using a digital densitometer (Paar, Austria).

2.8 Thin Layer Chromatography

In order to determine whether free or esterified cholesterol was effluxed into the medium. Macrophages were loaded with either [^{3}H]-acetylated LDL or [^{3}H]-oxidized LDL, washed, and then treated with HDL_3-containing medium. The efflux medium was lipid extracted (322) and dried under nitrogen. Lipids were separated by thin-layer chromatography (TLC) on silica TLC plates using a solvent system of hexane : diethyl ether : acetic acid (80 : 20 : 1, vol/vol). Bands were visualized with iodine vapor and zones corresponding to cholesterol, cholesterol oleate, and 7-ketocholesterol were scraped, mixed with Aquasol scintillation fluid, and counted in a Beckman LS5000CE scintillation spectrometer.

2.9 Statistical Analysis

Statistical significance was determined using a two-sample student's t-test for paired data. A value of $p < 0.05$ was considered to indicate a significant difference. Unless otherwise indicated, all data were expressed as the mean ± standard deviation of the mean.

Chapter 3 Results and Discussion

There is substantial evidence suggesting a role for LDL oxidation in the development of atherosclerosis. The biologic effects of oxidized LDL on macrophages include accelerated uptake of oxidized LDL (210, 246, 247), lipid accumulation (128, 238) and ultimately, foam cell formation (94, 217, 247). As described in the introduction, a hallmark feature of atherosclerotic lesions is the presence of oxidized LDL-containing foam cells in the arterial intima. Most of the lipid within foam cells is derived from plasma LDL cholesterol (11). The first step in reverse cholesterol transport is the movement of cholesterol out of cells onto lipoprotein acceptors in the interstitial fluid. When compared to macrophages loaded with acetylated LDL, cells loaded with oxidized LDL exhibit impaired efflux of cholesterol to an extracellular acceptor such as HDL (300). The objective of this thesis was to gain a better understanding of the mechanism whereby oxidized LDL causes impaired efflux of cholesterol from macrophages. After determining the degree of cholesterol efflux in cells loaded with either oxidized LDL or acetylated LDL, we investigated the relative importance of the lipid component and the protein component of the lipoproteins involved in the process of lipid accumulation and efflux. In addition, the subcellular location of both oxidized LDL-derived and acetylated LDL-derived cholesterol was identified in macrophages before and after HDL-mediated cholesterol efflux.

3.1 Cholesterol content of modified LDL

Acetylation and 24-hour copper oxidation are well-described LDL modifications that are known to result in recognition by scavenger receptors and foam cell formation (217). Copper-mediated oxidation of LDL results in profound alterations of both the lipid and protein compositions of LDL, including fragmentation of apolipoprotein B, hydrolysis of phosphatidylcholine, and loss of esterified cholesterol (210, 330). Consequently, cells loaded with acetylated LDL accumulated large quantities of cholesterol esters, whereas cells loaded with oxidized LDL accumulated a much smaller proportion of esterified cholesterol. As mentioned in the introduction, studies with oxidized LDL have used either mildly oxidized LDL or extensively oxidized LDL. There is a lack of evidence for the presence of extensively oxidized LDL in human atherosclerotic plaques. Mildly oxidized LDL has been extracted from human atheroma (331), but it was found to contain 7-ketocholesterol, which is a product of extensive, not mild, LDL oxidation (330, 332, 333). Such findings have made it difficult to deduce the degree of *in vitro* LDL oxidation that is most physiologically relevant. The oxidized LDL discussed in this thesis is extensively oxidized.

The composition of modified LDL's determines their atherogenicity. For this reason, it is useful to analyze the protein and cholesterol content of the principal modified LDL's discussed in this thesis (Table 5).

Table 5 Relative composition and electrophoretic mobility of modified LDL

	FC/protein (μg cholesterol per mg protein)	TC/protein (μg cholesterol per mg protein)	R_f value (relative to native LDL)
native LDL	0.39±0.04	1.64±0.10	1
unlabelled acetylated LDL	0.40±0.06	1.38±0.25	4.77
radiolabelled acetylated LDL	0.37±0.09	1.55±0.19	4.77
unlabelled oxidized LDL	0.39±0.03	0.87±0.11*	4.09
radiolabelled oxidized LDL	0.28±0.07*	0.56±0.14*	3.09

FC indicates Free Cholesterol; TC indicates Total Cholesterol; and R_f is the relative electrophoretic mobility in agarose. In this instance, the modified LDL's were radiolabelled after being either acetylated or oxidized; the radiolabelled LDL's contained [^{3}H]-cholesteryl linoleate. Protein and cholesterol contents were determined in the manner described in Chapter 2. Data represent the mean ± S.D. of three analyses of a single lipoprotein preparation. * $p < 0.01$ when compared to native LDL (by paired two-sample student's t-test).

From the values in Table 5, it can be seen that the composition of acetylated LDL is not significantly different from the composition of unmodified LDL. In agreement with previous reports, oxidized LDL was found to contain significantly less cholesterol ester than both native and acetylated LDL (211, 309, 334). The loss of this hydrophobic core of lipids results in a shift of the average hydrated density from 1.033 g/ml to 1.070 g/ml (211). The proportion of free cholesterol remains essentially unchanged in the unlabelled oxidized LDL. It is possible that a portion of the difference in the cholesterol ester content of oxidized LDL compared to native LDL is due not only to an actual difference in cholesterol ester content but also due to a difference in detection. The enzymatic assays that were used to measure cholesterol do not measure oxidized cholesterols as well as they detect unmodified

cholesterol. In fact, we have found that the assay underestimates the 7-ketocholesterol content by a factor of about eight.

Acetylated and oxidized LDL's have a greater R_f value than native LDL because these chemical modifications involve derivatization of lysine groups by adducts that neutralize the positively charged ε-amino groups on apolipoprotein B (11, 210). The R_f value of acetylated LDL did not change after being labelled with [^{3}H]-cholesteryl linoleate. However, labelling oxidized LDL particles by this technique does alter them to some extent. Radiolabelled oxidized LDL ran as a broad band with a lower R_f value than unlabelled oxidized LDL (Figure 7). This suggests that the radiolabelling procedure, which involves exchange with a microemulsion (308), may strip some of the oxidatively modified apo B fragments off the LDL particles. We observed a similar effect when oxidized LDL was incubated with native LDL, so the microemulsions themselves do not appear to be the cause of this. Moreover, the uptake of radiolabelled oxidized LDL by macrophages was only slightly lower than that of unlabelled oxidized LDL, and the cholesterol from radiolabelled oxidized LDL was retained within the cells to the same extent that unlabelled oxidized LDL was. Hence, oxidized LDL that is radiolabelled in this fashion can serve as a useful tool both to measure the degree of cholesterol efflux from oxidized LDL-loaded cells and to determine the subcellular localization of cholesterol delivered by oxidized LDL.

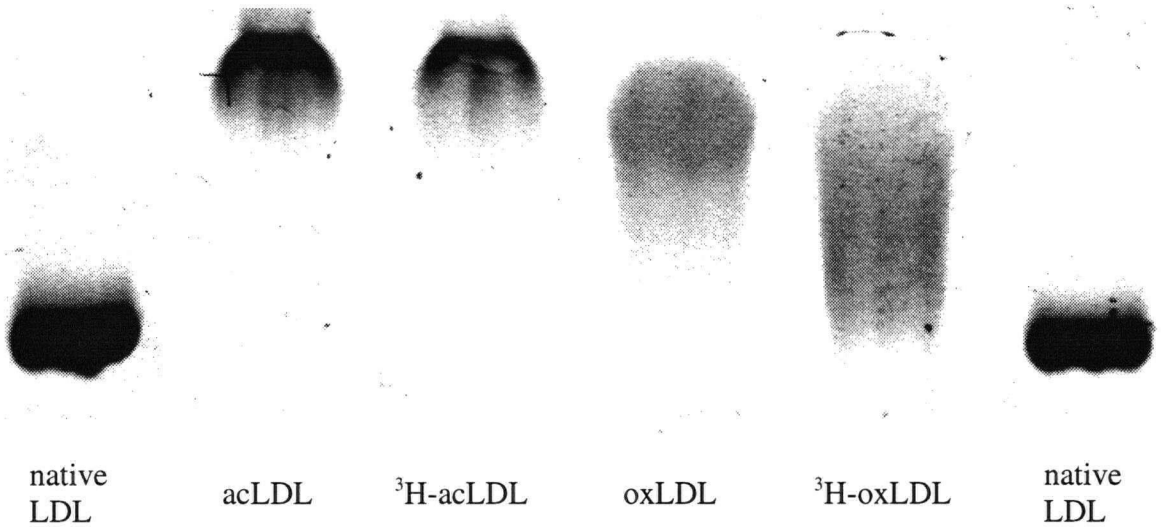


Figure 7 Agarose gel electrophoresis of labelled and unlabelled modified LDL's

Abbreviations are as follows: LDL, low density lipoprotein; acLDL, acetylated LDL; ^{3}H-acLDL, acetylated LDL that has been labelled with [^{3}H]-cholesteryl linoleate; oxLDL, oxidized LDL; and ^{3}H-oxLDL, oxidized LDL that has been labelled with [^{3}H]-cholesteryl linoleate. The R_f values for these lipoproteins are given in Table 5.

3.2 Oxidized LDL causes impaired efflux of cholesterol from Macrophages

In order to observe meaningful levels of cholesterol efflux, we found that at least a two-fold increase in total cholesterol compared to the baseline levels was required. To establish optimal conditions for cholesterol-loading of macrophages and subsequent measurement of cholesterol efflux, cells were loaded with oxidized LDL for 24 hours and then subsequently treated for an additional 12 hours with 75 μg/ml HDL_3. Figure 8 shows the results of a representative experiment out of three.

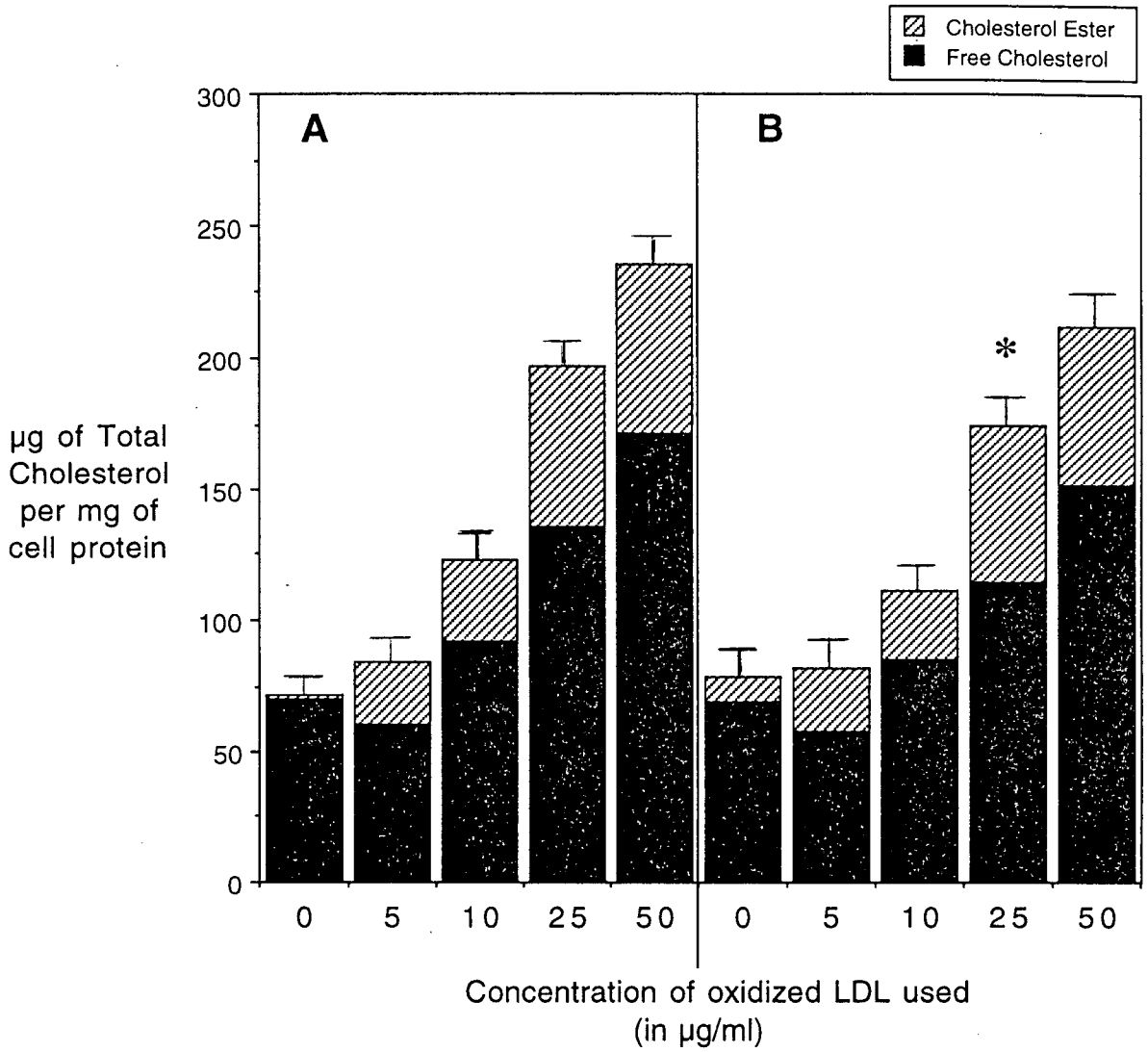


Figure 8 Oxidized LDL concentration is proportional to lipid accumulation.

Each bar represents the micrograms of total cholesterol per milligram of cell protein; within each bar the solid area represents the free cholesterol content of the cells, and the hatched area represents the cholesterol ester content of the cells. Panel A depicts macrophages that were loaded for 24 hours with 0, 5, 10, 25 or 50 µg/ml of oxidized LDL. Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of 75 µg/ml HDL_3. At these levels, the greater the oxidized LDL concentration, the greater the cholesterol accumulation within the cells. With respect to HDL-mediated cholesterol efflux, there was no significant decrease in total cholesterol in any of the plates, except for the plates that were loaded with 25 µg/ml oxidized LDL (* $p < 0.05$) compared to its counterpart which was not exposed to HDL_3. The values are mean ± S.D. for triplicate plates from one of three experiments with similar results.

At the end of the first 24 hour incubation in the presence of varying concentrations of oxidized LDL, half of the cells were extracted and assayed for protein and cholesterol content, and the remaining plates were washed and then treated with HDL_3 for an additional 12 hours, after which they too were processed in the same manner as the first set. It can be seen that there was no significant increase in total cholesterol after 24 hours of incubation with 5 μg/ml oxidized LDL. The cells loaded with 10 μg/ml oxidized LDL exhibited a less than two-fold increase in total cholesterol; those loaded with 25 μg/ml oxidized LDL exhibited a nearly three-fold increase in total cholesterol; and finally, the macrophages loaded with 50 μg/ml oxidized LDL exhibited a greater than three-fold increase in total cholesterol. Although, the cholesterol efflux from the plates loaded with 25 μg/ml is statistically significant ($p < 0.05$), it still amounts to only 11.2% of the total cholesterol in the lipid-loaded cells. The degree of cholesterol efflux in the cells loaded with 10 μg/ml or 50 μg/ml oxidized LDL was also approximately 10% of the total cholesterol in the lipid-loaded cells. There was a 2.8% decrease in total cholesterol in the cells loaded with 5 μg/ml oxidized LDL.

In order to keep the cells viable during these long incubations, the medium was supplemented with 0.5 mg/ml bovine serum albumin (BSA) in all experiments described in this thesis. It was found that 5% fetal bovine serum (FBS) was also effective in maintaining viability; however, FBS has the disadvantage of containing HDL, and it was found to be capable of bringing about efflux during the initial 24 hour "loading" of modified lipoproteins. Although lipoprotein-deficient serum has been used by others in such experiments (300), we did not find it to be effective in maintaining cell viability. Even with the BSA-supplementation of the medium, we found that oxidized LDL concentrations greater than 65

μg/ml appeared to be cytotoxic to macrophages and were associated with blebbing, cell detachment, and a decrease in the proportion of cells that excluded trypan blue (data not shown). Since 50 μg/ml of oxidized LDL was not cytotoxic to the cells and it resulted in massive accumulation of cholesterol, it was deemed to be the most appropriate concentration to be used in determining the efficiency of cholesterol efflux from these lipid-loaded cells.

In all experiments discussed in this thesis, cells were plated either in 35 mm diameter (Falcon) or 100 mm diameter (Corning) tissue culture plates at a density of either 6.0×10^6 cells/plate or 2.0×10^7 cells/plate, respectively. The total protein per plate was similar for both the acetylated LDL and the oxidized LDL-treated cells. In many cases, the control plates had lower cell numbers and consequently lower protein values at the end of the 24 hour incubation compared to the plates loaded with modified lipoproteins. The protein values did not change appreciably during the final 12 hour incubation with HDL_3. Although most of the control cells remained small and rounded throughout the experiment, the cells loaded with modified lipoproteins underwent morphological changes. Macrophages loaded with either acetylated LDL or oxidized LDL exhibited a substantial increase in size and they adhered to tissue culture plates better than control cells did. In addition, macrophages loaded with oxidized LDL developed long cytoplasmic projections.

In order to determine the effect of incubation time on cholesterol accumulation and efflux, macrophages were loaded with 50 μg/ml of oxidized LDL for 0, 6, 12, 18, and 24 hours, and then subsequently treated with 75 μg/ml HDL_3 for an additional 12 hours. Figure 9 shows the results of a representative experiment out of three.

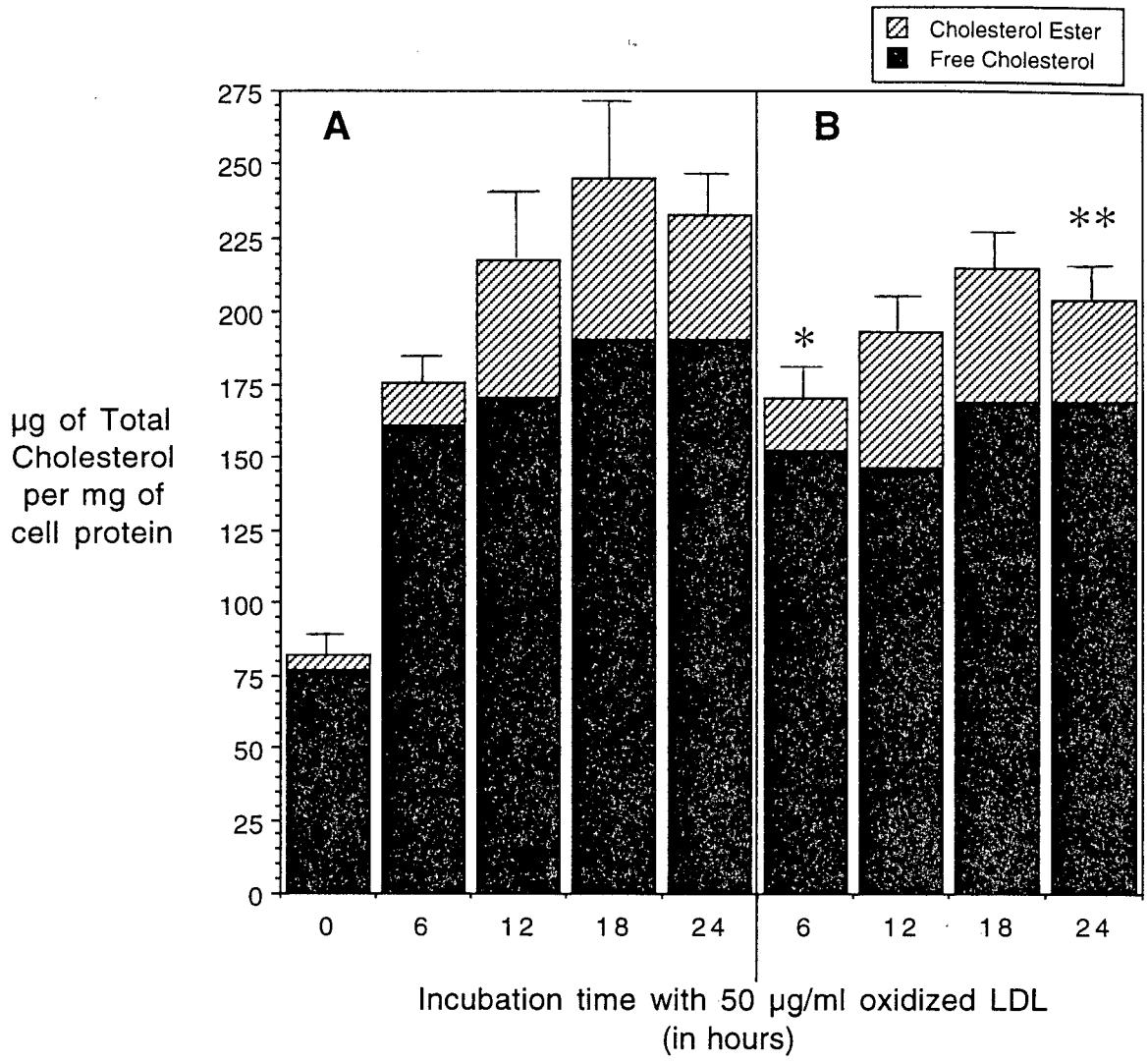


Figure 9 Effect of incubation time on lipid accumulation and efflux.

