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The Scavenger Cell Pathway for Lipoprotein Degradation: Specificity of the Binding Site That Mediates the Uptake of Negatively-Charged LDL by Macrophages

Michael S. Brown, Sandip K. Basu, J.R. Falck, Y.K. Ho, and Joseph L. Goldstein

Departments of Molecular Genetics and Internal Medicine, University of Texas Health Science Center, Dallas, Texas 75235

Macrophages isolated from a variety of organs in several animal species exhibit high affinity binding sites that recognize chemically modified proteins. One of these binding sites recognizes human plasma low density lipoprotein (LDL) in which the positive charges on the epsilon-amino groups of lysine have been removed or neutralized by chemical modification, thus giving the protein an enhanced negative charge. Effective treatments include reaction of LDL with organic acid anhydrides (acetylation or maleylation) and reaction with aldehydes, such as treatment with malondialdehyde. After the negatively-charged LDL binds to the surface receptor sites, it is rapidly internalized by the macrophages by endocytosis and hydrolyzed in lysosomes. The liberated cholesterol is re-esterified in the cytoplasm, producing massive cholesteryl ester deposition. The binding site for negatively-charged LDL has been demonstrated so far only on macrophages and other scavenger cells. It is not expressed in cultured fibroblasts, smooth muscle cells, lymphocytes, or adrenal cells. In addition to its affinity for acetylated LDL and malondialdehyde-treated LDL, the macrophage site binds a variety of polyanions. It exhibits a particularly high affinity for certain sulfated polysaccharides (dextran sulfate and fucoidin), certain polynucleotides (polyinosinic acid and polyguanylic acid), polyvinyl sulfate, and maleylated albumin. It is possible that the site that binds negatively-charged LDL may be responsible for the massive accumulation of cholesteryl esters that occurs in vivo in macrophages and other scavenger cells in patients with high levels of circulating plasma LDL.

Key words: **receptor-mediated endocytosis, acetylated LDL, malondialdehyde, polynucleotides, familial hypercholesterolemia, atherosclerosis**

INTRODUCTION

When low density lipoprotein (LDL) accumulates to high levels in human plasma, the lipoprotein's cholesterol is deposited at widespread sites in the body. This deposition is

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particularly striking in tissues of patients with homozygous familial hypercholesterolemia (FH). These patients do not catabolize circulating LDL efficiently because their cells have a genetic deficiency in a surface receptor that normally mediates the uptake and degradation of the lipoprotein. As a result, LDL accumulates to extremely high levels in plasma [1].

In tissues of patients with homozygous FH, LDL-derived cholesterol deposits both inside and outside cells. The most prominent intracellular deposits are found in macrophages and other scavenger cells [2, 3]. These cells become so swollen with cholesteryl ester droplets that they are called "foam cells." Inasmuch as macrophages accumulate massive amounts of LDL-cholesterol in patients who have a genetic inability to produce LDL receptors, we postulated several years ago that macrophages must express a different mechanism for taking up plasma LDL. This postulated mechanism was termed the "scavenger cell pathway" because it occurred primarily in macrophages and other cells whose function was to scavenge and degrade excess extracellular molecules [4].

MACROPHAGE MONOLAYERS AS A MODEL SYSTEM FOR STUDYING THE SCAVENGER CELL PATHWAY

In searching for the mechanism by which scavenger cells accumulate LDL-cholesterol, the initial approach was to establish an in vitro model system that could be studied biochemically [5, 6]. For this purpose, resident macrophages were isolated from the peritoneal cavity of unstimulated mice and were maintained in an active state as monolayers in Petri dishes according to the methods developed by Cohn and co-workers [7]. Our initial experiments showed that the freshly isolated mouse macrophages did not take up or degrade ^{125}I-labeled-LDL with high affinity [5]. As a result, LDL produced no increase in the cholesteryl ester content of macrophages, even when the lipoprotein was added

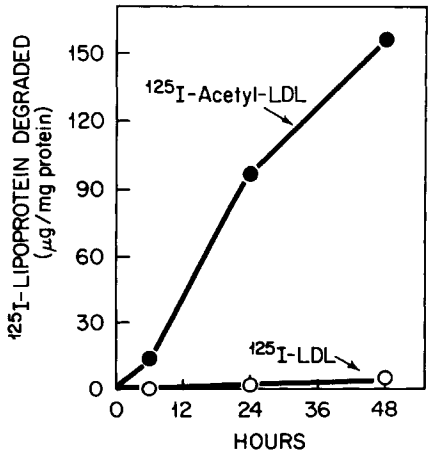

Fig. 1. Degradation of ^{125}I-acetyl-LDL (•) and ^{125}I-LDL (○) by mouse peritoneal macrophages. Macrophage monolayers were prepared as previously described [18]. Each 35-mm dish of approximately 1 × 10^6 adherent cells received 1 ml of medium containing 10% human lipoprotein-deficient serum and 10 μg protein/ml of either ^{125}I-acetyl-LDL (70,000 cpm/μg protein) (•) or ^{125}I-LDL (65,000 cpm/μg protein) (○). After incubation at 37°C for the indicated time, the amount of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material excreted by duplicate dishes of cells was measured [5]. Human LDL was prepared by sequential ultracentrifugation and was radiolabeled with ^{125}I as previously reported [13]. Acetyl-LDL and ^{125}I-labeled acetyl-LDL was prepared as described [13].

at high levels [5, 6]. Clearly, mouse peritoneal macrophages, in contrast to parenchymal and connective tissue cells from mice and other species, express few if any LDL receptors. If LDL were to deposit its cholesterol in macrophages, the lipoprotein would have to be altered in such a manner that it could be recognized by some other macrophage receptor system.

