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NONOXIDATIVE MODIFICATIONS OF LIPOPROTEINS IN ATHEROGENESIS

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ABSTRACT

The key initiating event in atherosclerosis is the retention of plasma lipoproteins in the subendothelial matrix. Subsequently, a series of biological responses to this retained material leads to specific molecular and cellular processes that promote lesion formation. There is considerable evidence that many of these biological responses, notably macrophage cholesteryl ester loading (foam cell formation), require subendothelial modification of the retained lipoproteins. Oxidation of lipoproteins is one such modification that likely occurs in vivo and promotes certain atherogenic events, but oxidation cannot explain all aspects of atherogenesis, including certain elements of macrophage foam cell formation. For this reason, there has been renewed interest in other modifications of lipoproteins that may be important in atherogenesis. This review addresses five such lipoprotein modifications, namely aggregation, glycation, immune complex formation, proteoglycan complex formation, and conversion to cholesterol-rich liposomes. The focus is on the evidence that these modifications occur in atherosclerotic lesions and on the potential role of these modified lipoproteins in atherogenesis, with an emphasis on macrophage foam cell formation.

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INTRODUCTION

The first experimental evidence linking plasma cholesterol levels with the development of atherosclerotic lesions in rabbits was reported approximately 80 years ago (3). Since then, extensive data has confirmed this association in other experimental animal models and, most important, in man (37). One of the strongest associations for the development of atherosclerosis in man is the level of plasma low-density lipoprotein (LDL) (37), and so there has been much interest in the mechanisms linking plasma LDL levels with atherogenesis. A theory that nicely fits some of the most compelling experimental data is the response-to-retention model, which states that the key initiating event in atherogenesis is the retention of LDL and other atherogenic lipoproteins in the subendothelial matrix, chiefly proteoglycans, of susceptible regions of the arterial tree (107, 108). Once retained, specific biological responses to the lipoproteins lead to biochemical and cellular events that promote atherogenesis, such as endothelial cell alterations, chemotaxis and cholesterol loading of macrophages, and smooth muscle cell migration and proliferation (107, 108). The strongest evidence in support of this model is recent data showing that a single amino acid alteration in apolipoprotein B-100, the protein moiety of LDL, can prevent LDL attachment to arterial-wall proteoglycans (8). What is remarkable is that mice that overexpress this mutant apoB-100 develop very little atherosclerosis compared with wild-type littermates despite equally high cholesterol levels (9).

One of the most prominent and pathologically important responses to subendothelial lipoprotein retention is cholesteryl ester (CE) loading of macrophages (foam cell formation) (19, 22, 49, 82, 88). CE loading occurs when lipoproteins or possibly other cholesterol-rich particles are internalized by macrophages (11, 97). Typically, the original CE of the internalized lipoproteins is hydrolyzed in lysosomes, and the resulting free cholesterol is reesterified by the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), leading to accumulation of cytoplasmic CE droplets (11, 97). Lesional macrophages, particularly in advanced atherosclerosis, also accumulate some of the original, unhydrolyzed lipoprotein CE in lysosomes (36). It is interesting that native plasma LDL, the levels of which correlate strongly with atherogenesis (above), is a poor inducer of macrophage foam cell formation in vitro (11). The explanation of

this finding is related both to decreased uptake of LDL due to partial down-regulation of the LDL receptor on foam cells (11) and to intracellular cholesterol metabolic events that do not favor cholesterol esterification (97). Moreover, native remnant-like lipoproteins from the plasma of apolipoprotein E knockout mice, a robust model of atherosclerosis and lesional foam cell formation, also fail to substantially load cultured macrophages with CE; the explanation appears to be related to the absence of apolipoprotein E, a receptor ligand, on these lipoproteins (26). Much effort, therefore, has been devoted to finding physiologically relevant modifications of LDL that could promote macrophage foam cell formation and perhaps other atherogenic processes.