Each bar represents the micrograms of total cholesterol per milligram of cell protein; within each bar the solid area represents the free cholesterol content of the cells, and the hatched area represents the cholesterol ester content of the cells. Panel A depicts macrophages that were loaded with 50 µg/ml oxidized LDL for 0, 6, 12, 18 or 24 hours. Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of 75 µg/ml HDL_3. Maximal lipid accumulation occurred at 18 hours of lipid-loading, but the cholesterol concentration in the cells loaded for 24 hours was not significantly lower. With respect to HDL-mediated cholesterol efflux, there was a significant decrease in total cholesterol in two of the conditions. The plates loaded for 6 hours and then exposed to HDL_3 exhibited a significant decrease (* $p < 0.01$) in total cholesterol compared to the corresponding plates that were not treated with HDL_3. Similarly, the plates loaded for 24 hours and then exposed to HDL_3 exhibited a significant decrease (** $p < 0.001$) in total cholesterol compared to the corresponding plates that were not treated with HDL_3. The values are mean ± S.D. for triplicate plates from one of three experiment with similar results.

At the end of the first incubation in the presence of 50 μg/ml oxidized LDL, half of the cells were extracted and assayed for protein and cholesterol content, and the remaining plates were washed and then treated with HDL_3 for an additional 12 hours, after which they were processed in the same manner as the first set. It can be seen from Figure 9 that cholesterol accumulation increases as a function of duration of exposure to oxidized LDL up to a maximum of 18 hours, where it plateaus. All of the cells loaded with oxidized LDL exhibited a two- to three-fold increase in total cholesterol. Compared to the cells that did not undergo the 12 hour "efflux incubation", the cells that were exposed to HDL_3 after being loaded with oxidized LDL for 6, 12, 18, and 24 hours exhibited a decrease in total cholesterol of 3%, 11%, 12%, and 12%, respectively. However, statistically significant decreases in total cholesterol were observed only in the cells loaded for 6 hours (* $p < 0.01$) and 24 hours (** $p < 0.001$). Since an incubation time of 24 hours resulted in a massive accumulation of cholesterol, it was deemed to be the most appropriate concentration to be used in determining the efficiency of cholesterol efflux from these lipid-loaded cells. Longer incubations times, such as 48 hours, have been used for such experiments, under different conditions (335); however, we have found that increasing the incubation time beyond 24 hours did not result in a greater accumulation of cholesterol within these macrophages, but it did lead to a decrease in viability (data not shown).

The ability of high density lipoproteins to remove excess cholesterol from cells is well established (31, 114), but how this occurs has been disputed (77, 336). However, it is becoming increasingly accepted that removal of cellular cholesterol occurs by at least two pathways: one involving aqueous diffusion, and another promoted by cellular interaction with apolipoproteins (114). The role of plasma HDL as the preferential physiological

acceptor for cholesterol from extrahepatic cells *in vivo* has been strongly supported by epidemiologic and *in vitro* studies (23, 25, 27, 28, 80, 249, 337-349). Compared to other HDL fractions separated by density, HDL_3 has been proposed to be the major promoter of cholesterol efflux, and it is for this reason that HDL_3 was used in all of the experiments presented in this thesis (80-82). In order to observe meaningful levels of cholesterol efflux, the HDL_3 concentration must not be a rate-limiting step. To determine the most appropriate concentration of HDL_3 to incubate the cells with, several different concentrations were used and the efflux of radiolabelled cholesterol into the medium from lipid-loaded cells was measured (Figure 10).

At the end of the 24 hour incubation in the presence of 50 μg/ml radiolabelled acetylated LDL or 50 μg/ml radiolabelled oxidized LDL, the cells were washed and then incubated for an additional 12 hours in the presence of varying concentrations of HDL_3. From Figure 10, we can see that cholesterol efflux increases as a function of HDL_3 concentration up to a maximum of 60 μg/ml HDL_3, where it plateaus. The release of [^{3}H]-cholesterol into the medium was paralleled by its loss from the cells (data not shown). We see impaired efflux of cholesterol from the cells loaded with oxidized LDL compared to those loaded with acetylated LDL. It is interesting to note that there was a small degree of [^{3}H]-cholesterol efflux in the absence of HDL; it occurred undoubtedly by simple diffusion from the cell membrane to the BSA in the medium. Kritharides et al. reported that maximal efflux of cholesterol from lipid-loaded cells occurred at apolipoprotein A-I concentrations as low as 20 μg/ml (300). We, on the other hand, found that HDL_3 concentrations of 40 to 60 μg/ml were required to achieve maximal cholesterol efflux from lipid-loaded cells. This difference may, in part, be due to the higher total cholesterol content of our cells prior to

treatment with HDL_3. We deemed 75 μg/ml HDL_3 to be an appropriate concentration to be used in determining the efficiency of cholesterol efflux from these lipid-loaded cells. While this is more than an order of magnitude lower than the concentration of HDL in human plasma, it is well above the estimated K_m for HDL-mediated cholesterol efflux in cultured murine macrophages (300).

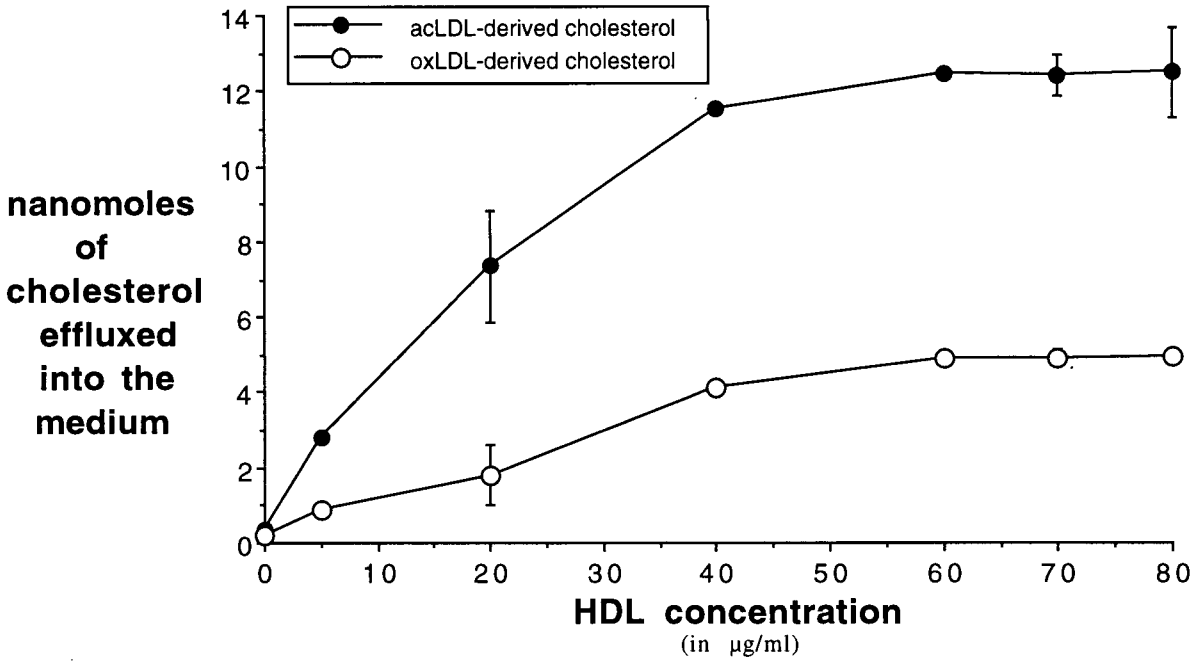


Figure 10 Concentration-dependence of HDL-mediated cholesterol efflux.

Each point represents the mean dpm's of [^{3}H]-cholesterol measured in the medium after 12 hours of exposure to HDL_3. In this experiment, cells were loaded with 50 μg/ml of either radiolabelled acetylated LDL or radiolabelled oxidized LDL. Then, the cells were washed and exposed to varying concentrations of HDL_3 for 12 hours, at the end of which, the radioactivity in the medium was measured. For this experiment, LDL was radiolabelled with [^{3}H]-cholesteryl linoleate before being either acetylated or oxidized. The values are mean ± S.D. for triplicate plates from one representative experiment out of three with similar results. Error bars not visible are within the plot symbols.

In order to determine the effect of HDL incubation time on cholesterol efflux, macrophages were loaded for 24 hours with 50 μg/ml of either radiolabelled acetylated LDL or radiolabelled oxidized LDL, and then subsequently treated with 75 μg/ml HDL_3 for 6, 12, and 24 hours; efflux of [^{3}H]-cholesterol into the medium was measured. Figure 11 shows the results of one representative experiment out of two.

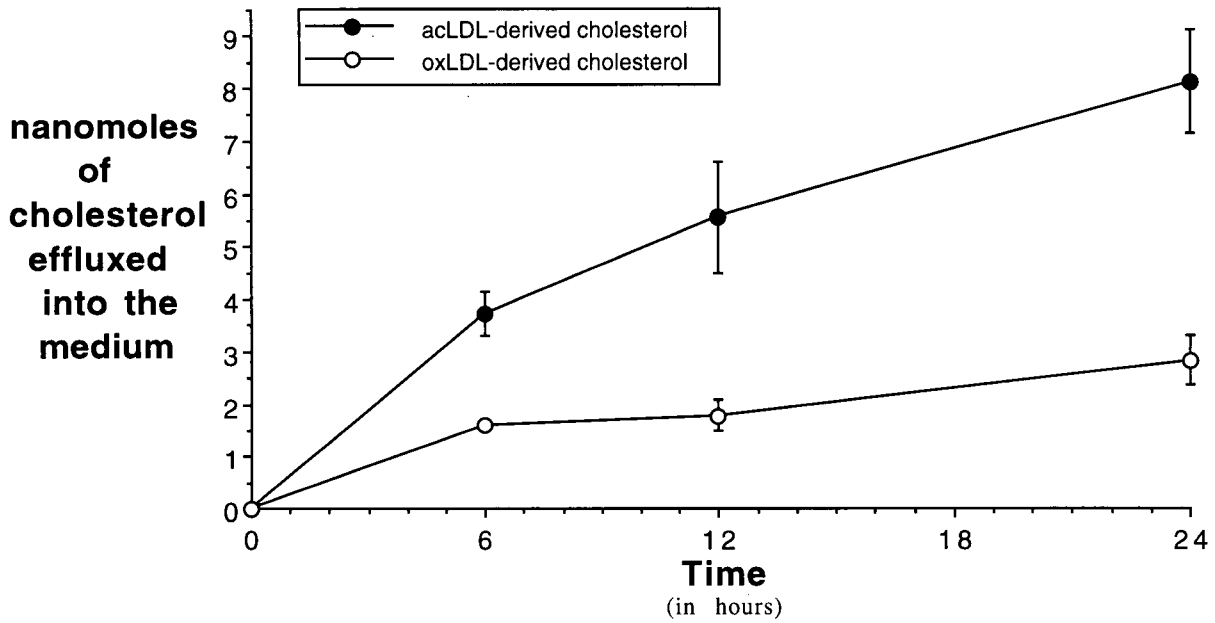


Figure 11 Effect of HDL incubation time on cholesterol efflux.

Each point represents the mean dpm's of [^{3}H]-cholesterol measured in the medium after exposure to HDL_3. In this experiment, cells were loaded with 50 μg/ml of either radiolabelled acetylated LDL or radiolabelled oxidized LDL. Then, the cells were washed and exposed to 75 μg/ml of HDL_3 for 6, 12 or 24 hours, at the end of which, the radioactivity in the medium was measured. For this experiment, LDL was radiolabelled with [^{3}H]-cholesteryl linoleate before being either acetylated or oxidized. The values are mean ± S.D. for triplicate plates from one representative experiment out of two with similar results. Error bars not visible are within the plot symbols.

At the end of the 24 hour incubation in the presence of 50 μg/ml radiolabelled acetylated LDL or 50 μg/ml radiolabelled oxidized LDL, the cells were washed and then incubated with 75 μg/ml HDL_3 for an additional 6, 12 or 24 hours. From Figure 11, we can

see that there was a progressive, time-dependent appearance of [^{3}H]-cholesterol in medium containing 75 μg/ml HDL_3, and it did not appear to be complete by 24 hours. This finding is supported by the literature (300). Since an HDL incubation time of 12 hours resulted in a substantial efflux of cholesterol from acetylated LDL-loaded cells, whereas an incubation time or 24 hours was associated with a decrease in viability (data not shown), 12 hours was deemed to be the optimal duration to be used in determining the efficiency of cholesterol efflux from these lipid-loaded cells.

The transport pathways through which cholesterol is recycled between the endoplasmic reticulum and the plasma membrane are poorly characterized (350-354). It has been suggested that cholesterol removed from cells by long-term incubations (> 6 hours) with HDL may pass through the Golgi apparatus (355, 356). In order to further characterize HDL-mediated cholesterol efflux, we explored the possible involvement of the Golgi by treating acetylated LDL-loaded macrophages with an inhibitor of Golgi transport before exposing them to HDL_3. Essential to the many cellular functions that take place within the central vacuolar system (which consists of the ER, Golgi apparatus, secretory vesicles, endosomes, and lysosomes) is membrane traffic, which mediates the exchange of components between different organelles (357-359). Brefeldin A (BFA) inhibits anterograde transport from the endoplasmic reticulum to the Golgi apparatus (360) and in contrast, it enhances retrograde transport from the Golgi apparatus to the endoplasmic reticulum (361). Although these have been the best characterized actions of BFA, its effects are not limited to the Golgi apparatus. For example, one of the hallmarks of the morphological effects of BFA is the rapid and dramatic induction of tubules not only in the Golgi apparatus, but also in peripheral organelles including endosomes, lysosomes, and the trans-Golgi network (362-

364). The effects of BFA are concentration dependent, as high concentrations of BFA are cytotoxic to cells. In our experiments, we found that concentrations greater than 3.5 μg/ml were cytotoxic.

In this experiment, macrophages were loaded for 24 hours with no lipoprotein (control) or 50 μg/ml acetylated LDL. Representative cultures were then washed with PBS and removed for analysis. After being washed with PBS, the remaining plates were treated for 30 minutes with either 2.25 μg/ml Brefeldin A (dissolved in ethanol) or an equivalent volume of ethanol alone. Finally, these plates were exposed to 75 μg/ml HDL_3 for 12 hours. Prior to incubation with acetylated LDL, cells had only 63 μg free cholesterol/mg cell protein and the esterified cholesterol content of these cells was negligible. After 24 hours, total cholesterol increased to 326 μg cholesterol/mg cell protein — a five-fold augmentation over the baseline level. Figure 12 depicts the degree of cholesterol efflux observed in each case.

We found that treating acetylated LDL-loaded cells with inhibitors of Golgi transport completely abolished the ability of HDL_3 to stimulate cholesterol efflux. This is in agreement with the findings of Mendez et al. (355, 356). It has been suggested that free cholesterol is transported from the endoplasmic reticulum and/or lysosomes to the Golgi apparatus and then assembled in phospholipid-rich vesicles that are subsequently transported to the cell surface. Upon fusion with the plasma membrane, these vesicles may form discrete membrane domains with unique compositions of lipids and proteins, similar to what has been described for caveolae (365, 366).

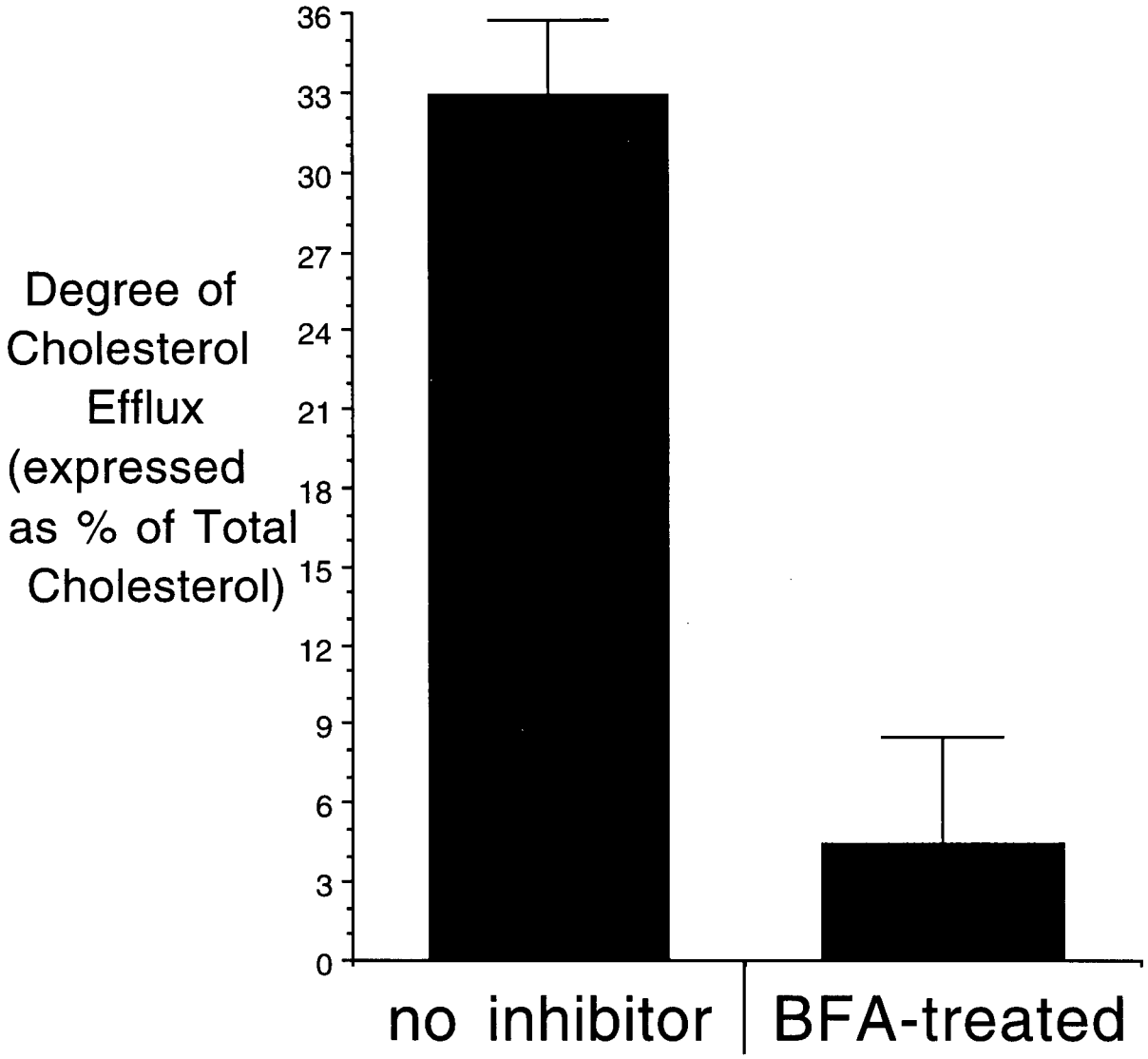


Figure 12 Comparison of cholesterol efflux in normal and Brefeldin A-treated cells.

Each bar represents the percent decrease in total cholesterol after HDL-mediated cholesterol efflux in cells that had been loaded with acetylated LDL. Prior to exposure to HDL, cells were treated for 30 minutes with either 2.25 μg/ml of Brefeldin A (in ethanol) or the equivalent volume of ethanol alone. The acetylated LDL-loaded cells that had not been exposed to Brefeldin A exhibited a significant ($p < 0.0001$) decrease in total cholesterol upon exposure to HDL_3. Furthermore, there was a statistically significant ($p < 0.05$) difference in the degree of cholesterol efflux in the BFA-treated cells compared to the untreated cells. Each value represents the mean percentage difference ± SD from triplicate plates from one of two experiments with similar results.

When resident peritoneal macrophages isolated from CD-1 mice were incubated for 24 hours with either 50 μg/ml acetylated LDL or 50 μg/ml oxidized LDL, there was an increase in both esterified cholesterol and free cholesterol compared to control cells which were given no lipoproteins during the same time period. After the initial 24 hours incubation, cells were washed and then exposed to 75 μg/ml HDL_3 for 12 hours. This resulted in a significant (* $p < 0.0001$) decrease in total cholesterol from the acetylated LDL-loaded cells compared to the acetylated LDL-loaded cells that had not been exposed to HDL_3. In contrast, there was no significant decrease in total cholesterol in the oxidized LDL-loaded cells compared to the oxidized LDL-loaded cells that had not been exposed to HDL_3. Figure 13 shows the results of one representative experiment out of thirty-six.