At the time that these macrophage studies were initiated, it was known that macrophages have a propensity to take up and degrade proteins that have been denatured by treatment with nitrosoguanidine or formaldehyde [8–12]. When injected intravenously into mice or rats, these denatured proteins are rapidly cleared from the circulation, appearing in hepatic Kupffer cells [8–10]. Moreover, in vitro studies of rat peritoneal macrophages [11], rat yolk sac [10], and rat Kupffer cells [12] showed rapid uptake and degradation of ^{125}I-labeled formaldehyde-treated albumin.

These experiments suggested that denaturing treatments would enhance LDL uptake by macrophages in vitro. Accordingly, we treated LDL particles with a variety of denaturing agents. Treatment of the lipoprotein with many chemicals, including urea and formaldehyde, failed to convert LDL into a form that was taken up by macrophages. Moreover, other denaturing treatments, such as exposure to acid, repeated freeze-thawing, and extensive heating, were similarly ineffective. One chemical reaction, acetylation, was successful.

RECEPTOR-MEDIATED ENDOCYTOSIS OF ACETYLATED LDL IN MACROPHAGES

In the earlier studies of LDL metabolism in human fibroblasts, we had observed that acetylation of the lysine residues of LDL caused the lipoprotein to lose its ability to bind to the LDL receptor. As a result, fibroblasts and other parenchymal cells failed to degrade ^{125}I- labeled acetyl-LDL with high affinity [13]. In the acetylation reaction, acetyl groups are attached to the lysine residues of LDL, neutralizing the positive charges and increasing the net negative charge of the protein. Since parenchymal and connective tissue cells would no longer recognize the negatively-charged lipoprotein, we next asked whether macrophages would. Would macrophages take up and degrade acetylated LDL? And would this degradation lead to the deposition of cholesteryl esters in these cells?

Figure 1 shows an experiment in which freshly isolated mouse peritoneal macrophages were incubated with ^{125}I-labeled native LDL and ^{125}I-acetyl-LDL. The macrophages degraded the protein component of the ^{125}I-acetyl-LDL completely and released the ^{125}I-label into the culture medium in the form of ^{125}I-monoiodotyrosine and ^{125}I-iodide [5]. Non-acetylated ^{125}I-LDL was taken up and degraded by the macrophages at rates that were 20-fold less than those for ^{125}I-acetyl-LDL (Fig. 1). Consistent with these findings was the observation of Mahley and co-workers, who found that the rate of uptake and degradation of acetoacetylated LDL by canine peritoneal macrophages was 8- to 10-fold higher than that observed for native LDL [14].

The uptake and degradation of acetyl-LDL by macrophages led to a several-hundred-fold increase in the content of esterified cholesterol in the cell (Fig. 2). Values as high as 300 μg of esterified cholesterol per mg of cell protein were routinely reached. On the other hand, the free cholesterol content of the cells rose much more modestly, from 20 to 55 μg per mg of cell protein [6]. Non-acetylated LDL, which was not taken up or degraded with high affinity, did not increase the cellular content of free or esterified cholesterol in macrophages (Fig. 2 and [5, 6]).

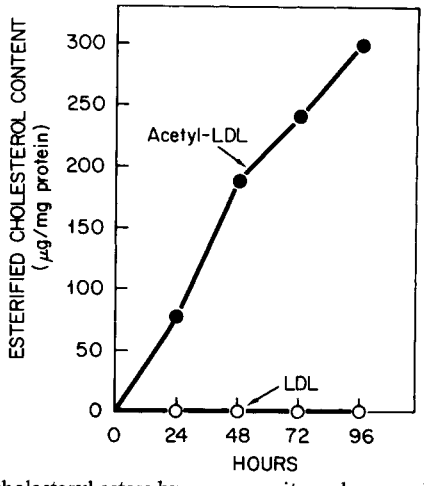

Fig. 2. Accumulation of cholesteryl esters by mouse peritoneal macrophages incubated with acetyl-LDL (•) and LDL (○). Macrophage monolayers were prepared as previously described [6]. Each 60-mm dish of approximately 3 × 10^6 adherent cells received 2 ml of medium containing 10% fetal calf serum and 25 μg protein/ml of either acetyl-LDL (•) or native LDL (○), and the cells were incubated at 37°C. Fresh medium of identical composition was added every 24 hours. At the indicated time, duplicate dishes of cells were harvested for measurement by gas-liquid chromatography of their content of esterified cholesterol [6].

