review

Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions

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Abstract **Initiation of atherosclerosis is characterized by accumulation of aggregates of small lipid droplets and vesicles in the extracellular matrix of the arterial intima. The droplets and vesicles have features that suggest that they are formed from modified plasma-derived low density lipoprotein (LDL) particles. A variety of hydrolytic enzymes and prooxidative agents that could lead to extracellular assembly of LDL-derived droplets and vesicles are present in the arterial intima. In fact, in vitro studies have demonstrated that extensive oxidation of LDL and treatment of LDL with either proteolytic or lipolytic enzymes will induce LDL aggregation and fusion and treatment of LDL with cholesterol esterase will cause formation of vesicles. Fusion of LDL particles proceeds faster in vitro when they are bound to components of the extracellular matrix derived from the arterial intima, such as proteoglycans, and, depending on the type of modification, the strength of binding of modified LDL to the matrix components may either increase or decrease. In the present article, we discuss molecular mechanisms that provide clues as to how aggregated lipid droplets and vesicles may be derived from modified LDL particles. We also describe how these modified forms of LDL, by means of their trapping to the extracellular matrix, may lead to extracellular lipid accumulation in the arterial intima.** —Öörni, K., M. O. Pentikäinen, M. Ala-Korpela, and P. T. Kovanen. **Aggregation, fusion, and vesicle formation of modified low density lipoprotein particles: molecular mechanisms and effects on matrix interactions.** ***J. Lipid Res.*** **2000.** 41: **1703–1714.**

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Accumulation of low density lipoprotein (LDL)-derived cholesterol in the inner layer of the arterial wall, the intima, leads to the development of atherosclerotic lesions. In the arterial intima, the dense extracellular matrix (ECM) forms an organized tight network [reviewed by Wight (1)] that has the potential to bind lipoproteins. The major components of the vascular ECM are collagens, elastin, and proteoglycans (PGs). The PGs include versican, which can form multimeric aggregates with hyaluronan, the small dermatan sulfate-rich PGs decorin and biglycan, and cell surface heparan sulfate PGs (e.g., syndecans and glypicans). Many of the non-PG components of the ECM appear to be partially covered by certain PGs. Thus, decorin and possibly also biglycan bind to collagen fibrils (2), and at least biglycan (3) and heparan sulfate PGs (2) are associated with elastin.

Increasing evidence points to the relevance of the interaction between LDL and PGs in the arterial intima [reviewed by Camejo et al. (4) and Hurt-Camejo et al. (5)]. Interestingly, PGs may also be involved in interactions between LDL and other components of the ECM. Thus, Nievelstein-Post et al. (6) showed that LDL-derived lipid droplets are associated with collagen via small PGs tethered to the collagen fibrils. This notion is supported by in vitro results demonstrating that decorin can link LDL to collagen (7). The atherosclerotic potential of the LDL-PG interaction was directly assessed in transgenic mice. Thus, Borén et al. (8) were able to express in transgenic mice mutated human apolipoprotein B-100 (apoB-100) that interacted poorly with PGs. Exposure of these animals to diet-induced hypercholesterolemia caused significantly delayed atherosclerosis as compared with mice expressing normal human apoB-100 (9). This finding supports the idea that retention of LDL to PGs is important in the development of early lesions in atherosclerosis, which has consistently been promoted by Camejo et al. and recently reintroduced as the response-to-retention hypothesis by Williams and Tabas (10, 11).

Abbreviations: AAPH, 2,2′-azobis(2-amidino-propane)hydrochloride; apoB-100, apolipoprotein B-100; DAG, diacylglycerol; ECM, extracellular matrix; GAG, glycosaminoglycan; HNE, 4-hydroxynonenal; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; lysoPC, lysophosphatidylcholine; MDA, malondialdehyde; PC, phosphatidylcholine; PG, proteoglycan; PLA_2, phospholipase A_2; PLC, phospholipase C; SM, sphingomyelin; SMase, sphingomyelinase; VLDL, very low density lipoprotein.

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In addition to direct binding to PGs, facilitated binding of lipoproteins to PGs by bridging molecules such as lipoprotein lipase (LPL) has been suggested to play a role in lipoprotein retention in the arterial intima. This enzyme is present in the ECM of the arterial intima and is produced by the intimal macrophages and smooth muscle cells (12–14). Importantly, macrophage LPL expression promotes the development of atherosclerotic lesions in fat-fed mice (15), a finding that demonstrates that expression of LPL in the arterial intima can be proatherogenic, possibly because it facilitates binding of LDL to PGs.

EXTRACELLULAR LIPIDS IN THE ATHEROSCLEROTIC ARTERIAL INTIMA

Morphology and properties of extracellular lipid particles

In atherosclerotic plaques, the lipid deposits are both extracellular and intracellular (**Fig. 1**). The initial sign of atherogenesis is the appearance of small lipid droplets and vesicles in the subendothelial extracellular space (16). Interestingly, in areas that contained subendothelial macrophage foam cells (fatty streaks), no such extracellular lipid particles could be found (17), suggesting that the foam cells had ingested the lipid droplets.