At the end of the first 24 hour incubation in the presence or absence of modified lipoproteins, half of the cells were extracted and assayed for protein and cholesterol content, and the remaining plates were washed and then treated with HDL_3 for an additional 12 hours, after which they were processed in the same manner as the first set. As would be expected from the composition of these modified LDL's (Table 5), the acetylated LDL-loaded cells accumulated large quantities of cholesterol ester, as well as free cholesterol, whereas the oxidized LDL-loaded cells accumulated predominantly free cholesterol. The increase in total cholesterol in the acetylated LDL-loaded macrophages represented a five-fold augmentation over the baseline level. Similarly, the increase in total cholesterol in the oxidized LDL-loaded macrophages represented a greater than two-fold augmentation over the baseline level. In such experiments, we have demonstrated that mouse peritoneal macrophages incubated with either acetylated LDL or oxidized LDL typically exhibit a two-fold to six-fold augmentation of their total cholesterol compared to control (nonloaded) cells. Indeed, many

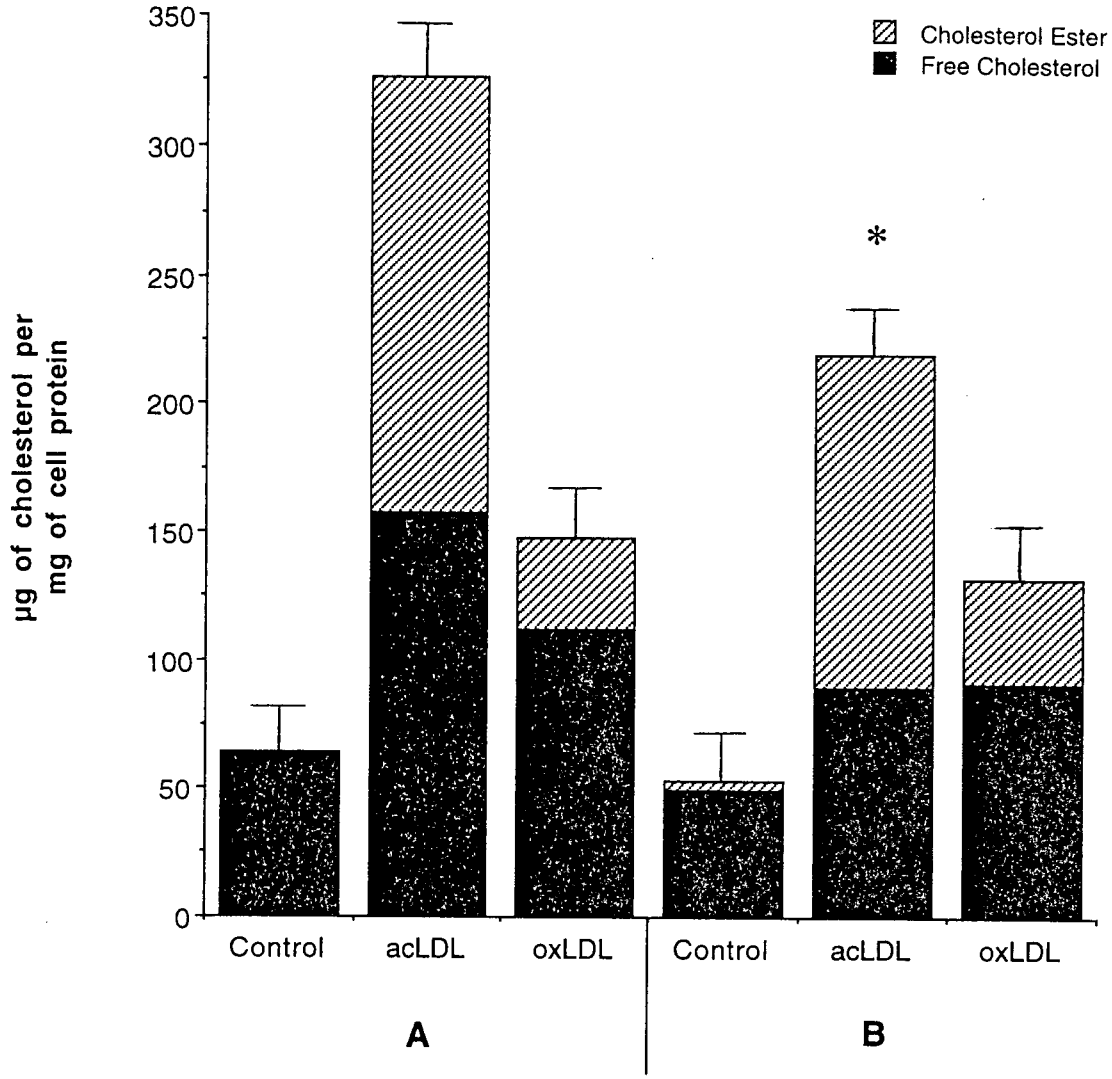


Figure 13 Total cholesterol content of cells after lipid-loading and exposure to HDL_3.

Each bar represents the micrograms of total cholesterol per milligram of cell protein; within each bar the solid area represents the free cholesterol content of the cells, and the hatched area represents the cholesterol ester content of the cells. Panel A depicts macrophages that were loaded for 24 hours with no lipoprotein, acetylated LDL, or oxidized LDL. Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of HDL_3. There was a massive accumulation of cholesterol in the macrophages loaded with modified LDL compared to the control cells. The acetylated LDL-loaded macrophages exhibited a significant (* $p < 0.0001$) decrease in total cholesterol upon exposure to HDL_3, whereas the decrease in cholesterol in the oxidized LDL-loaded cells was not statistically significant. Each value represents the mean ± S.D. for three determinations from one typical experiment out of thirty-six with similar results.

other studies have also reported that acetylated LDL and oxidized LDL stimulate significant (100-600 μg/mg cell protein) cholesterol accumulation in macrophages from many species (11, 300, 335, 367-370). In comparison to acetylated LDL-loaded cells, we have consistently found that oxidized LDL-loaded cells exhibit a significant impairment of HDL-mediated cholesterol efflux. This is in agreement with the findings of Kritharides et al. (300).

Because of the precision of the protein and cholesterol assays, the relatively small changes in cholesterol observed in oxidized LDL-loaded cells after treatment with HDL_3 often failed to achieve significance in individual experiments. However, when we compared the decrease in total cholesterol in oxidized LDL-loaded cells to the decrease measured in acetylated LDL-loaded cells, the difference was always statistically significant. When we averaged the data from experiments in which there was a nearly identical degree of loading, we found that the cholesterol efflux from oxidized LDL-loaded cells amounted to about 10% of the total cholesterol content of these cells (as depicted in Figure 14) — which is much lower than the approximately 30% decrease in total cholesterol observed in acetylated-LDL loaded cells that had been treated with HDL_3.

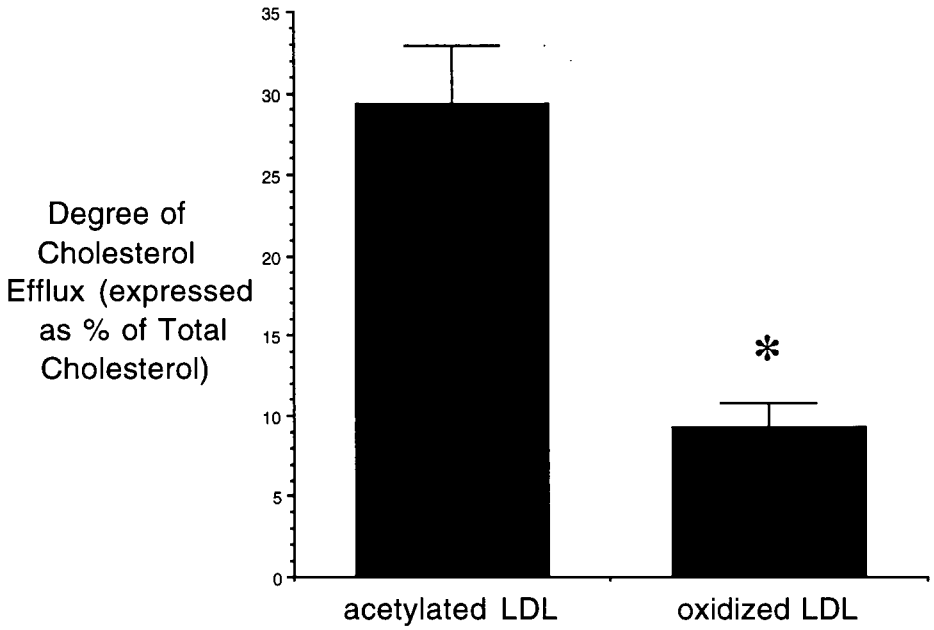


Figure 14 Decrease in total cholesterol after HDL_3-mediated cholesterol efflux.

Each bar represents the percent decrease in total cholesterol after HDL-mediated cholesterol efflux in cells that had been loaded with either acetylated LDL or oxidized LDL. Each value represents the mean ± S.E. of triplicate determinations from three experiments with similar results. The design of the experiments used to generate the data in this figure is the same as the experimental design of Figure 13. Macrophages were incubated for 24 hours with no lipoprotein, 50 μg/ml acetylated LDL, or 50 μg/ml oxidized LDL. Half of the plates were removed and the contents were lipid extracted and analyzed for protein and cholesterol content. The remaining plates were washed and then incubated for a further 12 hours with 75 μg/ml HDL_3. Finally, these cells were also lipid extracted and analyzed for protein and cholesterol content. There was a significant (* $p < 0.01$) difference between the degree of cholesterol efflux observed in the acetylated LDL-loaded cells compared to the degree of cholesterol efflux observed in the oxidized LDL-loaded cells.

3.3 Impairment of cholesterol efflux involves both lipid and protein modification of LDL

Oxidized LDL is known to be susceptible to aggregation (371). Like acetylated LDL and oxidized LDL, uptake of vortex-aggregated LDL also leads to cholesterol accumulation within macrophages. In order to rule out aggregation as a cause of impaired cholesterol efflux from macrophages, the efflux of cholesterol from aggregated LDL-loaded macrophages was measured. Figure 15 depicts the results from one such experiment.

At the end of the first 24 hour incubation in the presence or absence of modified lipoproteins, half of the cells were extracted and assayed for protein and cholesterol content, and the remaining plates were washed and then treated with HDL_3 for an additional 12 hours, after which they were processed in the same manner as the first set. The increase in total cholesterol in the acetylated LDL-loaded macrophages represented a greater than four-fold augmentation over the baseline level. Similarly, the increase in total cholesterol in both the vortex-aggregated LDL-loaded macrophages and the oxidized LDL-loaded macrophages represented a five-fold augmentation over the baseline level. The HDL-mediated efflux of cholesterol from the aggregated LDL-loaded cells was intermediate (31% of total) between that of the acetylated LDL-loaded cells (51%) and the oxidized LDL-loaded cells (5%). This suggests that aggregation of LDL may contribute to the impaired efflux of cholesterol from oxidized LDL-loaded cells. However, it does not appear to be the predominant cause of the impaired efflux.

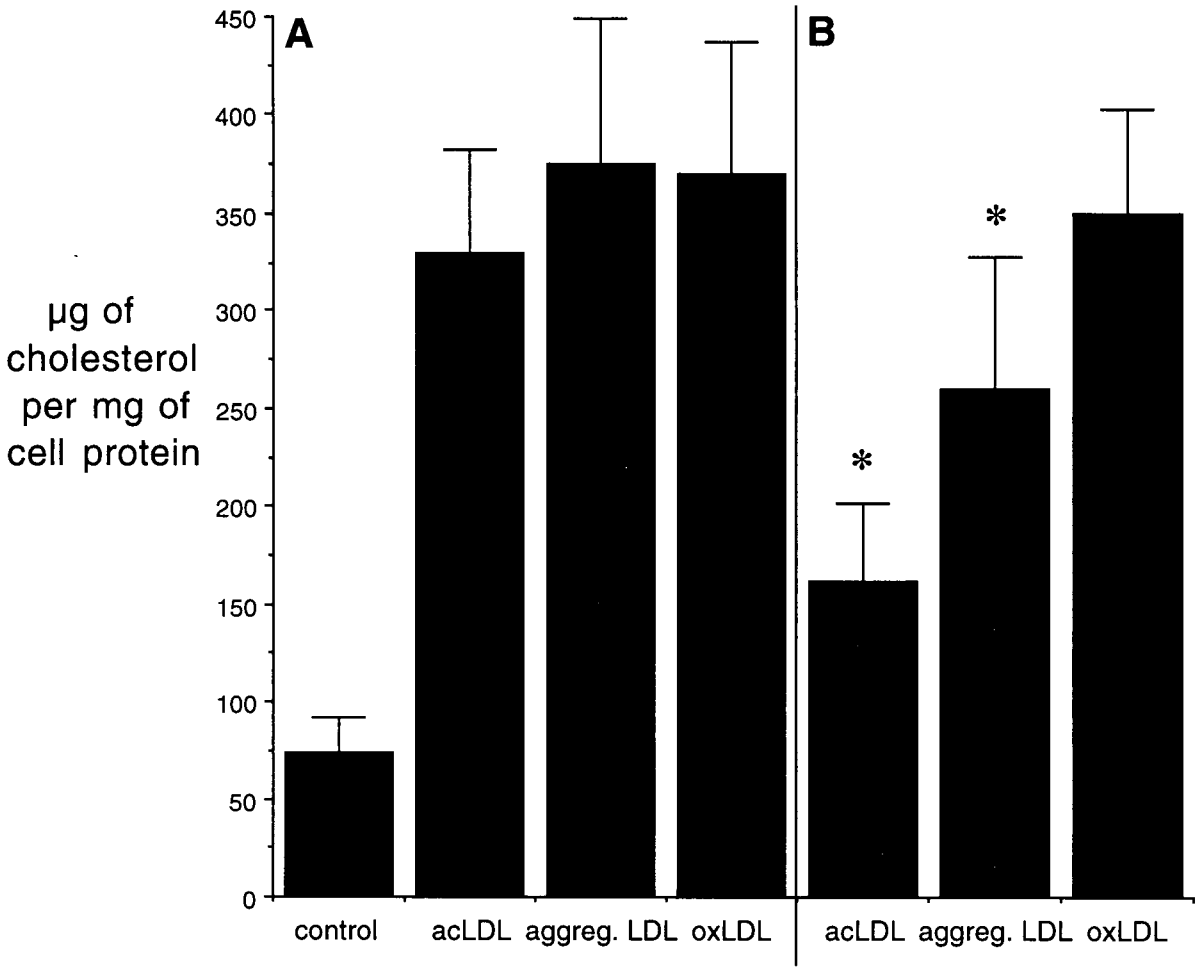


Figure 15 Accumulation and efflux of aggregated LDL-derived cholesterol.

Each bar represents the micrograms of total cholesterol per milligram of cell protein. Panel A depicts macrophages that were loaded for 24 hours with no lipoprotein, 50 μg/ml acetylated LDL (acLDL), 50 μg/ml oxidized LDL (oxLDL) or 50 μg/ml vortex-aggregated LDL (aggreg. LDL). Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of HDL_3. There was a significant accumulation of cholesterol in the macrophages loaded with modified LDL compared to the control cells. Both the acetylated LDL-loaded macrophages and the vortex-aggregated LDL-loaded macrophages exhibited a significant (* $p < 0.05$) decrease in total cholesterol upon exposure to HDL_3. Each value represents the mean ± S.D. for four determinations from a single experiment.

The impairment of efflux of cholesterol from macrophages loaded with oxidized LDL suggests that the metabolism of oxidized LDL is different from that of acetylated LDL. In fact, it has been reported that the protein moiety of oxidized LDL is hydrolyzed at a much slower rate than that of acetylated LDL (129, 295, 304). In order to compare the rate at which macrophages take up and degrade each of these modified lipoproteins, cells were loaded with ^{125}I-labelled modified LDL's, and then we determined the degradation of these lipoproteins as well as the cholesterol mass and radioactive LDL content of both loaded and effluxed cells. It has been debated whether extensive oxidation occurs *in vivo*. To determine whether mild oxidation could result in impairment of cholesterol efflux, we loaded cells with acetylated LDL that had been either mildly (5 hrs) or extensively (24 hrs) oxidized. It has been reported that the apolipoprotein component of oxidized LDL is resistant to lysosomal cathepsins, whereas the apolipoprotein component of acetylated LDL is hypersensitive to lysosomal cathepsins (129). The oxidation of acetylated LDL provides a molecule that contains an acetylated apolipoprotein component along with an oxidized lipid component. In acetylated LDL that has been subsequently oxidized, the apolipoprotein does get oxidized to some degree, but it is not oxidized nearly as extensively as oxidized LDL. Thus, it can serve as a useful tool because it allows us to deliver oxidized lipids without rendering the protein resistant to cathepsins. Figures 16, 17 and 18 depict the results from one of two experiments.

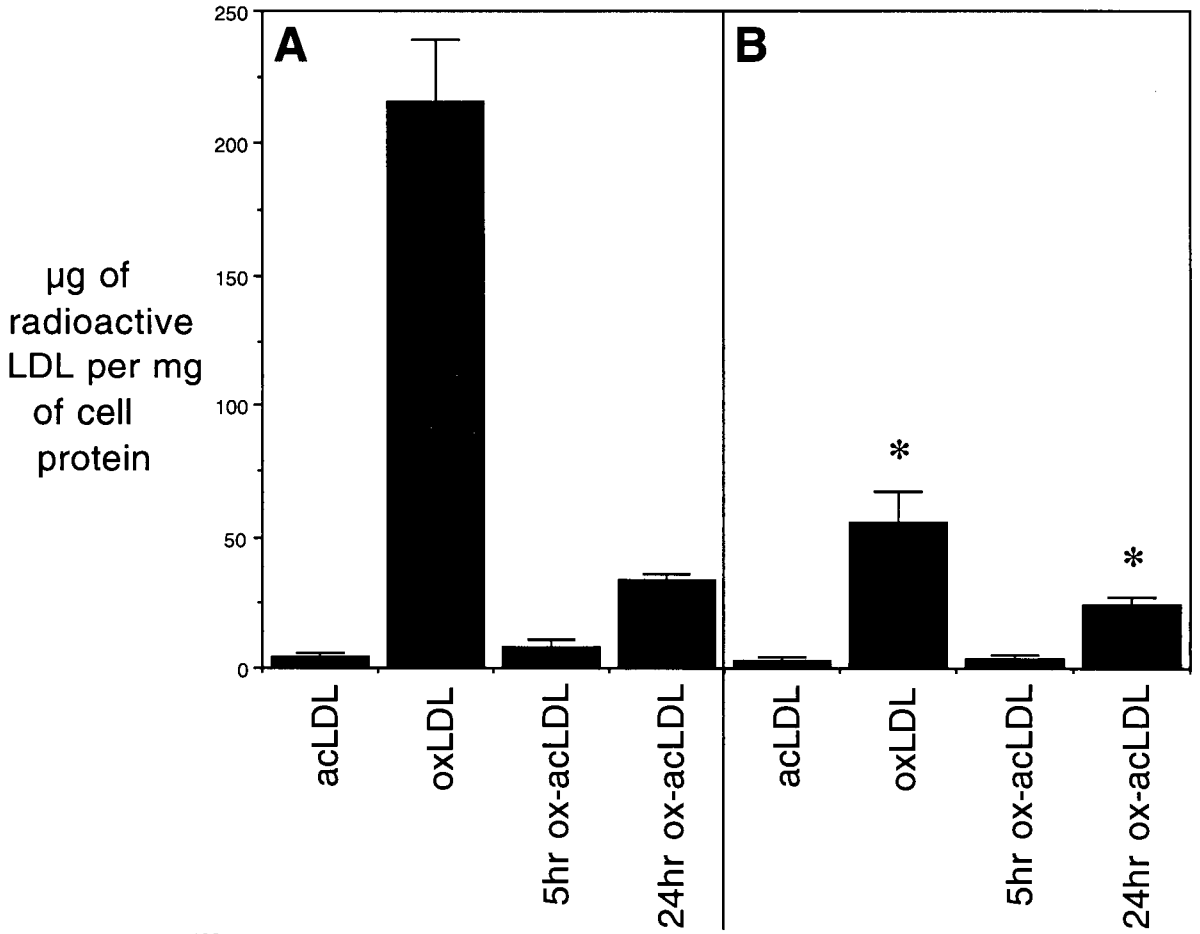


Figure 16 ^{125}I-labelled modified LDL content of loaded and effluxed cells.

Each bar represents the micrograms of radiolabelled modified LDL in the cells per milligram of cell protein. Panel A depicts macrophages that were loaded for 24 hours with 50 µg/ml of one of the following radiolabelled lipoproteins: acetylated LDL (acLDL), oxidized LDL (oxLDL), acetylated LDL that had been oxidized for 5 hours (5 hr ox-acLDL), or acetylated LDL that had been oxidized for 24 hours (24 hr ox-acLDL). Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of 75 µg/ml HDL_3. The most pronounced increase in radioactivity occurred in the cells loaded with oxidized LDL (oxLDL), followed by the cells loaded with 24 hr ox-acLDL. With respect to HDL-mediated cholesterol efflux, there was a statistically significant (* $p < 0.01$) decrease in radioactive LDL in two of the conditions: the plates loaded with oxidized LDL and then exposed to HDL_3 exhibited a significant decrease in radioactive LDL compared to the corresponding plates that were not treated with HDL_3; similarly, the plates loaded with 24 hr ox-acLDL and then exposed to HDL_3 exhibited a significant decrease in radioactive LDL compared to the corresponding plates that were not treated with HDL_3. The values are mean ± S.D. for triplicate plates from one of two experiments with similar results.