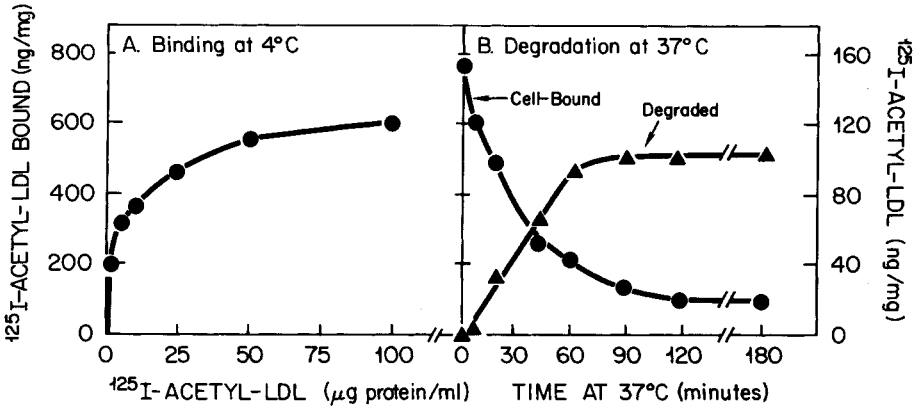

Fig. 3. A) Binding of ^{125}I-acetyl-LDL by mouse peritoneal macrophages at 4°C. Macrophage monolayers were prepared as previously described [5]. Each 35-mm dish of approximately 6 × 10^5 adherent cells received 1 ml of ice-cold medium containing 10% human lipoprotein-deficient serum and the indicated concentration of ^{125}I-acetyl-LDL (734 cpm/ng protein). After incubation for 2 hr at 4°C, the monolayers were washed extensively and the amount of ^{125}I-acetyl-LDL bound to the cells was determined in duplicate dishes [5]. B) Degradation at 37°C of ^{125}I-acetyl-LDL previously bound by mouse peritoneal macrophages at 4°C. Each dish was incubated for 2 hr at 4°C with 10 μg protein/ml of ^{125}I-acetyl-LDL (738 cpm/ng protein) as in Panel A, after which each monolayer was washed extensively [5]. Each dish then received 1 ml of warm medium containing 20 μg protein/ml of unlabeled acetyl-LDL, and all the dishes were incubated at 37°C. At the indicated interval, duplicate dishes were rapidly chilled to 4°C, the medium was removed, and its content of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material (▲) was measured. The total amount of ^{125}I-acetyl-LDL that remained associated with the cell (•) was also determined. (Data reprinted from [5]).

Uptake and degradation of acetyl-LDL was attributable to the binding of the lipoprotein to a specific receptor site on the macrophage surface [5]. Figure 3A shows an experiment in which macrophages were incubated with increasing concentrations of ^{125}I-acetyl-LDL at 4°C. The lipoprotein bound to a finite number of cell surface receptor sites. Half-maximal binding occurred at about 10 μg protein/ml. The number of surface binding sites for ^{125}I-acetyl-LDL was 20,000—40,000 per cell. This number was reduced by 90% when the macrophages were first incubated for 50 min at 37°C with either trypsin (250 μg/ml) or pronase (10 μg/ml) [5], suggesting that the binding site involves a protein molecule.

The fate of the surface-bound ^{125}I-acetyl-LDL was disclosed when the macrophages that had bound the lipoprotein at 4°C were washed and then warmed to 37°C (Fig. 3B). The amount of ^{125}I-acetyl-LDL associated with the cell rapidly declined, and all of the released radioactivity appeared as ^{125}I-labeled trichloroacetic acid-soluble products in the culture medium. The macrophages were quite efficient at internalizing and degrading the receptor-bound ^{125}I-acetyl-LDL; all of the cell-bound material was degraded within 60 minutes.

The receptor-bound ^{125}I-acetyl-LDL that was degraded by the macrophages was released either as ^{125}I-monoiodotyrosine or ^{125}I-iodide. The rapidity of this proteolysis and its completeness suggested that the ^{125}I-acetyl-LDL was internalized by endocytosis and degraded by the full complement of proteolytic enzymes that is contained within lysosomes [5]. This conclusion was further supported by the following additional data: 1) degradation did not occur at 4°C, a temperature at which endocytosis is completely inhibited; 2) at the same time that the protein component of acetyl-LDL was degraded, a stoichiometrically equal amount of lipoprotein-bound cholesteryl ester was hydrolyzed [6]; and 3) in the presence of chloroquine, an inhibitor of lysosomal function, hydrolysis of both the protein and cholesteryl esters was blocked, and intact acetyl-LDL accumulated progressively within cellular lysosomes [5, 6]. This intralysosomal acetyl-LDL was rapidly hydrolyzed when the chloroquine was removed from the medium (data not shown).

Although the protein and cholesteryl esters of acetyl-LDL were both hydrolyzed within the macrophage lysosomes, the fate of the degradation products differed. ^{125}I-Monoiodotyrosine and free ^{125}I-iodide released from the lysosomal hydrolysis of ^{125}I-acetyl-LDL were rapidly excreted into the culture medium. In contrast, when macrophages were incubated with acetyl-LDL in which the cholesteryl esters were labeled with [^{3}H] cholesteryl linoleate, only about 50% of the [^{3}H] cholesterol liberated from lysosomal hydrolysis of the [^{3}H] cholesteryl linoleate was excreted from the cell. The other 50% of this [^{3}H] cholesterol was retained within the cell [6].

Unlike the LDL receptor of fibroblasts and other parenchymal cells, the acetyl-LDL binding site on macrophages is not suppressed when the cellular cholesterol content rises [5, 6, 15]. This inability to suppress receptor activity explains why the macrophages accumulate massive amounts of cholesteryl ester when incubated with acetyl-LDL.

TWO-COMPARTMENT MODEL FOR CHOLESTERYL ESTER METABOLISM

The cholesterol that was retained by the macrophages did not remain in the free form. Rather, it was re-esterified and stored as cholesteryl esters. The synthesis of these cholesteryl esters was correlated with an increase in the activity of a membrane-bound fatty acyl CoA:cholesterol acyltransferase (ACAT) enzyme presumed to be located on the endoplasmic reticulum [6]. The stored cholesteryl esters remained in the cytoplasm. By

