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Macrophage Atherosclerosis WOW

LIPOPROTEIN METABOLISM **IN THE MACROPHAGE:** Implications for Cholesterol Deposition in 2 Years bitos, discovery of Aldihyde Modifii) Proten's site Atherosclerosis¹

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¹Abbreviations used: ACAT, acyl-CoA : cholesterol acyltransferase; apo, apoprotein; FH, familial hypercholesterolemia; HDL, high density lipoprotein; HDL, 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; B-VLDL, B-migrating very low density lipoproteins; WHHL rabbit, Watanabe Heritable Hyperlipidemic rabbit.

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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 <u>receptor-mediated endocytosis</u> (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, lipoproteinbound 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 [¹⁴C]oleate is added to the culture medium, the cells do not incorporate it into cholesteryl [¹⁴C]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 [¹⁴C]oleate into cholesteryl [¹⁴C]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 [¹⁴C]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 ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-acetyl-LDL is hydrolyzed and the label is excreted from the cell in the form of ¹²⁵I-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 ¹²⁵I-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 ¹²⁵I-acetyl-LDL is achieved at an acetyl-LDL concentration of 5 μ g pro-

²Although small amounts of ¹²⁵I-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 ¹²⁵I-LDI. 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 μ g 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 ¹²⁵I-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 ¹²⁵I-acetyl-LDL and ¹²⁵I-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, 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 ¹²⁵I-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 ¹²⁵I-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.

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

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

⁴Each monolayer of mouse peritoneal macrophages received 0.6 ml medium containing 0.2 mM [¹⁴C]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 [¹⁴C]oleate was determined by thin layer chromatography (8). The addition of either polyinosinic acid (30 µg/ml) or fucoidin (100 µg/ml) did not inhibit the formation of cholesteryl [¹⁴C]oleate in these experiments.

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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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