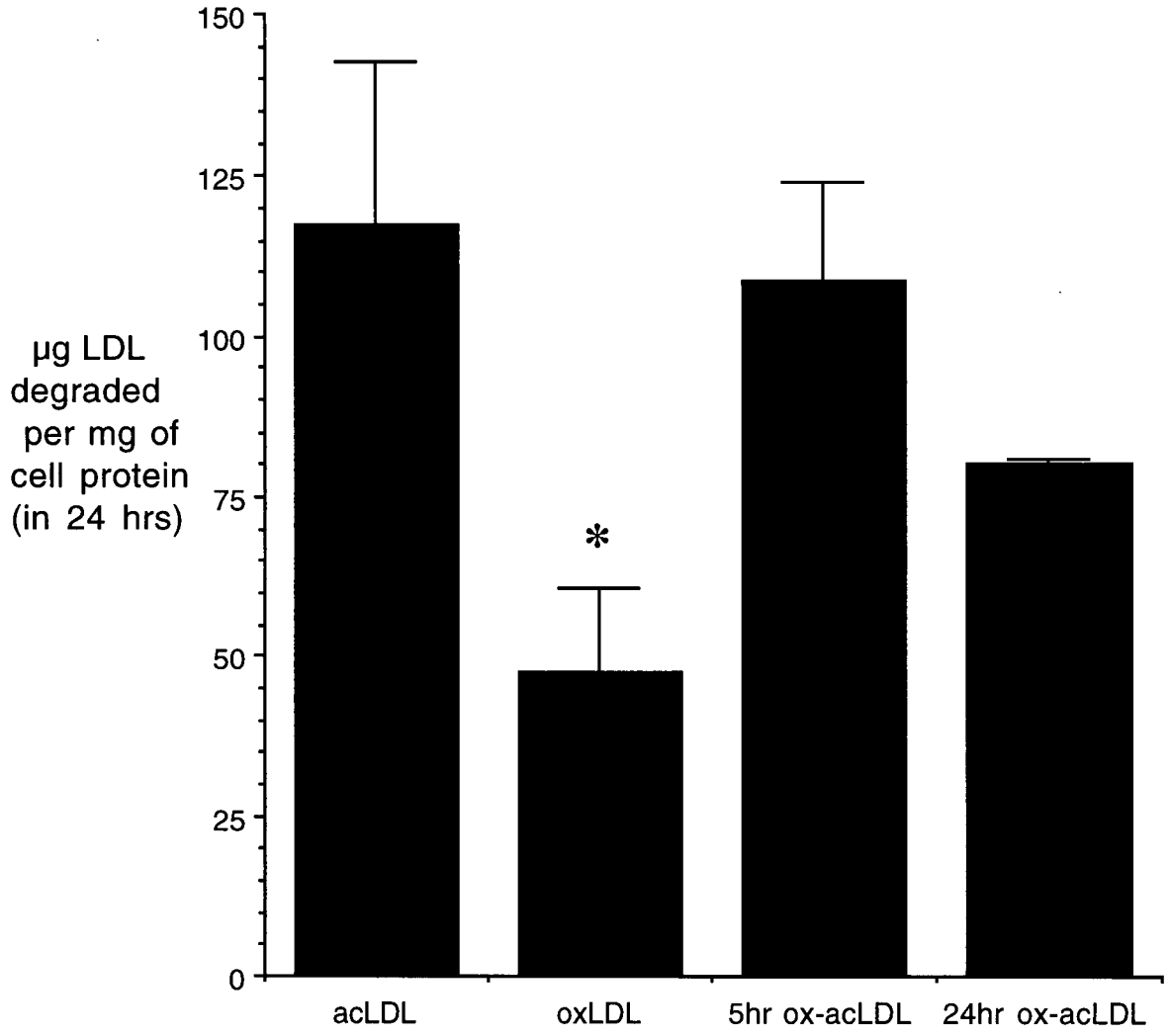


Figure 17 Degradation of ^{125}I-labelled modified LDL by macrophages.

Each bar represents the micrograms of radioactive LDL degraded per milligram of cell protein, over the course of 24 hours. Cells loaded with acetylated LDL (acLDL) exhibited the highest degree of LDL degradation, followed by the cells that had been loaded with acetylated LDL that had been oxidized for 5 hours (5 hr ox-acLDL). The cells loaded with acetylated LDL that had been oxidized for 24 hours (24 hr ox-acLDL) degraded one-third less LDL than the cells loaded with acLDL. Finally the cells loaded with oxidized LDL (oxLDL) degraded less than half as much LDL as the cells loaded with acLDL. However, only the oxLDL-loaded cells exhibited a statistically significant difference in LDL degradation compared to acLDL-loaded cells (* $p < 0.01$). The values are mean ± S.D. for triplicate plates from one of two experiments with similar results.

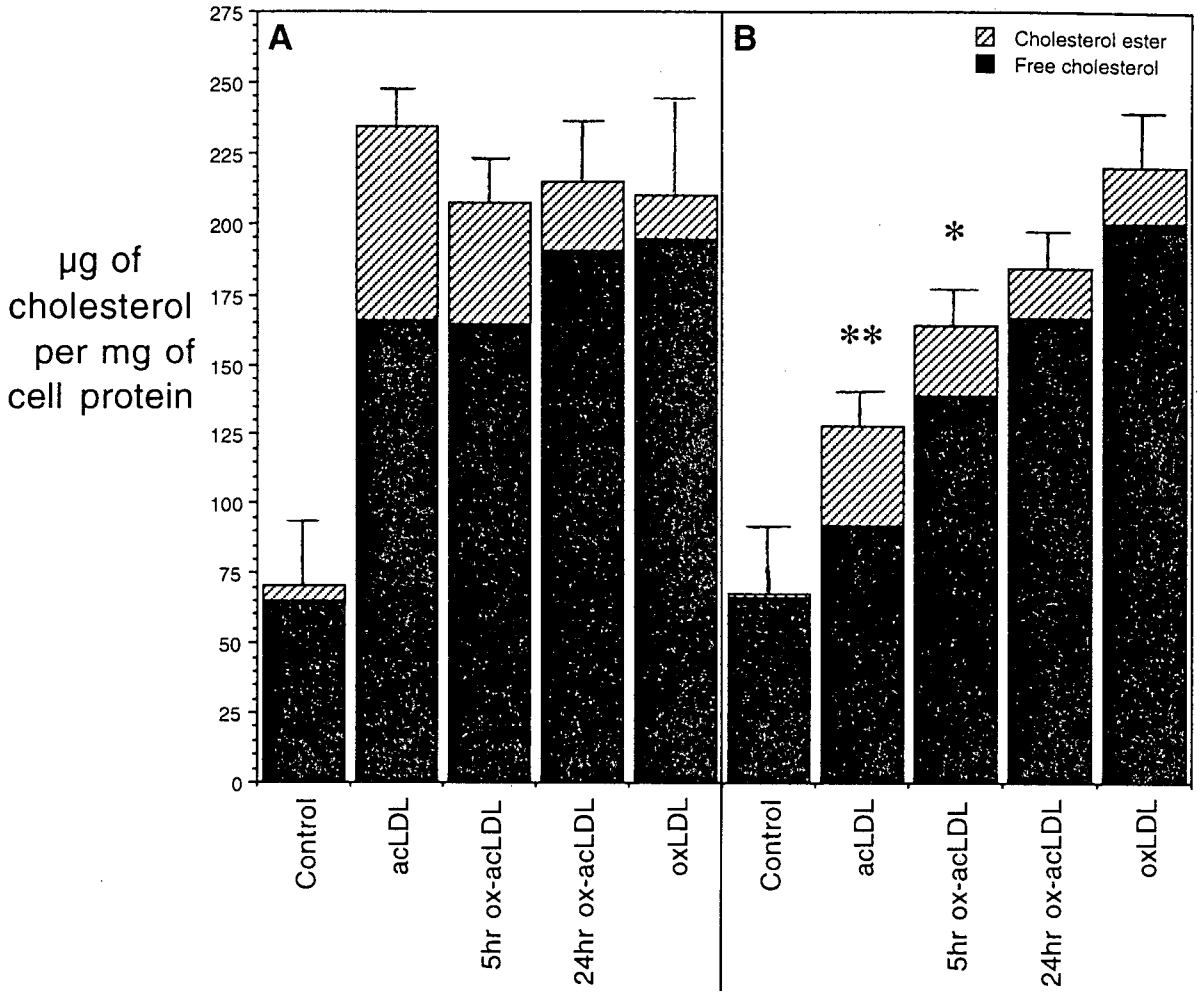


Figure 18 Accumulation and efflux of cholesterol from various modified LDL's.

Each bar represents the micrograms of total cholesterol per milligram of cell protein; within each bar the solid area represents the free cholesterol content of the cells, and the hatched area represents the cholesterol ester content of the cells. Panel A depicts macrophages that were loaded for 24 hours with either no lipoprotein or 50 µg/ml of one of the following: acetylated LDL (acLDL), acetylated LDL that had been oxidized for 5 hours (5hr ox-acLDL), acetylated LDL that had been oxidized for 24 hours (24hr ox-acLDL), or oxidized LDL (oxLDL). Panel B represents macrophages that were treated in the same manner as the cells in Panel A, only they underwent a further 12 hour incubation in the presence of 75 µg/ml HDL_3. Compared to the control cells, there was a statistically significant accumulation of cholesterol in all of the lipid-loaded macrophages. After treatment with HDL_3, there was a decrease in total cholesterol in all of the cells except the cells loaded with oxidized LDL. However, a statistically significant decrease in total cholesterol occurred in only two of the conditions: the plates loaded with acetylated LDL and then exposed to HDL_3 exhibited a significant decrease (** $p < 0.001$) in total cholesterol compared to the corresponding plates that were not treated with HDL_3;

similarly, the plates loaded with 5 hr ox-acLDL and then exposed to HDL_3 exhibited a significant decrease (* $p < 0.01$) in total cholesterol compared to the corresponding plates that were not treated with HDL_3. The values are mean ± S.D. for triplicate plates from one of two experiments with similar results.

Protein was labelled by radioiodination (311). In the experiment depicted in Figures 16 and 17, we measured the cellular content and degradation of modified apolipoprotein B. In Figure 16, we see a massive accumulation of ^{125}I-labelled oxidized apo B derived from oxidized LDL. In contrast, there was very little accumulation of ^{125}I-labelled acetylated apo B derived from acetylated LDL or acetylated LDL that had been oxidized for 5 hours. However, there was an appreciable accumulation of ^{125}I-labelled acetylated apo B derived from acetylated LDL that had been oxidized for 24 hours. Since we have seen massive accumulation of cholesterol in macrophages loaded with acetylated LDL, the results from this experiment provide further evidence that the protein component of acetylated LDL is metabolized differently from the lipid component; the results also confirm that the protein component of acetylated LDL is hypersensitive to lysosomal degradation and therefore, very little accumulation of acetylated apo B occurs. On the other hand, the large accumulation of oxidized apo B within the cells suggests that the protein component of oxidized LDL is resistant to lysosomal degradation, and that is why it accumulates within the macrophages. Following a 12 hour exposure to HDL_3, we saw a statistically significant decrease in radiolabelled modified apo B from the plates loaded with either oxidized LDL or acetylated LDL that had been oxidized for 24 hours. This may seem in opposition to our results which showed that oxidized LDL-derived macrophages exhibit impaired efflux of cholesterol; however, it must be recognized that although three-quarters of the accumulated oxidized apo B was removed from the cells upon treatment with HDL_3, the oxidized apo B that remained within the cells was still more than 20-fold higher than the acetylated apo B that remained

within the cells. Nevertheless, it is possible that the rapid decrease in cell-associated oxidized apolipoprotein B during the 12 hour "efflux" incubation involves exocytosis of undegraded oxidized LDL. If this were the case, the efflux medium would contain not only free cholesterol but also esterified cholesterol and oxysterols. To test this hypothesis, LDL was labelled with [^{3}H]-cholesteryl linoleate before being oxidized. Macrophages were treated with 50 μg/ml of this radiolabelled oxidized LDL for 24 hours, washed and then treated with 75 μg/ml HDL_3 for 12 hours. The efflux medium was removed, lipid extracted, and run on thin layer chromatography. The spots corresponding to free cholesterol, cholesterol oleate, cholesterol linoleate, and 7-ketocholesterol were scraped and counted on a Beckman LS5000CE scintillation spectrometer. The radioactivity of each spot is presented in Table 6 as the mean ± SD of triplicate plates from one single experiment.

Table 6 Sterol efflux into the medium from oxidized LDL-loaded macrophages

cholesterol	4230 ± 423 dpm's
cholesterol oleate	30 ± 18 dpm's
cholesterol linoleate	12 ± 12 dpm's
7-ketocholesterol	7 ± 19 dpm's

The same experiment was conducted using radiolabelled acetylated LDL, and although a decrease in cellular cholesterol ester content occurred, we found that only free cholesterol appeared in the medium, not cholesterol ester (data not shown). This is consistent with previous reports that any decrease in cholesterol ester content of cells is the result of hydrolysis of the ester rather than efflux of esterified cholesterol (347). Furthermore, the

appearance of only free cholesterol in the efflux medium opposes the hypothesis that exocytosis of undegraded oxidized LDL occurs.

In Figure 17, we see that the acetylated apo B is efficiently degraded but the oxidized apo B is not. Although the 5 hr ox-acLDL-loaded cells and the 24 hr ox-acLDL-loaded cells degraded less LDL than the cells loaded with acetylated LDL, the difference was not statistically significant. The significantly lower (* $p < 0.01$)) degradation of oxidized LDL-derived apo B compared to acetylated LDL-derived apo B is consistent with the retention of oxidized apo B within the cells. Thus, compared to apolipoprotein derived from acetylated LDL, apolipoprotein derived from oxidized LDL exhibits impaired degradation within macrophages, which results in accumulation within the cell. This experiment would be easier to interpret if the acetylated LDL degradation was not different from that of the acetylated LDL that had subsequently been oxidized, because that would clearly demonstrate that introducing oxidized lipids has no effect on the rapid metabolism of apolipoprotein from acetylated LDL. However, our data indicate that increasing the degree of oxidation of acetylated LDL from 5 hours of oxidation to 24 hours of oxidation resulted in poorer degradation and greater accumulation within the cells. Nevertheless, the data in Figures 16 and 17 does suggest that oxidized lipid may interfere with the normal degradation of the apolipoprotein component of LDL.

In Figure 18, we see the corresponding cholesterol content of the cells. There was an approximately three-fold augmentation of total cholesterol in all of the lipid-loaded cells compared to the control (nonloaded) cells. Here, again we see the characteristic impairment of cholesterol efflux from oxidized LDL-loaded macrophages, as compared to acetylated LDL-loaded macrophages. Introducing oxidized lipids into acetylated LDL resulted in

decreased efflux of cholesterol to HDL_3. Even mildly oxidized (5 hr) acetylated LDL resulted in impaired efflux of cholesterol compared to unoxidized acetylated LDL. Moreover, increasing the degree of oxidation of acetylated LDL resulted in a decrease in cholesterol efflux in the cells loaded with these modified LDL's. This suggests a prominent causal role for oxidized lipids in the impairment of cholesterol efflux.

In order to investigate the role of the oxidized protein component in the impairment of cholesterol efflux, cells were loaded with arachidonic acid-oxidation product LDL (A-OP LDL). This way, we were able to see the effects of delivering LDL that contained an oxidatively modified apolipoprotein component but not an oxidized lipid component. It has been proposed that aldehydic adducts on oxidized LDL that are unreactive at neutral pH become exposed at acidic pH and then covalently bind thiols on neighbouring proteins such as cathepsin B in lysosomes, thereby inducing cross-linking of proteins and consequently, enzyme inactivation (302). In order to test whether inactivation of such proteases by aldehydes in oxidized LDL plays a role in impairment of cholesterol efflux, we loaded macrophages with oxidized LDL that had been treated with sodium borohydride (to reduce the aldehydes to alcohols) and then determined whether the rate of efflux to HDL_3 was greatly enhanced. At the end of the first 24 hour incubation in the presence or absence of 50 μg/ml modified LDL's, representative cultures were extracted and assayed for protein and cholesterol content, and the remaining plates were washed and then treated with 75 μg/ml HDL_3 for an additional 12 hours, after which they were processed in the same manner as the first set. Prior to incubation, cells contained only 70 μg cholesterol/mg cell protein, nearly all of which was in the form of free (unesterified) cholesterol. After 24 hours, total cholesterol increased to 230 μg cholesterol/mg cell protein in the acetylated LDL-loaded

cells, 172 μg cholesterol/mg cell protein in the arachidonic acid-oxidation product LDL-loaded cells, 210 μg cholesterol/mg cell protein in the oxidized LDL-loaded cells, and 211 μg cholesterol/mg cell protein in the cells loaded with oxidized LDL that had been treated with sodium borohydride. The increase in total cholesterol in the A-OP LDL-loaded cells represented a 2.5-fold augmentation compared to the control cells; and there was an approximately three-fold augmentation of total cholesterol in all of the other lipid-loaded cells compared to control. The degree to which cholesterol efflux occurred in each of the conditions is represented in Figure 19.

Compared to acetylated LDL-loaded cells, the cells loaded with any of the other three modified LDL's resulted in decreased efflux of cholesterol to HDL_3. Only the decrease in total cholesterol from the acetylated LDL-loaded cells can be regarded as a statistically significant ($p < 0.05$)) decrease in total cholesterol, as assessed by student's t-test. In this experiment, we found that A-OP LDL-loaded cells exhibited a profound impairment of cholesterol efflux to HDL_3, which highlights the role of the oxidized apolipoprotein component in this phenomenon. Finally, quenching the aldehydes in oxidized LDL increased the rate of cholesterol efflux, suggesting that inactivation of certain lysosomal enzymes by aldehydes is a contributing factor in impairment of cholesterol efflux from oxidized LDL-loaded macrophages. Taken together, these results indicate that impairment of cholesterol efflux can be attributed to both the oxidized apolipoprotein component of oxidized LDL, as well as the oxidized lipid component. The latter claim is consistent with previous reports (372). It is clear that lipid and protein oxidation products, such as those found in oxidized LDL, can influence lysosomal metabolism of lipoproteins. For example, it has been

suggested that 7-ketocholesterol in oxidized LDL inhibits sphingomyelinase leading to a build-up of free cholesterol in lysosomes (92).

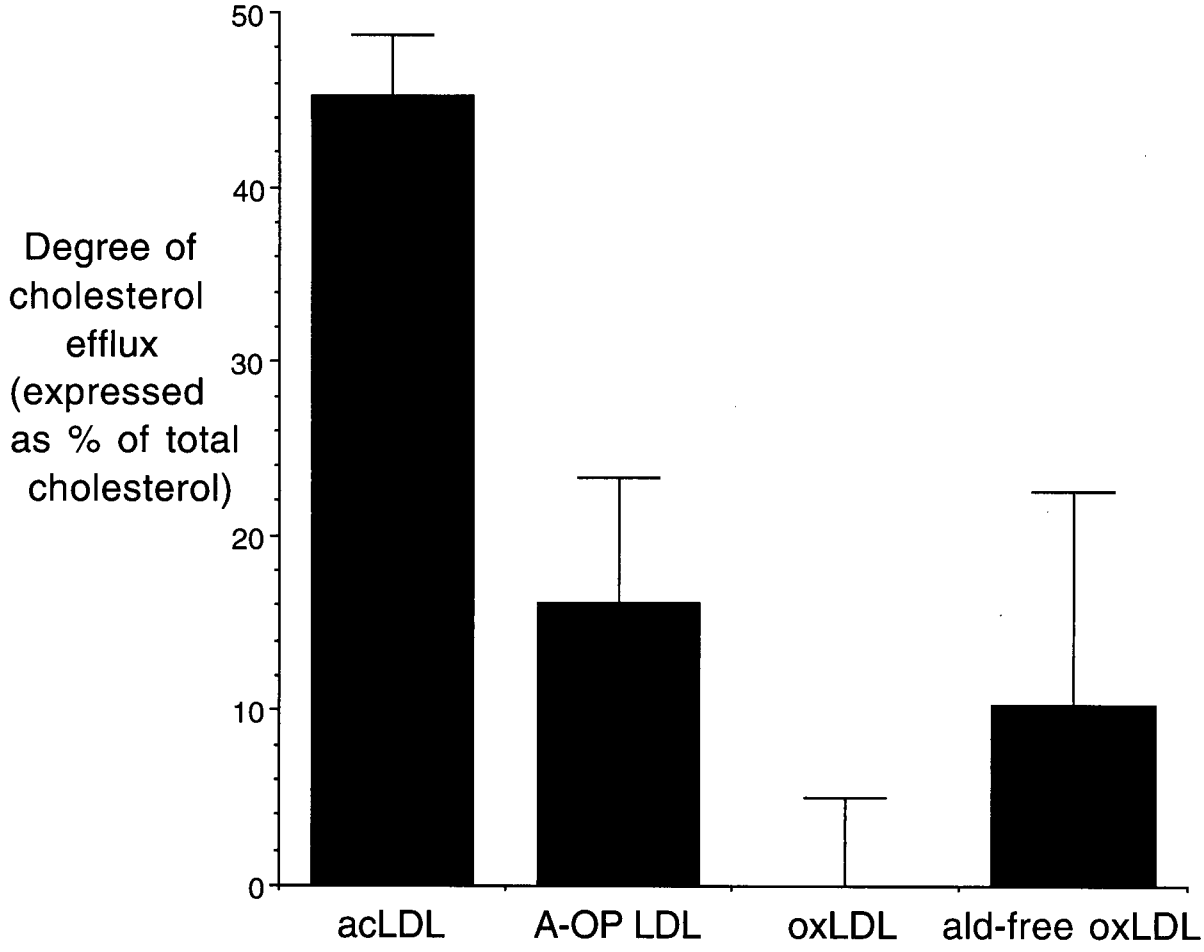


Figure 19 Decrease in cholesterol in cells loaded with $NaBH_4$-treated LDL and A-OP LDL.

Each bar represents the percent decrease in total cholesterol after HDL-mediated cholesterol efflux in cells that had been loaded with acetylated LDL (acLDL), arachidonic acid-oxidation product LDL (A-OP LDL), oxidized LDL (oxLDL), or aldehyde-free oxidized LDL (ald-free oxLDL). Each value is expressed as mean percentage decrease ± SD from triplicate plates from one of two experiments with similar results.

