

LIPOPROTEIN METABOLISM IN THE MACROPHAGE: Implications for Cholesterol Deposition in Atherosclerosis¹

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¹Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; apo, apoprotein; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HDL_c, a cholesterol-induced form of HDL containing apoprotein E in addition to apoprotein A-I; IDL, intermediate density lipoproteins; LDL, low density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; β -VLDL, β -migrating very low density lipoproteins; WHHL rabbit, Watanabe Heritable Hyperlipidemic rabbit.

PERSPECTIVES AND SUMMARY

Atherosclerotic plaques are filled with scavenger cells that have ingested large amounts of cholesterol and have become so stuffed with cholesteryl ester that they are converted into foam cells (1, 2). Most of these foam cells arise either from resident macrophages of the artery wall or from blood monocytes that enter the wall at sites of endothelial damage. Macrophages ingest and degrade cholesterol-carrying plasma lipoproteins that have leaked through damaged endothelium and penetrated into the tissue of the wall. When macrophages take up more lipoprotein cholesterol than they can excrete, the cholesterol is stored in the cytoplasm in the form of cholesteryl ester droplets. These droplets give the cytoplasm a foamy appearance in the electron microscope, thus accounting for the term foam cell.

The atherosclerotic plaque is a complicated structure. In addition to cholesterol-filled macrophages, the structure contains large numbers of proliferating smooth muscle cells and a large amount of extracellular material that includes sulfated glycosaminoglycans, collagen, fibrin, and cholesterol (3). Some of the smooth muscle cells contain cholesteryl ester droplets that resemble those of macrophage foam cells. In order to unravel such a complicated structure, in recent years scientists have begun to study the specialized properties of each of the cell types that comprise the lesion. For example, endothelial cells and smooth muscle cells were propagated *in vitro*, and their analyses identified several distinctive properties that might contribute to the initiation of atherosclerosis (reviewed in 3).

The macrophage, too, has come under study. Extensive investigations over the past five years disclosed that macrophages, isolated from the peritoneal cavity of mice and from the blood of man, possess mechanisms that allow them to take up and digest cholesterol-containing lipoproteins, to store the sterol, and to excrete it in large amounts when conditions permit (4-8). These mechanisms differ from those in other cell types, such as cultured fibroblasts and smooth muscle cells. Awareness of these special mechanisms for lipoprotein uptake made possible the conversion of macrophages into foam cells *in vitro* (4, 8). These studies shed new light on the possible mechanism for foam cell formation *in vivo*.

The uptake of lipoprotein-bound cholesterol in macrophages occurs through the process of receptor-mediated endocytosis (4-7). The initial event is the binding of the lipoprotein to a cell surface receptor. Although macrophages express few receptors for normal plasma lipoproteins, they exhibit abundant receptors for lipoproteins that have been altered by chemical derivitization (4) or by complexing with other molecules (5, 7). In addition, macrophages have receptors for at least one type of abnormal lipoprotein that accumulates spontaneously in plasma in hyperlipidemic states (6).

Most of the cholesterol in plasma lipoproteins is in the form of cholesteryl esters. Macrophages process these esters in a series of sequential reactions that take place in two cellular compartments (8, 9). Immediately after they enter the macrophage via receptor-mediated endocytosis, lipoprotein-bound cholesteryl esters are delivered to lysosomes (first cellular compartment) where they are hydrolyzed by an acid lipase. The liberated cholesterol crosses the lysosomal membrane and enters the cytoplasm (second cellular compartment) where it is re-esterified by a microsomal enzyme and stored in the cytoplasm as cholesteryl ester droplets.

The two-compartment pathway allows quantitative assay of the cellular uptake of cholesterol-rich lipoproteins without the need for radiolabeled lipoproteins. When incubated in the usual medium containing normal serum, macrophages do not take up lipoproteins at a high rate, and hence they do not synthesize cholesteryl esters (4, 8). Thus, when [^{14}C]oleate is added to the culture medium, the cells do not incorporate it into cholesteryl [^{14}C]oleate. However, when the cells are presented with a lipoprotein that they can ingest, cholesterol is liberated and then re-esterified, and this leads to a 100- to 200-fold increase in the rate of incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate (4, 8). All of the cholesterol-rich lipoproteins that enter macrophages were found to enhance cholesteryl ester synthesis in this fashion and hence stimulation of cholesteryl [^{14}C]oleate synthesis is used as a functional assay to measure lipoprotein uptake (4-8).

The cholesteryl esters stored in the cytoplasm of macrophage foam cells undergo a continual cycle of hydrolysis and re-esterification (9). Hydrolysis is mediated by a nonlysosomal esterase distinct from the lysosomal acid lipase. Re-esterification is mediated by a membrane-bound enzyme that transfers a fatty acid from fatty acyl coenzyme A to cholesterol. When the extracellular fluid contains a substance, such as high density lipoprotein (HDL), that is capable of binding cholesterol, the free cholesterol is not re-esterified or stored, but is excreted from the cell. When no cholesterol acceptor is available, the free cholesterol is re-esterified for storage, and the cycle of hydrolysis and re-esterification continues (9).

If macrophages metabolize lipoprotein cholesterol in the body as they do in tissue culture, then the cholesterol that they excrete may have two metabolic fates: (a) some of it may be transported directly to the liver where it is excreted from the body (the so called "reverse cholesterol transport") (10); and (b) some of it may be transferred to other lipoproteins, such as low density lipoprotein (LDL), that deliver it both to liver and to extrahepatic tissues for use in the synthesis of new plasma membranes and steroid hormones (11, 12). When macrophages excrete cholesterol, they simultaneously synthesize and secrete large amounts of apoprotein E (13, 14), a component of plasma lipoproteins that binds avidly to lipoprotein receptors. Secreted apo E and secreted cholesterol may associate with the

HDL present in the medium to produce a lipoprotein called HDL_c. When injected intravenously into animals, HDL_c is taken up rapidly by lipoprotein receptors on the surface of hepatocytes (11, 12). Thus, apo E may be synthesized by cholesterol-loaded macrophages in order to target the secreted cholesterol to the liver, thereby facilitating "reverse cholesterol transport" (14).

In this article, we review studies carried out over the last five years that have led to these new insights into the mechanisms for cholesterol uptake, storage, and excretion by macrophages. While the data were obtained almost exclusively from *in vitro* systems, they have important implications for macrophage function in the body and suggest how macrophages might go awry during the formation of foam cells in the atherosclerotic plaque.

UPTAKE OF LIPOPROTEIN-BOUND CHOLESTEROL BY MACROPHAGES

Macrophages can take up large amounts of cholesterol by two mechanisms: (a) by phagocytosis of whole cells or fragments of membranes containing cholesterol; or (b) by receptor-mediated endocytosis of plasma lipoproteins either in solution or complexed in insoluble form with other tissue constituents. The factors governing phagocytosis were discussed elsewhere (15). In this section we review the various systems for receptor-mediated endocytosis of cholesterol-containing lipoproteins.

The initial studies on receptor-mediated endocytosis of lipoproteins by macrophages, reported in 1979 by Goldstein et al (4), were carried out to resolve a paradox that emerged from studies of the LDL receptor. LDL receptors are present on a variety of nonmacrophage cells grown in tissue culture or taken directly from the body. The LDL receptors mediate the uptake and degradation of LDL by body cells and hence are an important determinant of the plasma LDL-cholesterol level (11). Subjects with homozygous familial hypercholesterolemia have a genetically determined total or near total deficiency of LDL receptors. Plasma LDL cannot penetrate into their cells with normal efficiency, and as a result the plasma LDL level rises. Despite their deficiency of LDL receptors, subjects with homozygous familial hypercholesterolemia nevertheless accumulate LDL-derived cholesteryl esters in macrophage foam cells at several sites in the body, notably in the arterial wall, causing atheromas, and in tendons, causing xanthomas (16). This clinical observation suggested that macrophages have some alternative mechanism for taking up LDL-cholesterol distinct from the LDL receptor. However, *in vitro* tissue macrophages take up native LDL at extremely slow rates and do not accumulate excessive cholesteryl esters, even when exposed to high concentrations of LDL for prolonged

periods of time (4). These paradoxical findings led to a search for altered forms of LDL that could be internalized by macrophages at rapid rates.

Receptor for Acetyl-LDL

The first plasma lipoprotein demonstrated to enter macrophages by receptor-mediated endocytosis was human LDL that had been reacted with acetic anhydride in vitro to form acetyl-LDL (4). These studies were conducted with monolayers of resident mouse peritoneal macrophages isolated by the classic techniques developed by Cohn and co-workers (reviewed in 17). Unlike most other cell types, normal tissue macrophages from the mouse and other species express few if any receptors for native LDL (4-6). When incubated with ^{125}I -labeled LDL in vitro, mouse peritoneal macrophages internalize only minimal amounts of the lipoprotein and do not increase cellular cholesterol content (4, 8).² In contrast, LDL that has been modified by chemical acetylation is taken up with extremely high efficiency by macrophages, resulting in massive cholesterol accumulation within the cells (4, 8).

BIOCHEMICAL PROPERTIES OF THE ACETYL-LDL RECEPTOR Studies with ^{125}I -labeled acetyl-LDL showed that the rapid uptake by mouse macrophages is mediated by an initial binding of the lipoprotein to a limited number of high affinity binding sites (20,000-40,000 sites/cell) that recognize acetyl-LDL but not native LDL (4, 18). Binding leads to rapid internalization of acetyl-LDL by endocytosis and delivery to lysosomes. Within 60 min, virtually all of the cell-bound ^{125}I -acetyl-LDL is hydrolyzed and the label is excreted from the cell in the form of ^{125}I -monoiodotyrosine (4). The receptor for acetyl-LDL is just beginning to be characterized biochemically. It is not yet clear whether it is a single molecular entity or is comprised of several different molecular species, each of which is capable of binding acetyl-LDL and mediating its rapid internalization by the cell. All of the surface binding sites for ^{125}I -acetyl-LDL are destroyed when the cells are treated briefly with low concentrations of trypsin or pronase (4), suggesting that all of the receptors are composed of protein. Half-maximal binding of ^{125}I -acetyl-LDL is achieved at an acetyl-LDL concentration of 5 μg pro-

²Although small amounts of ^{125}I -LDL are taken up and degraded by mouse peritoneal macrophages, this uptake does not appear to be mediated by the classic LDL receptor in that it is competitively inhibited nonspecifically by lipoproteins, such as acetyl-LDL [see Figure 2 A in (5)] and typical HDL (24), which do not bind to the LDL receptor. The nature of this nonspecific uptake process for ^{125}I -LDL by tissue macrophages is not clear; it may be related to the ability of lipoproteins to bind nonspecifically to a site on cell membranes that recognizes multiple lipoproteins, i.e. LDL, HDL, methyl-LDL, and acetyl-LDL (93, 105).

tein/ml at 4°C and 25 µg protein/ml at 37°C (4). Binding is not inhibited by EDTA (4), indicating that divalent cations are not essential.

Using the mouse macrophage cell line P388D₁ as a source of receptor, Via, et al (19) reported the partial characterization of a solubilized membrane protein that, after a 300- to 400-fold purification, shows the same affinity and binding specificity as does the acetyl-LDL receptor of intact cells. The detergent-receptor complex has a $M_r = 283,000$, an isoelectric point of 5.9, and a sedimentation coefficient of 6.55.