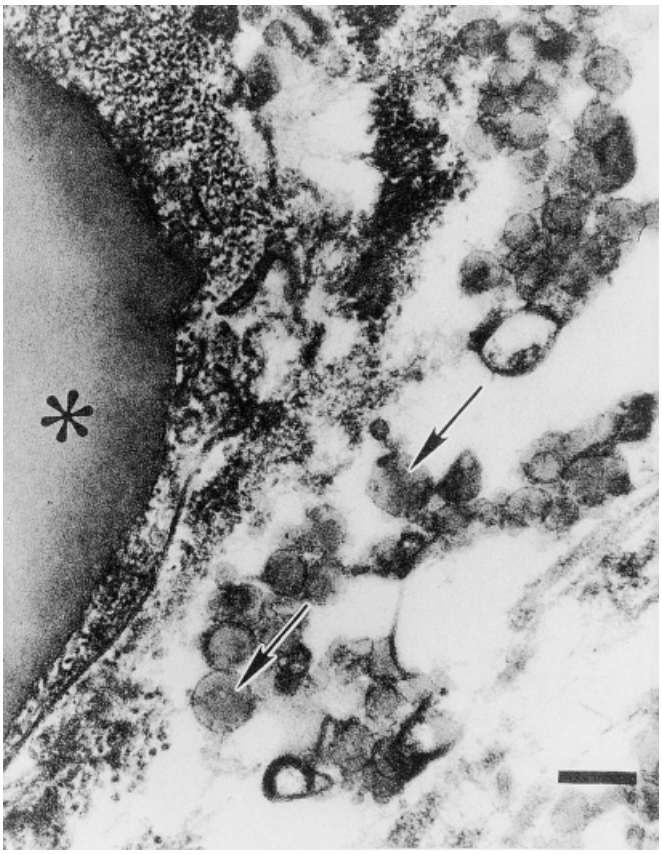

Fig. 1. Electron micrograph of human atherosclerotic lesion showing an intracellular lipid droplet (asterisk), extracellular lipid droplets (arrows), and lipid vesicles. Reproduced with permission from (18). Bar: 150 nm.

Advanced atherosclerotic lesions are characterized by an extracellular lipid core deep in the musculoelastic layer of the arterial intima (18). In lesions characterized by a paucity of macrophages and macrophage foam cells in the lesion cap, the core lipids consist mostly of small cholesteryl ester-rich lipid droplets. In contrast, in lesions in which macrophage foam cells are present in the cap, the deep lipid deposition is dominated by lipid vesicles rich in unesterified cholesterol and cholesterol crystals. These findings suggest that the cholesteryl ester-rich lipid droplets are formed directly from infiltrated LDL particles, whereas macrophages take part in the generation of extracellular lipid vesicles enriched in unesterified cholesterol.

Three types of extracellular lipid particles have been isolated from the atherosclerotic arterial intima: *i*) LDL-like particles, *ii*) small cholesteryl linoleate-rich lipid droplets, and *iii*) small lipid vesicles. Analysis of these lipid particles has shown that the arterial LDL-like particles resemble plasma LDL in many respects, but show signs of slight modification, whereas the lipid droplets and vesicles have features suggesting that they may be derived from plasma LDL by extensive modification.

Arterial LDL. ApoB-100 in LDL isolated from atherosclerotic arterial intimas of cadavers (19–22) and from fresh surgical samples (23, 24) has been reported to be fragmented to variable degrees. These findings suggested that the apoB-100 had been predisposed to breakdown by the enzymes or oxidants present in the arterial intima.

Arterial LDL particles have a slightly lower phospholipid content than plasma LDL (23). LDL isolated from atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits contained increased amounts of sphingomyelin (SM) and lysophosphatidylcholine (lysoPC) and decreased amounts of phosphatidylcholine (PC) (20), changes that might have resulted from oxidation or hydrolysis of PC by phospholipase A_2 (PLA_2). In addition, aggregated LDL isolated from human atherosclerotic lesions was enriched 10- to 50-fold in ceramide, a product of cleavage of SM by sphingomyelinase (SMase) (25). However, because the purity of the isolated aggregated LDL was not assessed, it cannot be determined whether the ceramide was enriched in LDL, or alternatively in other lipid particles such as vesicles that may have coisolated with the LDL.

Antibodies that recognize products of lipid peroxidation [malondialdehyde (MDA) and 4-hydroxynonenal (HNE)] cross-react with arterial LDL (26). In addition, arterial LDL has been shown to contain specific stable oxidation products. Thus, markers of oxidation by reactive nitrogen species (27), by metal ions (28), and by myeloperoxidase (29) have been detected in LDL isolated from atherosclerotic plaques. Lipoxygenase oxidation products have been detected in early atherosclerotic lesions (30, 31). In addition, epitopes have been detected in the arterial intima with antibodies against MDA-modified LDL (26, 32), HNE-modified LDL (32), oxidized phospholipids (33), hypochlorite-modified LDL (34), and nitrotyrosine (35). Furthermore, two monoclonal antibodies produced by Torzewski et al. (36), that reacted in

vitro with trypsin-modified LDL or with LDL modified by a combination of trypsin and cholesterol esterase, were shown to react with epitopes in the human atherosclerotic intima.

Arterial lipid droplets. Chemical analyses of the extracellular lipid droplets in human atherosclerotic lesions and their size suggest that the droplets originate directly from LDL particles. Thus, the extracellular lipid droplets are smaller (20−400 nm) than the intracellular lipid droplets of foam cells (>400 nm) and the chemical composition of their cholesteryl esters (linoleate rich) resembles that of plasma lipoproteins and not that of intracellular lipid droplets (oleate rich) (37, 38). Moreover, such extracellular lipid droplets can be generated from LDL in the arterial intima in vivo: aggregated lipid droplets were seen in the rabbit aortic intima 2 h after a bolus injection of a large quantity of human LDL into the animal (39). Similar droplet deposits were also observed in situ in isolated rabbit cardiac valves after incubation with human LDL for 4 h (6).

From minced human aortas, Chao et al. (40) isolated and purified cholesteryl linoleate-rich lipid particles of a size that corresponded to the lipid droplets detected microscopically, the average diameter of the particles being slightly greater than 100 nm. The particles did not contain immunoreactive apoB-100 and contained lower relative amounts of phospholipids (9%) and protein (9%) than plasma LDL (20 and 22%, respectively) (41). Extensive proteolysis and/or oxidation of the protein of LDL would explain both the loss of protein and the absence of immunoreactive apoB-100 in the lipid droplets. Indeed, LDL particles have been suggested to lose apoB-100 immunoreactivity on their deposition in human atherosclerotic lesions of various types (42).