3.4 Cholesterol delivered by oxidized LDL accumulates in lysosomes

It has been postulated that degradation of the entire oxidized LDL particle may be arrested in the lysosome, and this would certainly account for the observed impairment of cholesterol efflux (304). If this is the case, one would expect that cholesterol would be sequestered in the lysosomal compartment of oxidized LDL-loaded cells. It has been previously reported that the apolipoprotein B component of oxidized LDL accumulates in the lysosomes of macrophages (305). However, there are conflicting reports regarding the lysosomal accumulation of cholesterol in oxidized LDL-loaded cells (92, 295, 296, 299-301, 335). In order to reconcile the contradictory findings, we investigated the subcellular distribution of cholesterol in both oxidized LDL-loaded cells and acetylated LDL-loaded cells.

Since we hypothesized that the oxidized LDL-derived cholesterol would accumulate within the lysosomal fraction, after loading cells with oxidized LDL, we disrupted the plasma membranes, fractionated using a density gradient, and analyzed each fraction for lysosomal marker enzyme activity and [^{3}H]-cholesterol content. As a point of reference, each fraction was also measured for endoplasmic reticulum marker enzyme activity. Before loading the cells with modified lipoproteins, we determined the relative distribution of the marker enzymes in control (nonloaded) macrophages. The results from one representative experiment out of three are presented in Figures 20 through 24. The distribution in control cells is given in Figure 20.

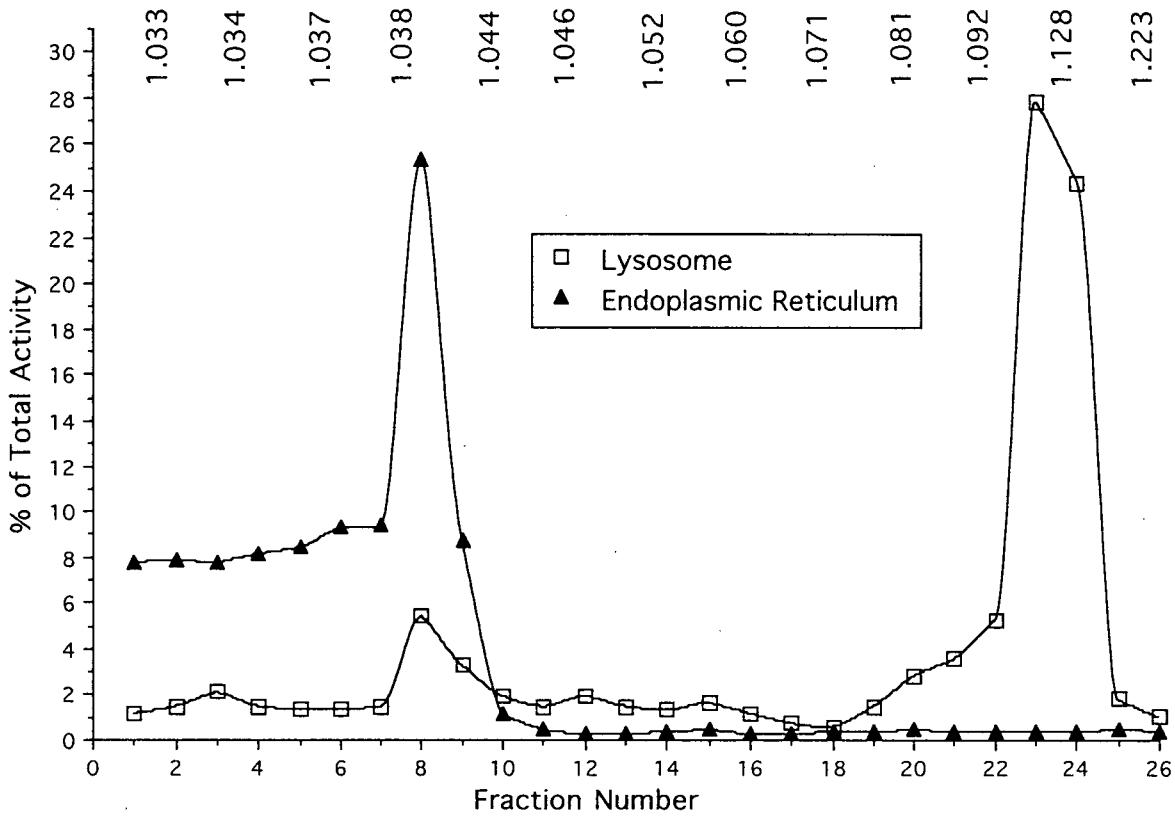


Figure 20 Distribution of ER and lysosome enzyme markers in control cells.

Each point represents the percentage of total enzyme activity in the fraction. In order to identify the lysosomal fraction, we assayed each fraction for acetylglucosaminidase. In order to identify the endoplasmic reticulum fraction, we assayed each fraction for neutral α-glucosidase. The density of the fractions (in grams/milliliter) is written across the top of the graph. Macrophages were incubated in the absence of lipoproteins for 24 hours. Then the plasma membranes were disrupted by nitrogen cavitation, and the nuclei, mitochondria, and intact cells were removed by centrifugation. Finally, the various subcellular fractions were separated by Percoll density gradient centrifugation.

In control (nonloaded) cells, we found that the endoplasmic reticulum fraction occurred at a density of approximately 1.040 g/ml, and the lysosomal fraction occurred at a density of approximately 1.100 g/ml. Figure 21 represents the distribution in macrophages that have been loaded for 24 hours with 50 µg/ml radiolabelled oxidized LDL.

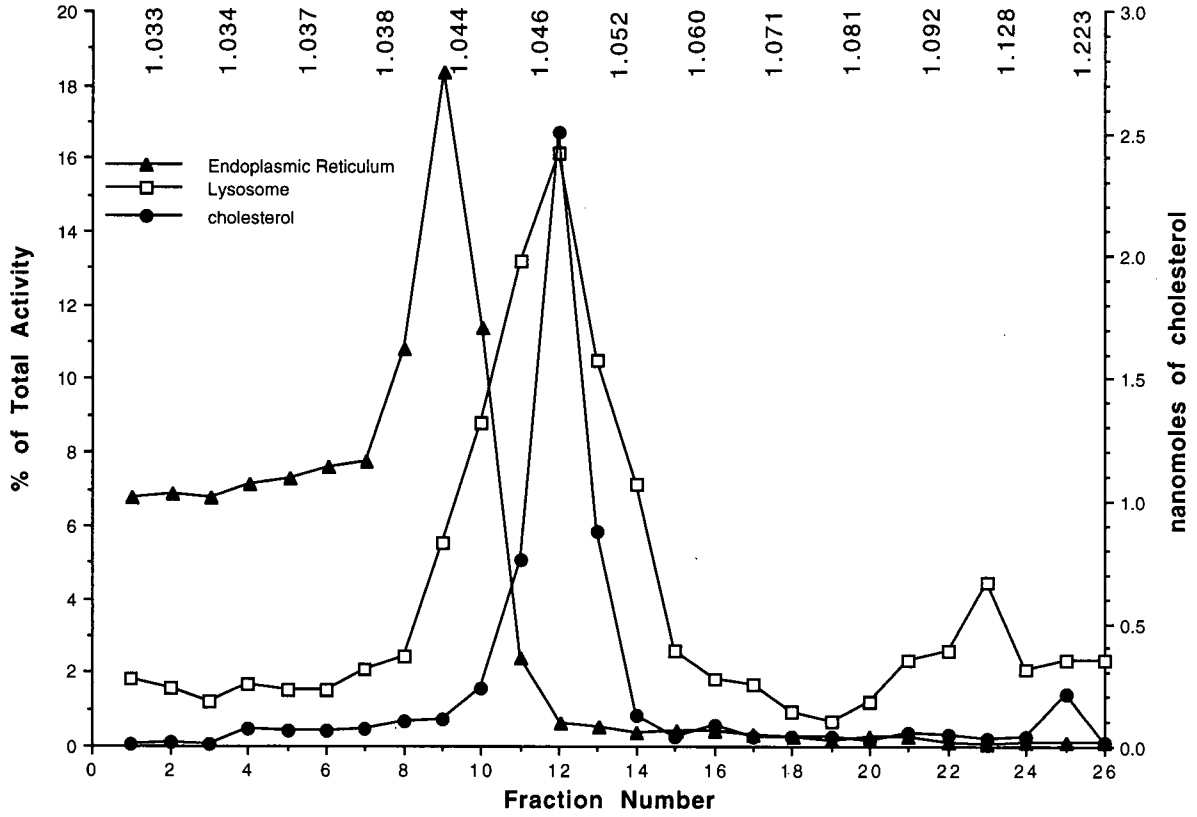


Figure 21 Distribution in oxidized LDL-loaded cells.

Each point represents the percentage of total enzyme activity in the fraction or in the case of radiolabelled cholesterol, each point represents the nanomoles of [^{3}H]-cholesterol in each fraction. Macrophages were incubated with 50 µg/ml radiolabelled oxidized LDL for 24 hours. Other methods are described briefly in the legend for the previous figure, and in detail in Chapter 2.

In macrophages loaded with oxidized LDL, we found that the endoplasmic reticulum fraction remained at a density of approximately 1.040 g/ml. However, the lysosomal marker

shifted down to approximately 1.050 g/ml. The cholesterol clearly concentrates in the fractions that contain high lysosomal marker activity, suggesting that the cholesterol from oxidized LDL is sequestered in lysosomes after the initial 24 hour incubation. Figure 22 represents the distribution in macrophages that have been loaded for 24 hours with 50 μg/ml radiolabelled oxidized LDL, followed by a 12 hour incubation with 75 μg/ml HDL_3.

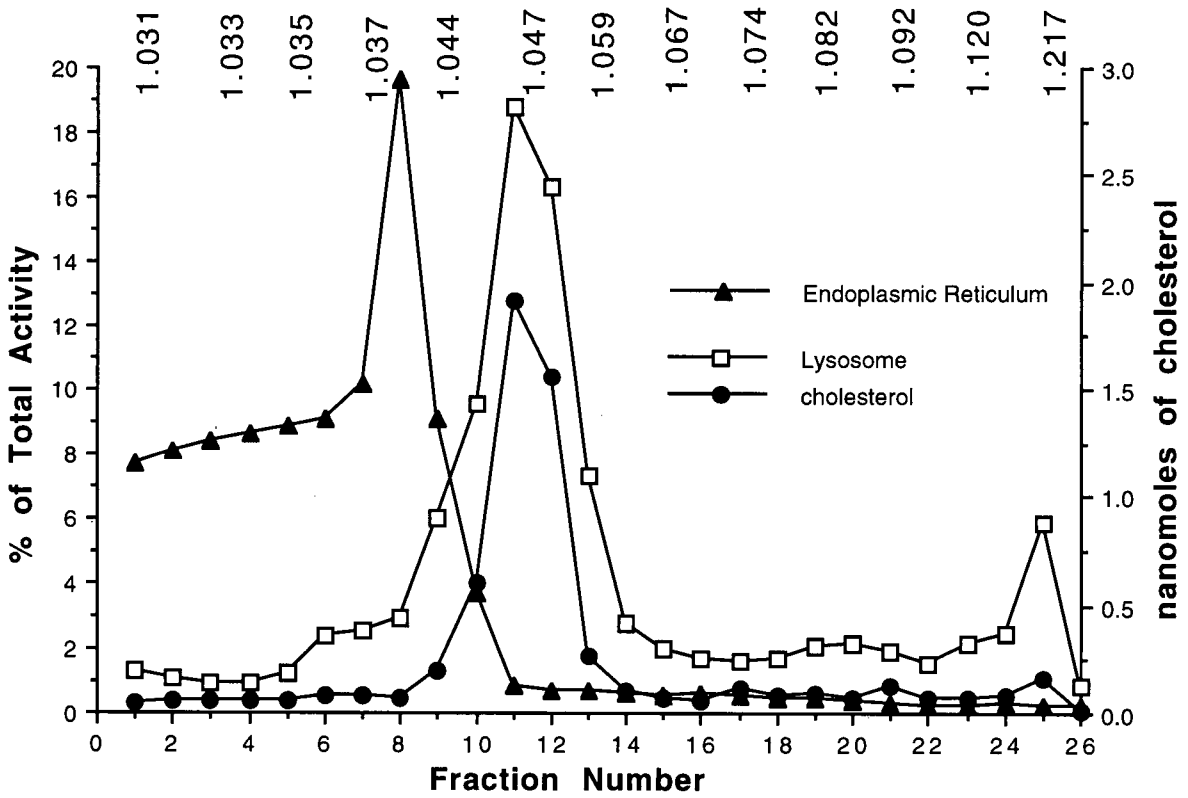


Figure 22 Distribution in oxidized LDL-loaded cells after HDL-mediated efflux.

Each point represents the percentage of total enzyme activity in the fraction or in the case of radiolabelled cholesterol, each point represents the nanomoles of [^{3}H]-cholesterol in each fraction. Macrophages were incubated with 50 μg/ml radiolabelled oxidized LDL for 24 hours, washed, and then treated for an additional 12 hours in the presence of 75 μg/ml HDL_3. Other methods are described briefly in the legend for figure 20, and in detail in Chapter 2.

In macrophages loaded with oxidized LDL and then treated with HDL_3, we found that the endoplasmic reticulum fraction remained at a density of approximately 1.040 g/ml and the lysosomal marker remained shifted down to approximately 1.050 g/ml. The cholesterol remained highly concentrated in the fractions that contained high lysosomal marker activity, suggesting that the cholesterol from oxidized LDL is sequestered in lysosomes even after incubation with HDL_3. From the cholesterol content we can see that less than one-quarter of the radiolabelled cholesterol is effluxed after being treated with HDL_3. Figure 23, represents the distribution in macrophages that have been loaded for 24 hours with 50 μg/ml radiolabelled acetylated LDL.

In macrophages loaded with acetylated LDL, we found that the endoplasmic reticulum fraction remained at a density of approximately 1.040 g/ml. However, the lysosomal marker shifted down to approximately 1.050 g/ml. Again, the radiolabelled cholesterol concentrated in the fractions that contained high lysosomal marker activity. However, very little cholesterol accumulated within the lysosomes compared to the radiolabelled cholesterol content of lysosomes in oxidized LDL-loaded cells. Figure 24, represents the distribution in macrophages that have been loaded for 24 hours with 50 μg/ml radiolabelled acetylated LDL, followed by a 12 hour incubation with 75 μg/ml HDL_3.

In macrophages loaded with acetylated LDL and then treated with HDL_3, we found that the endoplasmic reticulum fraction remained at a density of approximately 1.040 g/ml and the lysosomal marker remained shifted down to approximately 1.050 g/ml. Again, we found that there was a low level of radiolabelled cholesterol present in the fractions that contained high lysosomal marker activity even after incubation with HDL_3. However, from

the cholesterol content we can see that nearly two-thirds of the radiolabelled cholesterol was effluxed after being treated with HDL_3.

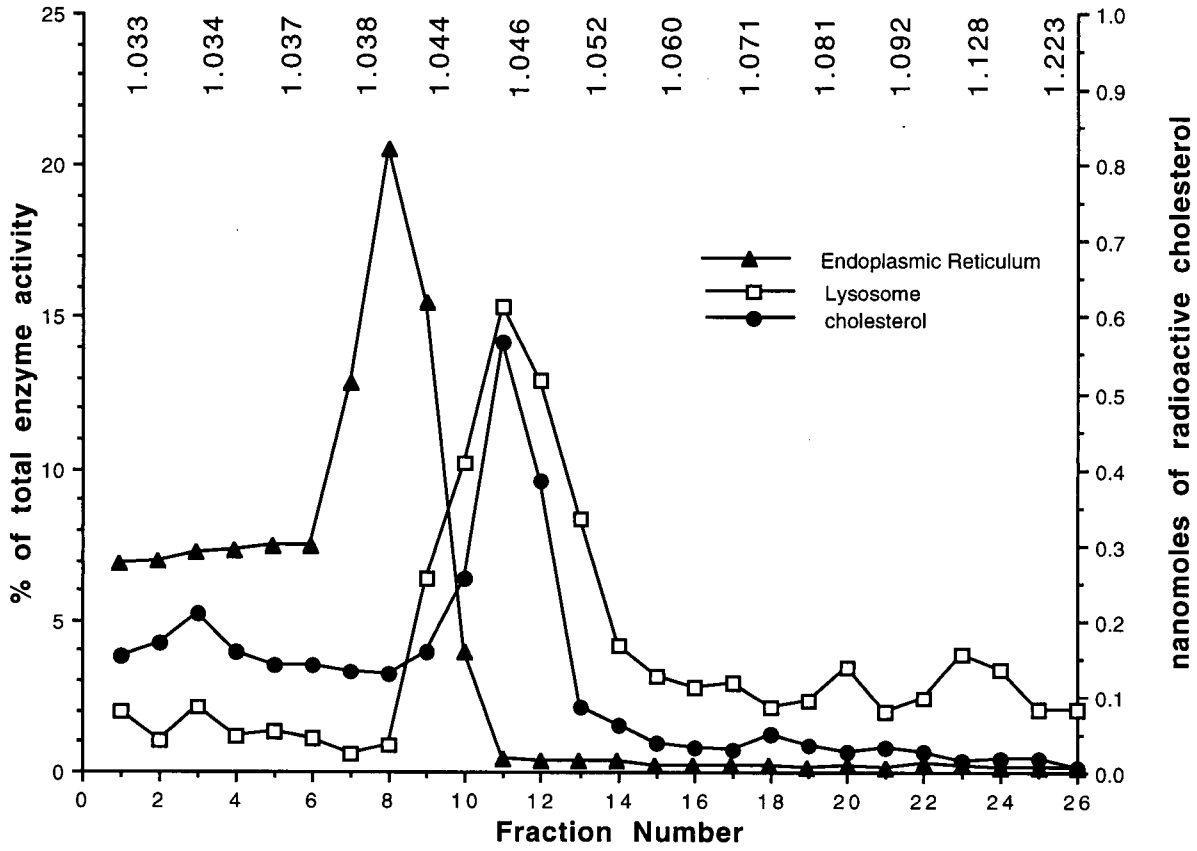


Figure 23 Distribution in acetylated LDL-loaded cells.

Each point represents the percentage of total enzyme activity in the fraction or in the case of radiolabelled cholesterol, each point represents the nanomoles of [^{3}H]-cholesterol in each fraction. Macrophages were incubated with 50 μg/ml radiolabelled acetylated LDL for 24 hours. Other methods are described briefly in the legend for figure 20, and in detail in Chapter 2.

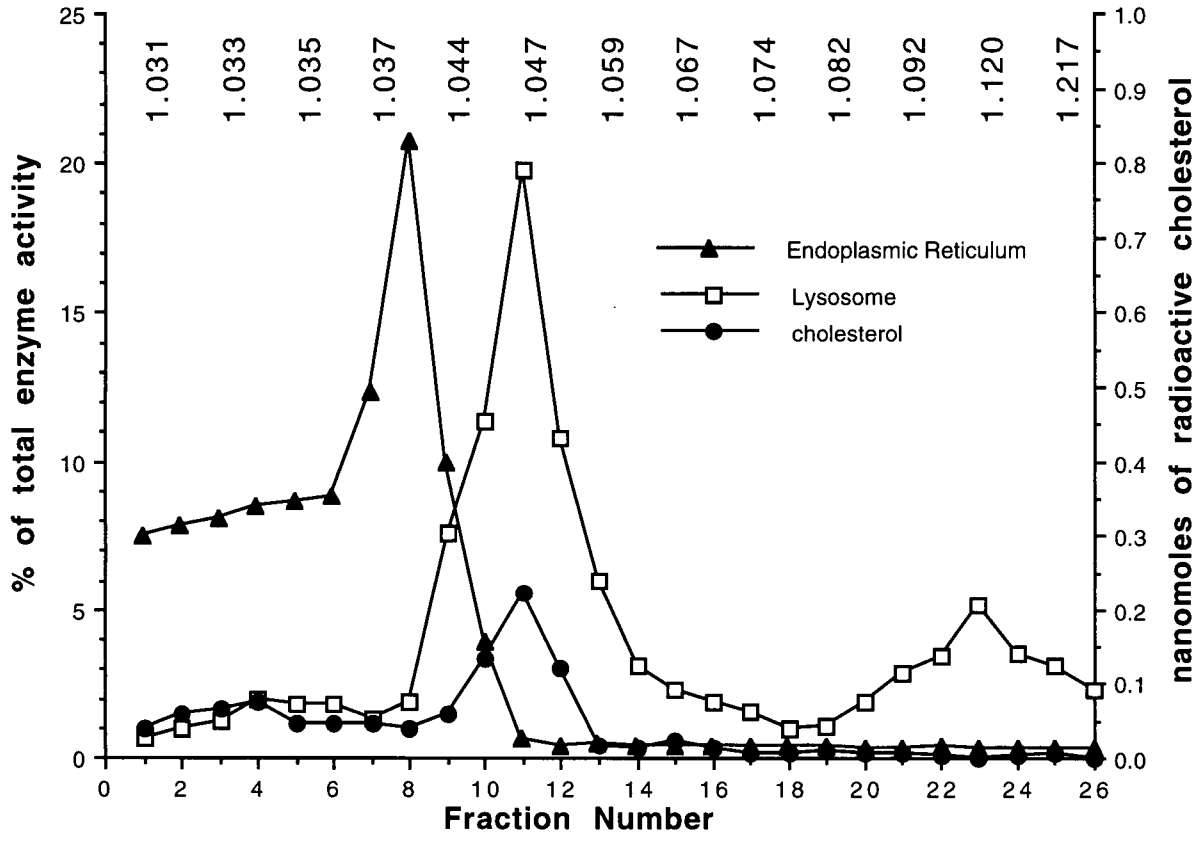


Figure 24 Distribution in acetylated LDL-loaded cells after HDL-mediated efflux.

