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MODIFIED LOW DENSITY LIPOPROTEINS IN THE PATHOGENESIS OF ATHEROSCLEROSIS

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Abstract. A fatty streak, the earliest atherosclerotic lesion consists mostly of lipid-laden macrophages. The mechanism leading to accumulation of cholestryl ester in macrophages *in vivo* is unknown, but it may result from the uptake of modified low density lipoproteins that enter from blood or are modified within the arterial wall. In this review I discuss the major types of modified low density lipoproteins with a special focus on their chemical nature.

INTRODUCTION

The earliest atherosclerotic lesion is considered by many researchers to be the fatty streak (44). These lesions are characterized by the presence of numerous cholesteryl esters laden cells called "foam cells" (40). Many if not most, of these foam cells, are believed to be derived from peripheral blood monocytes that have been recruited into arterial intima to become tissue macrophages (15). Despite intensive investigations, the molecular mechanism underlying lipid deposition within macrophages resulting in their transformation to foam cells remain still unknown.

Cholesterol and triglicerides are transported in the circulation as water-soluble non-covalently associated macromolecular complexes called lipoproteins (23). Density, composition and electrophoretic mobility have been used to divide lipoproteins into four major classes: chylomicrons, very-low-density lipoproteins (VLDLs), low-density-lipoproteins (LDLs), and high-density-lipoproteins (HDLs).

It is well established that cholesterol deposites in arteries stem primarily from LDL and that the level of plasma LDL correlate with an increased risk of atherosclerosis (33).

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A widely accepted structural model for human plasma LDL describes a spherical particle containing a hydrophobic core of cholesteryl esters and triglicerides surrounded by an amphipathic monolayer of phospholipid and cholesterol in which a single molecule of apolipoprotein B-100 (apo-B) is immersed (59). Apo-B plays a central role in the assembly and transport of LDL, and in the regulation of