Within the atherosclerotic lesions, lipid droplets have also been found that, in addition to apoB-100, also contain apoE (24, 43). The isolated droplets had diameters of about 35 nm and densities in the very low density lipoprotein (VLDL)-intermediate density lipoprotein (IDL) range, and, accordingly, they were suggested to have originated from infiltrated β-VLDL-like particles (which contain apoE) (24, 43), a possibility supported by the observation that human atherosclerotic lesions contain apoC-III, another component of plasma VLDL particles (44). However, unlike plasma VLDL and IDL, the isolated lipid droplets were poor in triglycerides and rich in cholesteryl esters (24, 43). Moreover, mice lacking macrophage apoE secretion also lacked apoE in atherosclerotic lesions (45, 46) suggesting that most of the apoE in the atherosclerotic intima is synthesized locally by intimal macrophages. Thus, if the lipid droplets containing apoB-100-apo-E acquired the apoE locally, they could actually be modified LDL particles.

Arterial lipid vesicles. Both in human atherosclerotic lesions and in arterial lesions of atherosclerosis-prone experimental animals, lipid vesicles appear in large numbers (47–49). However, the origin of the vesicles is not known. Their colocalization with apoB-100 in the arterial intima suggests that they are derived from infiltrated VLDL or LDL particles. Thus, they could have been generated directly from VLDL (50) or from LDL (41) during lipolysis of the core lipids of the particles. The absence of immunoreactive apoB-100 in vesicles isolated from human atherosclerotic lesions (51) indicates that, if the vesicles are derived from VLDL or LDL particles, apoB-100 is also extensively modified. Alternatively, the vesicles could have been released from foam cells, because lipid-loaded macrophages in culture have been shown to release phospholipids in the form of vesicles (52). Finally, their peculiar lipid composition (rich in SM and unesterified cholesterol) (40) resembles that of lysosome membranes, which makes dead cells a third possible source of their origin. Further experiments are needed to distinguish between these alternatives.

ENZYMES AND AGENTS POTENTIALLY CAPABLE OF MODIFYING LDL IN VIVO

In the arterial intima, there are several proteolytic and lipolytic enzymes and oxidants capable of modifying LDL (**Table 1**). Chymase and tryptase, two neutral proteases secreted by mast cells (53), and matrix metalloproteinases secreted by macrophages and smooth muscle cells (54) have been detected extracellularly in the arterial intima. In addition, the arterial intima contains plasma-derived plasmin (55), kallikrein (56, 57), thrombin (58), and lysosomal proteases (59, 60). Chymase of rat mast cells can extensively hydrolyze the apoB-100 component of LDL particles (61, 62) and lysosomal proteases of macrophages degrade apoB-100 at acidic pH (63). In addition, plasmin, kallikrein, thrombin (64), and several types of matrix metalloproteinases (K. Öörni and P. T. Kovanen, unpublished observation) are capable of cleaving apoB-100.

Secretory PLA_2 (65–67) and secretory SMase (25, 68, 69) have been found extracellularly in the arterial intima. The PLA_2 can hydrolyze PC molecules of LDL particles (70), and the SMase, although having an acidic pH optimum, can hydrolyze SM molecules of oxidized and PLA_2-treated LDL at neutral pH (71). The arterial intima also contains carboxyl ester lipase, a bile acid-dependent cholesterol esterase (72, 73). This enzyme is capable of hydrolyzing the lysophospholipids in oxidized LDL, and also, in the presence of cholate, the cholesteryl esters and triglycerides of LDL. Finally, macrophages and macrophage-derived foam cells in the vessel wall synthesize lysosomal acid lipase (74). Whether this enzyme is active in the intimal extracellular space is not known.

The arterial intima expresses enzymes capable of oxidizing LDL: 15-lipoxygenase (75), myeloperoxidase (76), and heme-oxygenase I (77). In addition, nitric oxide synthase (78) and NADPH oxidase (79), which may produce free radicals capable of oxidizing LDL, are expressed in the arterial intima. Atherosclerotic lesions also contain ceruloplasmin (80) and transition metals (80–83), both of which are potentially capable of oxidizing LDL.

TABLE 1. Presence of LDL-modifying enzymes and agents in the arterial intima

	Normal Intima	Atherosclerotic Intima	Comment	References
Proteases				
Chymase, tryptase	(+)	+	Secreted by intimal mast cells	53
Matrix metalloproteinases	(+)	+	Secreted by intimal macrophages, T cells, and smooth muscle cells; inactive or inhibited in normal intima	54
Plasmin	+	+	Mostly plasma derived	55
Kallikrein	(+)	+	Both plasma and tissue kallikrein present	56, 57
Thrombin	(+)	+	Mostly plasma derived; activity found in human intimal extracts	58
Lysosomal proteases	(+)	+	Mostly in macrophages	59, 60
Lipases				
Secretory PLA_2	−	+	Secreted by macrophages and smooth muscle cells; binds to ECM	65–67
Secretory SMase	−	+	Secreted by endothelial cells and macrophages; binds to ECM; in normal intima limited to the endothelium	25, 68, 69
Carboxyl ester lipase	?	+	Present in extracts of human atherosclerotic lesions; secreted in culture by endothelial cells and macrophages	72, 73
Lysosomal acid lipase	(+)	+	Activity found in human atherosclerotic lesions; associated mainly with macrophage foam cells	74
Oxidative agents				
Inducible nitric oxide synthase	−	+	Expressed in smooth muscle cells and macrophages	78
NADPH oxidase	−	+	Expressed in smooth muscle cells and macrophages	79
Lipoxygenases	(+)	+	Enzyme and lipoxygenase-modified lipids detected	30, 31, 75
Myeloperoxidase	−	+	Associated with macrophages; hypochlorite-modified proteins detected	34, 76
Heme-oxygenase I	−	+	Enzyme associated with endothelial cells and macrophages	77
Ceruloplasmin	−	+	Plasma protein, also expressed in macrophages	80
Free transitional metal ions	−	+	Present extracellularly in advanced atherosclerotic lesions	80–83

Symbols: +/(+), present/present to a lesser extent; −, absent; ?, not known.