DISTRIBUTION OF THE ACETYL-LDL RECEPTOR ON DIFFERENT CELL TYPES The acetyl-LDL receptor has been found on macrophages from every source and species so far tested. These include resident peritoneal macrophages from mice (4), rats (4), and dogs (20); Kupffer cells from guinea pigs (4) and rats (21); monocyte-derived macrophages from humans (4, 18, 22, 23); and established lines of mouse macrophage tumors such as IC21 cells (24), J774 cells (25), and P388D₁ cells (19). Activated and inflammatory macrophages produced by intraperitoneal injection of mice with a variety of agents (including thioglycollate, fetal calf serum, phytohemagglutinin, BCG, *Corynebacterium parvum*, and pyran copolymer) express roughly the same amount of acetyl-LDL receptor activity as do unstimulated resident macrophages (26). This is in contrast to other receptors, such as those for mannose-conjugated proteins, which vary markedly in number after several of these treatments (26). Conditioned medium from human lymphocyte cultures stimulated by concanavalin A reduces the ability of macrophages to degrade malondialdehyde-treated LDL, a lipoprotein that enters the cell via the acetyl-LDL receptor (27; see below). This suggests that lymphocytes produce a substance that suppresses the function of the acetyl-LDL receptor.

Pitas et al (28) provided a particularly striking demonstration of the cell specificity of the acetyl-LDL receptor. They made mixed cultures of human fibroblasts and mouse peritoneal macrophages and incubated them with lipoproteins that had been rendered fluorescent through incorporation of the lipophilic fluorescent dye 3,3'-dioctadecylindocarbocyanine. When the 3,3'-dioctadecylindocarbocyanine was incorporated into acetoacetylated LDL, which binds to the acetyl-LDL receptor but not to the LDL receptor, the macrophages became intensely fluorescent but the interspersed fibroblasts did not (28).

In contrast to the LDL receptor of nonmacrophage cells whose number is suppressed when cellular cholesterol accumulates to high levels (29), acetyl-LDL receptors remain constant in number even when macrophages have accumulated massive amounts of cholesterol (4). As a result of their failure to suppress the production of acetyl-LDL receptors, macrophages

incubated continuously with acetyl-LDL take up so much cholesterol that they are converted into foam cells *in vitro* (4, 8; see below).

In contrast to its apparently universal expression in macrophages, the acetyl-LDL receptor is generally absent from nonmacrophage cells, including cultured human fibroblasts, cultured human and bovine smooth muscle cells, freshly isolated human lymphocytes, human lymphoblasts, mouse Y-1 adrenal cells, and Chinese hamster ovary cells (4, 18). The one exception is cultured bovine endothelial cells, which express a small number of acetyl-LDL receptors and degrade ^{125}I -acetyl-LDL at 6% of the rate of resident mouse peritoneal macrophages (30). Endothelial cells are known to share other properties with macrophages, such as the presence of lipoprotein lipase (31, 32) and the ability to present antigens to T lymphocytes in an immunogenic form (30).

In contrast to tissue macrophages, which express acetyl-LDL receptors but virtually no LDL receptors, monocytes freshly isolated from the blood of normal subjects express receptors for both native LDL and acetyl-LDL (4, 18, 22, 23, 33, 34). After 5 days of culture *in vitro*, the activity of the acetyl-LDL receptor increases by as much as 20-fold and markedly exceeds (by more than 10-fold) the activity of the LDL receptor (33, 34). Cultured malignant macrophages such as J774 cells (25) and IC21 cells (24) express low levels of LDL receptors and high levels of acetyl-LDL receptors. Monocytes cultured from the blood of subjects with the homozygous form of familial hypercholesterolemia display normal acetyl-LDL receptor activity despite their genetic deficiency of receptors for native LDL (18, 34).

Figure 1 demonstrates the all-or-none difference in the ability of cultured human fibroblasts and mouse peritoneal macrophages to take up and degrade ^{125}I -acetyl-LDL and ^{125}I -LDL. This difference between acetyl-LDL receptors and LDL receptors is one of the most striking biologic differences between macrophage and nonmacrophage cells and implies an important role for the acetyl-LDL receptor in macrophage function *in vivo*.

LIGAND SPECIFICITY OF THE ACETYL-LDL RECEPTOR Acetylation of LDL removes positive charges from the ϵ -amino groups of lysine and thereby converts a weakly anionic lipoprotein into a strongly anionic one (35). The acetyl-LDL loses its ability to bind to the classic LDL receptor of nonmacrophage cells, but it remains precipitable by antibodies to native LDL (35). The enhanced net negative charge of acetyl-LDL is responsible for its binding to the macrophage acetyl-LDL receptor (4). Other chemical modifications that abolish positive lysine residues and increase LDL's net negative charge also convert the lipoprotein into a ligand for the acetyl-LDL receptor. Such ligands include acetoacetylated LDL (20), maleylated LDL (4), succinylated LDL (4), and malondialdehyde-treated LDL (18,

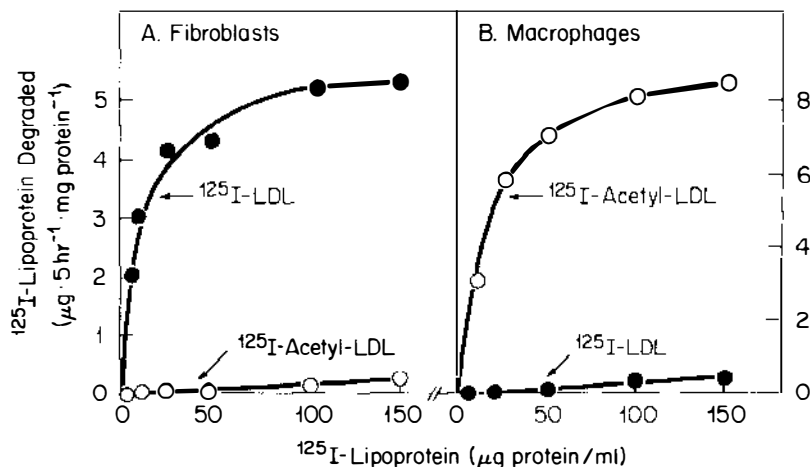


Figure 1 Differences in the uptake and degradation of ^{125}I -LDL (●) and ^{125}I -acetyl-LDL (○) by human fibroblasts (A) and mouse peritoneal macrophages (B). Monolayers of growing fibroblasts were incubated in lipoprotein-deficient serum for 48 hr prior to the experiments to induce maximal LDL receptors (29). Monolayers of freshly isolated macrophages were studied without prior incubation in lipoprotein-deficient serum (4). For degradation assays, each monolayer received medium containing 10% lipoprotein-deficient serum and the indicated concentration of either human ^{125}I -LDL (●) or ^{125}I -acetyl-LDL (○). After incubation for 5 hr at 37°C , the amount of ^{125}I -labeled acid-soluble material released into the medium was measured (4).

22). Reductive methylation of LDL, which modifies the lysine residues but does not remove their positive charge, fails to convert LDL into a species that will bind to the acetyl-LDL receptor (18).

Haberland et al (36) studied the stoichiometry of lysine modification of LDL. Using malondialdehyde as a lysine-modifying agent, they showed that binding to the acetyl-LDL receptor in human monocyte-derived macrophages occurs only above a threshold of 30 moles of malondialdehyde incorporated per mole of LDL protein. Additional incorporation of malondialdehyde (up to 60 moles/mole of LDL) produced no further increase in lipoprotein uptake. In contrast to this sharp threshold effect for ligand binding to the acetyl-LDL receptor, the ability of native LDL to bind to the classic LDL receptor was reduced gradually and in proportion to the number of malondialdehyde residues incorporated. High affinity binding of LDL to the LDL receptor disappeared entirely when 20 moles of malondialdehyde were incorporated per mole of LDL. When the incorporation of malondialdehyde was between 20 and 30 moles per mole of LDL, the particle would bind neither to the LDL receptor nor to the acetyl-LDL receptor (36).

The identity of the negatively charged residues on acetyl-LDL that mediate binding to the acetyl-LDL receptor is not known. LDL is a complex particle that contains negatively charged lipids as well as amino acids and carbohydrates. To simplify analysis of ligand-receptor interactions, experiments were performed with less complex polyanionic ligands that bind to the acetyl-LDL receptor and thus compete for the binding, uptake, and degradation of ^{125}I -acetyl-LDL. In general, acetylation of other proteins (such as albumin, gamma globulin, α -1-antitrypsin, transferrin, ferritin, ovalbumin, histones, ovomucoid, α -1-acid glycoprotein, and HDL) does not convert them into ligands for the acetyl-LDL receptor (37). However, maleylated albumin binds with high affinity (4). In contrast to acetylation, reaction with the dicarboxylic acid maleate not only removes positive charges on lysine residues but also adds additional negatively charged residues in the form of carboxyl groups. These experiments suggest that most native proteins, such as albumin, do not contain a sufficient number or arrangement of negatively charged residues to bind to the acetyl-LDL receptor, even when all of the positive charges on the available lysine residues have been obliterated. However, the addition of new negative charges in the form of maleate converts the molecule into a binding moiety. The unique aspect of LDL is that it contains sufficient negatively charged residues so that elimination of the positive lysine residues induces binding to the acetyl-LDL receptor without a requirement for additional negative charges. HDL behaves like albumin in that it requires maleylation in order to be recognized by the acetyl-LDL receptor (37).

The acetyl-LDL receptor also recognizes compounds in which the negative charges reside on noncarboxyl moieties (4, 18), such as sulfate (e.g. polyvinyl sulfate, dextran sulfate, and fucoidin) or phosphate (e.g. polyinosinic acid and polyxanthinylic acid). All binding polyanions have a high molecular weight. Low molecular weight polyanions (e.g. ATP and GTP) do not bind, as judged by their inability to compete for the uptake of ^{125}I -acetyl-LDL (37).

Table 1 lists a large number of compounds that were tested for binding to the acetyl-LDL receptor. Testing was performed by measuring the ability of each molecule to compete with ^{125}I -acetyl-LDL for uptake and degradation by the mouse peritoneal macrophage receptor. Multiple negative charges are necessary but not sufficient for receptor binding. Certain contrasts are striking. For example, certain polypurines (such as polyinosinic acid, polyguanylic acid, and polyxanthinylic acid) compete effectively for the binding of ^{125}I -acetyl-LDL, while another polypurine (polyadenylic acid) does not compete. Adenylic acid differs from the first three purines in that it has an amino group in place of a keto group at carbon 6. However, polyguanylic acid, which has an amino group at carbon 2, is recognized by

the acetyl-LDL receptor. Since the simple presence of an amino group is not sufficient to prevent binding, the configuration of the polymer is probably the important feature. Two polypyrimidines (polycytidylic acid and polyuridylic acid) do not compete for the binding of ^{125}I -acetyl-LDL. Other polyanions that do not compete for receptor binding include sulfated polysaccharides of relatively low molecular weight (such as heparin and chondroitin sulfate), polycolominic acid, polyphosphate chains containing up to 65 phosphates, and polyglutamic acid (4, 18).

From the results of the inhibitor studies shown in Table 1, we conclude that binding to the acetyl-LDL receptor requires not only a large number of negatively charged residues but also an arrangement that produces a high density of charge within specific regions of the molecule (18).