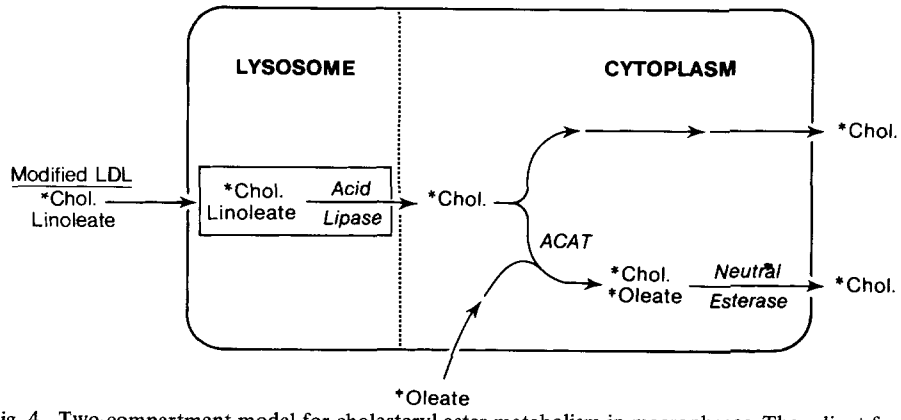

Fig. 4. Two-compartment model for cholesteryl ester metabolism in macrophages. The salient features of the model are discussed in the text. The hydrolysis and re-esterification of cholesteryl esters of modified LDL can be demonstrated experimentally by incubating macrophages with [^{14}C] oleate and acetyl-LDL radiolabeled with [^{3}H] cholesteryl linoleate and showing that the cholesteryl esters that accumulate within the cytoplasm contain [^{3}H] cholesterol and [^{14}C] oleate [6]. Abbreviations: *Chol., [^{3}H] cholesterol; ‡Oleate, [^{14}C] oleate; *chol. ‡Oleate, [^{3}H] cholesteryl [^{14}C] oleate; ACAT, acyl-CoA: cholesterol acyltransferase.

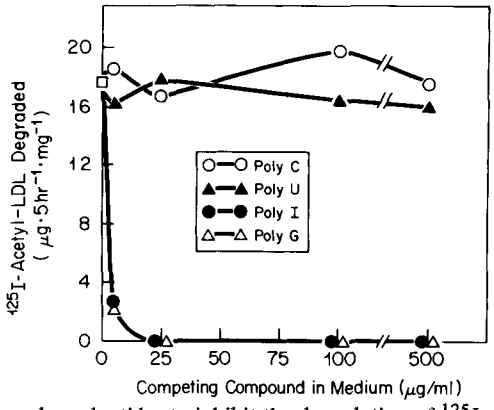

Fig. 5. Ability of various polynucleotides to inhibit the degradation of ^{125}I-acetyl-LDL by mouse peritoneal macrophages. Macrophage monolayers were prepared by the standard procedure described in the legend to Fig. 1. Each 35-mm dish received 1 ml of medium containing 10% human lipoprotein-deficient serum and 25 μg protein/ml of ^{125}I-acetyl-LDL (40,000 cpm/μg protein) and the indicated competing compound. After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material in the medium was determined in duplicate dishes [5]. Polynucleotides (K salt) were obtained from Sigma Chemical Co. The estimated M_r for the polynucleotides was > 100,000.

electron microscopy they were seen to form lipid droplets that were not surrounded by a membrane. These droplets reproduced the characteristic features of the "foam cells" seen in tissues of patients with high plasma LDL levels [6].

The sequential processes of lysosomal hydrolysis of incoming LDL-derived cholesteryl esters followed by microsomal re-esterification of the liberated cholesterol have been termed the two-compartment model for cholesteryl ester metabolism [6, 16]. Figure 4 illustrates the main features of this model. The major cholesteryl ester in LDL is cholesteryl linoleate. After the modified LDL enters the macrophage, its cholesteryl linoleate is delivered to lysosomes where the ester bond is hydrolyzed by the acid lipase enzyme. The liberated cholesterol passes across the lysosomal membrane and enters the cytoplasm where

it has two fates. Some of the cholesterol is excreted by the cell. In the case of peritoneal macrophages, about half of the cholesterol liberated from acetyl-LDL is excreted in this fashion [6]. The other half of the cholesterol interacts with the microsomal ACAT and is re-esterified. When the cells are incubated in the presence of exogenous oleate, the re-esterified cholesterol is stored in the cytoplasm as cholesteryl oleate droplets. If a different fatty acid is supplied to the cells, eg, stearate, the predominant cholesteryl ester formed is cholesteryl stearate.

An important aspect of this storage process is the finding that the stored cholesteryl ester droplets are not sequestered permanently by the macrophages. When the source of incoming cholesterol is removed, these stored cholesteryl esters are rapidly hydrolyzed, possibly by a nonlysosomal neutral esterase, and the free cholesterol is quickly excreted by the macrophages [6, 17]. The hydrolysis of the stored cholesteryl esters and the excretion of the resultant free cholesterol are both absolutely dependent on the presence in the culture medium of a substance that is capable of binding the insoluble cholesterol molecule. A variety of substances can fulfill this binding function, including red blood cells. Human high density lipoprotein (HDL) is particularly effective, whereas LDL is much less effective in removing cholesterol from the overloaded macrophages [17].

PROPERTIES OF THE ACETYL-LDL BINDING SITE

The macrophage binding site that recognizes acetyl-LDL is also capable of binding other macromolecules that, like acetyl-LDL, possess multiple negative charges. The binding site shows selectivity, however, in that only certain polyanions are bound. A striking illustration of this specificity is seen in Figure 5. In this experiment, macrophages were incubated with ^{125}I-acetyl-LDL in the presence of increasing concentrations of four different polynucleotides, each possessing a strong negative charge. The three purine polynucleotides, polyinosinic acid, polyguanylic acid, and polyxanthinylic acid, blocked ^{125}I-acetyl-LDL degradation at concentrations below 25 μg/ml with 50% inhibition occurring at 1.5–3 μg/ml (Table I). Direct binding studies performed with intact cells at 4°C demonstrated that this inhibition was due to competition with ^{125}I-acetyl-LDL for the binding site on the macrophage surface. That these polynucleotides were acting by binding to the cells and not by binding to the ^{125}I-acetyl-LDL was indicated by the finding that prior incubation of cells with either 20 μg/ml of polyinosinic acid or 20 μg/ml of polyguanylic acid at 4°C followed by washing the cells inhibited the subsequent ^{125}I-acetyl-LDL binding (10 μg protein/ml, 60 min, 4°C) by more than 90%.