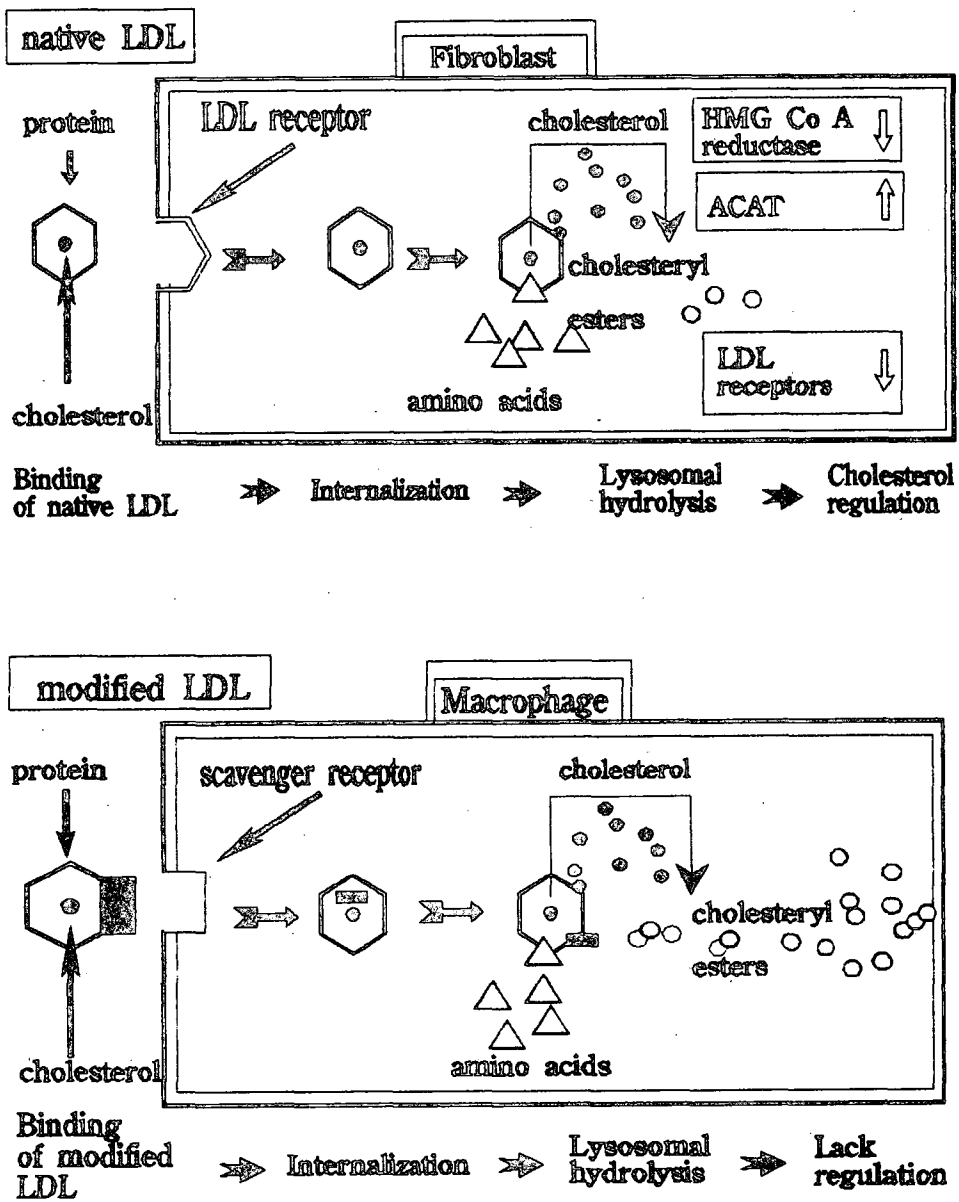

Fig. 1. LDL receptor and LDL-modified scavenger receptor metabolism. Adapted from Brown and Goldstein (1976).

circulating lipids levels. Apo-B appears to possess a very high affinity for lipids, and is the ligand responsible for the binding of LDL to the LDL receptors (9). There is strong evidence suggesting that lysine, arginine and histidine residues of apo-B are of crucial importance in mediating lipoprotein receptor binding, since ionic interaction exists between the positively charged groups of amino acid, and the negatively charged receptor (71). Once bound to the receptor, the LDL is internalized and delivered to the lysosome, where proteases degrade the apolipoproteins, and lipases hydrolise most of the cholesterol esters to free cholesterol, which is than released from the lysosome (20). Subsequently, the free cholesterol in some fashion reduces the activity of the rate-limiting enzyme of cholesterol synthesis i.e. beta-hydroxy--beta-methylglutaryl CoA reductase (HMG-CoA reductase), stimulates the enzyme leading to its own reesterification, acyl cholesterol coenzyme A: acyltransferase (ACAT) and downregulates the expression of LDL receptors.

The process of uptake and delivery to lysosome is therefore a regulated one that can be saturated: once the lysosome has sufficient cholesterol, the cholesterol synthesis is decreased, and the uptake of LDL ceases (5, 19).

Paradoxically, monocytes/macrophages possess few receptors for native LDL and degrade it poorly. Primary cultured macrophages do not accumulate lipid when incubated with even large concentrations of native LDL (60). So alternative mechanisms are necessary to explain the accumulation of cholesterol on macrophages. Indeed, macrophages have specific so called "scavenger receptors" which mediate the endocythosis of several forms of modified LDL (16, 17, 18). In contrast to the receptor for native LDL, the scavenger receptor is not down regulated by cellular cholesterol. This in turn results in lipid loading of these cells (Fig. 1). Some of the modifications to LDL that facilitate its uptake by macrophages are summarized in Table 1.

Table 1. Low-density lipoprotein modifications facilitating uptake by macrophages

I. Chemically modiffied LDL:
acetic anhydride-treated LDL (Acetylated-LDL),
malondialdehyde-treated LDL (MDA-LDL),
4-hydroxynonenal-treated LDL (HNE-LDL),
glutaraldehyde-treated LDL,
free radical oxidized LDL with Cu 2+ (Ox-LDL),
enzymes-treated LDL (desialylated-LDL), glycosylated LDL,
carbon disulfide-treated LDL (LDL-CS_2).
II. LDL-containing associates:
endotoxin-LDL complexes,
proteoglycan-LDL complexes, proteoglycan-LDL aggregates,
mast cell granule-LDL complexes,
antibody-LDL complexes,
heparin-fibronectin-collagen-LDL complexes,
bacterial polysaccharide-LDL complexes,
vortexed-LDL aggregates,
malondialdehyde-treated-LDL aggregates,
oxidized-LDL aggregates, HNE-LDL aggregates.
III. Cell modified LDL:
endothelial cells, arterial smooth muscle cells, and mononuclear blood cells oxidized LDL.

In principle three basic categories of LDL modifications can be distinguished: chemical modifications, formation of LDL containing associates and cell – mediated modifications.