FUNCTION OF THE ACETYL-LDL RECEPTOR IN VIVO Evidence that the acetyl-LDL receptor is expressed in macrophages in vivo comes from experiments in which ^{125}I -labeled acetyl-LDL (4), ^{125}I -labeled acetoacetylated LDL (20, 38) and ^{125}I -labeled succinylated LDL (39) were administered intravenously to mice, dogs, and rats, respectively. The modified lipoproteins were cleared from the plasma within minutes by macrophages (Kupffer cells) of the liver (38). The hepatic uptake of intravenously administered ^{125}I -acetyl-LDL in mice was blocked when the animals were injected

Table 1 ^{125}I -Acetyl-LDL binding site in mouse peritoneal macrophages

Effective competitors		Ineffective competitors	
Negatively charged compounds		Negatively charged compounds	
Polyvinyl sulfate	1 $\mu\text{g/ml}^a$	Polyadenylic acid, polycytidylic acid, poly-	
Polyinosinic acid	1.5	polyuridylic acid, heparin, chondroitin	
Polyguanylic acid	1.5	sulfates A and C, phosvitin, colominic	
Poly G:I (1:1)	2	acid (polysialic acid), polyphosphates	
Polyxanthinylic acid	3	($n = 65$), poly(D-glutamic acid)	
Dextran sulfate	3	Positively charged compounds	
Fucoidin	10	Lysozyme, spermine	
Bovine sulfatides	10	Glycoproteins	
Carrageenan	20	Mannan (yeast), thyroglobulin, fetuin,	
Maleylated LDL	20	orosomucoid, asialorosomucoid	
Poly I: poly C	50	Others	
Maleylated albumin	100	Acetylated proteins, including albumin,	
Maleylated HDL	150	γ -globulin, α -1-antitrypsin, transferrin,	
		ovalbumin, histones, ovomucoid, α -1-acid	
		glycoprotein, and HDL	
		Methylated LDL	

^a Values refer to the concentrations required for 50% inhibition of ^{125}I -acetyl-LDL binding.

simultaneously with fucoidin (4), confirming that the uptake was mediated by a saturable receptor with a specificity similar to the binding site demonstrated *in vitro*.

The normal ligand for the acetyl-LDL receptor *in vivo*, if any, is unknown. It has not yet been demonstrated that the receptor participates in the clearance of endogenous plasma lipoproteins, as opposed to injected modified lipoproteins. It is unlikely that acetyl-LDL would be formed extracellularly in the body since its biologic formation requires acetyl-CoA or some other donor of active acetate whose occurrence in extracellular fluid has not been demonstrated. Other functionally equivalent types of lysine modification reactions might take place extracellularly, however. For example, malondialdehyde is known to be secreted by platelets and macrophages as a by-product of the oxidation of arachidonic acid (40, 41). As originally shown by Fogelman, et al. (22), malondialdehyde can react with the lysines of LDL and convert the lipoprotein to a form that is recognized with high affinity by macrophages. Subsequent studies showed that the malondialdehyde-modified LDL was taken up by the acetyl-LDL receptor (18, 33). Chemical formation of malondialdehyde-LDL *in vitro* requires concentrations of malondialdehyde above 1 mM (18, 22, 33). This is several orders of magnitude higher than the malondialdehyde concentrations that are likely to occur in the body, even in platelet thrombi (42). Nevertheless, some local factor(s) operating within damaged tissues or platelet thrombi might increase the susceptibility of LDL to reaction with malondialdehyde.

Malondialdehyde is not the only substance derived from arachidonic acid that might modify the lysines of LDL. Table 2 lists several theoretical reactions that could occur between the ϵ -amino group of lysines in LDL and one or more metabolites generated normally *in vivo* during the conversion of arachidonic acid to thromboxanes and leukotrienes. Each of these reactions would convert LDL to a more negative form that could be recognized by the acetyl-LDL receptor.

In addition to the arachidonic acid-derived aldehydes listed in Table 2, there are several other naturally occurring aldehydes that might react with the lysine residues of LDL in extracellular fluids. One of these is glucose. Glucosylated-LDL was shown to be present in the plasma of patients with diabetes mellitus (43-45). When LDL is reacted with glucose *in vitro*, the lysine residues are modified to such an extent that the LDL loses its ability to bind to the native LDL receptor of fibroblasts (44, 46, 47). However, this modified LDL is not taken up by macrophages via the acetyl-LDL receptor, perhaps because an insufficient number of lysine residues is accessible to modification by glucose (36, 44).

In addition to chemical derivitization, the lysines of LDL could be altered enzymatically through the action of lysyl oxidase, an extracellular enzyme

Table 2 Arachidonic acid metabolites that could theoretically react with lysine residues of LDL so as to eliminate their positive charge

Pathway of Arachidonic acid metabolism	Reactive Metabolite	Chemical reaction with Lysine	Example
Cyclooxygenase	Malondialdehyde	Imination (Schiff base)	$\text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}_3^+ + \text{H}-\text{C}(=\text{O})-\text{CH}=\text{CH}-\text{OH} \longrightarrow \text{H}-\text{N}=\text{CH}-\text{CH}(\text{OH})-\text{CH}_2-(\text{CH}_2)_2-\text{C}$ <p>Protein-bound Lysine Malondialdehyde</p>
Cyclooxygenase	Prostaglandin A	Michael addition	$\text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}_3^+ + \text{Prostaglandin A} \longrightarrow \text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}-\text{Prostaglandin A}$ <p>Protein-bound Lysine Prostaglandin A</p>
Lipoxygenase	5-HPETE	Oxidation by active oxygen species	$\text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}_3^+ + \text{5-HPETE} \longrightarrow \text{C}-(\text{CH}_2)_2-\text{C}(=\text{O})-\text{H} \text{ or } \text{C}-(\text{CH}_2)_2-\text{CH}(\text{OH})-\text{NH}_2$ <p>Protein-bound Lysine 5-HPETE</p>
Lipoxygenase	Leukotriene A ₄	Alkylation	$\text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}_3^+ + \text{Leukotriene A}_4 \longrightarrow \text{C}-(\text{CH}_2)_2-\text{CH}_2-\text{NH}-\text{Leukotriene A}_4$ <p>Protein-bound Lysine Leukotriene A</p>

This table was prepared by J. R. Falck. 5-HPETE, 5-hydroxyperoxyeicosatetraenoic acid.

that oxidizes the ϵ -amino group of certain lysine residues to allysine, an aldehyde intermediate that is essential to the cross-linking of collagen fibrils (48). Whether such oxidation of LDL's lysines would lead to its uptake by the acetyl-LDL receptor is unknown. Lysyl oxidase occurs in highest concentrations in the extracellular fluid of tendons and aorta (48). Interestingly, these are precisely the sites at which the most pronounced accumulation of LDL-cholesterol occurs in macrophages in vivo (16). A role for lysyl oxidase is rendered less likely by the belief that the enzyme is specific for collagen and elastin (48); it has not been shown to react with LDL. Oxidation of protein-bound lysine residues can also occur chemically in the presence of hydrogen peroxide, a peroxidase enzyme, and a hydrogen acceptor (49).

A recent exciting development was the demonstration by Henriksen et al (25) that cultured endothelial cells can convert human LDL into a form that is recognized by the acetyl-LDL receptor of macrophages. This modification follows incubation of human LDL with any one of several types of cultured cells: rabbit aortic endothelial cells, human umbilical vein endothelial cells, or aortic smooth muscle cells from guinea pig or swine (25, 50). It is not produced by the incubation of LDL with conditioned medium from these cultures or by incubation with intact cultured fibroblasts or red blood cells. The modified LDL has an increased density, owing to a decrease in its content of free and esterified cholesterol (50). It also has an enhanced electrophoretic mobility, presumably due to an increased negative charge (25). The chemical nature of this modification is unknown; malondialdehyde was not detectable (25).

Although the acetyl-LDL receptor was discovered on the basis of its ability to bind modified LDL, its function in vivo may involve pathways other than lipoprotein metabolism. Maleylated proteins, which enter macrophages through the acetyl-LDL receptor (4), were shown to stimulate markedly the secretion of three neutral proteases: neutral caseinase, plasminogen activator, and cytolytic proteinase (51). These findings suggest that the acetyl-LDL receptor may play a regulatory role in the inflammatory response. In this regard, it is striking that many of the ligands for the acetyl-LDL receptor are potent stimulators of interferon secretion, e.g. polyinosinic acid, polyvinyl sulfate, dextran sulfate, fucoidin, and polyinosinic acid:polycytidylic acid (52). Whether the activity of these agents is dependent upon binding to the acetyl-LDL receptor has not been explored.

Receptor for LDL/Dextran Sulfate Complexes

LDL has long been known to form soluble and insoluble complexes with sulfated polysaccharides, both naturally occurring and synthetic (53). In 1979 Basu et al (5) reported that complexes of LDL and dextran sulfate are

taken up with high affinity by mouse peritoneal macrophages, apparently as a result of binding to saturable and high affinity surface receptor sites. As with acetyl-LDL, the protein component of the ^{125}I -LDL/dextran sulfate complexes is rapidly degraded in lysosomes and the cholesterol is retained by the cell for processing and storage (5; see below).

The LDL/dextran sulfate binding site has so far been found only on macrophages, including not only mouse peritoneal macrophages but also cultured mouse IC21 cells (24) and human monocyte-derived macrophages (27, 33, 34). A similar uptake does not occur in other cell types such as human fibroblasts (5). In fact, dextran sulfate inhibits the receptor-mediated endocytosis of LDL in fibroblasts because LDL that is complexed to dextran sulfate can no longer bind to the native LDL receptor (29).

The stoichiometry of the LDL/dextran sulfate complex entering macrophages is not known. When the two ligands are present in the culture medium in a 10:1 mass ratio, the maximal rate of ^{125}I -LDL degradation occurs at an LDL concentration of 250 $\mu\text{g/ml}$ and a dextran sulfate concentration of 25 $\mu\text{g/ml}$ (5). Recently, the converse experiment was performed, and the results demonstrated that LDL greatly increases the uptake of [^3H]dextran sulfate by macrophages (54), confirming that the two substances enter the cell as a complex.

Whereas dextran sulfate of $M_r = 500,000$ is effective in promoting the uptake of LDL, dextran sulfate of lower M_r (i.e. 40,000) is ineffective (5). A variety of naturally occurring sulfated glycosaminoglycans (including chondroitin-6-sulfate, chondroitin-4-sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, and heparin) do not stimulate uptake and degradation of ^{125}I -LDL (5), even though many of these compounds are known to form tight complexes with LDL (53). Although purified heparin is not effective, a heparin-containing proteoglycan isolated from rat skin, $M_r > 900,000$ (55), stimulates the uptake and degradation of human ^{125}I -LDL in mouse macrophages by two-fold (56). These data suggest that stimulation of LDL uptake in macrophages requires the formation of large complexes.

The nature of the cell surface binding site for LDL/dextran sulfate is not known. Monocyte-derived macrophages isolated from the blood of subjects with homozygous familial hypercholesterolemia take up and degrade ^{125}I -LDL/dextran sulfate complexes with high affinity and at a normal rate under conditions in which no high affinity uptake and degradation of native ^{125}I -LDL is detected, thus providing genetic evidence that the binding site is distinct from the LDL receptor (34). Competition studies show that the binding site for LDL/dextran sulfate is also distinct from the acetyl-LDL binding site (5). Thus, uptake and degradation of ^{125}I -LDL/dextran sulfate is not inhibited by incubation with polyinosinic acid up to concentrations of 500 $\mu\text{g/ml}$, although uptake and degradation of ^{125}I -acetyl-LDL is com-

pletely inhibited at $< 5 \mu\text{g/ml}$ (5). Similar differential inhibitory results are obtained with fucoidin, an inhibitor of acetyl-LDL binding (5). Polycations, such as spermine, spermidine, and putrescine, prevent the formation of the ^{125}I -LDL/dextran sulfate complex in vitro and thereby prevent dextran sulfate from stimulating ^{125}I -LDL degradation by intact macrophages (5). The potency of these agents declines in the following order: spermine $>$ spermidine $>$ putrescine.