In contrast to the above three purine polynucleotides, two pyrimidine polynucleotides, polycytidylic acid and polyuridylic acid, failed to block the binding or degradation of ^{125}I-acetyl-LDL at concentrations as high as 500 μg/ml (Fig. 5). The difference between purines and pyrimidines was not absolute, however. For example, another purine polynucleotide, polyadenylic acid, was also ineffective in inhibiting the binding, uptake, and degradation of ^{125}I-acetyl-LDL (Table I).

Considered together, these data suggest that the effectiveness of a polypurine as an inhibitor depends on having a keto group (eg, polyinosinic acid, polyguanylic acid, polyxanthinylic acid) rather than an amino group (polyadenylic acid) at the six position of the purine base. In other experiments, we found that a random copolymer consisting of equal amounts of guanylic acid and inosinic acid (Poly G, I [1:1]) was extremely effective in blocking the degradation of ^{125}I-acetyl-LDL (50% inhibition at 2 μg/ml). On the other hand, the double stranded polynucleotide, polyinosinic acid:polycytidylic acid (Poly I:Poly C) was considerably less effective than polyinosinic acid (Table I).

TABLE I. ^{125}I-Acetyl-LDL Binding Site in Macrophages

Effective competitors		Ineffective competitors
Negatively charged compounds		**Negatively charged compounds**
polyvinyl sulfate	1 μg/ml[a]	polyadenylic acid
polyinosinic acid	1.5	polycytidylic acid
polyxanthinylic acid	3	polyuridylic acid
polyguanylic acid	1.5	heparin
poly G,I (1:1)	2	chondroitin sulfate, A and C
poly I:poly C	50	phosvitin
dextran sulfate	3	colominic acid (polysialic acid)
fucoidin	10	polyphosphates (n = 65)
carragheenan	20	poly (D-glutamic acid)
bovine sulfatides	10	
maleylated LDL	20	**Positively charged compounds**
maleylated albumin	100	lysozyme
		spermine
		Glycoproteins
		mannan (yeast)
		thyroglobulin
		fetuin
		orosomucoid
		asialo-orosomucoid
		Others
		acetylated albumin
		methylated LDL

[a]K_i: these values refer to the concentrations required for 50% inhibition.

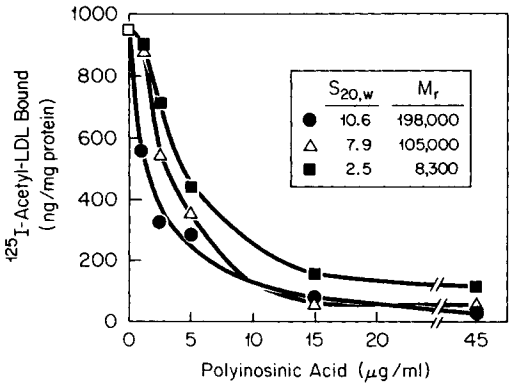

Fig. 6. Inhibition of the binding of ^{125}I- acetyl-LDL by mouse peritoneal macrophages at 4°C: Effect of polyinosinic acid of different molecular weights. Macrophage monolayers were prepared by the standard procedure described in the legend to Fig. 1. Each 35-mm dish of approximately 1×10^6 adherent cells received 1 ml of ice-cold medium containing 10% human lipoprotein-deficient serum, 10 μg protein/ml of ^{125}I-acetyl-LDL (200,000 cpm/μg protein), and the indicated compound. After incubation for 2 hr at 4°C, the monolayers were washed extensively, and the amount of ^{125}I-acetyl-LDL bound to the cells was determined in duplicate dishes [5]. Each preparation of polyinosinic acid was obtained from P.L. Biochemicals. The M_r for each compound was estimated from its $S_{20,w}$ value according to the formula, $M_r = 1100\ S_{20,w}^{2.2}$ [21].

The potency of polyinosinic acid in inhibiting the binding of ^{125}I-acetyl-LDL was independent of the molecular weight within the range from 8,300 to 198,000 (Fig. 6). The specificity of the polyinosinic acid effect on ^{125}I-acetyl-LDL binding was suggested by two control experiments: 1) the polynucleotide failed to block the proteolytic degradation of ^{125}I-LDL/dextran sulfate complexes, which enter macrophages by binding to a site different from the acetyl-LDL binding site [18]; and 2) the polynucleotide failed to block the uptake and degradation of canine β-migrating very low density lipoproteins (β-VLDL), a lipoprotein that binds to a macrophage receptor site distinct from the acetyl-LDL site [15].

In addition to its inhibition by certain polynucleotides, the binding of ^{125}I-acetyl-LDL was also inhibited competitively by polyvinyl sulfate and by a group of polyanionic sulfated polysaccharides, including dextran sulfate (a synthetic glucose-containing polymer), fucoidin (a naturally occurring fucose-containing polymer), and carragheenan (a naturally occurring galactose-containing polymer). On the other hand, other sulfated polysaccharides having less negative charge density, including heparin and chondroitin sulfates A and C, failed to compete for ^{125}I-acetyl-LDL binding ([5] and Table I).

Certain chemically modified proteins with enhanced negative charge also bound to the acetyl-LDL binding site. For example, maleylated albumin competed for the binding and uptake of ^{125}I-acetyl-LDL, whereas acetylated albumin did not [5]. Inasmuch as maleic acid is a dicarboxylic acid, maleylation of each lysine residue adds one negative charge to a protein in addition to removing one positive charge. Apparently, maleylation of albumin is required because the naturally occurring negative charges in this protein are insufficient to achieve binding to the macrophage binding site, even when the positive charges on the lysine are eliminated by acetylation. As expected, methylation of the lysine residues of LDL, which does not change the net charge of the lipoprotein, failed to convert it to a form that is recognized by the acetyl-LDL binding site (Table I).