Each point represents the percentage of total enzyme activity in the fraction or in the case of radiolabelled cholesterol, each point represents the nanomoles of [^{3}H]-cholesterol in each fraction. Macrophages were incubated with 50 μg/ml radiolabelled acetylated LDL for 24 hours, washed, and then treated for an additional 12 hours in the presence of 75 μg/ml HDL_3. Other methods are described briefly in the legend for figure 20, and in detail in Chapter 2.

In this experiment, we found that loading cells with either oxidized or acetylated LDL resulted in a shift in the density of the lysosomal fraction, compared to control cells. In these instances, the lysosomal fraction became less dense after taking up so much lipoprotein-derived cholesterol. Even after the 12 hour incubation with HDL_3, we found that the lysosomal marker remained shifted down to a density of approximately 1.050 g/ml. In the case of oxidized LDL-loaded cells this is not surprising since less than one-quarter of the

radiolabelled cholesterol was effluxed during the incubation with HDL_3. However, in the case of acetylated LDL-loaded cells, the lysosomal content of cholesterol decreased significantly from 0.57 nanomoles to 0.22 nanomoles, but the lysosomal marker density did not shift up to a higher density. The only explanation that we can provide for this is that during the processing of acetylated LDL-derived cholesterol, lighter endosomes may have fused with the lysosomal compartment containing lipid and the lysosomal marker. Nevertheless, these results provide further evidence that macrophages loaded with oxidized LDL exhibit impaired efflux of cholesterol compared to macrophages loaded with acetylated LDL. Moreover, the cholesterol delivered by oxidized LDL is sequestered in a lysosomal compartment. However, this is in sharp contrast to the findings of Kritharides et al. (300), who reported that there was equilibration of cholesterol to all membrane pools in murine macrophages loaded with oxidized LDL. The contrast between this and our observations may be based on differences in the analytical methods employed to fractionate the cells and detect cholesterol. Kritharides et al. arbitrarily combined the collected fractions into five major fractions representing large ranges in density, in which the density range of 1.035 g/ml to 1.050 g/ml was labelled the endosomal fraction and the density range of 1.050 g/ml to 1.095 g/m was labelled the lysosomal fraction. In our experience, the fractions containing lysosomal enzyme activity in oxidized LDL-loaded cells form a sharp peak between the density range of 1.045 g/ml and 1.055 g/ml. They reported a large proportion of free cholesterol and 7-ketocholesterol in both their endosomal and lysosomal fractions; this is in agreement with our data which demonstrates that most of the oxidized LDL-derived cholesterol is found in the 1.050 g/ml density fraction. Unlike, Kritharides et al., we did not find any accumulation of cholesterol in the density range 1.020 g/ml to 1.035 g/ml. This may

be explained by the fact that they measured the distribution of all cholesterol in the cell, whereas we measured only the radiolabelled cholesterol delivered by oxidized LDL. Our results also differ from those of Yancey et al., who found that mouse peritoneal macrophages stored most of their oxidized LDL-derived cholesterol (71%) within cytoplasmic inclusions (301, 335). In contrast to mouse peritoneal macrophages, Yancey et al. reported that pigeon and THP-1 macrophages stored most (60%-90%) of their oxidized LDL-derived cholesterol in lysosomes, whereas the bulk (64%-88%) of acetylated LDL cholesterol was stored in cytoplasmic inclusions (301, 335). It is difficult to reconcile the findings of Yancey et al. with our data because we used extensively oxidized LDL for our work, whereas they used only mildly oxidized LDL.

Our identification of the lysosomal accumulation of cholesterol in oxidized LDL-loaded macrophages is in agreement with the findings of Maor et al. (92, 299), who studied this phenomenon in the J-774 A.1 murine macrophage-like cell line. Moreover, immunolocalization and ultrastructural studies also suggest that oxidized LDL accumulates in the lysosomes of both cultured macrophages and human atherosclerotic tissues (295, 296).

Because acetylated LDL does not exist *in vivo,* there have been few reports on the subcellular distribution of cholesterol in acetylated LDL-loaded cells. In disagreement with the findings of Yancey et al., we found that there was a moderate accumulation of radiolabelled cholesterol in the lysosomes of acetylated LDL-loaded cells (301, 335). It is difficult to surmise an explanation for the contrast between their work and ours. However, it may be based on differences in the analytical methods or culture conditions used. We used subcellular fractionation followed by assays for lysosomal and endoplasmic reticulum enzyme markers to determine the subcellular distribution of acetylated LDL-derived [^{3}H]-

cholesterol. They, on the other hand, used electron microscopy and cytochemistry to determine the subcellular distribution of lipid; specifically, they stained cells for the lysosomal enzyme marker, acid phosphatase, and then analyzed electron micrographs to determine whether lipid associated with acid phosphatase-positive lysosomes (301). Alternatively, it is possible that the conditions under which they cultured the cells may have had an effect. They loaded their cells with medium containing 100 μg/ml acetylated LDL and 1% FBS, for 48 hours; we loaded our cells for only 24 hours, with medium containing 50 μg/ml acetylated LDL and 0.5 mg/ml BSA. It is possible that the longer incubation time in the presence of FBS redistributed the lipid within the cell as it was being effluxed from the cell by FBS. This would also explain their very low levels of lipid accumulation compared to the levels that we achieve in 24 hours.

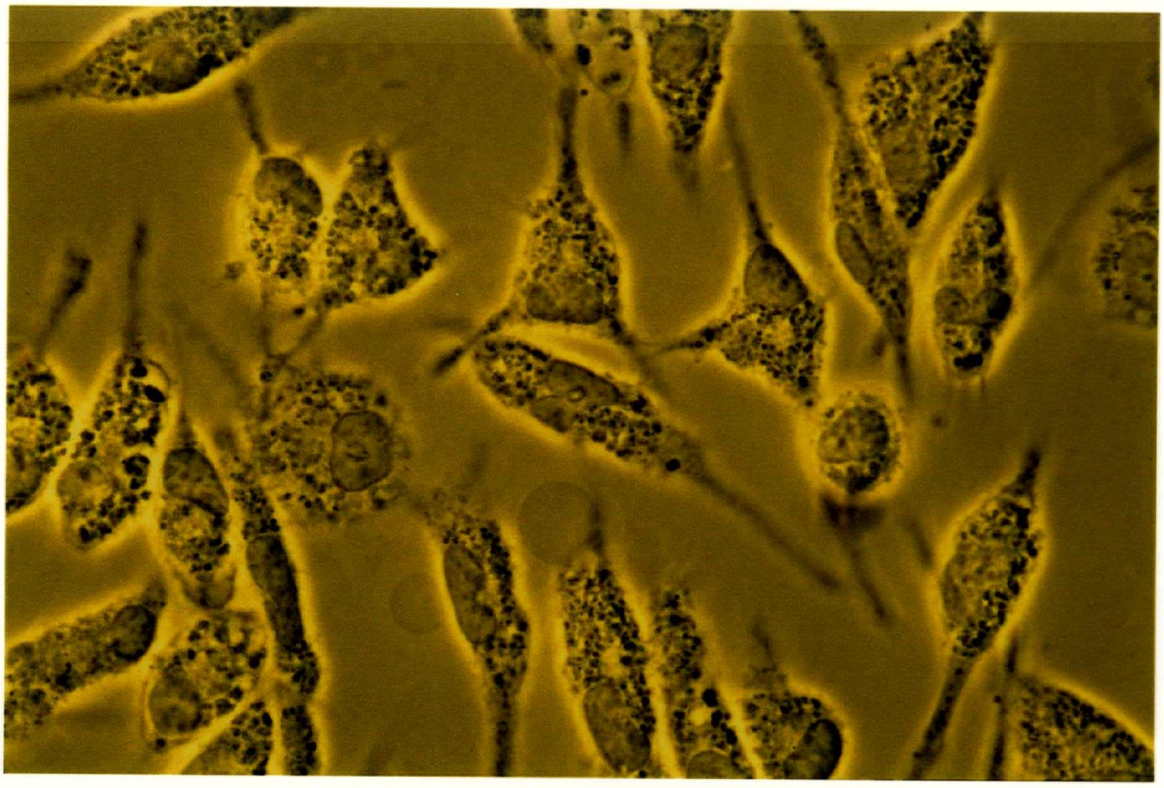
There are no reports of the subcellular distribution of cholesterol in modified LDL-loaded cells that have been treated with a cholesterol acceptor such as HDL. We found that subcellular distribution of cholesterol in oxidized LDL-loaded macrophages that had been treated with HDL_3 was identical to the distribution of cholesterol in untreated oxidized LDL-loaded macrophages. This is in keeping with the observed impairment of cholesterol efflux from these cell. Surprisingly, when the same analysis was done on cells that had been loaded with acetylated LDL and then treated with HDL_3, it was found that the distribution was again similar to that of untreated acetylated LDL-loaded cells. We would have expected the lysosomal compartment in acetylated LDL-loaded macrophages to become denser as cholesterol was released to HDL_3. Since this did not occur, we surmise that lighter endosomes may have fused with the lysosomal compartment containing lipid and the lysosomal marker, during the efflux of cholesterol from the cell. Despite the lack of change

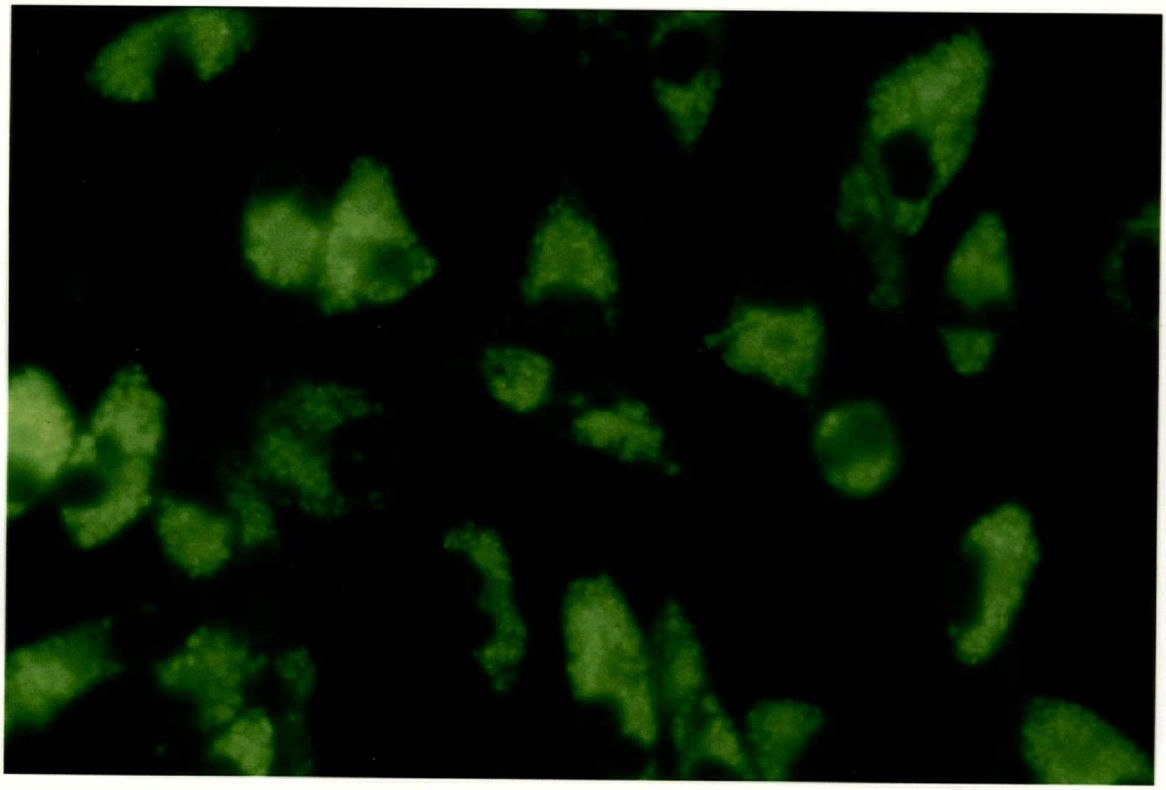
in the subcellular distribution of cholesterol after HDL-mediated cholesterol efflux, it can be seen that the acetylated LDL-loaded cells released nearly two-thirds of their radiolabelled cholesterol to HDL_3, whereas the oxidized LDL-loaded cells released less than one-quarter of their radiolabelled cholesterol to HDL_3. This provides further evidence that macrophages loaded with oxidized LDL exhibit impaired efflux of cholesterol compared to macrophages loaded with acetylated LDL.

To seek morphologic confirmation of the cell fractionation results indicating that oxidized LDL accumulates within macrophages and oxidized LDL-derived lipids are inefficiently effluxed in the presence of HDL_3, we loaded cells with fluorescently-labelled oxidized LDL and then observed the decrease in fluorescence, if any, after treating them with HDL_3. Figure 25 depicts a typical example of macrophages loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled oxidized LDL. Figure 26 depicts an example of macrophages that were loaded under the same conditions as the cells in Figure 25, only the cells in Figure 26 underwent a further 12 hour incubation with 75 μg/ml HDL_3. These pictures were taken from one of three experiments. The caption for Figure 25 is provided below, but the photographs corresponding to this caption are on page 89.

Figure 25 Macrophages loaded with NBD-C_6-sphingomyelin-labelled oxidized LDL.

Both pictures were taken under 400 × magnification. Cells were loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled oxidized LDL, washed, and then fixed onto microscope slides. The photograph on the top of the next page was taken in the phase mode of the microscope, and the photograph on the bottom is the corresponding fluorescent micrograph. The NBD-C_6-sphingomyelin appears green.

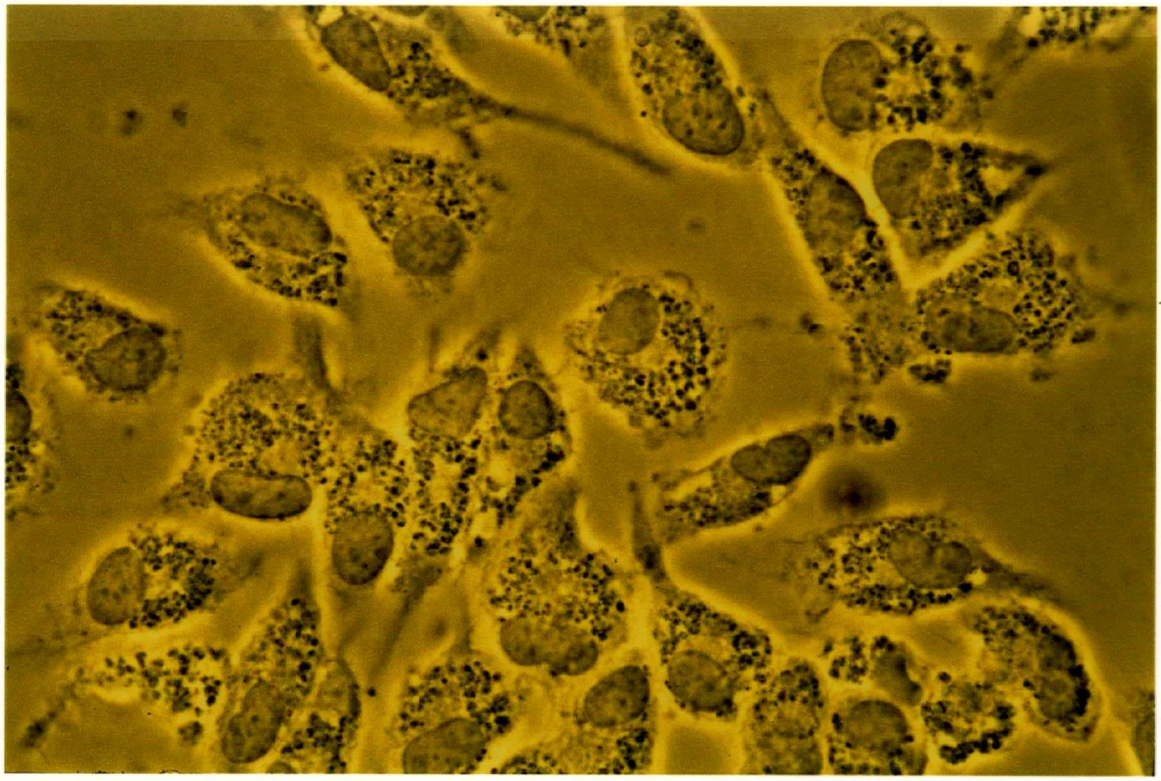


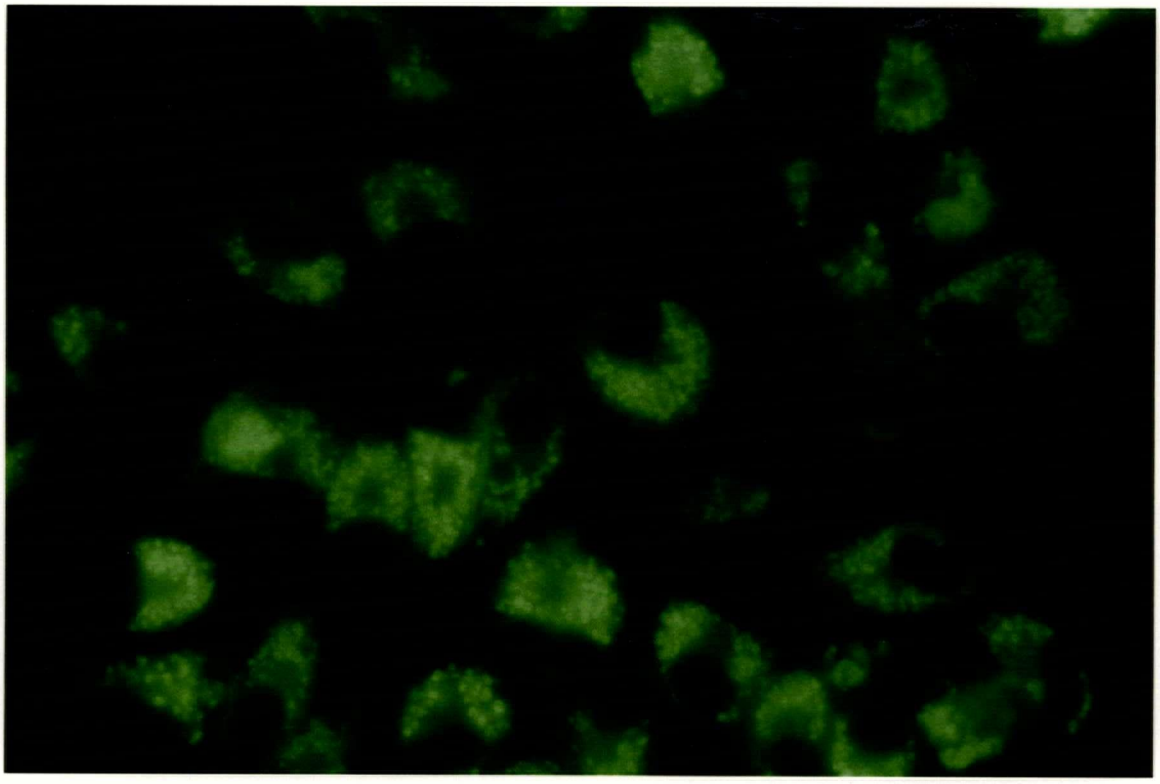


The caption for Figure 26 is provided below, but the photographs corresponding to this caption are on page 91.

Figure 26 Macrophages treated with fluorescent oxidized LDL followed by HDL_3.

Both pictures were taken under 400 × magnification. Cells were loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled oxidized LDL, washed, treated for an additional 12 hours with 75 μg/ml HDL_3, washed again, and then fixed onto microscope slides. The photograph on the left was taken in the phase mode of the microscope, and the photograph on the right is the corresponding fluorescent micrograph. The NBD-C_6-sphingomyelin appears green.





Comparing photographs of fluorescence in cells is highly subjective because the degree of exposure affects the apparent fluorescence intensity of the cells. Furthermore, photobleaching of the fluorescent probe does occur the longer it is exposed to light. It is for this reason that both the amount of time that each slide was exposed to light and the exposure time of the film were kept constant for each photograph in order to ensure comparable photographs were taken. After examining numerous slides under the microscope, we are confident that there appeared to be little or no decrease in fluorescence after HDL-mediated cholesterol efflux. However, in cells loaded for 24 hours with NBD-C_6-sphingomyelin-labelled acetylated LDL, very little NBD-C_6-sphingomyelin accumulated within the cells because unlike oxidized LDL derived sphingomyelin, acetylated LDL-derived sphingomyelin is rapidly hydrolyzed within macrophages and the water-soluble fluorescent label is also expelled from the cell. Figure 27 depicts a typical example of macrophages loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled acetylated LDL. Cells that were loaded with fluorescently-labelled acetylated LDL and subsequently treated with HDL_3 contained little or no NBD-C_6-sphingomyelin, as we would expect (photographs not shown). These results suggest that, in addition to cholesterol, sphingolipids delivered by oxidized LDL are also poorly degraded and consequently accumulate within oxidized LDL-loaded cells.