Since the arterial wall and other interstitial spaces contain large amounts of sulfated proteoglycans (57), it is tempting to speculate that LDL forms complexes with these proteoglycans in vivo and that these complexes are then taken up by macrophages in a manner analogous to the uptake of LDL/dextran sulfate complexes in vitro. Whether such an event does occur in vivo remains speculative (see below).

Receptor for β -VLDL

In 1980, Goldstein et al (6) reported that mouse peritoneal macrophages express a surface binding site that mediates the uptake and lysosomal degradation of β -migrating very low density lipoproteins (β -VLDL). To date, β -VLDL are the only naturally occurring plasma lipoproteins known to bind to macrophage receptors (6, 58). Although these lipoproteins are not normally present in detectable amounts in the plasma of humans or animals, Mahley et al (58, 59) showed them to accumulate in the plasma of a variety of species, including dogs, rats, rabbits, and monkeys, when the animals are fed a high cholesterol diet. β -VLDL are also present in plasma of humans with the genetic disease familial dysbetalipoproteinemia, also called type 3 hyperlipoproteinemia (60).

Upon ultracentrifugation of plasma, β -VLDL are found in the same density range as VLDL ($d < 1.006 \text{ g/ml}$). In contrast to normal VLDL, which have a triglyceride-rich core, the β -VLDL have a core composed largely of cholesteryl ester. Whereas normal VLDL contain apoproteins B, E, and C, β -VLDL particles contain predominantly apo B and apo E, with markedly reduced amounts of apo C. In contrast to normal VLDL, which have pre- β mobility on agarose gel electrophoresis, the β -VLDL particles show β mobility (59, 61).

β -VLDL particles are thought to represent exaggerated forms of remnant particles normally created during the catabolism of the triglyceride-rich lipoproteins, chylomicrons and VLDL. After the triglycerides of chylomicrons and VLDL are hydrolyzed by lipoprotein lipase, the particles are converted into cholesteryl ester-rich remnant lipoproteins that are rapidly removed by the liver (11, 61). In cholesterol-fed animals and in patients with familial dysbetalipoproteinemia, the normal hepatic clearance mechanism is either overloaded or functions inefficiently. As a result, the rem-

nant particles remain in plasma where they grow in size and become even further enriched in cholesteryl esters to form β -VLDL (60, 61).

Mouse peritoneal macrophages have surface receptors that specifically bind β -VLDL but not VLDL (6, 58). Similar receptors are present on macrophages derived from human monocytes (58, 62). The β -VLDL receptor differs from the acetyl-LDL receptor since it does not bind acetyl-LDL, fucoidin, or polyinosinic acid, all of which bind to the acetyl-LDL receptor (6).

BIOCHEMICAL PROPERTIES OF THE β -VLDL RECEPTOR In many respects the β -VLDL receptor resembles the LDL receptor of human fibroblasts. For example, both receptors are under feedback regulation. When fibroblasts are induced to accumulate large amounts of cholesterol, they suppress production of LDL receptors (29). Similarly, when mouse peritoneal macrophages (6) or human monocyte-derived macrophages (62) are loaded with cholesterol, the number of β -VLDL receptors is reduced. However, there are sufficient differences to indicate that the macrophage β -VLDL receptor and the fibroblast LDL receptor are probably different molecules. Thus, although the LDL and β -VLDL receptors both bind β -VLDL (58), binding of ^{125}I -labeled β -VLDL to the LDL receptor of human fibroblasts is competitively inhibited by excess unlabeled LDL, while binding of ^{125}I -labeled β -VLDL to the β -VLDL receptor in mouse peritoneal macrophages is not inhibited significantly by LDL (6, 58). Monocytes from patients with familial hypercholesterolemia cannot produce LDL receptors because of a defect in the gene for the LDL receptor (63). Yet, these mutant monocytes produce normal amounts of β -VLDL receptors (58, 62). Additional evidence for two different receptors comes from comparative studies of the effect of exogenously added apo E on the uptake of VLDL by the β -VLDL receptor of macrophages and by the LDL receptor of fibroblasts (see below).

LIGAND SPECIFICITY OF THE β -VLDL RECEPTOR Based on Scatchard analysis, the K_d for ^{125}I - β -VLDL binding to mouse peritoneal macrophages at 4°C is about $1\ \mu\text{g protein/ml}$ (64). The component of β -VLDL recognized by the macrophage receptor has been difficult to elucidate. When the proteins of β -VLDL are modified by reductive methylation, binding to the β -VLDL receptor is abolished, as judged by the loss of the ability to stimulate cholesteryl ester formation in macrophages (64). This experiment strongly suggests that the protein component is involved in binding. As mentioned above, the proteins of β -VLDL consist almost entirely of apo-proteins B and E. Yet, lipoproteins containing only apo B (LDL) or only apo E (apo E-HDL_c) fail to inhibit the uptake and degradation of ^{125}I - β -

VLDL by the macrophage β -VLDL receptor (6, 58, 65), suggesting that neither apo B or apo E alone mediates binding. Moreover, normal ^{125}I -VLDL, which contain both apo B and apo E, fail to bind to the macrophage β -VLDL receptor (6). Thus, it has not been possible to determine which, if any, of the proteins on β -VLDL mediate binding. Binding of ^{125}I - β -VLDL is not competitively inhibited by ligands for other known macrophage receptors, such as yeast mannans (6).

Fainaru et al (66) recently subfractionated dog β -VLDL particles by agarose gel chromatography into two discrete populations. One population, designated Fraction I, was larger in diameter (90–300 nm) than Fraction II (20–70 nm). On paper electrophoresis, Fraction I remained at the origin, whereas Fraction II had β -mobility. Both Fractions I and II contained predominantly apoproteins B and E. However, Fraction I contained an equal mixture of high and low M_r forms of apoprotein B, whereas Fraction II contained only the high M_r form. On the basis of these findings, Fainaru et al suggested that the Fraction I component of canine β -VLDL was of intestinal origin, i.e. it was derived from intestinal chylomicrons. In contrast, Fraction II particles were postulated to be of hepatic origin (66). This hypothesis was supported by the finding that fasting for 48 hr led to a near disappearance of Fraction I particles from plasma, but did not lower the amount of Fraction II in dogs previously fed cholesterol. Both Fractions I and II bound to the β -VLDL receptor on mouse peritoneal macrophages, as evidenced by their ability to deliver cholesterol to cells and thereby stimulate the incorporation of $[^{14}\text{C}]$ oleate into cholesteryl $[^{14}\text{C}]$ oleate (66). Fraction I was 3- to 15-fold more active than Fraction II in stimulating cholesteryl ester synthesis when added at comparable cholesterol levels, suggesting that the β -VLDL receptor may interact preferentially with intestinally derived remnant particles.

Plasma from two patients with familial dysbetalipoproteinemia were also found to contain two fractions of β -VLDL (66). These fractions corresponded in size and composition to Fractions I and II of dog plasma. In both the dog and human samples, the Fraction I lipoprotein had a much lower protein-to-cholesterol ratio than did Fraction II. As with the canine samples, the human Fraction I material was several-fold more potent than Fraction II in stimulating cholesteryl ester synthesis in mouse peritoneal macrophages (66).

Gianturco et al (67) showed that chylomicrons and VLDL isolated from hypertriglyceridemic patients bind to the β -VLDL receptor of mouse peritoneal macrophages. Chylomicrons and VLDL from normal humans failed to bind or be taken up by the macrophages. Uptake of ^{125}I -VLDL from the hypertriglyceridemic patients was not inhibited competitively by acetyl-LDL, but was inhibited competitively by rabbit β -VLDL to a much

greater degree than by LDL, suggesting that the hypertriglyceridemic VLDL particles were binding to the β -VLDL receptor. Uptake of hypertriglyceridemic VLDL by the cells delivered triglyceride and produced a massive increase in the triglyceride content of the macrophages. The apo-proteins of hypertriglyceridemic VLDL that mediate binding to the β -VLDL receptor are unknown. No difference in protein composition was found between normal VLDL, which does not bind to this binding site, and hypertriglyceridemic VLDL, which binds with high affinity (67).

The addition of exogenous apo E to hypertriglyceridemic VLDL markedly limits its ability to be taken up by the β -VLDL receptor of macrophages (68). On the other hand, the addition of apo E to normal VLDL, which does not bind to either the β -VLDL receptor of macrophages or the LDL receptor of fibroblasts, results in a particle that is efficiently internalized by the LDL fibroblast receptor (69). Thus, incorporation of exogenous apo E inhibited binding to the β -VLDL receptor and stimulated binding to the LDL receptor. The mechanism for these effects is unknown.

Although hypertriglyceridemic VLDL and β -VLDL appear to bind to the same site on the macrophage surface and although the uptake mechanisms appear to be similar, the final result of the uptake process is different in the case of the two different lipoproteins. Since β -VLDL contain predominantly cholesteryl ester, uptake of this particle causes cholesteryl ester accumulation in the cells (6; see below). In contrast, uptake of hypertriglyceridemic VLDL leads to degradation of the internalized triglyceride within lysosomes, release of fatty acids, and re-esterification of the fatty acids within the cytoplasm to form triglycerides (67). Thus, although in both cases the cells develop Oil Red-O-positive inclusions, the lipids comprising these inclusions differ.

FUNCTION OF THE β -VLDL RECEPTOR IN VIVO As mentioned above, the β -VLDL receptor appears designed to bind remnants of chylomicron or VLDL metabolism that have circulated in plasma for an abnormally long time and are excessively rich in cholesteryl esters or triglycerides. The macrophage receptor thus may function as a backup mechanism to clear remnant lipoproteins when they are not removed promptly by their normal receptors on hepatocytes or other cells. Evidence that the macrophage receptor functions in vivo is so far indirect. One line of evidence stems from the observation by Mahley (59) that cholesterol-fed dogs develop massive cholesteryl ester deposition in macrophages throughout the body. This deposition becomes marked only when the plasma cholesterol level exceeds 750 mg/dl. Similarly, β -VLDL becomes a predominant lipoprotein only when the plasma cholesterol level exceeds 750 mg/dl. The simultaneous appearance of circulating β -VLDL and cholesterol-loaded macrophages

suggests that β -VLDL may be depositing its cholesterol in macrophages, presumably as a result of uptake via the β -VLDL receptor.

The second line of evidence for function of the β -VLDL receptor in vivo comes from studies in which ^{125}I - β -VLDL are injected into animals. These β -VLDL particles are removed from the circulation with extreme rapidity (70–72). Canine Fraction I particles are removed much more rapidly than Fraction II particles (66). The plasma clearance of β -VLDL is markedly reduced when the β -VLDL are reductively methylated, a reaction known to prevent the binding of β -VLDL to the macrophage receptor (64). Interpretation of these in vivo clearance experiments is clouded, however, because in addition to binding to the macrophage β -VLDL receptor, these particles also bind to LDL receptors which are present on hepatocytes and in extrahepatic tissues (58, 72). Binding of β -VLDL to the LDL receptor as well as to the β -VLDL receptor is inhibited by reductive methylation (64, 73). Thus, the rapid disappearance of β -VLDL from the plasma could be a consequence of its binding to LDL receptors on hepatocytes, rather than to β -VLDL receptors on macrophages. Indeed, Kovanen et al (72) showed that feeding of cholesterol to rabbits results in a marked diminution in the number of β -VLDL binding sites on liver membranes and a corresponding decrease in the rate of uptake of β -VLDL by the liver in vivo. The in vitro binding sites measured in these studies represented LDL receptors rather than β -VLDL receptors of macrophages, because the binding of ^{125}I - β -VLDL was susceptible to inhibition by LDL (72). Thus, at present, it is not possible to conclude definitively that β -VLDL receptors on macrophages participate in the clearance of β -VLDL from plasma.