OVERVIEW OF ATHEROGENIC LIPOPROTEIN MODIFICATIONS

From the initial search for atherogenic lipoprotein modifications emerged the vast area of oxidative modification of lipoproteins (89, 90). Many groups have now shown (*a*) that epitopes of oxidized LDL exist in atherosclerotic lesions, (*b*) that oxidized LDL can be readily internalized by macrophages, (*c*) that oxidized LDL can induce proatherogenic changes in endothelial cells, and (*d*) that in cholesterol-fed rabbits, antioxidants are antiatherogenic (89). Major questions remain, however, about the oxidation hypothesis. For example, in some recently reported human trials (18, 68, 76, 91), as well as in studies with LDL receptor-deficient rabbits (21) and murine models of atherosclerosis (7, 115), protection of plasma LDL with antioxidants was not associated with a decrease in atherosclerosis. In addition, there may be mechanisms other than oxidation of lipoproteins that lead to the generation of oxidized lipids in lesions (35). Furthermore, some of the endothelial changes attributed to oxidized LDL, including the induction of monocyte adhesion molecules, may occur in the presence of native LDL (1, 117).

Regarding the important process of macrophage foam cell formation, a widely held concept is that oxidized lipoproteins universally stimulate cholesterol esterification in cultured macrophages. One on hand, LDL subjected to relative mild oxidation conditions in vitro has been recently reported to cause lysosomal CE accumulation (i.e. original lipoprotein CE without reesterification) in human macrophages and cytoplasmic CE inclusions (i.e. reesterified cholesterol) in mouse macrophages after prolonged incubation (111). Several groups of investigators, however, have shown that macrophages incubated with LDL oxidized under other conditions accumulate mostly unesterified cholesterol (40, 62, 77, 80). This phenomenon may be due to a decrease in the CE content of the lipoprotein as a result of oxidation (80) or to decreased export of free cholesterol (FC) from lysosomes (32, 56, 59). Enrichment of oxidized

LDL in vitro with cholesterol enhances the ability of the lipoprotein to accumulate CE (24), but there is no evidence that this occurs in vivo. Mice deficient in class A scavenger receptors, one of the receptors for oxidized LDL (44, 89), have reduced foam cell lesions (95). This observation, however, by no means rules out a role for nonoxidative modifications of LDL. First, certain forms of nonoxidatively modified LDL are also internalized by class A scavenger receptors (e.g. 6, 50, 103, 112). Second, although foam cell lesions are reduced in these mice, they are still prominent (95). Although the presence of foam cells in these mice might suggest a role for other receptors for oxidized LDL in foam cell formation in vivo, an equally plausible interpretation is that a significant portion of foam cell formation involves other types of modified lipoproteins.

LIPOPROTEIN AGGREGATION

Aggregated lipoproteins are prominent in atherosclerotic lesions (4, 25, 28, 65, 92) and are among the most potent inducers of CE loading of cultured macrophages (30, 38, 94, 99, 110). In addition, processes that promote lipoprotein aggregation before or during retention dramatically increase the amount of lipoprotein retained (69, 98), which, in turn might amplify the atherogenic responses to retention (107, 108). The mechanisms responsible for increased retention as a result of aggregation include increased binding of the aggregates to subendothelial matrix (69, 98) and decreased efflux of the aggregates from the arterial wall due to their increased size (cf 67, 101).

Several different methods have been employed to document the presence of lipoprotein aggregates in atherosclerotic lesions. Hoff & Morton (28) isolated apoB-100–containing particles from human atherosclerotic lesions using immunoaffinity chromatography and showed by gel filtration that a significant portion of these particles were either aggregated or fused. Similar data were obtained using LDL isolated by ultracentrifugation from human atherosclerotic lesions (86, 92) and from the lesions of atherosclerotic apolipoprotein E knockout mice (4). When examined, the aggregated lesional LDL fraction was always a potent inducer of macrophage foam cell formation (28, 92). Furthermore, Hoff & O'Neil (29) showed that the monomeric fraction of human lesional LDL was more susceptible than plasma LDL to aggregation when the lipoproteins were concentrated in vitro. The existence of aggregated LDL in atherosclerotic lesions was further demonstrated by Frank and colleagues (20, 65, 66) using an entirely different method, namely freeze-etch electron microscopy of rabbit aortic intima. Frank & Fogelman (20) showed that the aortic intima of Watanabe heritable hyperlipidemic (WHHL) and cholesterol-fed rabbits contained aggregated lipid particles bound to subendothelial matrix. Nievelstein et al (65) injected a bolus of human LDL into normocholesterolemic rabbits

and, remarkably, found matrix-bound aggregated and fused LDL in the intima as early as 2 h after the LDL injection (Figure 1).