TYPES OF MODIFICATIONS OF LOW DENSITY LIPOPROTEINS

Chemically modified LDL

Chemical modifications of LDL *in vitro* that were first studied in detail include acetylation, acetoacetylation, and malondialdehyde treatment (16, 18). All of these modifications increase the net negative charge of the LDL by abolishing the positive charge of the amino groups of lysine, resulting probably in a conformational shifts in the apo-B 100. The affinity of modified LDL to the scavenger receptor depends upon the kind and the extense of chemical modification of amino acid residues of apo-B. The same reagents that modify lysine residues are more efficient than others in producing molecules that are recognized by the specific scavenger receptor. The modification of only 16% of lysine residues in LDL by malondialdehyde, and 30% of lysine residues in LDL modified by acetoacetylation leads to scavenger receptor recognition, while approximately two thirds of the lysine residues need to be modified by acetylation with acetic anhydride in order to achieve a similar degree of scavenger receptor recognition (21, 22, 71).

Various other forms of chemically modified LDL were produced *in vitro*. It is noteworthy that carbon disulfide (CS_2), a solvent used in various industrial processes, also interacts *in vitro* with LDL (41). The reaction of CS_2 with apo-B gives rise to the formation of dithiocarbammates from amino groups of the lysine resulting in the modification of LDL which leads to a shift to the scavenger pathway. These results are consistent with the fact that CS_2 intoxication accelerates the appearance of atherosclerotic lesions in persons working in the viscose rayon fiber industry, chronically exposed to carbon disulfide (68). Like carbon disulfide, other pollutants or their reactive metabolites (e.g., α, β unsaturated aldehydes, nitrifying compounds), able to bind to amino groups, sulfhydryl groups or/and tyrosine residues of apo-B might make LDL recognizable by scavenger receptors of macrophages, thus may induce the formation of foam cells.

Among modifications leading to the LDL uptake by macrophages, oxidation is of particular interest. The existing experimental data show, that in contrast to some other chemical modifications (e.g., acetylation), oxidation could and most likely does occur *in vivo* (2, 48, 52, 55). LDL becomes oxidized in a cell-free system suplemented with trace amount of certain redox active transition metal ions (Cu2+, Fe2+) (12, 64). Other reducting agents able to oxidize LDL *in vitro* include sulfur containing amino acids, or cigarette smoke extract (54, 75). LDL can be also oxidatively modified *in vitro* by a combined action of two purified enzymes: lipooxygenase and phospholipase-A2 (61).

Oxidatively modified LDL (Ox-LDL) is characterized by the increase in density and net negative charge, loss of esterified cholesterol, hydrolysis of phosphatidylcholine and partial degradation of apo-B 100.

Oxidation of LDL is a free radical process in which the polyunsaturated fatty acids of LDL, in the presence of suitable transition metals (Cu2+, Fe2+), are degraded in a lipid peroxidation process to a variety of highly reactive products

including lipid hydroperoxides (12). A subsequent decomposition of lipid hydroperoxides is always accompanied by formation of a great variety of aldehydes. Clearly identified were 4-hydroxynonenal, 4-hydroxyoctenal, 4-hydroxyhexenal, propanal, butanal, pentanal, hexanal, octanal, 2,4-hepatadienal, and malondialdehyde (MDA) (12, 11). Some of these aldehydes can bind covalently to apo-B 100 particularly through the lysine residues, and modify the structural and functional properties of LDL (12, 26, 32). The reactive oxygen species generated during oxidation of LDL may also convert histidine and proline residues to negatively charged aspartic acid (from His) and glutamic acid (from Pro) residues (12).

Contrary to other chemically modified LDL, Ox-LDL can injure or kill cells grown in culture, including endothelial cells, vascular smooth muscle cells, and skin fibroblasts. Recently it has been shown that toxin of Ox-LDL, probably lipid hydroperoxides and/or oxysterols, resides in the lipid phase and the susceptibility of Ox-LDL depends upon the phase of the cell cycle and the level of intracellular glutatione (24, 38, 39, 43).

Of great recent interest has been a proposal that glycosylated LDL, LDL(a), LDL-endotoxin complexes, cholesterol-rich LDL and the LDL modified through interaction with proteoglycans are chemically unstable, that renders them open to attack by free radicals generated by peroxidation of LDL lipids or to undergo further direct chemical modification by oxidation (6, 28, 57).