Receptors for Cholesteryl Ester/Protein Complexes from Atherosclerotic Plaques

In addition to the receptors for modified or abnormal plasma lipoproteins, mouse peritoneal macrophages express binding sites that mediate the uptake of cholesteryl ester/protein complexes isolated from atherosclerotic plaques of human aortas (7). This uptake leads to a marked stimulation of cholesteryl [^{14}C]oleate synthesis and a marked cellular accumulation of cholesteryl esters (7; see below).

The biochemical elements of the aortic cholesteryl ester/protein complexes that promote macrophage cholesteryl ester accumulation have been defined in only a preliminary way. The bulk of the active complexes were excluded on a Biogel A-50m column and floated in the ultracentrifuge in the density range of 1.006–1.063 g/ml, indicating that they consisted of large lipid/protein aggregates (7). The complexes contained 19% free cholesterol, 35% esterified cholesterol, 11% phospholipid, 4% triglyceride, and 31% protein (7), a composition roughly similar to that of LDL. It is likely

that the active complexes also contained sulfated glycosaminoglycans as found by Berenson and co-workers in studies of similar material (57, 74). The protein component of the complexes was important for uptake by macrophages since pronase treatment abolished their ability to stimulate cholesterol esterification (7).

Some of the aortic cholesteryl ester/protein complexes were retained on an anti-apoprotein B affinity column, which suggested that they contained immunoreactive apo B (7). However, the material that did not adhere to the affinity column was just as active as the retained material in stimulating cellular cholesteryl [^{14}C]oleate synthesis (7), showing that apo B was not required, at least in an immunoreactive form.

The aortic cholesteryl ester/protein complexes appeared to enter macrophages by binding to high affinity saturable sites on the cell surface. Uptake was prevented in an apparent competitive fashion by certain polyanions, such as polyinosinic acid, dextran sulfate, and fucoidin, but not by polycytidylic acid (7). This pattern of competition is similar to that displayed by the receptor for acetyl-LDL (4, 18). In direct competition experiments, however, the aortic extract competed to only a small degree for the uptake and degradation of ^{125}I -acetyl-LDL (7). It was concluded that the binding site for the aortic complexes was similar but not identical to that for acetyl-LDL (7).

Macrophages showed specificity at two levels in interacting with the aortic complexes. First, there was ligand specificity in that other types of cholesteryl ester/protein complexes, such as those isolated from liver and adrenal glands, were not taken up by macrophages, as monitored by their inability to stimulate cellular cholesteryl [^{14}C]oleate synthesis when added to the medium at comparable cholesterol concentrations (7). Second, there was receptor specificity in that other cell types such as cultured human fibroblasts failed to take up the cholesteryl ester-rich particles of the aortic extract (7).

In early studies, Werb & Cohn (75, 76) showed that mouse peritoneal macrophages take up particulate complexes formed by sonicating mixtures of albumin and cholesteryl ester. Goldstein et al (7) formed such complexes in the presence of ^{125}I -labeled albumin and showed that the complexes bound with high affinity to a surface binding site at 4°C . Surface binding was inhibited completely by polyinosinic acid and fucoidin, partially by acetyl-LDL, and not all by polycytidylic acid (7, 77). However, the albumin/cholesteryl ester complexes did not inhibit the binding of ^{125}I -acetyl-LDL (7). Thus, the albumin/cholesteryl ester complexes, like the aortic complexes, bind to a high affinity site that resembles but is not identical to the acetyl-LDL receptor. Interestingly, ^{125}I -albumin, in the absence of cholesteryl ester, was not taken up or degraded with high affinity by the macrophages (7).

The simplest interpretation of the above experiments is that macrophages express a family of receptors that recognize cholesteryl ester/protein complexes. One of these is the receptor for acetyl-LDL. Other receptors bind cholesteryl ester/protein complexes from aorta and cholesteryl ester/albumin complexes, but not acetyl-LDL. These receptors share the striking property that binding and uptake are inhibited by polyinosinic acid (but not by polycytidylic acid) and by sulfated polysaccharides such as fucoidin and dextran sulfate. However, cross-competition studies indicate that the receptors for acetyl-LDL differ from those for cholesteryl ester/protein complexes.

PROCESSING AND STORAGE OF LIPOPROTEIN-BOUND CHOLESTEROL BY MACROPHAGES

All of the lipoproteins that bind to receptors on the surface of macrophages appear to be processed in a similar fashion (4–7). All are rapidly internalized at 37°C, apparently by endocytosis, and then delivered to lysosomes where the protein and cholesteryl ester components are hydrolyzed. The liberated cholesterol is released from the lysosome and subsequently re-esterified in the cytoplasm or excreted from the cell, depending on whether or not a cholesterol acceptor is present in the culture medium. The most detailed studies were conducted with acetyl-LDL as a model (4, 8, 9, 18, 78), and these are described below.

Endocytosis and Lysosomal Hydrolysis

When ^{125}I -labeled acetyl-LDL is allowed to bind to the macrophage surface at 4°C and the cells are subsequently warmed to 37°C, the surface-bound lipoprotein is internalized and degraded with extreme efficiency (4). Within 30 min at 37°C, 50% of the initial cell-bound radioactivity is degraded and the major degradation product, ^{125}I -monoiodotyrosine, is excreted from the cell (4).

In the presence of low concentrations of the lysosomal inhibitor chloroquine (20–75 μM), the endocytic uptake of the ^{125}I -acetyl-LDL continues, but degradation is blocked (4, 8). As a result, large amounts of undigested ^{125}I -acetyl-LDL accumulate within the cell, demonstrating that degradation normally occurs in lysosomes. When binding of the ^{125}I -acetyl-LDL to the surface receptor is inhibited by compounds such as fucoidin and polyinosinic acid, cellular uptake and degradation are reduced by more than 95%, indicating that uptake and degradation require binding of the ^{125}I -acetyl-LDL to its receptor (4, 18).

In an extensive series of studies, Brown et al (8, 9) traced the fate of the cholesteryl ester component of acetyl-LDL within macrophages. These studies were carried out with the use of reconstituted lipoproteins, prepared according to the technique of Krieger et al (79). By this technique acetyl-LDL particles are lyophilized in the presence of starch and extracted with heptane to remove cholesteryl ester and free cholesterol. The core of the lipoprotein is then reconstituted by addition of [^3H]cholesteryl linoleate in heptane. The resultant particle, designated *r*-[^3H -cholesteryl linoleate]acetyl-LDL, is taken up by macrophages in the same way as acetyl-LDL, and the [^3H]cholesteryl ester component is hydrolyzed (8). In the presence of chloroquine, hydrolysis of the [^3H]cholesteryl linoleate is inhibited, and unhydrolyzed [^3H]cholesteryl linoleate accumulates within the cell (8). Double-label studies using ^{125}I -acetyl-LDL and *r*-[^3H -cholesteryl linoleate]acetyl-LDL indicate that the protein and cholesteryl ester components are taken up and hydrolyzed in amounts that are proportional to their occurrence in the lipoprotein particle, i.e. the particle appears to be taken up and delivered to lysosomes intact (8).

Cytoplasmic Re-esterification and Hydrolysis of Lipoprotein-Derived Cholesteryl Esters

The studies with *r*-[^3H -cholesteryl linoleate]acetyl-LDL show that the [^3H]cholesterol released after lysosomal hydrolysis does not remain as free cholesterol, but rather is rapidly re-esterified (8). The re-esterification reaction was demonstrated by two approaches. First, macrophages were incubated with *r*-[^3H -cholesteryl linoleate]acetyl-LDL in the presence of unlabeled oleate. At intervals the cells were extracted and the [^3H]cholesteryl esters were fractionated on silver nitrate-coated thin layer chromatogram sheets, which separate cholesteryl oleate and cholesteryl linoleate. At early time points (less than 2 hr), most of the cellular [^3H]cholesteryl esters were composed of [^3H]cholesteryl linoleate. Some free [^3H]cholesterol also was formed. By 2 hr, the levels of intact [^3H]cholesteryl linoleate and free [^3H]cholesterol had reached a steady state plateau. Subsequently, there was a marked accumulation of [^3H]cholesteryl oleate, indicating hydrolysis of the entering [^3H]cholesteryl linoleate with subsequent re-esterification of the free [^3H]cholesterol to form [^3H]cholesteryl oleate. In the presence of chloroquine, the hydrolysis of [^3H]cholesteryl linoleate was inhibited. As a result, [^3H]cholesteryl linoleate continued to accumulate in the cells without reaching a plateau, and there was no generation of free [^3H]cholesterol or of [^3H]cholesteryl oleate (8).

The second approach used to demonstrate the hydrolysis and re-esterification mechanism consisted of incubating macrophages with *r*-[^3H -cholesteryl linoleate]acetyl-LDL in the presence of [^{14}C]oleate (8). During

the initial 2 hr period, all of the rise in cholesteryl esters within the cell could be attributed to [^3H]cholesteryl linoleate. At this point the cholesteryl ester fraction contained little [^{14}C]oleate. However, after 2 hr the rise in [^3H]cholesterol in the ester fraction was paralleled by an equimolar rise in the [^{14}C]oleate content of the cholesteryl fraction. Thus, after 2 hr all of the increase in cholesteryl ester represented newly synthesized cholesteryl oleate (8).

The above two experiments documented that the cholesteryl esters of acetyl-LDL (predominantly cholesteryl linoleate) are hydrolyzed in lysosomes and that the resultant free cholesterol is rapidly re-esterified (primarily with oleate). Figure 2 shows the marked stimulation in the synthesis (Panel A) and accumulation (Panel B) of cholesteryl esters that occurs in macrophages incubated with acetyl-LDL. Fucoidin, which inhibits binding to the acetyl-LDL receptor, prevents the uptake of acetyl-LDL and thereby prevents the lipoprotein from delivering cholesterol to the cells (Figure 2B).

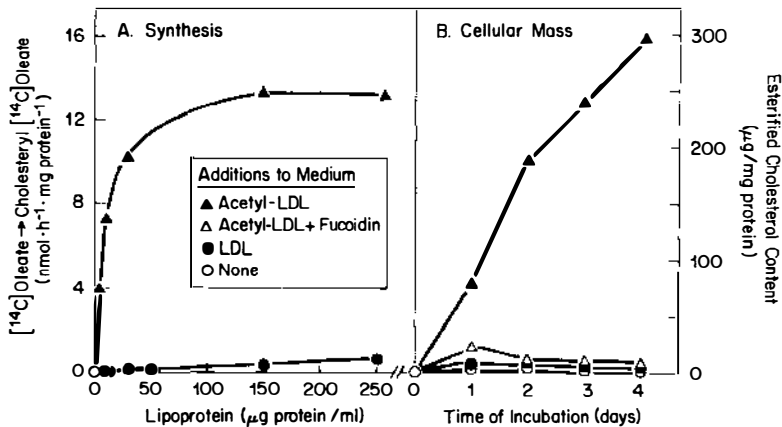


Figure 2 Accumulation of cholesteryl esters in mouse peritoneal macrophages incubated with acetyl-LDL. *Panel A:* Stimulation of cholesteryl [^{14}C]oleate formation. Monolayers of macrophages were incubated for 2 days in lipoprotein-deficient serum and then with the indicated concentration of either native LDL (\bullet) or acetyl-LDL (\blacktriangle) 5 hr at 37°C . Each monolayer was then pulse-labeled for 2 hr with 0.1 mM [^{14}C]oleate bound to albumin and the cellular content of cholesteryl [^{14}C]oleate was measured by thin layer chromatography (8). *Panel B:* Time course of cholesteryl ester accumulation. Monolayers of macrophages received medium containing lipoprotein-deficient serum and one of the following additions: \circ , none; \bullet , $25 \mu\text{g protein/ml}$ of native LDL; \blacktriangle , $25 \mu\text{g protein/ml}$ of acetyl-LDL; or \triangle , $25 \mu\text{g protein/ml}$ of acetyl-LDL plus $50 \mu\text{g/ml}$ of fucoidin. At the indicated time, the monolayers were harvested and their content of esterified cholesterol was measured by gas-liquid chromatography (8).