Of considerable interest is the observation that poly(D-glutamic acid) and polyphosphates (n = 65) do not compete for the ^{125}I-acetyl-LDL binding site [5]. Considered together with the other evidence of specificity discussed above, these data indicate that multiple negative charges are necessary for a substance to bind to the acetyl-LDL binding site, but they are not sufficient. Effective competing compounds must also have some specific configuration of negative charges. The nature of this configuration is not known.

DEGRADATION OF MALONDIALDEHYDE-TREATED LDL IN MACROPHAGES

The finding of a specific macrophage binding site that recognizes lysine-modified, negatively charged LDL has stimulated a search for naturally occuring reactions that might modify the lysines of LDL, removing the positive charge and converting the lipoprotein to a high-uptake form for macrophages. Although the initial studies were performed with acetyl-LDL, acetylation of the lipoprotein in the extracellular fluid does not seem likely to occur.

In addition to reacting with anhydrides of organic acids, the primary amino groups of lysine can also react with aldehydes, leading to the formation of Schiff bases with varying degrees of stability. Fogelman and co-workers first reported that treatment of LDL with malondialdehyde modified the lipoprotein in such a way that it stimulated cholesteryl ester accumulation in human monocytes [19]. From a physiological point of view, the importance of the malondialdehyde reaction derives from the fact that malondialdehyde is produced by platelets as a byproduct of arachidonate metabolism that arises during the

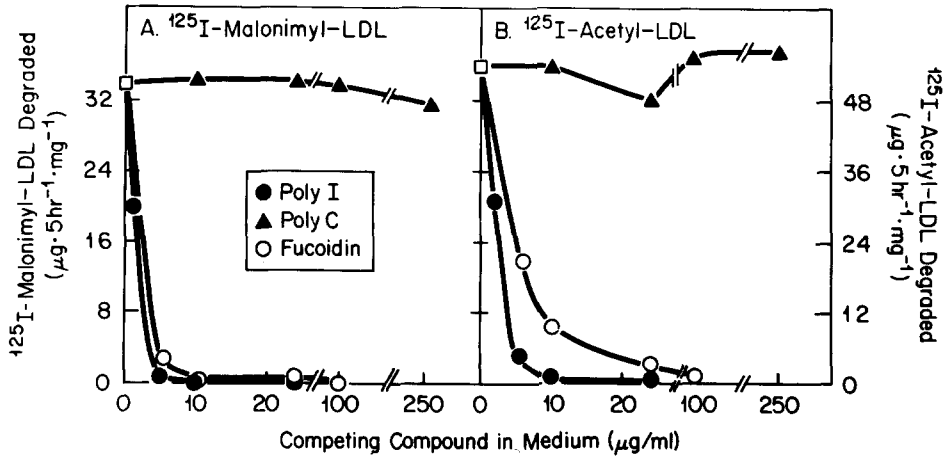

Fig. 7. Ability of various negatively-charged compounds to inhibit the degradation of ^{125}I-malonimyl-LDL (A) and ^{125}I-acetyl-LDL (B) by mouse peritoneal macrophages. Macrophage monolayers were prepared by the standard procedure described in the legend to Fig. 1. Each 35-mm dish received 1 ml of medium containing 10% human lipoprotein-deficient serum and 25 μg protein/ml of either ^{125}I-malonimyl-LDL (225,000 cpm/μg protein) (A) or ^{125}I-acetyl-LDL (185,000 cpm/μg protein) (B) and the indicated compound. After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material in the medium was determined in duplicate dishes [5]. ^{125}I-Malonimyl-LDL was prepared by incubating ^{125}I-LDL with malondialdehyde. The reaction was carried out for 18 hr at 37°C in a final volume of 200 μl containing 0.15 M sodium acetate (pH 6), 2.5 mg protein/ml of ^{125}I-LDL, and 7.5 mM malondialdehyde. The malondialdehyde solution was prepared by mild acid hydrolysis of malondialdehyde bis-(diethylacetal) according to the method of Kwon and Watts [22] and was purified as the sodium salt according to the method of Marnett et al [23].

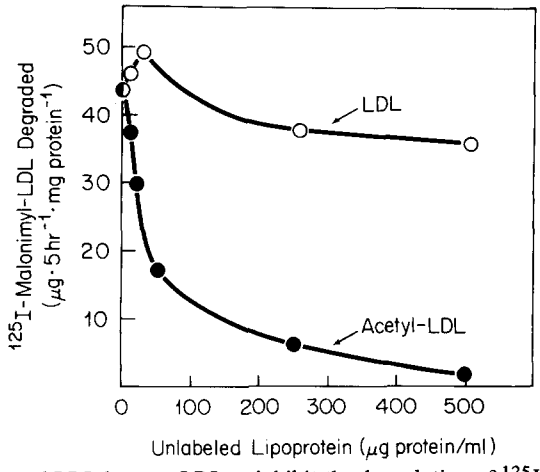

Fig. 8. Ability of acetyl-LDL but not LDL to inhibit the degradation of ^{125}I-malonimyl LDL by mouse peritoneal macrophages. Macrophage monolayers were prepared by the standard procedure described in the legend to Figure 1. Each 35 mm dish received 1 ml of medium containing 10% human lipoprotein-deficient serum and 25 μg protein/ml of ^{125}I-malonimyl-LDL (84,000 cpm/μg protein) and the indicated concentration of either unlabeled acetyl-LDL (•) or unlabeled native LDL (○). After incubation for 5 hr at 37°C, the amount of ^{125}I-labeled trichloroacetic acid-soluble (non-iodide) material in the medium was determined in duplicate dishes (5). ^{125}I-Malonimyl-LDL was prepared as described in the legend to Figure 7.

synthesis of thromboxanes [20]. If liberated in sufficient amounts at certain sites, such as regions of thrombosis, the malondialdehyde might modify LDL and other proteins to such an extent that they are taken up and degraded by macrophages through the acetyl-LDL binding site.