LDL-Containing associates

Other *in vitro* modification of LDL that could possibly be involved in the uptake and accumulation of lipoprotein cholesterol by cells of the arterial wall includes soluble and insoluble complexes between apo- B and sulfated proteoglycans (29). The uptake of these LDL-sulfated polysaccharide complexes is mediated by scavenger receptors, which are similar but not identical with binding sites for acetyl-LDL. A potential importance of this type of lipoprotein modification is emphasized by the fact that sulfated polysaccharides are abundant in the extracellular matrix of the arterial wall (49).

Recently it has been found that some types of *in vitro* modified LDL [vortexed LDL, Ox-LDL, 4-hydroxynonenal LDL, malondialdehyde-treated LDL and proteoglican-LDL complex], show an increased tendency towards aggregation and that this aggregation is a necessary step in the process of cholesteryl ester accumulation within cultured human aortic smooth muscle cells (26, 27, 34, 66, 70).

In addition to LDL aggregates, "associates" between LDL and various connective tissue components, such as collagenase– resistent debris, heparin, elastin, fibronection, collagen and heparin-fibrinonectin-denaturated complexes seem to be able to induce lipid accumulation in macrophages (14, 51, 58, 63). LDL association with exocytosed mast cell granules, LDL-antibody complexes, and LDL compounded with bacterial polysaccharide evokes the same effect (36, 37, 47, 69).

The mechanisms responsible for the uptake of LDL aggregates and LDL associates by macrophages are probably the phagocytosis for aggregated particles and, the scavenger receptor for monomeric particles (27). However, other forms of modification leading to other uptake mechanisms may also be operative.

Cell mediated modification of LDL

Cell mediated *in vitro* modification of LDL is responsible for monocytes/macrophages, endothelial cells from human umbilical vein and rabbit aorta, bovine and human smooth muscle cells (7, 46, 65). Recently, a number of studies have demonstrated that reactive oxygen species generated by activated macrophages and neutrophiles can oxidize native lipoprotein (8). All these cells oxidize LDL *in vitro* in a mode similar to the oxidation catalyzed by transition metals in cell-free system. Both procedures result in the same changes in the LDL physicochemical properties.

An exact mechanism by which cells modify LDL remains unknown. However, sufficient evidence exists that some processes are strong enough to be included in the chain of events leading to the formation of modified LDL. It has been postulated that cellular modification depends on the release of superoxide radicals by cells (25). Superoxide, the one-electron-reduced form of molecular oxygen, is a reactive oxygen species able to initiate lipid peroxidation in the presence of transition metals. The strongest evidence for the involvement of superoxide radicals came from observations of inhibiton of the LDL-modifying action of a wide variety of cells by superoxide dismutase. The role of superoxide radicals in oxidation of LDL by cells is further supported by studies which showed that activated monocytes or neutrophils were much more effective than the resting cells (25). An exact nature of reducting agent required in a cell system able to produce superoxide radicals, is still uncertain. Sulfur containing aminoacids (L-cysteine) is likely to be one of such reducing agents, since it is able to undergo oxidation-reduction reactions as well as to enter and leave cells by specialized transport systems (10).

The alternative oxidizing pathways have been investigated and it is tempting to speculate that certain types of cells have on their surface an enzyme that initiates oxidation of LDL. This cellular modification of LDL may involve lipooxygenase(s), and it has been shown that lipooxygenase inhibitors block modification by endothelial cells and macrophages. However, more recently the involvement of lipooxygenases in modification of LDL has been questioned (62).

OTHER PROPERTIES OF MODIFIED LDL THAT MAY ENHANCE ATHEROGENESIS

The importance of a chemical modification of LDL has been demonstrated in many other processes involved in atherogenesis. In addition to cytotoxicity and altered recognition, the *in vitro* modifed LDL appears to produce other biological effects. The Ox-LDL can be a chemoattractant for monocytes, accelerating the recruitment of these cells into the arterial wall (56). The chemotactic factor has been identified as lysophosphatidylochline derived from oxidized LDL. It is also known that Ox-LDL inhibits macrophage migration *in vitro*, facilitating continued uptake of the modified lipoprotein (55, 56). Furthermore, oxidized LDL is more active than native LDL in promoting platelet aggregation; the poorly oxidized LDL is able to stimulate endothelial cell prostanoid synthesis by smooth muscle cells in culture, whereas high levels of lipid peroxides are inhibitory (1). In addition, it has been shown that oxidized LDL causes endothelium-dependent coronary contraction which, impairs endothelial cell vasodilator function *in vitro*; it blocks the release of endothelial derived relaxing factor and inhibits the relaxation in response to