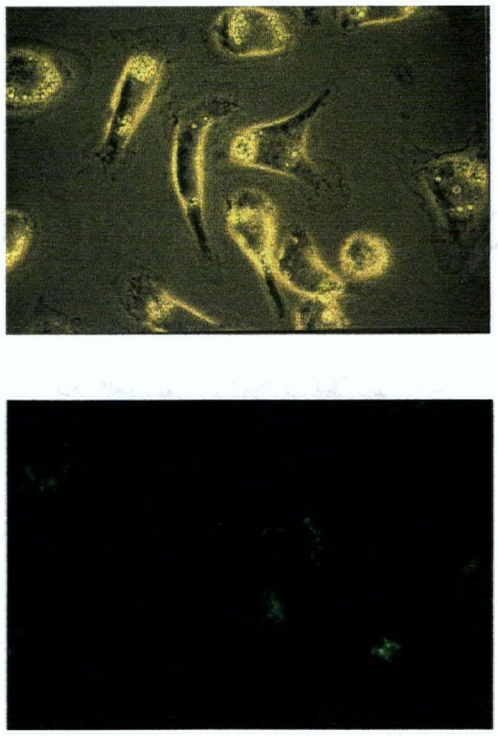


Figure 27 Macrophages loaded with NBD-C_6-sphingomyelin-labelled acetylated LDL.

Both pictures were taken under 400 × magnification. Cells were loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled acetylated LDL, washed, and then fixed onto microscope slides. The photograph on the top of the next page was taken in the phase mode of the microscope, and the photograph on the bottom is the corresponding fluorescent micrograph. The NBD-C_6-sphingomyelin appears green.

3.5 HDL-mediated cholesterol efflux

Because interstitial fluid contains a greater concentration than plasma of lipoprotein-unassociated apolipoprotein A-I (373) and because cholesterol-laden fibroblasts release cholesterol to an apo A-I-only-containing particle (374), it has been proposed that apo A-I may be involved in the initial steps of cholesterol efflux. Moreover, purified apolipoprotein A-I has been shown to promote cholesterol efflux *in vitro* (375). Very little efflux of cholesterol from oxidized LDL-loaded cells occurs, compared to cells loaded with acetylated

LDL. In order to determine whether the residual cholesterol efflux from oxidized LDL-loaded cells is apolipoprotein-dependent, lipid-loaded cells were treated with either HDL_3 or purified apo A-I. In this experiment, control cells contained only 80 μg total cholesterol/mg cell protein. After 24 hours, total cholesterol increased to 276 μg cholesterol/mg cell protein in the acetylated LDL-loaded cells, and 335 μg cholesterol/mg cell protein in the oxidized LDL-loaded cells. As would be expected from the composition of these modified LDL's (Table 5), the acetylated LDL-loaded cells accumulated large quantities of cholesterol ester, as well as free cholesterol, whereas the oxidized LDL-loaded cells accumulated predominantly free cholesterol. The increase in total cholesterol in the acetylated LDL-loaded macrophages represented an approximately 3.5-fold augmentation over the baseline level. Similarly, the increase in total cholesterol in the oxidized LDL-loaded macrophages represented an approximately four-fold augmentation over the baseline level. In such experiments, the degree of cholesterol accumulation varied from two-fold to six-fold, but in all cases, the acetylated LDL-loaded cells effluxed significant quantities of cholesterol, whereas the oxidized LDL-loaded cells exhibited impaired efflux of cholesterol. Some efflux of cholesterol from the acetylated LDL-loaded cells occurred in the absence of apo A-I or HDL_3. In this case, the BSA in the medium acted as the cholesterol acceptor and cholesterol efflux occurred by passive diffusion from the cells to the BSA. Figure 28 depicts the degree of cholesterol efflux observed in this experiment.

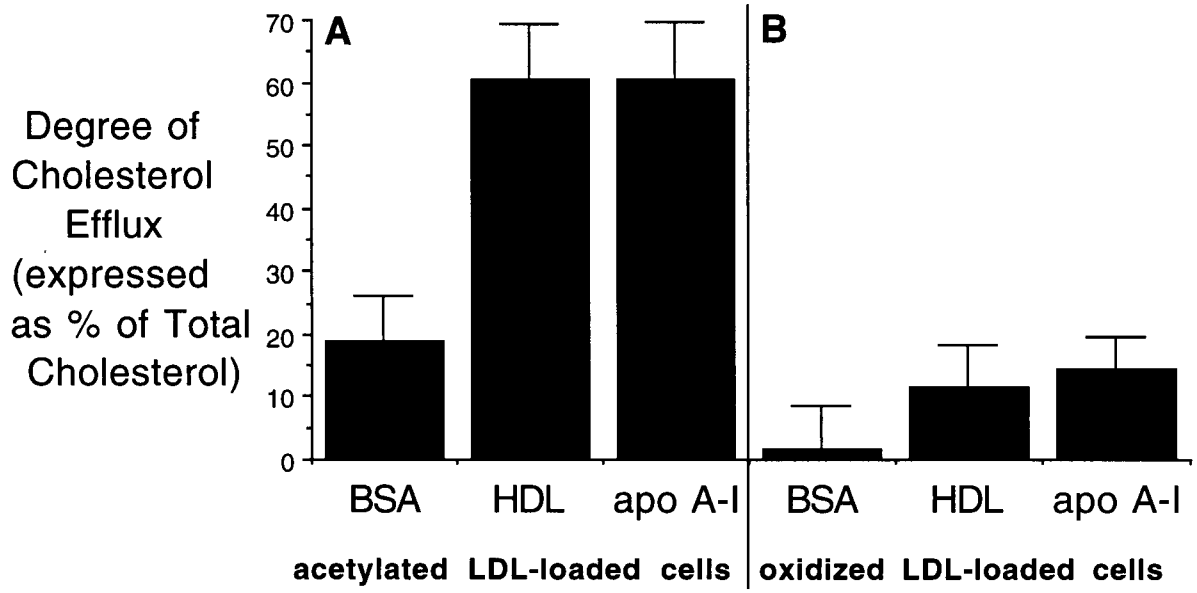


Figure 28 Decrease in total cholesterol after treatment with apo A-I or HDL_3.

Each bar represents the percent decrease in total cholesterol after cholesterol efflux to BSA, HDL_3, or apo A-1, in cells that had been loaded with either acetylated LDL (Panel A) or oxidized LDL (Panel B). Each value represents the percentage difference of the means ± SD from triplicate plates from one of two experiments with similar results.

Once again, we see the striking difference in cholesterol efflux from cells loaded with oxidized LDL compared to cells loaded with acetylated LDL. It has been shown previously that using apolipoproteins such as apo A-I to mediated cholesterol efflux from cultured cells results in the formation of HDL-like particles in the culture medium (75, 78, 375-383). Our data indicated that HDL_3 was equally as effective as apolipoprotein A-I at bringing about cholesterol efflux from macrophages loaded with either acetylated LDL or oxidized LDL. This raises the question of the role of the lipid component of HDL_3 in cholesterol efflux. As discussed in Chapter 1 (Section 1.2), there are two proposed mechanisms of efflux: one involving passive diffusion of cholesterol from the cell and the other involving apolipoprotein binding to the cell surface. In order to determine the importance of HDL

lipids in bringing about cholesterol efflux, loaded cells were treated with either HDL_3 or an equivalent amount of trypsinized HDL_3. The equivalent amount of trypsinized HDL was calculated by determining the phosphorus content of both native HDL and the trypsinized HDL preparation. To ensure that the apolipoprotein component had been adequately digested, the trypsinized HDL sample was run on SDS-polyacrylamide gel electrophoresis (Figure 29).

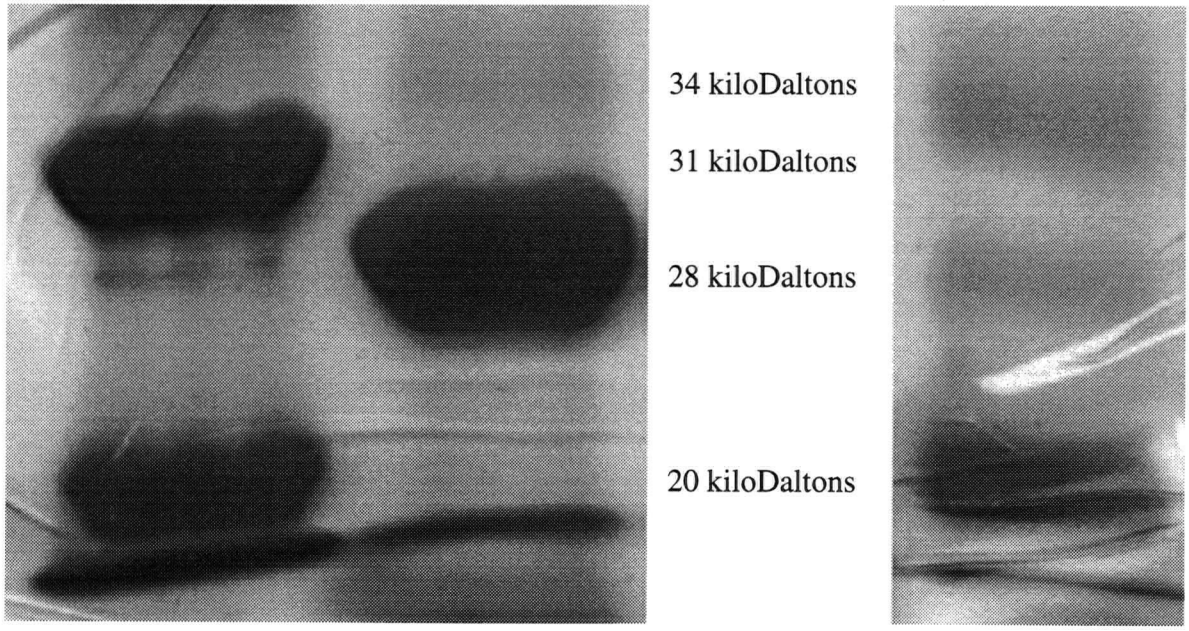


Figure 29 SDS-PAGE of native HDL_3 and trypsinized HDL_3.

Each band in the gel represents a protein of a particular molecular weight; the molecular weights are labelled on the gel. Each lane is labelled on the bottom of the gel. The molecular weight of apolipoprotein E is 34 kDa. The molecular weight of apolipoprotein A-I is 28 kDa. It can be seen from the lanes containing trypsinized HDL_3 that there is a nearly complete absence of intact apolipoprotein A-I.

In Figure 29, we see that the trypsinized HDL_3 preparation contained small amounts of intact apolipoprotein E. However, it was a suitable preparation to use because nearly all of the apolipoprotein A-I was degraded. Macrophages loaded with either oxidized LDL or acetylated were treated with either native HDL or trypsinized HDL, and the degree of cholesterol efflux was measured. Control cells contained only 77 μg total cholesterol/mg cell protein. After 24 hours, total cholesterol increased to 147 μg cholesterol/mg cell protein in the acetylated LDL-loaded cells, and 144 μg cholesterol/mg cell protein in the oxidized LDL-loaded cells. The increase in total cholesterol in both the acetylated LDL-loaded cells and the oxidized LDL-loaded cells represented an approximately two-fold augmentation over the baseline level. There was a slight decrease in total cholesterol in all of the cells treated with trypsinized HDL_3, but it was not statistically significant. Figure 30 depicts the degree of cholesterol efflux observed in this experiment.

Once again, we see the striking difference in cholesterol efflux from cells loaded with oxidized LDL compared to cells loaded with acetylated LDL. The data from Figure 30 suggests that HDL lipids are responsible for less than one-third of the efflux observed when lipid-loaded cells are treated with native HDL_3. This is in agreement with previous reports that cholesterol efflux from plasma membranes is mediated by HDL lipids, whereas removal of excess intracellularly accumulated cholesterol is mediated by HDL apolipoproteins (77). Furthermore, our results are consistent with the hypothesis that HDL apolipoproteins must bind to a cell surface receptor in order to stimulate translocation of excess intracellular cholesterol to the cell surface where it becomes accessible for removal by HDL (53, 65, 67, 78).

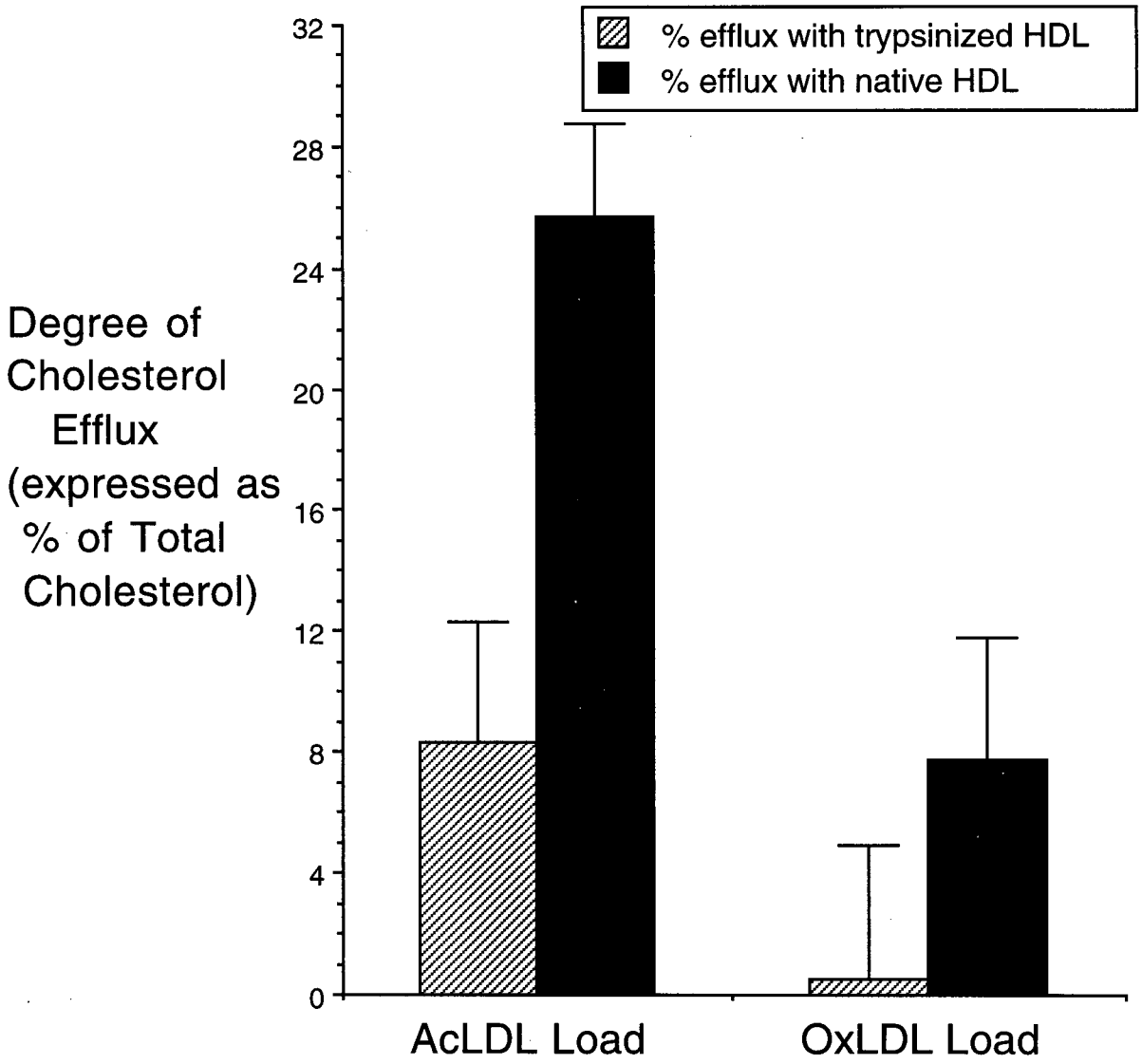


Figure 30 Decrease in total cholesterol after treatment with trypsinized HDL_3.

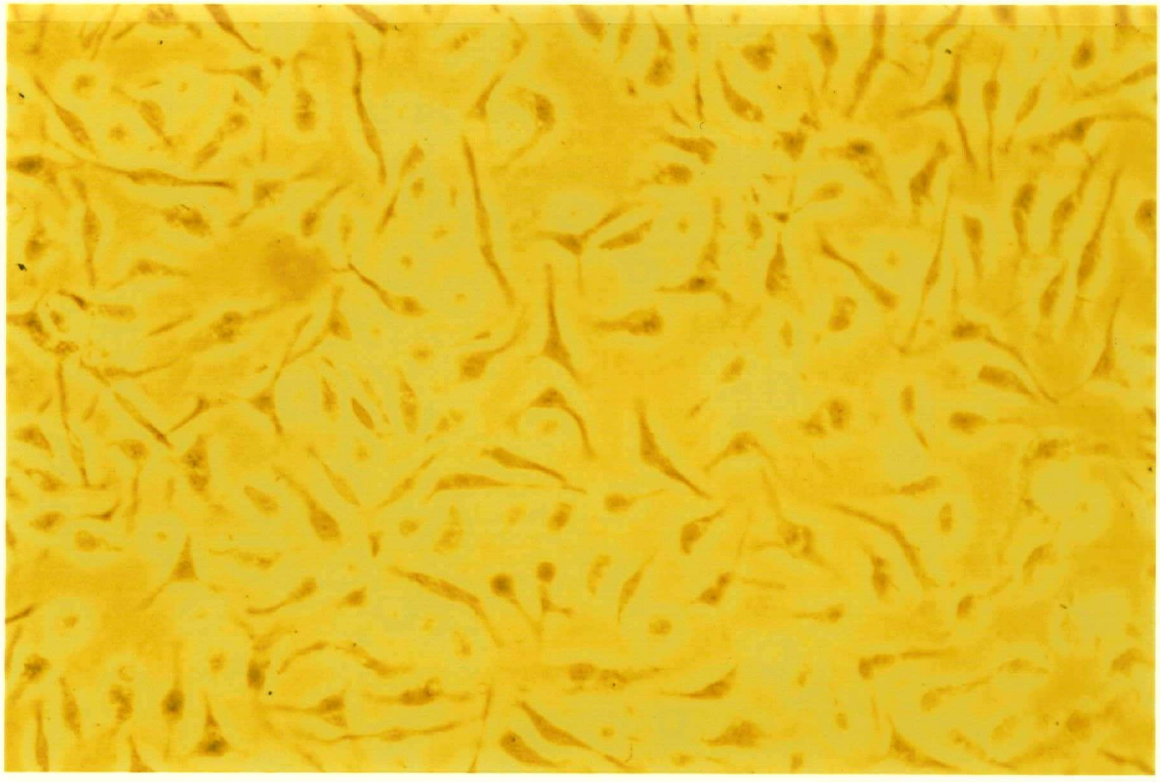
Each bar represents the percent decrease in total cholesterol after cholesterol efflux to trypsinized HDL_3 or native HDL_3, in cells that had been loaded with either acetylated LDL (acLDL) or oxidized LDL (oxLDL). Each value represents the mean percentage difference ± SD from triplicate plates from one of three experiments with similar results.

Some of our early experiments showed substantial efflux of cholesterol from oxidized LDL-loaded cells. Subsequently, we were not able to verify these results. Nevertheless, these unexpected results prompted us to investigate possible mechanisms whereby oxidized

LDL-derived cholesterol could be effluxed to HDL-containing medium. In addition to the two mechanisms of HDL-mediated cholesterol efflux discussion in Chapter 1 (Section 1.2), another pathway has been reported in differentiated enterocytes (384, 385) and rat peritoneal macrophages (386). These reports demonstrated that HDL_3 was bound specifically to these cells, leading to a subsequent intracellular passage and resecretion through a process of retroendocytosis effecting the efflux of cellular cholesterol. To investigate whether retroendocytosis was involved in HDL-mediated cholesterol efflux from cultured mouse peritoneal macrophages, lipid-loaded cells were treated with fluorescently-labelled HDL_3. This experiment allowed us to determine whether the mechanism of cholesterol efflux involves uptake and resecretion of HDL_3. Figure 31 depicts the control for this experiment: macrophages were treated for 24 hours with BSA-containing medium only, followed by a 12 hour incubation with 75 μg/ml Texas Red HDL_3. Figure 32 depicts an example of macrophages that were loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled oxidized LDL, washed, and then treated with 75 μg/ml Texas Red HDL_3 for an additional 12 hours. These pictures were taken from one of three experiments. The caption for Figure 31 is provided below, but the photographs corresponding to this caption are on page 100.

Figure 31 Control macrophages treated with Texas Red HDL.

Both pictures were taken under 100 × magnification. Cells were incubated for 24 hours with medium in the absence of lipoproteins, washed, and then treated with 75 μg/ml Texas Red HDL_3 for 12 hours. Finally, the cells were washed and fixed onto microscope slides. The photograph on the top of the next page was taken in the phase mode of the microscope, and the photograph on the bottom is the corresponding fluorescent micrograph. Texas Red HDL appears red.