LDL that has been reacted in vitro with high concentrations of malondialdehyde (above 1 mM) undergoes a net loss of positive charges and shows enhanced electrophoretic mobility in agarose gels at pH 8, like acetylated LDL (data not shown). This modified LDL is designated malonimyl-LDL. Figure 7 shows that ^{125}I-malonimyl-LDL is degraded by mouse peritoneal macrophages at a rate that is comparable to the degradation rate for ^{125}I-acetyl-LDL. The ^{125}I-malonimyl-LDL appears to enter the cell via the same binding site that mediates the uptake of ^{125}I-acetyl-LDL, as evidenced by the finding that polyinosinic acid and fucoidin compete equally for both processes (Fig. 7). In contrast, polycytidylic acid does not inhibit the degradation of either of the two lysine-modified lipoproteins (Fig. 7). Moreover, as shown in Fig. 8, unlabeled acetyl-LDL blocks the uptake and degradation of ^{125}I-malonimyl-LDL; half-maximal inhibition of the degradation of ^{125}I-malonimyl-LDL (25 μg protein/ml) was achieved by unlabeled acetyl-LDL in the range of 15 μg protein/ml. Unlabeled native LDL at concentrations as high as 500 μg protein/ml showed no ability to inhibit the uptake and degradation of ^{125}I-malonimyl-LDL by macrophages (Fig. 8).

The cholesteryl esters that enter macrophages within malonimyl-LDL appear to be metabolized by the same two-compartment process that is used for acetyl-LDL (Fig. 4). Table II shows an assay that measures the last step in that process, ie, the re-esterification of the lipoprotein-derived cholesterol. Addition of malonimyl-LDL to macrophages stimulated this reaction by more than 100-fold as measured by the incorporation of exogenous [^{14}C] oleate. This stimulation was blocked by fucoidin and polyinosinic acid, confirming that the reaction requires uptake of the malonimyl-LDL via the surface acetyl-LDL binding site. These inhibitory effects of fucoidin and polyinosinic acid appear specific to the acetyl-LDL binding site. These compounds do not block the stimulation of cholesterol re-esterification that is elicited by either LDL/dextran sulfate complexes [18] or by canine β-VLDL [15], each of which enters macrophages via a different binding site.

TABLE II. Stimulation of Cholesteryl Ester Synthesis in Mouse Peritoneal Macrophages By Malonimyl-LDL and Its Inhibition by Fucoidin and Polyinosinic Acid

Addition to medium	Incorporation of [^{14}C] Oleate into Cholesteryl [^{14}C] Oleate
	nmol 7 hr^{-1} · mg protein^{-1}
None	0.39
Malonimyl-LDL, 188 μg protein/ml	56.4
Malonimyl-LDL, 188 μg protein/ml + fucoidin, 100 μg/ml	0.75
Malonimyl-LDL, 188 μg protein/ml + polyinosinic Acid, 10 μg/ml	1.2

Monolayers of mouse peritoneal macrophages were prepared by the standard procedure described in the legend to Figure 1. Each 35-mm dish received 1 ml of medium containing 0.2 mM [^{14}C] oleate-albumin (10,600 cpm/nmol) and the indicated addition. After incubation for 7 hr at 37°C, the amount of cholesteryl [^{14}C] oleate formed by the cells was determined [5]. Malonimyl-LDL was prepared as described in the legend to Figure 7.

TABLE III. Stimulation of Cholesteryl Ester Synthesis by LDL and Acetyl-LDL in Lymphocytes and Cultured Monocytes From a Normal Subject and a Patient With Homozygous FH

Genotype	Lipoprotein addition to medium	Incorporation of [^{14}C] oleate into cholesteryl [^{14}C] oleate	
		Lymphocytes	Cultured monocytes
		nmol 6 hr^{-1} · mg protein^{-1}	
Normal	None	0.08	1.0
Normal	LDL	2.8 (35)[a]	2.1 (2.1)[a]
Normal	Acetyl-LDL	0.08 (1)	10.9 (10.9)
FH Homozygote	None	0.13	0.79
FH Homozygote	LDL	0.14 (1.1)	0.72 (0.9)
FH Homozygote	Acetyl-LDL	0.16 (1.2)	7.7 (9.7)

Mononuclear cells were isolated from the peripheral blood of a healthy subject and a patient with homozygous FH by the method of Böyum [25], and the monocytes and lymphocytes were separated by differential adherence to plastic [26]. The monocytes (adherent cells) and lymphocytes (non-adherent cells) were incubated separately at 37°C in a humidified CO_2 (5%) incubator. The lymphocytes were incubated in suspension for 3 days in RPMI 1640 medium containing 10% (v/v) human lipoprotein-deficient serum [26]. The monocytes were incubated as monolayers for 5 days in RPMI 1640 medium containing 10% autologous human serum with fresh medium added on day 3 [26]. For experiments, the lymphocytes (2.9×10^6 cells/dish) and monocytes (2.0×10^6 adherent cells/dish) were incubated with 1 ml of medium containing 0.2 mM [^{14}C] oleate-albumin (10,800 cpm/nmol) and the indicated lipoprotein at a final concentration of 100 μg protein/ml. After incubation for 6 hr at 37°C, the amount of cholesteryl [^{14}C] oleate formed by the cells was determined [5]. Each value represents the average of duplicate incubations.

[a]The value in parenthesis indicates the *-fold* stimulation by either LDL or acetyl-LDL.

The malondialdehyde used in the above studies was prepared by mild acid hydrolysis of malondialdehyde bis-(diethylacetal) [22] and was purified as the sodium salt [23]. In the in vitro studies performed in our laboratory to date, high concentrations of malondialdehyde (above 1 mM) have been required to convert LDL into a high uptake form for macrophages. These concentrations are probably several orders of magnitude higher than those that result from malondialdehyde production by platelets in vitro [24]. For this reason, further studies will be required to determine whether platelets in vivo can modify LDL through malondialdehyde generation.