stimulants (72). The data also revealed that the oxidation of LDL plays an important role in influencing gap-junctional communication between vascular smooth muscle cells (76). The disruption of the growth control by Ox-LDL results in an uninhibited multiplication of these cells leading to atherosclerotic lesions. Finally, recent data concerning the role of macrophages in inflammatory reactions indicate that Ox-LDLs (but not Ac-LDL) suppress nitric oxide synthase activity, suggesting that the functional activity of lipid loaded macrophages may depend upon the type of modified lipoproteins (31).

EVIDENCE OF LDL MODIFICATION *IN VIVO*

Recent studies carried out by several laboratories, suggest that oxidation is involved in LDL modification in the body. This hypothesis is supported by several kinds of investigations: a) treatment of Watanable heritable hyperlipidemic (WHHL) rabbits with probucol (antioxidant) can prevent progression of atherosclerosis (35, 73), b) antioxidant Butylated Hydroxytoluene (BHT) protects against cholesterol-induced atherosclerosis in rabbits (3), c) oxidation-specific lipid-protein adducts are present in WHHL rabbit and human lesions (52, 74), d) LDL isolated from human or WHHL rabbits aortic lesions show physical, chemical and biological changes indicative of oxidation (74), e) products of lipid peroxidation appear in the form of ceroids present in atheromatous plaques (45), f) an oxidatively modified LDL subfraction (5−20%) is present in normal human plasma (2), g) autoantybodies against oxidized LDL have been noticed in both human and rabbit sera (52), however, no oxidized LDL has been detected by immunological methods, h) LDL derived from hypercholesterolemic patients demonstrate a greater propensity for oxidation *in vitro* than normal LDL (42).

In the light of these observations, it is possible that, LDL accumulated in the arterial intima may undergo some structural modifications, responsible for functional changes, some of which are relevant to atherogenesis.

LDL is protected against oxidation by its endogenous antioxidants, e.g. vitamin E, carotenoids and others. Oxidation of polyunsaturated fatty acids in LDL is preceded by a sequential loss of these antioxidants in the sequence: α-tocopherol, τ-tocopherol, lycopene, and β-carotene (12).

Natural protectors against peroxidation of lipoproteins in plasma are vitamin C, vitamin E and vitamin A: their levels can be modified by a diets. A possible relationship between the plasma vitamin E level and the risk of atherosclerosis has been the subject of several epidemiological studies. Some researchers found an inverse correlation between the plasma level of the vitamin E and the incidence of ischemic heart disease, while others did not show any relation (30). However, more recently a number of studies have indicated that vitamin C acts as a synergist with vitamin E by sparing vitamin E (4). The data of studies *in vitro* and *ex vivo* show that vitamin E is an important but not only one parameter which determines the oxidation resistance of LDL. Additional studies (e.g. supplementation with β-carotene) should be made to elucidate the importance of other antioxidants for the oxidation resistance of LDL.

LDL MODIFICATIONS AND THE RETICULOEDOTHELIAL SYSTEM

Modified LDLs injected intravenously into rats are rapidly and almost completely removed from plasma by the liver (67). Using the isolated perfused rat liver, it was possible to demonstrate that the acetoacetylated LDL, acetylated LDL, and oxidized LDL taken up by nonparenchymal cells of the liver consist mainly of the endothelial cells line and Kupffer cells, which form the largest reservoir of macrophages in the body (50).

The presence of scavenger receptors on these cells, combined with their strategic anatomical localization, form an important protection system against the uptake of modified LDL by macrophages of arterial wall. This system will only be efficient if the uptake is coupled with a complete processing of the modified LDL and secretion of the metabolic products from the cell.

In the animal body, many of foreign compounds undergo a wide range of metabolic transformations, catalysed by enzymes of the endoplasmatic reticulum of the liver (53). These compounds are absorbed from the blood of the hepatic sinusoids, into hepatic parenchymal cells and are then transferred, as metabolites or conjugates, into the bile or return to blood of the sinusoids, to be ultimately excreted by the kidney. It is tempting to suggest that these foreign compounds can interfere in the internalization and/or catabolism of some modified LDL by endothelial sinusoid cells line.

The biology of this phenomen is little known and presents an important area for further studies.

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