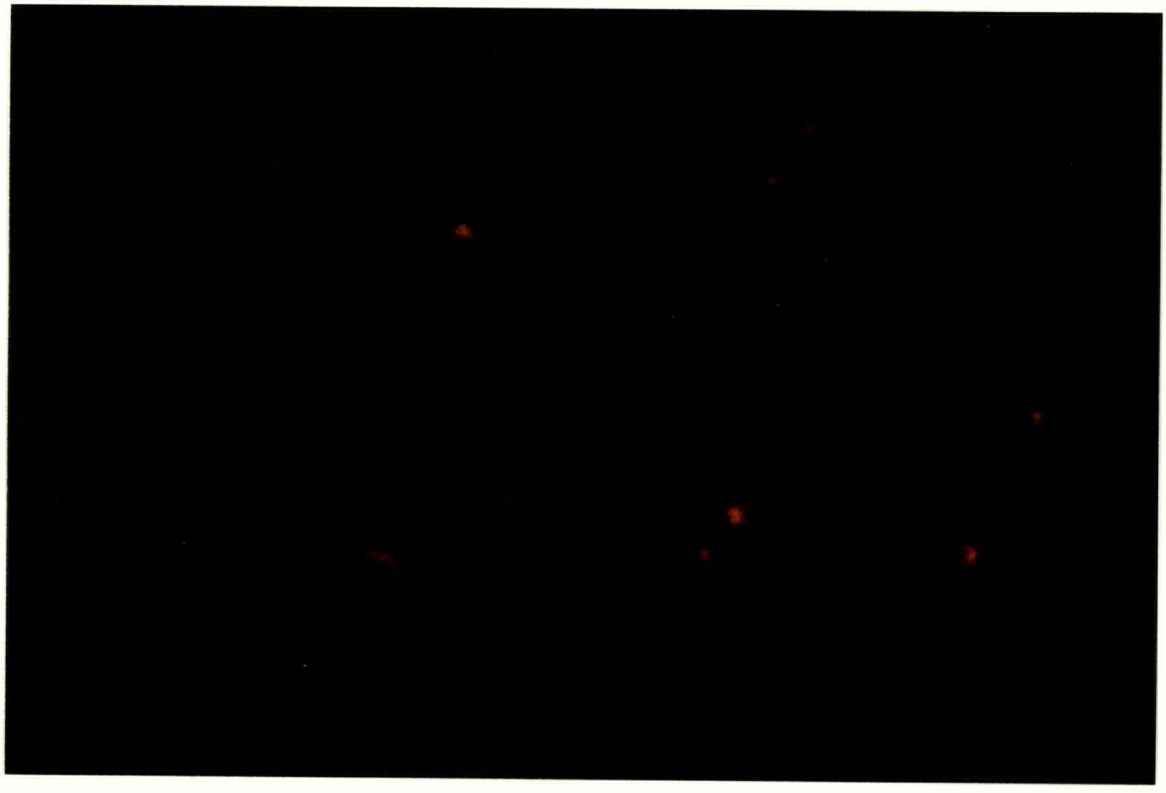
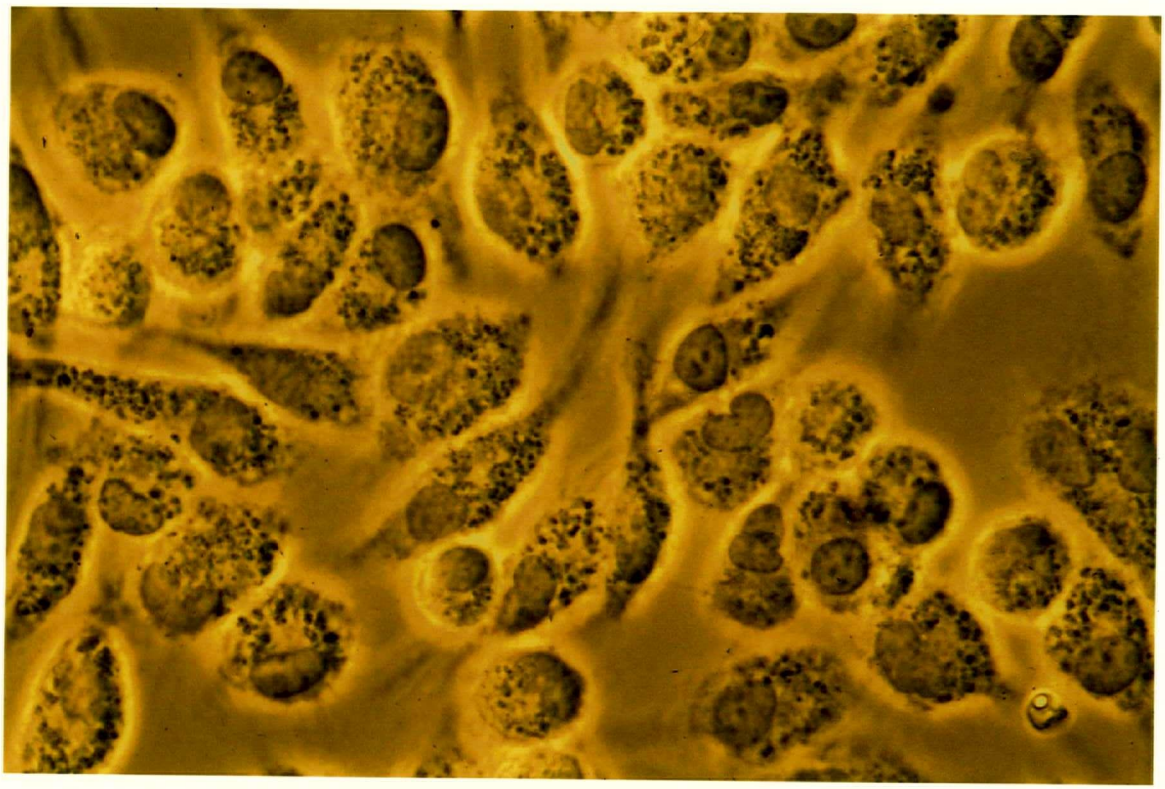
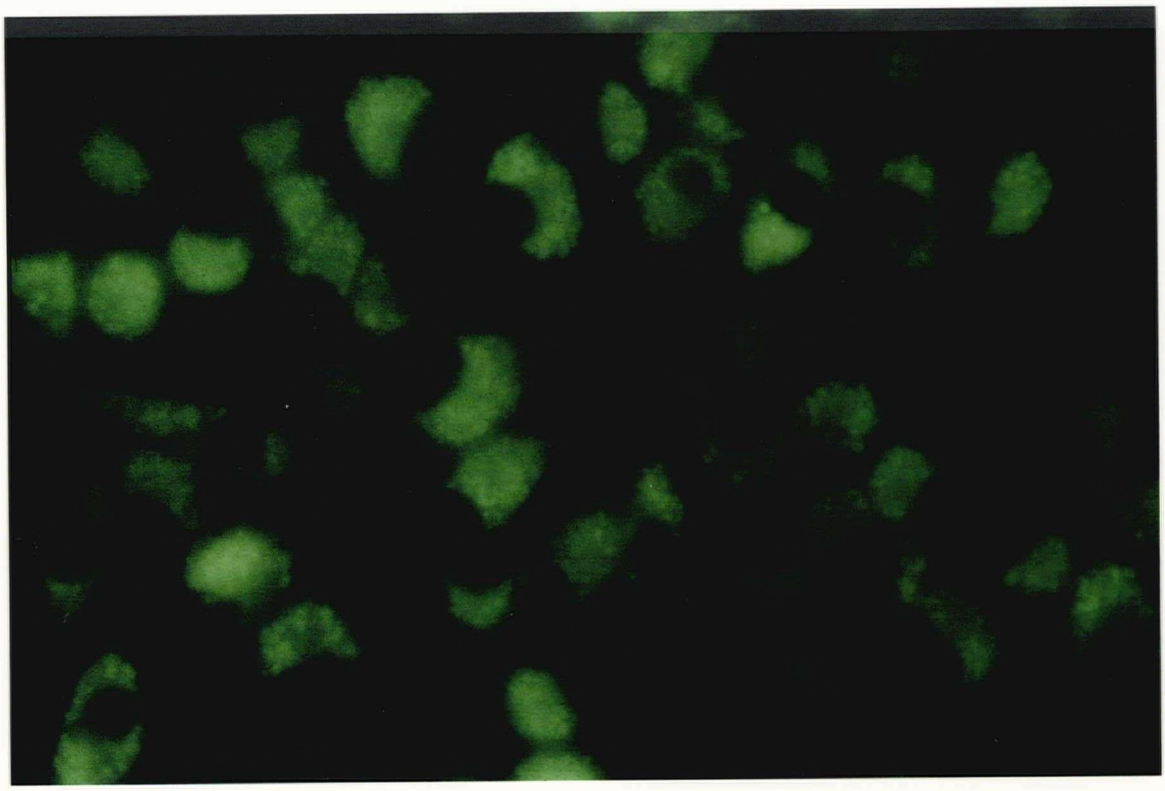
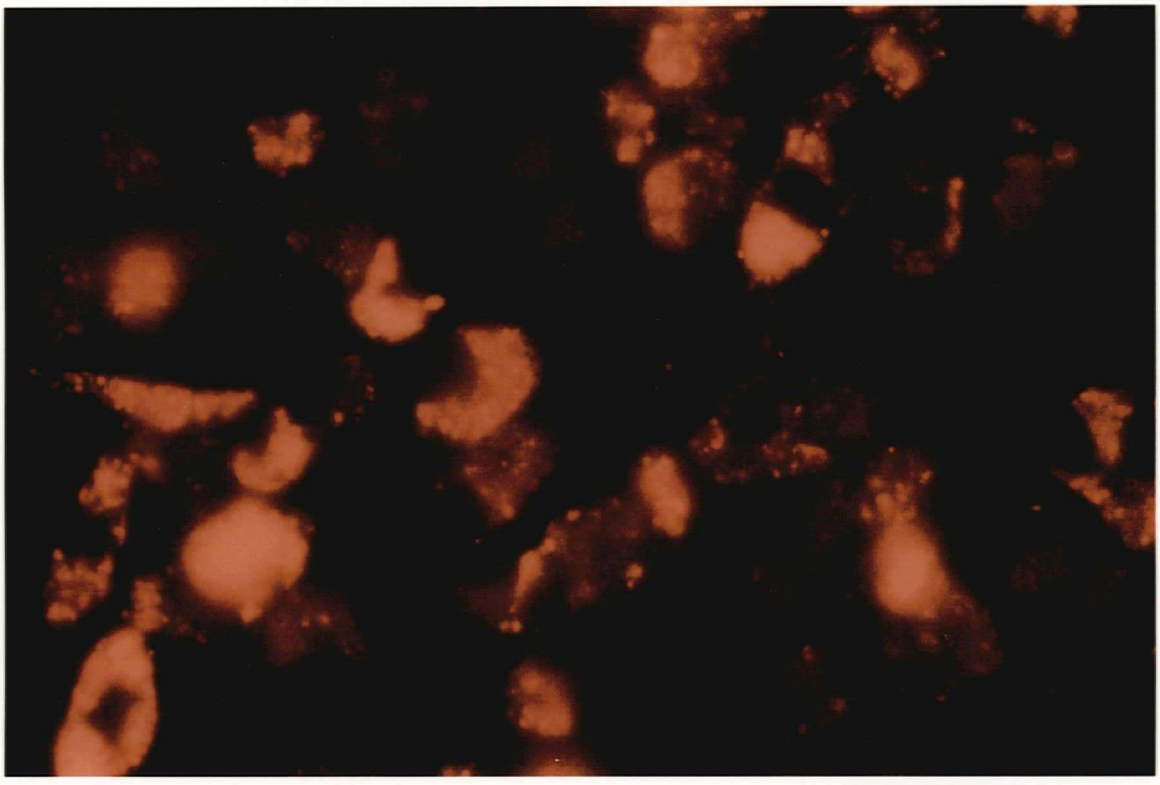


Figure 32 OxLDL-loaded macrophages treated with Texas Red HDL.

All three pictures were taken under 400 × magnification. Cells were loaded for 24 hours with 50 μg/ml NBD-C_6-sphingomyelin-labelled oxidized LDL, washed, treated for an additional 12 hours with 75 μg/ml Texas Red HDL_3, washed again, and then fixed onto microscope slides. The photograph below was taken in the phase mode of the microscope. The photographs on the next page are the corresponding fluorescent micrographs taken using the fluorescein filter set (top) and the rhodamine filter set (bottom). The NBD-C_6-sphingomyelin appears green. Texas Red HDL appears red







We found that control (non-loaded) cells took up very little HDL_3, whereas cells loaded with either acetylated LDL (photographs not shown) or oxidized LDL took up a substantial amount of HDL_3. In some cases, lipid loaded cells that had been exposed to Texas Red HDL were washed and incubated in the absence of lipoproteins for an additional 12 hours. It appeared that there was minimal decrease in Texas Red HDL fluorescence in the cells that were treated with no lipoproteins during this "chase" period; however, it was impossible to come to any meaningful conclusions about this "chase" incubation because there was a significant decrease in cell viability during this additional incubation (photographs not shown).

Since HDL_3 was taken up by lipid-loaded cells but not by control (nonloaded) cells, retroendocytosis could not be ruled out as a possible mechanism of cholesterol efflux in these cells. To quantify this phenomenon, we measured the retention and degradation of HDL_3 in lipid-loaded and control (nonloaded) cells. Macrophages were incubated for 24 hours with no lipoprotein (control), 50 μg/ml acetylated LDL (acLDL) or 50 μg/ml oxidized LDL (oxLDL). Then, they were washed and treated for an additional 12 hours with ^{125}I-labelled native HDL_3. Finally, we measured both the HDL retained within the cells as well as the HDL that had been degraded during the 12 hour incubation. Figure 33 depicts the results of a representative experiment out of three.

Iodine labels proteins by associating with tyrosine rings. In the experiment depicted in Figure 33, we measured the cellular content and degradation of HDL. We found that after 12 hours of incubation with 75 μg/ml HDL_3, both the lipid-loaded and control (nonloaded) macrophages retained approximately 0.76 μg of HDL_3 per mg of cell protein. Rogler et al. reported a lower (0.40 μg/mg of cell protein) retention of HDL_3 (385). However, they used

enterocytes (Caco-2) and they incubated their cells with HDL_3 for only three hours, so this may account for the conflicting results.

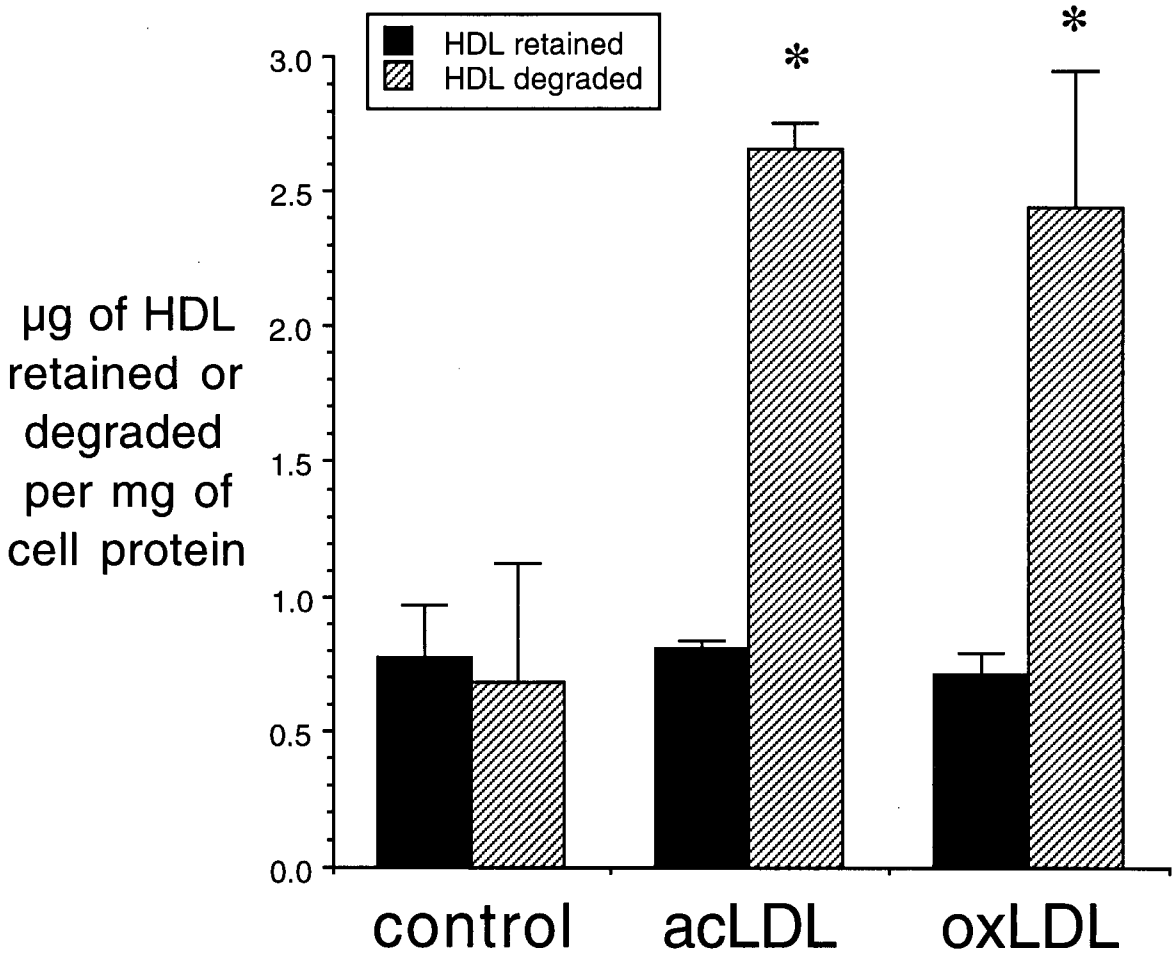


Figure 33 HDL retention and degradation by macrophages.

Each bar represents the micrograms of radioactive HDL retained (solid bar) or degraded (hatched bar) per milligram of cell protein at the end of a 12 hours incubation with ^{125}I-HDL_3. Before treating the macrophages for 12 hours with 75 μg/ml radiolabelled HDL, the cells were incubated for 24 hours with no lipoprotein (control), 50 μg/ml acetylated LDL (acLDL), or 50 μg/ml oxidized LDL (oxLDL). There was no statistically significant difference in the retention of HDL in the lipid-loaded cells compared to the control cells. There was a statistically significant difference in the degradation of HDL in the in the lipid-loaded cells compared to the control cells (* $p < 0.05$). However, there was no statistically significant difference in the degradation of HDL in the acLDL-loaded cells compared to the oxLDL-loaded cells. The values are mean ± S.D. for triplicate plates from one of three experiments with similar results.

The pathway of retroendocytosis described by Rogler et al. does not involve degradation of HDL (385); in fact, intact HDL particles are a requirement for this pathway. In contrast to previous reports (385, 386), we found that degradation of HDL_3 by nonloaded cells was not negligible. Again, the discrepant results may be attributed to the difference in the cell type used as well as the different incubation times. However, it was clear from our findings that HDL_3 retroendocytosis did not occur in our experiments. The most interesting findings came from the lipid-loaded cells, which exhibited a nearly four-fold increase in the degradation of HDL_3 compared to control cells (* $p < 0.05$). This suggests that uptake of either oxidized LDL or acetylated LDL results in upregulation of an internalizing receptor for HDL. Han et al. have reported that exposure to either oxidized LDL or acetylated LDL resulted in marked induction of CD36 mRNA expression in both J774 cells and mouse peritoneal macrophages (177). However, they also found that exposure to native LDL increased the expression of CD36 mRNA (177). As outlined in Table 3, CD36 is capable of binding to and internalizing oxidized LDL. The scavenger receptor class B type I (SR-BI) is the only known HDL receptor which can mediate selective uptake of cholesteryl esters from HDL (without apolipoprotein uptake). Fluiter et al. reported that rats that were fed a high-cholesterol diet for two weeks exhibited decreased SR-BI expression in parenchymal cells, while the expression in Kupffer cells increased (387). Our finding that lipid-loaded cells exhibit increased degradation of HDL may represent upregulation of SR-BI expression, and it is plausible (although untested) that under these culture conditions, this receptor could mediate uptake of the entire HDL particle by macrophages, not just selective uptake of cholesteryl esters from HDL as in liver and adrenal cells.

In summary, the results of the present study demonstrate that oxidized LDL-loaded macrophages exhibit impaired efflux of cholesterol. This impairment of HDL-mediated cholesterol efflux involves both the oxidized lipid component and the oxidized apolipoprotein B moiety of oxidized LDL. Furthermore, cholesterol delivered by oxidized LDL is sequestered in lysosomes. With regards to the mechanism of HDL-mediated cholesterol efflux in modified LDL-loaded mouse peritoneal macrophages, our data suggest that exocytosis of undegraded lipoproteins does not occur, nor does HDL_3 retroendocytosis. It is possible that the impairment of cholesterol efflux from oxidized LDL loaded cells influences the formation and persistence of the foam cell phenotype *in vivo* and may therefore contribute to the atherogenicity of oxidized LDL.

Chapter 4 Conclusions

The data in this study present compelling evidence that macrophages loaded with oxidized LDL have a reduced ability to release cholesterol to HDL, compared to cells loaded with acetylated LDL. Oxidation of LDL results in profound alterations of both the lipid and protein compositions of LDL, including fragmentation of apolipoprotein B, hydrolysis of phosphatidylcholine and loss of esterified cholesterol. We have found that both the oxidized lipid portion and the oxidized apolipoprotein component are involved in the observed impairment of HDL-mediated cholesterol efflux from macrophages loaded with oxidized LDL. In addition, the data also provide evidence that oxidized LDL-derived cholesterol is sequestered within lysosomes. This implicates a possible defect in cholesterol metabolism and transport in foam cells of atherosclerotic lesions. Finally, our work has demonstrated that HDL-mediated cholesterol efflux from lipid-loaded mouse peritoneal macrophages involves neither HDL_3 retroendocytosis, nor exocytosis of undegraded oxidized LDL. These findings not only increase our understanding of the role of oxidized LDL in lipid accumulation, but they also give insight into the physiologically-relevant process of HDL-mediated cholesterol efflux. To fully understand how oxidized LDL causes impairment of cholesterol efflux from macrophages, further investigations are required. Future studies should focus on the identification of the specific oxidized protein and oxysterol components responsible for the impaired metabolism of cholesterol. Whether oxidized LDL is responsible for cholesterol accumulation in atherosclerotic lesions is unknown and will be a challenge to determine. However, the present findings provide impetus to look at the

metabolism and transport of cholesterol within cells isolated from atheromas, and also to further characterize the mechanism of HDL-mediated cholesterol efflux.

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