MOLECULAR MECHANISMS OF AGGREGATION AND FUSION

To clarify molecular mechanisms by which the various types of LDL modifications lead to self-aggregation and fusion of LDL particles, the molecular composition and structure of the particles are briefly described. LDL particles have a hydrophobic core consisting of nonpolar lipids (mostly cholesteryl esters and some triglycerides) and a shell, which is a monolayer containing phospholipids, unesterified cholesterol, and a single copy of apoB-100 protein. Each LDL particle contains about 1,600 esterified cholesterol molecules, 700 phospholipid molecules, 600 unesterified cholesterol molecules, and 170 triglyceride molecules (84). The main phospholipids in LDL are PC and SM, which constitute about 70% (500 molecules) and 30% (200 molecules) of the phospholipids, respectively. The distribution between the surface and core components is not absolute: up to 30% of the unesterified cholesterol appears to be located in the particle core and 2–6% of the cholesteryl esters and triglycerides are within the surface monolayer (85). Specific interactions between the surface molecules appear to lead to the formation of domains on the LDL surface: there is evidence that apoB-100 is irreversibly associated with a fraction of the PC molecules (85–87), whereas cholesterol interacts preferentially with SM (88, 89).

Modification of the surface structure of an LDL particle can result in loss of particle stability. This, again, will affect interactions between the particles and can lead to their aggregation and subsequent fusion. Indeed, it appears that extensive modification of a single class of surface components of the LDL particles alone is sufficient to trigger their aggregation and/or fusion. Aggregation of LDL brings the surfaces of different LDL particles into contact, but does not unite the particles, and thus does not change the size of the individual particles. If particle modifications are sufficiently extensive, energetic stabilization will result in subsequent fusion of the attached particles. It is important to note that particle aggregation is, in principle, a reversible reaction, whereas particle fusion is an irreversible phenomenon (**Fig. 2**).

Proteolytic modifications

Proteolytic degradation of apoB-100 of LDL has been shown to cause aggregation and fusion of LDL particles (**Table 2**). Particle fusion was originally observed on the surface of isolated rat mast cell granule remnants (62). In this system, LDL particles are bound to the heparin PGs of the granule remnants while two granule remnant proteases, chymase and carboxypeptidase A, also bound to the heparin PGs, degrade the apoB-100 of the bound LDL. Later, it was shown that mere fragmentation of apoB-100 does not induce LDL fusion: only after peptide fragments have been released from the particles do they become sufficiently unstable for initiation of fusion (64).

ApoB-100 is an important structural component of the LDL surface (90, 91) and therefore even partial loss of apoB-100 from the surface of LDL particles is likely to lead to reorganization of the surface, and secondarily also of core lipids. Indeed, loss of protein fragments from LDL appears to lead to loosening of the surface, because cholesterol esterase is able to hydrolyze the core lipids of LDL only after apoB-100 has been hydrolyzed, for example, by

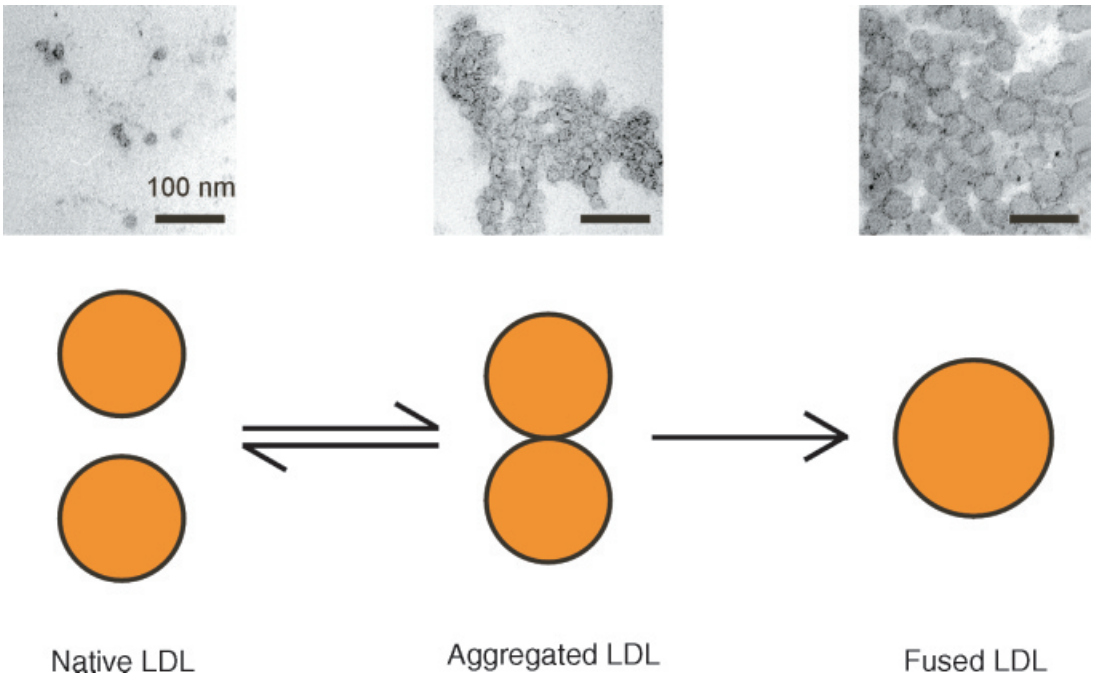

Fig. 2. Aggregation and fusion of LDL. When two modified LDL particles collide, they may remain attached to each other (aggregation of LDL). Such aggregated particles may fuse into a larger particle (fusion of LDL). Note that aggregation is in principle a reversible phenomenon, whereas fusion is irreversible. Samples of native, aggregated, and fused LDL particles were prepared for thin-section electron microscopy as described in ref. (96). Osmium-tannic acid-paraphenylenediamine staining, original magnification: ×30,000. Bars: 100 nm.