The mechanism of the cholesterol re-esterification reaction was studied in cell-free homogenates prepared from macrophages incubated in the absence or presence of acetyl-LDL (8). The cell-free homogenates were incubated with [^{14}C]oleate in the presence of ATP, magnesium, and coenzyme A. Homogenates of the cells that had been incubated with acetyl-LDL incorporated [^{14}C]oleate into cholesteryl [^{14}C]oleate at a rate that was 20-fold higher than that of homogenates of cells not incubated with acetyl-LDL. The cholesterol esterifying enzyme was associated with membranes, since it was recovered in the pellet after centrifugation at $100,000 \times g$. The reaction was totally dependent on ATP and coenzyme A, indicating that it was mediated by an acyl-CoA:cholesterol acyltransferase (ACAT) enzyme (8).

The cholesterol derived from lysosomal hydrolysis appears to be the component of acetyl-LDL that stimulates the ACAT enzyme, but the mechanism of this stimulation is not known. A similar stimulation of ACAT activity occurs when human fibroblasts take up and hydrolyze native LDL and thereby liberate free cholesterol (29). The activity of the ACAT enzyme can be enhanced in a similar fashion if intact cells are simply incubated with cholesterol or oxygenated derivatives of cholesterol that are dissolved in ethanol (29). However, the stimulation of the ACAT reaction is not simply a result of the provision of excess cholesterol substrate, since addition of cholesterol to the membranes *in vitro* does not reproduce the stimulation observed when acetyl-LDL or cholesterol is incubated with intact cells *in vivo* (8). In fibroblasts the stimulation of ACAT activity is not blocked by cycloheximide, indicating that LDL-derived cholesterol does not induce synthesis of new enzyme molecules, but rather activates pre-existing ACAT (29). Experiments with cycloheximide are not yet reported in macrophages.

Stimulation of ACAT in macrophages requires lysosomal hydrolysis of the acetyl-LDL-derived cholesteryl esters. If the acetyl-LDL is allowed to accumulate intact in lysosomes in the presence of chloroquine, no stimulation of ACAT activity occurs (6–8). However, if the cells are then washed free of extracellular acetyl-LDL and of chloroquine, the acetyl-LDL that has accumulated in lysosomes is hydrolyzed and the liberated cholesterol stimulates the ACAT reaction (6, 7). Similar effects of chloroquine on cholesteryl ester metabolism were obtained in macrophages incubated with β -VLDL (6) and human aortic extracts prepared from atherosclerotic plaques (7).

The cholesteryl esters that are synthesized by the ACAT enzyme accumulate in the cytoplasm of the cell as cholesteryl ester droplets. By electron microscopy, the cytoplasmic cholesteryl esters are not surrounded by a typical bilaminar membrane, but rather appear as discrete lipid droplets (8).

Figure 3 shows a polarized light micrograph of macrophages that were incubated with acetyl-LDL. At low power, the cytoplasm of the cells is packed with birefringent crystals of cholesteryl ester. The morphologic appearance of these cells, which were produced in culture dishes *in vitro*, is remarkably similar to that of macrophage foam cells that occur *in vivo* in atherosclerotic plaques.

The Cholesteryl Ester Cycle

Figure 4 shows a two-compartment model proposed by Brown, Ho & Goldstein (9) to account for the metabolism of cholesteryl esters in mouse peritoneal macrophages. Lipoprotein-bound cholesteryl esters that enter the macrophage via receptor-mediated endocytosis are delivered to lysosomes (first cellular compartment), where the cholesteryl esters are hydrolyzed. The liberated cholesterol leaves the lysosomes and enters the cytoplasm (second cellular compartment), where it has two fates. Some of the cholesterol is immediately excreted. The remainder of the excess cholesterol is re-esterified by the ACAT enzyme and accumulates in the cytoplasm as cholesteryl ester droplets. Although these droplets appear inert in the light and electron microscope, their cholesteryl esters are in a dynamic



Figure 3 Polarized light microscopy of macrophages incubated with acetyl-LDL. Monolayers of mouse peritoneal macrophages were incubated for 3 days with 25 μg protein/ml of acetyl-LDL (8). The cytoplasm is packed with birefringent crystals of cholesteryl ester. *Magnification:* X 1000. Richard G. W. Anderson kindly provided this photograph.

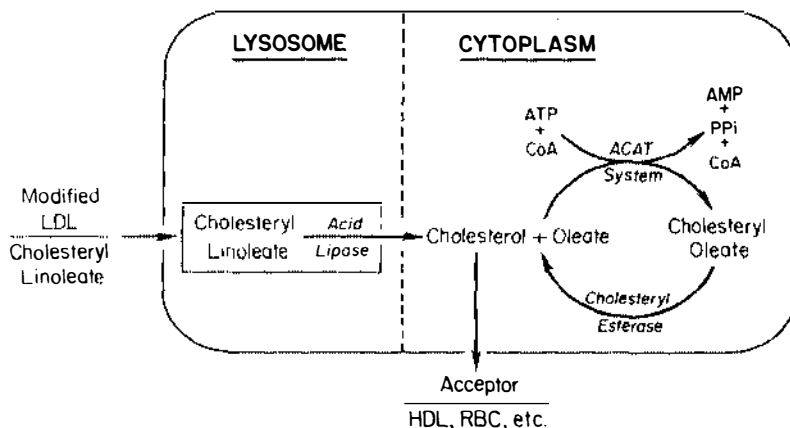


Figure 4 Two-compartment model for cholesteryl ester metabolism in macrophages, illustrating the cytoplasmic cholesteryl ester cycle. The salient features of the model are discussed in the text. RBC, red blood cells. Reproduced with permission from the *Journal of Biological Chemistry* (9).

state, continually undergoing a cycle of hydrolysis and re-esterification, which has been termed the cholesteryl ester cycle.

The cholesteryl ester cycle was demonstrated in several types of double-label experiments (9). Macrophages were incubated with acetyl-LDL in the presence of [^3H]oleate, so that the cells accumulated cytoplasmic cholesteryl ester droplets containing cholesteryl [^3H]oleate. The cells were then placed in medium free of acetyl-LDL and containing [^{14}C]oleate in place of [^3H]oleate. Under these conditions the cellular content of cholesteryl [^3H]oleate declined steadily, falling by about 50% in 24 hr. This loss in cholesteryl [^3H]oleate was balanced almost exactly by an increase in the content of cholesteryl [^{14}C]oleate. Thus, although the total cholesteryl oleate content of the cells did not change significantly, there was a continual hydrolysis of the cholesteryl [^3H]oleate and re-esterification of the cholesterol with [^{14}C]oleate (9).

Hydrolysis of the cytoplasmic cholesteryl [^3H]oleate was shown to be mediated by an enzyme distinct from the lysosomal acid lipase (9). Macrophages were induced to form radiolabeled cytoplasmic cholesteryl esters by incubation with *r*-[^3H -cholesteryl linoleate]acetyl-LDL. After hydrolysis and re-esterification, the [^3H]cholesterol accumulated in the cytoplasm as [^3H]cholesteryl oleate. The acetyl-LDL was then withdrawn from the medium and the cells were further incubated in the absence or presence of chloroquine and in the presence of HDL. In the absence of chloroquine, the cytoplasmic [^3H]cholesteryl esters were hydrolyzed, and the [^3H]cholesterol was excreted from the cell. In the presence of chloroquine, hydrolysis and excretion were unaffected.

If the culture medium does not contain HDL or another acceptor for cholesterol, the free cholesterol released from the hydrolysis of cytoplasmic cholesteryl esters is re-esterified, completing the cholesteryl ester cycle (Figure 4). The re-esterification reaction appears to be catalyzed by the same microsomal ACAT enzyme that esterifies the lipoprotein-derived cholesteryl esters. This conclusion is based on experiments with progesterone, a known inhibitor of the microsomal ACAT reaction (80). These experiments employed a double-labeled protocol (9). Macrophages were incubated with acetyl-LDL in the presence of [^3H]oleate, so that they formed cytoplasmic cholesteryl esters containing cholesteryl [^3H]oleate. They were then switched to medium containing [^{14}C]oleate. In the absence of progesterone, the cholesteryl [^3H]oleate esters were hydrolyzed, and cholesteryl [^{14}C]oleate esters were synthesized, with no change in the total amount of cholesteryl ester. In the presence of progesterone, hydrolysis of the cholesteryl [^3H]oleate proceeded as before. However, progesterone prevented the re-esterification with [^{14}C]oleate. As a result, cholesteryl [^{14}C]oleate did not accumulate, and the total amount of cellular cholesteryl esters fell (9).

SECRETION OF CHOLESTEROL AND APOPROTEIN E BY MACROPHAGES

Cholesterol Excretion Dependent on Cholesterol Acceptors

Macrophages that have stored cytoplasmic cholesteryl esters after incubation with acetyl-LDL cannot secrete this cholesterol when incubated in serum-free medium. In the absence of serum, the cholesteryl esters are continually hydrolyzed and the cholesterol is re-esterified, as mentioned above. This cholesteryl ester cycle is interrupted in a striking fashion when the culture medium contains serum or other substances that are capable of binding cholesterol (9). Werb & Cohn (75, 76) originally showed that an acceptor molecule is required for the excretion of cholesterol by macrophages. They studied cells that were cholesterol-loaded by incubation with albumin/cholesteryl ester complexes. While a variety of cholesterol acceptors can perform this removal function, the most detailed studies were carried out with human HDL₃ with a density of 1.125–1.215 g/ml (9, 78).

Direct demonstration of HDL's action in promoting cholesterol excretion from macrophages has come from mass measurements of the free and esterified cholesterol content of cells that had been loaded with cholesterol by incubation with acetyl-LDL. Ho et al (78) showed that HDL produced a marked decline in the cellular content of esterified cholesterol, the level falling from 250 to 50 μg sterol/mg cellular protein within 24 hr, while the free cholesterol content dropped only slightly, from 30 to 20 μg sterol/mg cellular protein. The cholesteryl ester content of the cells decreased progressively with increasing HDL concentrations until it reached a minimum at

an HDL concentration of 100–200 μg protein/ml. Although the bulk of the HDL-mediated decline was attributable to a decline in esterified cholesterol, all of the cholesterol that was excreted into the culture medium was in the free (i.e. nonesterified) form (78). This conclusion was based on experiments in which macrophages were incubated with *r*-[^3H -cholesteryl linoleate]acetyl-LDL so that the cytoplasmic cholesteryl esters were radiolabeled. The subsequent addition of HDL led to a marked decline in the cellular content of [^3H]cholesteryl esters, yet all of this sterol was recovered in the medium as nonesterified [^3H]cholesterol (9, 78).