Mechanisms of LDL Aggregation

What is the mechanism of LDL self-aggregation in vivo? Many in vitro treatments of LDL lead to aggregation (25), such as vortexing (38), extensive hydrolysis by phospholipase C (94), thiolation (64), and modification by flavonoids (75). The most physiologically plausible mechanisms, however, include extensive oxidation (4, 31), hydrolysis by sphingomyelinase (72, 83, 86, 110), and proteolysis by mast cell proteases (72). All three processes cause a combination of LDL aggregation and fusion, which is similar to what is observed in vivo

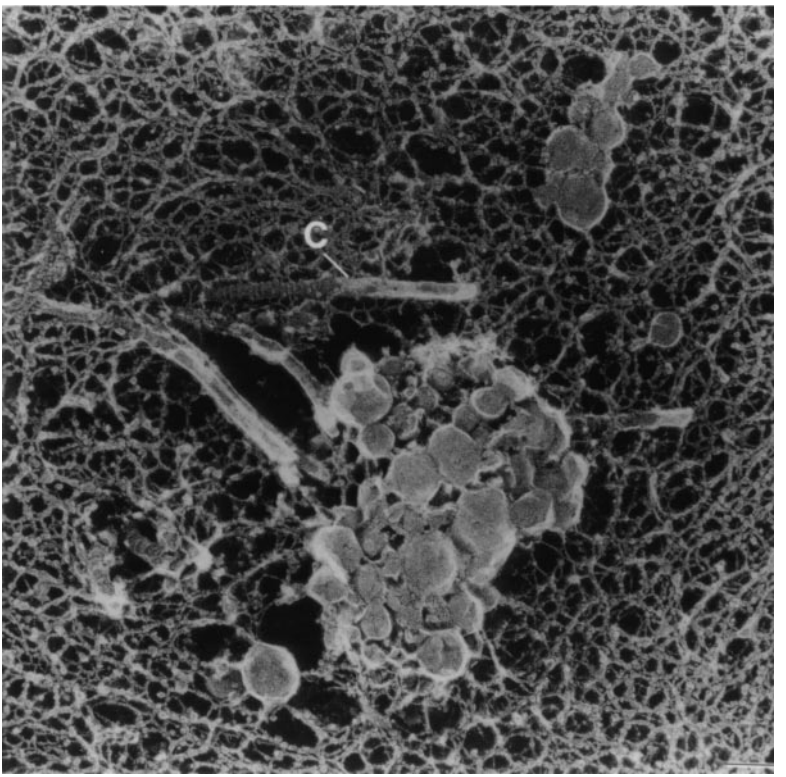

Figure 1 Electron photomicrograph of a freeze-etch replica from rabbit aorta intima 2 h after bolus injection of human low-density lipoproteins. The aggregated particles are surrounded by matrix and collagen fibrils (*C*). Reprinted from Reference 65 with permission from Dr. Joy Frank and the American Heart Association.

(72). Although oxidation may play a role in advanced lesions, it is unlikely that the extensive oxidation needed to cause LDL aggregation can explain the results of Frank and colleagues, i.e. aggregation occurring as early as 2 h in an otherwise normal aorta (see above). Similarly, proteolysis by mast cell proteases may be important in advanced atheromata (below) but cannot explain lipoprotein aggregation in early lesions, i.e. before the entry of mast cells. A form of sphingomyelinase (SMase), however, is secreted by endothelial cells and could play a role in early lesion development (see below).

MAST CELL–MEDIATED MODIFICATION OF LDL Kovanen has investigated the role of mast cell–mediated modifications of LDL in foam cell formation (43). Mast cell granules contain heparin-bound chymase and carboxypeptidase A. The number of mast cells and the amount of secretion of mast cell granules increase as atherosclerosis progresses (43). In rat serosal mast cells in culture, he demonstrated the following series of events (42). Upon secretion of mast cell granules, LDL becomes bound to the heparin proteoglycan component of the granule remnants. The proteoglycan-bound LDL then becomes a potent inducer of foam cell formation from both macrophage and smooth muscle cells. It appears as if the proteoglycan-LDL complexes themselves can lead to foam cells via scavenger receptor–mediated phagocytosis (see below; see also 50). Foam cell formation is greatly promoted, however, by proteolysis of the apolipoprotein B-100 of LDL by chymase and carboxypeptidase A from granule remnants (41), which causes LDL fusion and aggregation. This mechanism also may explain the presence in atheromata of CE-rich droplets that appear to be derived from LDL yet immunostain poorly for apolipoprotein B-100 (27, 73).