trypsin (92, 93). During apoB-100 proteolysis, some of the core lipids may also rise to the surface of the particles, which enhances their hydrophobicity. This could provide a mechanistically plausible explanation for the fusion of proteolyzed particles. This idea is supported by the finding that, at low temperature (15°C), when the cholesteryl esters of the LDL particle core are in a relatively ordered rigid state, proteolyzed LDL particles are resistant to fusion (94).

Lipolytic modifications

Sphingomyelinase. SMase hydrolyzes SM molecules in LDL particles, yielding water-soluble phosphocholine molecules, which are released, and ceramide molecules, which are retained in the particles. Treatment of LDL with SMase has been shown to induce aggregation and fusion of lipolyzed LDL particles (95–97), with aggregation and fusion resulting from the increase in the ceramide content of the particles (25) (Table 2). Ceramide, in contrast to unhydrolyzed SM, is known to induce lateral phase separation into regions of gel phases in PC bilayers, ceramide being largely partitioned in the resultant gel phase (98, 99). Once formed, the ceramide-enriched domains may act as nonpolar spots at the surface of the particles and lead initially to particle aggregation through hydrophobic associations. At low temperature, the rigid core lipids can inhibit lateral diffusion of phospholipids in the surface monolayer (100), and so should at least partially inhibit the formation of the ceramide-enriched microdomains. Indeed, lipolysis of LDL with SMase at 15°C and subsequent incubation of the modified particles at this temperature do not lead to aggregation or fusion of the particles. However, subsequent incubation of the lipolyzed particles at 37°C results in their aggregation and fusion (K. Öörni and P. T. Kovanen, unpublished observation).

TABLE 2. Relative abilities of different modifications to trigger aggregation, fusion, and vesicle formation of LDL particles

Modifying Agent	Aggregation	Fusion	Vesicle Formation	References
Proteases	+	+++	−	62, 64, 96
Sphingomyelinase	+++	++	−	25, 95–97
Phospholipase A_2	++	+	−	97, 106
Phospholipase C	+++	+++	−	110, 111
Cholesterol esterase	+	+	+++	92, 93
Oxidants	++	+	+	21, 96, 115–119

Phospholipase A_2. PLA_2 catalyzes hydrolysis of fatty acid esters in the *sn*-2 position of diacylglycerol phospholipids. If LDL is treated with PLA_2 in the absence of lipid-binding proteins, such as albumin, the lipolytic products, lysoPC and fatty acid molecules, accumulate in the LDL particles. However, in the presence of physiologic albumin concentrations, most of the fatty acid and some of the lysoPC molecules are transferred from LDL to albumin (101). Lipolysis of LDL with PLA_2 in the presence of albumin leads to conformational changes in the apoB-100 component and reorganization of lipids (101, 102). Therefore, it is not surprising that lipolysis of LDL by PLA_2 leads to aggregation of the lipolyzed LDL particles. However, lipolysis of LDL by PLA_2 does not lead to particle fusion (97) (Table 2). In fact, by electron microscopy the lipolyzed LDL particles appear slightly smaller than the native particles (97, 103, 104). In the smaller particles, the proportion of core lipids

in the surface monolayer increases and (105) likely leads to a surface lipid environment that is more rigid and has less lateral mobility than that of native particles (102). Therefore, although the surface hydrophobicity of such modified LDL particles must increase their tendency to aggregate, the enhanced structural rigidity of the particles may stabilize the aggregates and preclude particle fusion.

Interestingly, heparin, whether present before, during, or after lipolysis, triggers fusion of the PLA_2-lipolyzed LDL particles (Table 2) (106). Also, in the presence of PGs human secretory PLA_2 induces fusion of LDL (J. K. Hakala and P. T. Kovanen, unpublished observation). The finding that heparin treatment before lipolysis is able to trigger fusion of the lipolyzed LDL particles suggests that heparin causes irreversible changes in the LDL particles. Indeed, interaction between LDL particles and glycosaminoglycans (GAGs) induces irreversible changes in the conformation of apo B-100, which increase the exposure of the lysine- and arginine-containing segments (107), and decreases the organization of the core and surface regions of the particles (107, 108).

Phospholipase C. Phospholipase C (PLC) hydrolyzes phospholipids into phosphocholine and diacylglycerol. After treatment with PLC, phosphocholine is released from the LDL particles, whereas the hydrophobic DAG appears able to reside in both the surface and the core of the particles (109). PLC treatment of LDL results in both aggregation and fusion of the particles (110, 111) (Table 2). The aggregation and fusion of the PLC-treated LDL particles appears to be due to formation of hydrophobic surface domains on the LDL particles. Consistently, the amphipathic apolipoproteins apoA-I and apoE (112) and *Manduca sexta* apolipophorin III (111), which bind to the particles and potentially cover the newly formed hydrophobic domains, inhibit PLC-induced aggregation and fusion of LDL particles. PLC-induced enrichment of DAG alone seems insufficient to induce PLC-dependent aggregation and fusion, because induction of LDL particle aggregation and fusion requires an increase in the neutral lipid content of the LDL particles of more than 18% (113), which exceeds the amount potentially formed by PLC in LDL particles.