The above findings suggest that macrophages are unable to excrete cholesteryl esters and therefore excrete only free cholesterol. As discussed above, the cell's cholesteryl esters are continually undergoing hydrolysis and re-esterification in a cholesteryl ester cycle. HDL could increase the generation of free cholesterol by one of two means: (a) it could increase the rate of hydrolysis of cholesteryl esters; or (b) it could prevent the re-esterification reaction (Figure 4). These possibilities were distinguished in a series of double-label experiments (9). Macrophages were incubated with acetyl-LDL in the presence of [^3H]oleate so that they accumulated cytoplasmic cholesteryl [^3H]oleate. After loading, the [^3H]oleate was replaced with an equal concentration of [^{14}C]oleate. As described above, when this second incubation was performed in the absence of HDL, a decline in cholesteryl [^3H]oleate was balanced by an increase in cholesteryl [^{14}C]oleate, indicating that hydrolysis and re-esterification were occurring at equal rates. When HDL was present in the medium, the cellular content of cholesteryl [^3H]oleate declined at the same rate, but [^{14}C]oleate was no longer incorporated into cholesteryl [^{14}C]oleate. This experiment demonstrated that HDL causes a net hydrolysis of cholesteryl esters by preventing the re-esterification reaction and not by stimulating the hydrolysis reaction. Consistent with this interpretation was an experiment in which macrophages were incubated with acetyl-LDL and then further incubated in medium with or without HDL. The cells were harvested for measurement of *in vitro* ACAT activity. The cells incubated with HDL had a 60% reduction in the activity of the cholesterol-esterifying enzyme (9).

The mechanism by which HDL blocks cholesterol re-esterification is not known precisely, but it must be related to the known ability of HDL to remove cholesterol from cell membranes (81–83). It seems likely that HDL removes free cholesterol from the macrophage plasma membrane and this depletion allows the membrane to bind the cholesterol that is generated within the cytoplasm from the hydrolysis of cholesteryl esters. The shift of this hydrolyzed cholesterol from the cytoplasm to the cell membrane would prevent its re-esterification. After binding to the plasma membrane, the cholesterol would be removed by HDL and this would facilitate a continu-

ous net hydrolysis and excretion of cholesterol (Figure 4). When no cholesterol acceptor is available, the plasma membrane becomes saturated with cholesterol. The cytoplasmic cholesterol cannot enter the plasma membrane and therefore it is re-esterified by the ACAT enzyme.

In the absence of a cholesterol acceptor, about 50% of the stored cholesteryl esters are hydrolyzed and re-esterified each day in a type of futile cycle (9). Inasmuch as the ACAT enzyme uses a fatty acyl-CoA derivative that requires ATP for its synthesis, each turn of the cholesteryl ester cycle has the net effect of breaking down one molecule of ATP to AMP and pyrophosphate (Figure 4).

HDL is an effective acceptor for membrane cholesterol because its outer shell contains a low ratio of cholesterol to phospholipid as compared with most cell membranes (10). The cholesterol:phospholipid ratio is kept low in vivo by the combined action of the plasma enzyme lecithin:cholesterol acyl-CoA transferase (LCAT), which esterifies the surface cholesterol of HDL and initiates its transfer into the core of HDL (10), and of a plasma cholesteryl ester transfer protein, which promotes the transfer of cholesteryl esters from HDL to other plasma lipoproteins (85). Under the conditions of the macrophage experiments in vitro, HDL can accept cholesterol from cell membranes without a requirement for LCAT (9, 78). Presumably if the incubations were continued long enough in the absence of LCAT, the surface of HDL would become saturated with free cholesterol and such HDL would cease to be an effective cholesterol acceptor. In vitro, HDL has not been shown to become saturated with cholesterol, perhaps because the amount of HDL present in the medium is large relative to the amount of cholesterol that is excreted. Large amounts of HDL are used because the experiments were designed to maximize the rate of cholesterol removal.

In addition to HDL, other substances can remove cholesterol from cholesterol-loaded macrophages. For example, the lipoprotein-deficient fraction of serum (density > 1.215 g/ml) is quite effective (78). The two major proteins of this fraction, albumin and gamma globulin, have no cholesterol-removing activity when tested as isolated proteins (78). It is possible that the cholesterol-removing activity of lipoprotein-deficient serum resides in trace amounts of the apoproteins of plasma lipoproteins, such as apo A-I, which are known to be present in that fraction (84). High concentrations of casein or thyroglobulin (i.e. above 1 mg/ml) can also remove cholesterol from cholesterol-overloaded macrophages (78). The activity of these two proteins persists after lipid extraction, suggesting that the protein component itself, and not attached phospholipid, is responsible. Presumably these proteins have a small ability to bind cholesterol that is sufficient to promote cholesterol excretion when the proteins are present at high concentrations. Human erythrocytes have an exceptionally potent ability to remove chole-

terol from cholesterol-overloaded macrophages (78). Five μl of packed washed erythrocytes per ml of culture medium were as effective as maximal concentrations of whole serum or HDL.

At least two lipophilic agents do not appear to remove cholesterol from cholesterol-overloaded macrophages. One is human LDL and the other is a liposome preparation composed of egg phosphatidylcholine with or without sphingomyelin (78). LDL has a much higher ratio of cholesterol to phospholipid than does HDL, and this presumably accounts for its inability to remove large amounts of cholesterol from cells. The inability of phospholipid liposomes to facilitate removal is more difficult to understand. Such liposomes bind cholesterol, and in other cell systems they are effective in cholesterol removal (86). The failure of the liposomes in the macrophage experiments may be due to the sensitivity of these cells to the toxic effects of phosphatidylcholine. Concentrations of phosphatidylcholine above 200 $\mu\text{g/ml}$ were not tolerated by the macrophages and hence could not be tested (78).

Macrophages that have been loaded with cholesteryl ester by incubation with β -VLDL have the same requirements for cholesterol excretion as do the cells loaded with acetyl-LDL. Thus, HDL reduces the synthesis of cholesteryl esters and lessens their accumulation in cells incubated with β -VLDL (65).

Synthesis and Secretion of Apoprotein E in Response to Cholesterol Loading

Overloading macrophages with cholesterol stimulates the cells to synthesize and secrete large amounts of apo E, a normal constituent of plasma lipoproteins. This surprising observation was made initially by Basu et al (13, 14), who incubated mouse peritoneal macrophages with acetyl-LDL and then pulse-labeled the cells with [^{35}S]methionine. The cholesterol-loaded cells secreted a protein that floated in the ultracentrifuge at a density $< 1.215 \text{ g/ml}$, indicating that it was complexed with lipid. The molecular weight of this protein (35,000) and its isoelectric point corresponded to that of authentic mouse plasma apo E. Moreover, the ^{35}S -labeled apoprotein was precipitated by an antibody directed against apo E (13, 14). In the absence of cholesterol loading, macrophages synthesized and secreted small amounts of apo E. Secretion was stimulated 24-fold when the cells were subjected to prior incubation with acetyl-LDL or other sources of cholesterol (14). Replacement of the cholesteryl esters of acetyl-LDL with triglycerides abolished the ability of these lipoproteins to stimulate apo E secretion (14). Under conditions of maximal cholesterol loading, the synthesized apo E represented more than 10% of the total protein secreted by macrophages and more than 2% of the total protein synthesized (13, 14).

The identity of macrophage apo E was confirmed by genetic studies performed with human monocytes (14). These studies took advantage of the known genetic polymorphism of apo E in humans. The apo E locus in humans comprises three common alleles, apo ϵ^2 , ϵ^3 , and ϵ^4 , whose products (E-2, E-3, and E-4) are distinguishable by isoelectric focussing (87, 88). The apo E secreted by human monocytes contained more sialic acid residues than the corresponding plasma apo E and therefore exhibited a more acidic isoelectric point on two-dimensional gels (14). However, a correspondence between monocyte and plasma apo E became apparent when the proteins from both sources were treated with neuraminidase to remove the charge heterogeneity created by sialic acid. After such neuraminidase treatment, it could be shown that blood monocytes from subjects homozygous for the ϵ^3 allele produced a protein that corresponded in size and isoelectric point to the E-3 protein isolated from the plasma of the same individual (14). Similarly, blood monocytes from an individual heterozygous for the ϵ^2 and ϵ^3 alleles synthesized and secreted proteins corresponding to both E-2 and E-3 (14).

Electron microscopic studies with negative staining techniques revealed that the macrophage apo E was secreted in the form of disc-like structures that measured 180 Å in diameter and 30 Å in width (14). These discs resembled the phospholipid/protein discs known to be secreted by perfused rat liver and designated as "nascent HDL" (89). Nascent HDL contains apo A-I as well as apo E (89), but the macrophage discs contain only apo E (14). Nascent HDL consists of a lamellar bilayer of phospholipid. The proteins are thought to circumscribe the edges of the discs, thereby stabilizing the structure (90). In plasma, these lamellar discs are converted into spherical pseudomicellar particles by the insertion of cholesteryl ester between the lamellae (91). The esters are synthesized by the plasma enzyme LCAT, which transfers a fatty acid from the 2-position of lecithin to the 3-hydroxyl position of cholesterol. The free cholesterol substrate for the LCAT reaction can come from the cholesterol on the surface of the nascent HDL particle or it can come from free cholesterol extracted from plasma membranes or from other lipoproteins (10).

The apo E/phospholipid discs secreted by macrophages do not carry large amounts of cholesterol out of the cell. As described above, cholesterol-loaded macrophages secrete large amounts of cholesterol only when an exogenous cholesterol acceptor, such as HDL, is added to the culture medium. Yet the cells synthesize and secrete nearly as much apo E in the absence of HDL as in the presence of HDL (13). In the absence of HDL, the apo E/phospholipid discs are secreted, but there is little secretion of cholesterol (13, 78), indicating that the discs do not carry significant amounts of cholesterol out of the cell. The converse is also true. Cholesterol

excretion occurs even when secretion of apo E is blocked by incubation of the cells with monensin (91a), an ionophore that prevents the movement of secreted proteins from the Golgi apparatus to the plasma membrane (92). Thus, the fundamental mechanisms for secretion of apo E and cholesterol are different.

The above results suggest that secretion of apo E is neither necessary nor sufficient to mediate the excretion of large amounts of cholesterol by macrophages. The apo E must perform some other function. To integrate this information, Basu et al (14) proposed the model shown in Figure 5. In this model, cholesterol leaves the macrophages by binding to the surface of HDL. Apo E is secreted by the cells through an independent mechanism in the form of phospholipid discs. In the presence of plasma LCAT, cholesterol adsorbed to HDL is esterified. During this process, apo E may transfer from the disc to the HDL particle; alternatively, the cholesteryl esters and apo A-I of HDL may transfer to the core and surface of the apo E/phospholipid discs, respectively. By either mechanism, a large spherical particle would be formed that contains a core of cholesteryl esters and a coat containing apoproteins A-I and E (Figure 5).

Mahley (59) described a lipoprotein particle fitting the above description. This lipoprotein, termed HDL_c, is present in small amounts in plasma of normal man and most animals. Its concentration rises markedly after cholesterol feeding, especially in dogs, rabbits, and swine (59). HDL_c contains a core of cholesteryl ester and a coat of apo A-I and apo E. When administered intravenously to normal animals, HDL_c is rapidly taken up by hepatocytes through receptor-mediated endocytosis that is triggered by receptors that bind apo E (12, 59). This uptake suggests that the function of

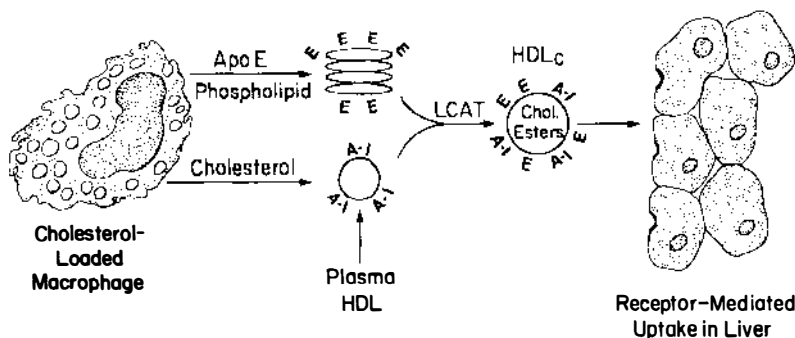


Figure 5 Working model for the role of apo E secretion in mediating "reverse cholesterol transport," i.e. the transport of cholesterol from cholesterol-loaded macrophages to the liver. The salient features of the model are discussed in the text. Adapted with permission from the *Journal of Biological Chemistry* (14).