UPTAKE OF ACETYL-LDL AND LDL BY CULTURED HUMAN MONOCYTES AND LYMPHOCYTES

Human lymphocytes that are isolated from the bloodstream and incubated in lipo-lipoprotein-deficient serum develop high levels of LDL receptor activity [27]. As a result of this induction of LDL receptors, the subsequent addition of native LDL produced a 35-fold increase in the rate of cholesteryl ester synthesis as monitored by the incorporation of exogenous [^{14}C] oleate into cholesteryl [^{14}C] oleate (Table III). Lymphocytes isolated from patients with homozygous FH fail to develop LDL receptors [27]. When LDL was added to these mutant cells, no stimulation of cholesteryl [^{14}C] oleate synthesis occurred (Table III). Acetyl-LDL failed to stimulate cholesteryl [^{14}C] oleate synthesis in either the normal or the FH homozygote lymphocytes (Table III), indicating that lymphocytes express LDL receptors, but not the acetyl-LDL binding site.

In contrast to the behavior of lymphocytes, monocytes isolated from the same normal subject and cultured for five days in vitro acted like the mouse peritoneal macrophages in that they expressed acetyl-LDL binding sites. Thus, as shown in Table III, the addition of acetyl-LDL to monocytes produced an 11-fold increase in the rate of cholesteryl [^{14}C] oleate synthesis. A similar response to acetyl-LDL was seen in monocytes from an FH homozygote. Thus, the acetyl-LDL binding site is present in monocytes from individuals who lack the genetic capacity to produce LDL receptors. The cultured monocytes from a normal subject, but not an FH homozygote, also showed significant stimulation of cholesterol esterification by LDL. These data suggest that cultured monocytes can express small numbers of LDL receptors in addition to acetyl-LDL binding sites.

SUMMARY: COMPARISON OF THE LDL RECEPTOR PATHWAY AND THE SCAVENGER CELL PATHWAY FOR LIPOPROTEIN DEGRADATION

As summarized in Table IV, the scavenger cell pathway differs from the LDL receptor pathway in three important respects: 1) the cell type that expresses each pathway; 2) the specificity of the surface binding sites; and 3) the fate of the cholesterol liberated from the degraded lipoprotein. With regard to cell type, the LDL receptor pathway has been demonstrated in a wide variety of cultured parenchymal and connective tissue cells and in freshly isolated lymphocytes [26]. None of these cells expresses significant numbers of acetyl-LDL binding sites. In contrast, tissue macrophages from several sources uniformly express only a small number of LDL receptors, if any, even when the cells are incubated for several days in lipoprotein-deficient serum [5, 14, 15]. Instead, tissue macrophages from all sources so far tested express large numbers of binding sites that recognize acetyl-LDL and lead to the internalization and degradation of the modified lipoprotein ([5] and Table V). Thus, the possession of acetyl-LDL binding sites appears to be a differentiated property of the macrophage that is related to its scavenger cell function. Cultured human blood monocytes, as mentioned above, appear to express both LDL receptors and acetyl-LDL receptors.

With regard to the specificity of the surface binding sites, the LDL receptor of parenchymal and connective tissue cells specifically recognizes apoprotein B or apoprotein

TABLE IV. Cellular Pathways for LDL Degradation

	LDL Receptor pathway	Scavenger cell pathway
Cell type	Parenchymal and connective tissue	Macrophages
Binding sites	LDL receptor recognizes Apo B and Apo E	Three different binding sites recognize: 1) Lysine-modified LDL (acetylated, maleylated, malondialdehyde-treated) 2) LDL/sulfated polysaccharide complexes 3) LDL/anti-LDL complexes
Fate of cholesterol	Used for synthesis of membranes and steroid hormones	Some is excreted Some is reesterified and stored as cholesteryl ester droplets (2-compartment model)

E [1, 14]. In contrast, macrophages possess at least three binding sites that can mediate the efficient uptake and degradation of modified LDL (Table IV). The first is the acetyl-LDL binding site that is reviewed herein. In addition to recognizing acetyl-LDL, this site binds other lysine-modified preparations, including maleylated LDL and malondialdehyde-treated LDL. Macrophages also possess two additional binding sites that mediate the uptake and degradation of LDL. One of these sites recognizes complexes of LDL and certain sulfated polysaccharides, most notably dextran sulfate [18]. This binding site is not inhibited by either acetyl-LDL or polyinosinic acid, indicating that it is distinct from the acetyl-LDL binding site [18]. The other site recognizes LDL that is attached to specific anti-LDL antibodies (unpublished observations). This latter site is probably the well described macrophage receptor that binds the Fc portion of antigen-antibody complexes.

Finally, in fibroblasts and parenchymal cells the cholesterol liberated from the receptor-mediated uptake of LDL is used for synthesis of membranes and steroid hormones (Table IV). When these requirements are satisfied, the number of LDL receptors is repressed. Thus, cells will not overaccumulate cholesterol through the LDL receptor pathway [28]. In contrast, the scavenger cell uptake mechanisms show no such regulation. About half of the cholesterol that enters macrophages through this pathway is excreted, and the other half is re-esterified and stored as cholesteryl ester droplets. Since the acetyl-LDL binding sites are not regulated, the amount of cholesteryl ester that accumulates can be enormous [5, 6].

The major unanswered question at present is whether any of these scavenger cell mechanisms actually function in vivo and whether they are responsible for the deposition of LDL-cholesterol in scavenger cells of xanthomas and atheromas that occur in patients with familial hypercholesterolemia. Given the availability of the in vitro systems for studying lipoprotein-macrophage interactions that have been developed in the last several years, it should be possible in the near future to answer this important question.

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