Oxidative modification

Mildly oxidized LDL particles have a tendency to aggregate (Table 2). This phenomenon has been consistently observed in studies of different oxidants, such as copper, 2,2′-azobis(2-amidino-propane)hydrochloride (AAPH), and hypochlorite (21, 114, 115). Extensive oxidation of LDL by copper ions results in loss of particle integrity, demonstrated by both aggregation and fusion of LDL particles and formation of vesicles and membranous structures (Table 2) (21, 96, 114–119). In contrast to the other LDL modifications, which modify either the protein or the lipid component of the LDL particle, oxidative modifications of LDL modify both components. Thus, lipid peroxides are formed that decompose further into aldehydes capable of reacting with apoB-100 and ultimately lead to oxidative degradation of amino acids of apoB-100 and cleavage of apoB-100 into peptides (84).

What, then, are the critical oxidative changes in LDL that can lead to aggregation and fusion of LDL particles? Changes in lipids during oxidation resemble those caused by lipolytic enzymes. Thus, oxidation initially attacks unsaturated fatty acids of surface phospholipids, which can then be hydrolyzed by the endogenous PLA_2 of LDL particles. As discussed above, the generated lysophospholipids and free fatty acids can be transferred to albumin, leading to changes in the LDL surface that increase the tendency of LDL to aggregate. Extensive oxidation, in turn, has been shown to decrease the mobility of the surface phospholipids, to disturb lipid-apoB-100 interactions, and, at later stages, to increase the polarity of the lipid phase (120–122). Oxidative changes in apoB-100 also play a role in LDL aggregation and fusion. Aldehydes, which react with apoB-100, have been shown to be able to cause LDL to aggregate (21). In addition, other oxidative changes in apoB-100 occur, because oxidation by hypochlorite, which readily modifies apoB-100 but results in the production of only minor amounts of MDA, also leads to aggregation of LDL. Moreover, during oxidation the hydrophobic regions of apoB-100 are preferentially degraded (123) and relocated to the particle surface (120). Thus, oxidation causes a number of changes in both the lipids and apoB-100 of LDL, which may eventually lead to loss of particle stability with ensuing aggregation and fusion.

Modification by cholesteryl ester hydrolysis

Hydrolysis of the cholesteryl esters of LDL with fungal cholesterol esterase produces small lipid droplets and vesicles from LDL particles (92, 93) (Table 2). If the particles are additionally treated with neuraminidase, the morphologic changes increase still further (93). Because cholesteryl ester hydrolysis requires some disruption in the apoB-100 component of LDL particles (92, 93), it appears that, in native LDL, the number of surface-penetrating cholesteryl ester molecules accessible to the enzyme is insufficient for the proper functioning of the enzyme.

Hydrolysis of the hydrophobic cholesteryl ester core of LDL, with concomitant generation of large amounts of unesterified cholesterol, must increase the hydrophilic character of the particles. Moreover, loss of core hydrophobicity must lead to an energetically unfavorable situation for the hydrophobic fatty acid chains of the surface phospholipids. This, combined with the tendency of unesterified cholesterol molecules to be incorporated into membrane-like structures, would explain the generation of liposomes and complex multilamellar structures from cholesterol esterase-treated LDL particles (92, 93).

INTERACTION OF MODIFIED LDL PARTICLES WITH PROTEOGLYCANS

Binding of LDL to PGs is mediated by ionic interactions between the negatively charged sulfate and carboxyl groups of the GAGs and the positively charged amino acid

residues in apoB-100 (124, 125). ApoB-100 contains multiple PG-binding sites (126, 127), but, interestingly, genetic modification of one of the sites (amino acid residues 3359–3369) is sufficient to decrease the binding of LDL to PGs significantly (8). The strength of the interaction between apoB-100 and GAGs depends on the properties of both the LDL particles and the GAG chains. The effects of the size and charge of the GAG chains have been reviewed by Jackson, Busch, and Cardin (128) and Camejo et al. (4). We have been studying how enzymatic and oxidative changes in LDL particles influence the interaction between LDL and PGs, notably how LDL modification affects the strength of binding and how this binding affects LDL modification.

Binding of modified LDL to proteoglycans

Small dense LDL particles have higher affinity for PGs than do large LDL particles (129, 130). This has been suggested to depend on differences in the conformation of apoB-100 in LDL particles of different sizes (131). Indeed, the conformation of apoB-100 has been shown to change, especially in the carboxy terminus of the protein, as large LDL particles are converted to smaller LDL (132). Interestingly, PLA_2 modification of LDL has been shown to produce small dense LDL particles with increased affinity for PGs and GAGs (104). Moreover, patients with rheumatoid arthritis, who have high levels of circulating PLA_2, also have increased levels of small dense LDL particles with increased binding affinity for PGs (133). Also, LDL particles isolated from patients with coronary heart disease bind to PGs and GAGs more avidly than does LDL from control patients (134, 135).