HDL_c is to transport cholesterol to the liver. However, the origin of plasma HDL_c is unknown.

Since cholesterol-loaded macrophages secrete both apo E/phospholipid discs and cholesterol in the presence of HDL, it is attractive to speculate that these secreted products are assembled extracellularly into HDL_c, as depicted in Figure 5. The HDL_c would then carry the excreted cholesterol to the liver, where receptor-mediated uptake would occur by virtue of the apo E component. Inasmuch as the actual formation of HDL_c has not been demonstrated in macrophage cultures, the model in Figure 5 must be considered hypothetical. Nevertheless, the model has heuristic value in that it unites many of the ideas and observations of Glomset & Norum (10) on the role of LCAT in plasma cholesteryl ester formation, the findings of Mahley, Innerarity, and co-workers (12, 93) on the role of HDL_c in delivering cholesterol to the liver, and the work of Brown, Goldstein and co-workers (4, 11, 14) on cholesterol metabolism in macrophages. Additional work will be necessary to determine whether the assembly of HDL_c is accomplished in the manner suggested in Figure 5.

IMPLICATIONS FOR FOAM CELL FORMATION IN ATHEROSCLEROSIS

The *in vitro* studies of isolated macrophages disclosed many new properties of these scavenger cells relevant to cholesterol metabolism, and raised new questions about the role of macrophages in atherosclerosis. The studies demonstrated that macrophages are specifically adapted for internalizing, storing, and secreting large amounts of lipoprotein-cholesterol. Several types of lipoprotein receptors were identified on the surface of macrophages, and a two-compartment pathway for the storage of cytoplasmic cholesteryl ester droplets was defined. The extraordinary ability of the cholesterol-loaded macrophage to excrete large amounts of cholesterol and apo E was also disclosed.

The evidence that these mechanisms function *in vivo* is so far indirect. When plasma lipoprotein levels rise, macrophages of the artery wall accumulate cytoplasmic cholesteryl ester droplets (1, 2) that are morphologically identical to those of foam cells created *in vitro* (8). Such cholesteryl ester droplets are rich in cholesteryl oleate (8, 94, 95), indicating that they represent re-esterified cholesterol and suggesting that the two-compartment model shown in Figure 4 operates *in vivo*. Moreover, the foam cells of atherosclerotic plaques in experimental animals (95–97) and humans (94, 98) were shown to incorporate [¹⁴C]oleate into cholesteryl [¹⁴C]oleate at a high rate. Again, this finding is explained by the two-compartment model delineated in the *in vitro* macrophage studies.

There is also growing evidence to indicate that macrophages *in vivo* express the same types of lipoprotein receptors that were demonstrated *in vitro*. Foam cells from atherosclerotic plaques of cholesterol-fed rabbits were recently shown to contain active acetyl-LDL receptors and β -VLDL receptors (99). Direct demonstration that these and other receptors function in the uptake of plasma lipoprotein-cholesterol in the arterial wall will require treatment of animals with specific inhibitors that prevent this uptake and thereby prevent foam cell formation *in vivo*.

The plasma concentration of apo E rises in cholesterol-fed animals, and apo E-containing lipoproteins, such as HDL_c, appear in the plasma (59). Even though cholesterol-loaded macrophages can synthesize apo E *in vitro* (13, 14), one cannot yet conclude that these cells are an important source for plasma HDL_c *in vivo*. In this regard, it would be instructive to measure the synthesis of apo E in explants of aortas from atherosclerotic animals and humans.

The finding that HDL facilitates cholesterol excretion by macrophages (9, 78) may be relevant to the epidemiologic observation that high levels of plasma HDL are correlated with a reduced frequency of atherosclerotic complications in man (100). The excretion of cholesterol by macrophages in the artery wall *in vivo* may be limited by the availability of HDL. The concentration of HDL in the arterial wall is unknown, as is the route by which it enters and leaves. Whether an increase in the plasma level of HDL would lead to a higher arterial level of HDL and whether this would speed the removal of cholesterol from macrophages is a question that seems worthy of study.

The scavenging of lipoprotein-cholesterol by macrophages appears to be a protective mechanism that functions to rid the interstitial space of excessive lipoproteins. By this formulation, foam cell formation in atherosclerosis would result when this protective mechanism becomes overwhelmed, either because the amount of plasma lipoprotein-cholesterol that enters the arterial wall is too great for the macrophages to process, or because the ability of the macrophages to excrete cholesterol becomes limited. Entry of lipoproteins into the arterial wall could be controlled by: (a) lowering the lipoprotein level in plasma; or (b) improving the integrity of the endothelium. Excretion of cholesterol from macrophages might be enhanced by: (a) increasing the concentration of HDL in plasma (and presumably its concentration in the arterial wall); or (b) improving the ability of HDL to act as a cholesterol acceptor by increasing the efficiency of the LCAT and cholesteryl ester transfer protein reactions that lower the cholesterol content of HDL.

The question arises as to whether macrophages make any contribution to the pathogenesis of atherosclerosis other than by scavenging cholesterol.

Macrophages produce factors that stimulate the growth of smooth muscle cells, which form a major part of the bulk of the atherosclerotic plaque (3, 101). Macrophages also synthesize and secrete lipoprotein lipase (31, 32), which might liberate toxic fatty acids and triglycerides from plasma lipoproteins locally within the artery wall. Macrophages also secrete a host of other biologically active molecules, such as prostaglandins and proteases (102). The influence of macrophage cholesterol accumulation on all of these secretory events needs to be explored. Maleylated albumin, which enters macrophages through the acetyl-LDL receptor (4), stimulates the secretion of several proteases (51). If lipoproteins act similarly, then they might trigger a vicious cycle in vivo in which lipoprotein entry activates macrophages to secrete factors that lead to additional damage to the artery wall, which in turn leads to additional lipoprotein entry, etc.

The Foam Cell in Familial Hypercholesterolemia

The studies of lipoprotein metabolism in macrophages were initiated to explain the paradoxical finding that familial hypercholesterolemia (FH) patients whose cells lack receptors for LDL can nevertheless accumulate lipoprotein-derived cholesteryl esters in macrophages (4). The studies revealed a variety of receptors that might mediate the macrophage uptake of lipoprotein cholesterol in these patients. As mentioned above, only one of these receptors operates on a naturally occurring lipoprotein and that is the receptor for β -VLDL (6, 58). Recent studies of FH in man and in rabbits have begun to provide evidence that particles resembling β -VLDL are present in the circulation of affected individuals and that these particles may be an important source of macrophage cholesteryl esters.

The rabbit studies have dealt with a strain known as Watanabe Heritable Hyperlipidemic (WHHL) rabbits (103). These rabbits have a mutation in the gene for the LDL receptor that is analogous to the mutation in human FH. When present in the homozygous form, this mutation leads to a near complete deficiency of LDL receptors in tissues such as liver, adrenal, and cultured fibroblasts (104–106). As a result of this deficiency, LDL is removed slowly from the circulation and accumulates to massive levels in plasma (107). In these respects the homozygous WHHL rabbits resemble humans with homozygous FH (16).

In addition to binding LDL, the LDL receptor is known to bind particles such as β -VLDL and intermediate density lipoproteins (IDL) that contain apo E as well as apo B (11). IDL particles are remnant lipoproteins formed during the metabolism of VLDL in man and animals on normal diets. In normal rabbits IDL are rapidly removed from the circulation in the liver, apparently by binding to LDL receptors (108). When ^{125}I -labeled VLDL is injected into the circulation of homozygous WHHL rabbits, the VLDL is

converted to IDL by lipoprotein lipase, but the IDL is not removed normally from the circulation (108). Hence, these mutant rabbits accumulate cholesterol-rich particles in the VLDL and IDL density classes as well as in LDL (109). Similar findings were made in studies of the turnover of ^{125}I -VLDL in humans with homozygous FH (110). The VLDL and IDL that accumulate in WHHL plasma are similar, though not identical, to the β -VLDL particles that accumulate in cholesterol-fed animals (109), especially to the Fraction II subfraction of these particles (66).

The cholesterol-rich VLDL and IDL particles from WHHL rabbits bind to a receptor on macrophages that appears to be the same as the β -VLDL receptor. As a result of this binding, VLDL and IDL particles from WHHL rabbits stimulate cholesteryl oleate synthesis and storage (Table 3).

The above data raise the possibility that cholesterol-rich VLDL and IDL particles, in addition to modified LDL, may constitute major sources of cholesterol in the atherosclerotic foam cells of FH homozygotes and perhaps of heterozygotes as well (although elevated IDL levels have not been well documented in heterozygotes). Zilversmit (111) proposed that IDL particles and other forms of remnant lipoproteins are the primary cause of atherosclerosis in cholesterol-fed animals; these lipoproteins may contribute to the atherosclerosis in FH as well.

Table 3 Stimulation of cholesteryl ester formation in mouse peritoneal macrophages by lipoproteins from normal and WHHL rabbits^a

Source of lipoproteins	Lipoprotein fraction added to medium	Concentration in medium		[^{14}C]oleate \rightarrow cholesteryl [^{14}C]oleate (nmol/mg protein)
		Protein ($\mu\text{g/ml}$)	Cholesterol ($\mu\text{g/ml}$)	
—	None	0	0	0.26
Normal rabbit (2% cholesterol diet)	β -VLDL (d < 1.006)	5	50	16.5
		30	300	61.3
		360	300	0.30
Normal rabbit (chow diet)	VLDL (d < 1.006)	60	50	0.24
	IDL (d 1.006–1.019)	40	50	2.4
		240	300	8.9
	LDL (d 1.019–1.063)	40	50	0.30
		240	300	0.90
WHHL rabbit (chow diet)	VLDL (d < 1.006)	14	50	15.0
		84	300	25.4
	IDL (d 1.006–1.019)	20	50	3.7
		120	300	12.6
	LDL (d 1.019–1.063)	40	50	0.15
		240	300	0.68

^a Each monolayer of mouse peritoneal macrophages received 0.6 ml medium containing 0.2 mM [^{14}C]oleate bound to albumin and the indicated concentration of the indicated lipoprotein fraction. After incubation for 7.5 hr at 37°C, the cellular content of cholesteryl [^{14}C]oleate was determined by thin layer chromatography (8). The addition of either polyinosinic acid (30 $\mu\text{g/ml}$) or fucoidin (100 $\mu\text{g/ml}$) did not inhibit the formation of cholesteryl [^{14}C]oleate in these experiments.

Among FH patients (both heterozygotes and homozygotes), there is considerable variation in the rate of progression of atherosclerosis, despite uniformly elevated LDL levels. The suggestion was made that those FH heterozygotes who have low HDL levels are more susceptible to atherosclerosis than those who have higher HDL levels (112, 113). VLDL, the precursor of IDL, is known to vary inversely with HDL levels. FH heterozygotes with low HDL levels may also have high IDL levels and the high IDL level may be the aggravating factor in atherosclerosis rather than the low HDL level.

The studies of macrophage lipoprotein metabolism have raised many questions concerning the role of lipoproteins in atherosclerosis. Further studies should throw new light on the biochemical mechanisms responsible for foam cell formation.

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