We examined the effects of lipolytic and proteolytic modifications on the binding of LDL to human aortic PGs immobilized on affinity columns (**Fig. 3**) (97, 136). On the PG column, the binding of the small PLA_2-treated LDL particles was similar to that of untreated LDL particles. Also, mere lipolysis of LDL with SMase did not appear to affect the strength of binding of the particles to PGs. With a gel mobility shift assay, Sartipy et al. (104) found that the affinity of the small PLA_2-treated LDL to PGs was increased. The difference is likely to be due to the higher sensitivity of the gel mobility shift assay in detecting small changes in affinity. In contrast, aggregated and fused lipolyzed particles bound to the PGs more tightly in the affinity column than native LDL. This is not surprising, because there is more than one copy of apoB-100 per LDL aggregate or per fused LDL particle. It is, however, surprising that, of the proteolyzed particles, not only the fused but also the unfused LDL particles bound to the PGs more avidly than native LDL (136). In an attempt to find an explanation for this observation, we examined how LDL modifications affect a special population of lysine residues, "active lysine residues," of apoB-100. The active lysine residues have been suggested to mediate the binding of LDL to PGs and they can be studied with nuclear magnetic resonance spectroscopy (137). Interestingly, despite the loss of lysine-containing peptide fragments from the proteolyzed LDL particles, the number of active lysine residues was not decreased in the proteolyzed LDL particles. In fact, there was an increase in the number of active lysine residues in the fused proteolyzed (136). Similarly, the number of active lysine residues was increased in the fused lipolyzed LDL particles (97). Thus, during particle fusion, conformational changes in the proteolyzed apoB-

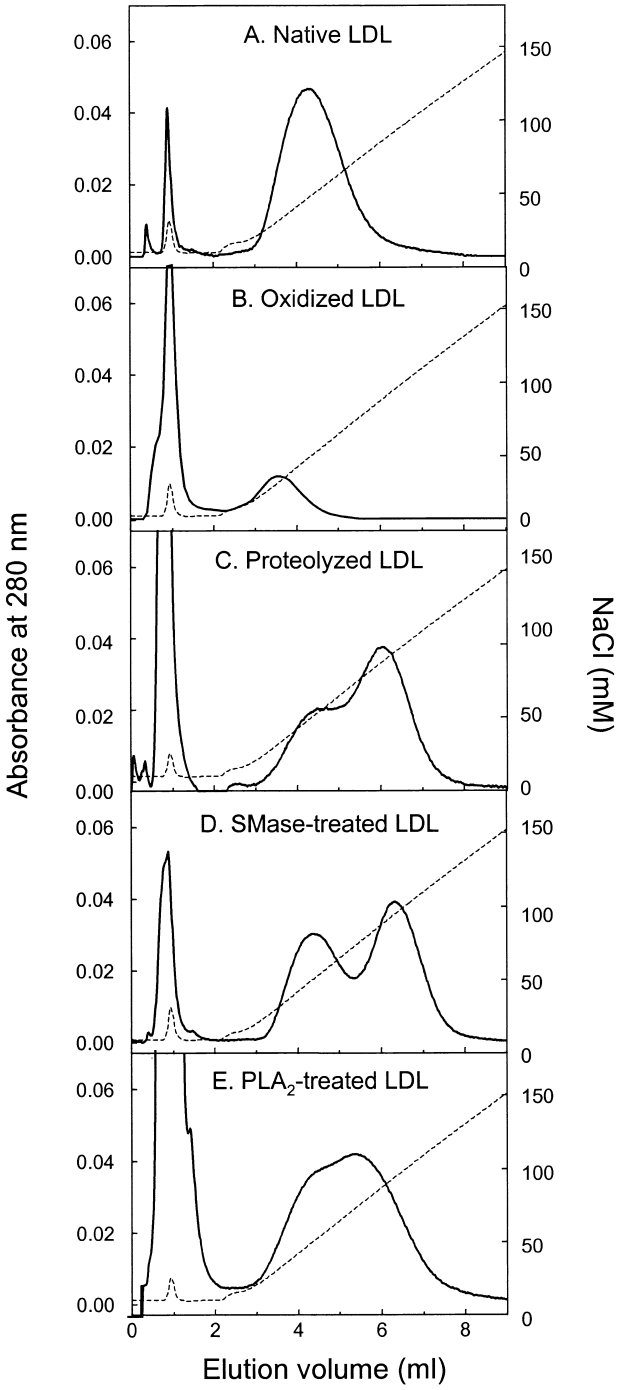

Fig. 3. Affinity chromatography of native and modified LDL on a human aortic proteoglycan column. Samples corresponding to 10–20 μg of LDL (A), LDL oxidized with copper sulfate for 6 h (B), LDL treated with α-chymotrypsin for 16 h (C), LDL treated with SMase for 1 h (D), and LDL treated with PLA_2 for 1 h (E) were applied to a 1-ml proteoglycan HiTrap column and eluted with a linear gradient of 0–250 mM NaCl. Adapted from refs. (97), (136), and (140).

100 fragments and in the apoB-100 of the lipolyzed particles appear to occur. These changes may either expose some shielded PG-binding regions in the apoB-100 or bring positively charged regions of apoB-100 together to form a new PG-binding site.

During oxidation, the decomposition products of LDL lipids, such as MDA and HNE, are known to react with the lysine residues of apoB-100, thereby neutralizing them (138). Therefore, it is not surprising that MDA modification of LDL blocks the binding of LDL to heparin (139). Consistently, we found that oxidation of LDL in vitro decreased the binding of LDL to arterial PGs (Fig. 3B) (140). Regardless of the method of oxidation used (copper sulfate, AAPH, mouse peritoneal macrophages, soybean lipoxygenase), it was found that the higher the degree of LDL oxidation, the fewer lysine residues of apoB-100 remained unmodified and the weaker was the ability of the LDL particles to bind to PGs. Indeed, it appeared that modification of the lysine residues during oxidation was a necessary and sufficient prerequisite for reducing binding strength of LDL to PGs (140). In addition to reducing the amount of LDL bound to PGs, oxidative modifications can even release LDL from GAGs (141).

Interaction of modified LDL with proteoglycans in the presence of lipoprotein lipase

LPL, in addition to its ability to hydrolyze the triglycerides of postprandial triglyceride-rich lipoproteins, has been shown to possess a nonenzymatic function, that is, to link VLDL and LDL to GAGs and various cell surface receptors [reviewed by Goldberg (142)]. In contrast to the situation with PGs, oxidation increases the affinity of LDL for LPL (143). We performed a series of experiments to explore the mechanisms by which native and modified LDL are linked to decorin PG by LPL, and found that, in contrast to native LDL, which preferentially bound to monomeric, catalytically inactive LPL, oxidized LDL bound to the active dimeric LPL, regardless of the method used to oxidize the LDL (**Fig. 4**) (144). Binding to dimeric LPL did not appear to involve apoB-100, because reconstituted LDL devoid of apoB-100 also bound to dimeric LPL. Moreover, extensive proteolysis of apoB-100 led to binding of LDL to dimeric LPL, suggesting that apoB-100 actually hindered binding of LDL to LPL. Because each type of oxidation studied is known to cause some modification of apoB-100, it is possible that such perturbations of apoB-100 structure can overcome its shielding ability, and so allow oxidized LDL to bind to the dimeric form of LPL.

Effects of proteoglycans on LDL modification

As discussed above, binding of LDL to GAGs induces irreversible changes in the conformation of apoB-100 and in the organization of LDL lipids: exposure of the arginine- and lysine-containing segments of apoB-100 increases (107) and organization of the core and surface regions of the particles decreases (107, 108). These GAG-induced modifications of LDL promote atherogenic modifications of LDL. Thus, binding of LDL to PGs increases the rate of proteolysis of apoB-100 and of subsequent fusion of the proteolyzed particles in vitro (145). Similarly, formation of soluble complexes between LDL and PGs or GAGs increases the rate of LDL proteolysis by trypsin and, to a lesser extent by the proteinase V8 (107). The increased rate of apoB-100 degradation was suggested to depend on GAG-induced exposure of lysine- and arginine-rich apoB-100 segments and on a less marked effect on segments rich in aspartate and glutamate. In addition, the sensitivity of LDL to oxidation increases after binding and subsequent release of LDL from chondroitin sulfate PGs and GAGs (146). PGs may also participate in lipolytic modification of LDL: binding of secretory PLA_2 to decorin PG increases its enzymatic activity (147) and PLA_2-treated LDL particles undergo fusion only if the particles have been bound to GAGs (106). Finally, a variety of enzymes can bind to GAGs via their "heparin-binding domains." This may enhance the availability of LDL to the enzyme: proteases that are bound to GAGs, such as mast cell chymase, preferentially hydrolyze LDL particles also bound to GAGs (148).

CONCLUDING REMARKS

The atherosclerotic arterial wall contains a number of enzymes and agents capable of modifying LDL particles.

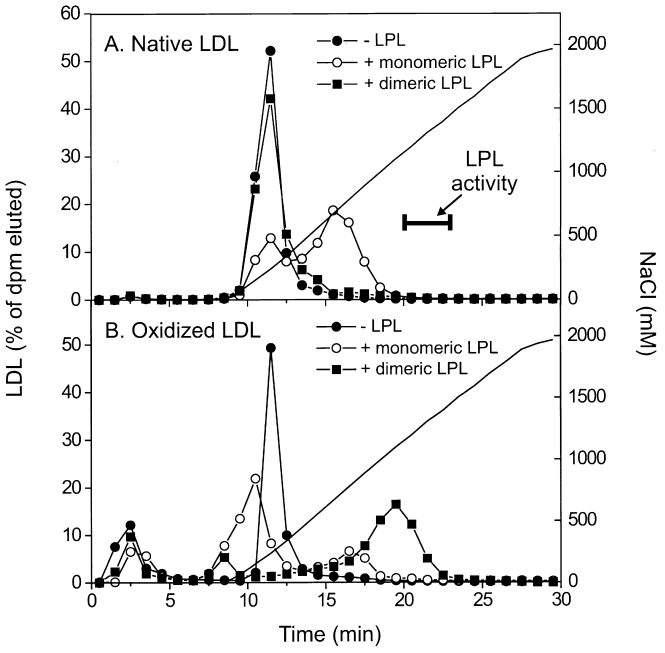

Fig. 4. Affinity chromatography of native (A) and Cu^{2+}-oxidized (B) LDL on heparin columns in the absence and presence of monomeric and dimeric LPL. Radiolabeled native LDL and LDL oxidized with copper sulfate for 18 h at 37°C were applied to heparin columns and eluted with a gradient of 0–2 M NaCl (−LPL). In the indicated experiments, this was preceded by loading of the columns with 100 μg of LPL inactivated by incubation with guanidium HCl (+ monomeric LPL) or with 100 μg of LPL followed by washing of the columns with 1 M NaCl to release monomeric, but not dimeric, LPL (+ dimeric LPL). The bar in (A) shows elution of dimeric LPL with catalytic activity. Adapted from ref. (144).

Accordingly, the presence of aggregated lipid droplets and vesicles within the ECM of the arterial wall can be attributed to extracellular modification of infiltrated plasma LDL particles. Initial retention of LDL in the ECM appears to be a prerequisite for LDL modification. Interestingly, the ECM appears to play an active role in the modification of LDL on account of its ability to modulate the activity of various enzymes toward LDL. The modifications differ significantly in their effects on LDL-matrix interaction, which is likely to be one determinant of the ability of intimal cells to process modified LDL. Because LDL modifications are known to create a variety of proinflammatory molecules, the significance of LDL modifications may not be limited to lipid accumulation per se. Thus, understanding of the molecular mechanisms of LDL modifications also aids in understanding of the biology of atherogenesis. In addition to LDL, also other apoB-100-containing lipoproteins that are retained in the arterial intima, such as small VLDL and lipoprotein[a], can be modified by the intimal enzymes and agents. Therefore, modification of these lipoproteins may also turn out to be important in the development of atherosclerotic lesions.

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