HYDROLYSIS OF LDL BY SMASE Hydrolysis of approximately 25% of the sphingomyelin (SM) of LDL to ceramide in vitro by bacterial SMase leads to the formation of aggregated and fused particles that are excellent inducers of macrophage foam cell formation (72, 98, 110) (Figure 2). Two lines of evidence suggest that a mammalian arterial-wall SMase may carry out a similar reaction in atherosclerotic lesions in vivo. First, the aggregated fraction of LDL extracted from human atherosclerotic lesions is enriched in ceramide, whereas monomeric lesional LDL and matched plasma LDL are not (86). Because ceramide transfers poorly onto lipoproteins, this finding most likely indicates that the LDL was directly hydrolyzed by a SMase. Second, when [^{3}H]SM-labeled LDL was incubated with strips of rabbit aorta, a portion was retained in the subendothelium [similar to what happens during early atherogenesis in vivo (see above)]; this retained portion, but not unretained [^{3}H]SM-labeled LDL, contained [^{3}H]ceramide (86).

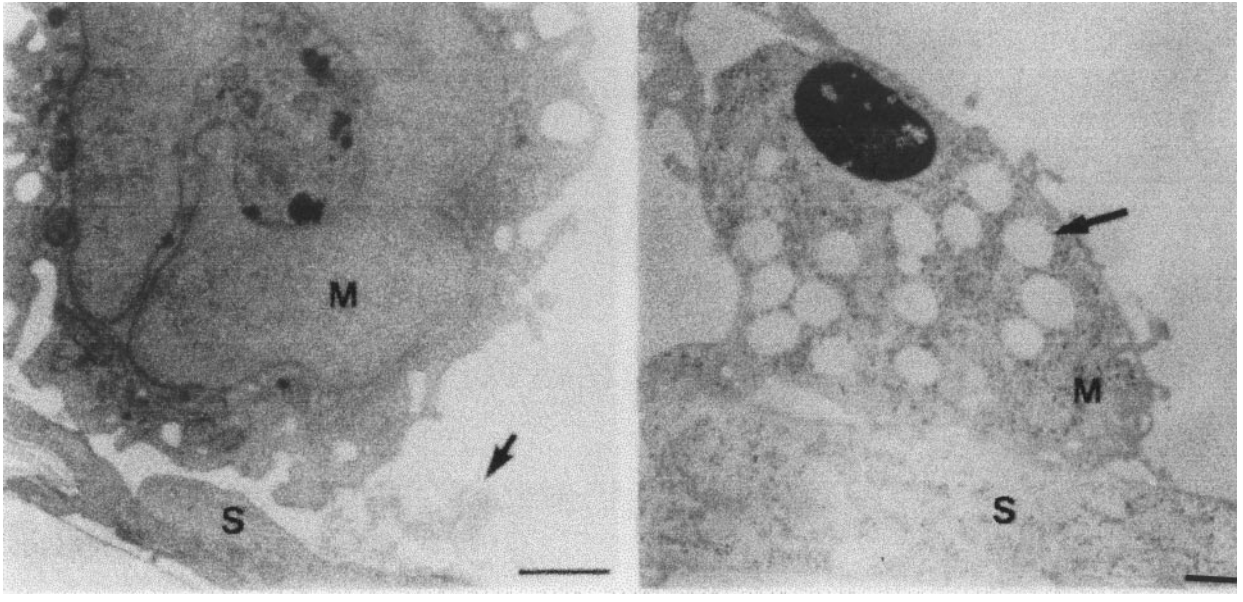

Figure 2 (*Left*) Electron photomicrograph of a macrophage (*M*) 1 h after being plated on top of sphingomyelinase-induced low-density lipoprotein (LDL) aggregates (*arrow*) retained on the surface of a smooth muscle cell (*S*). (*Right*) Photo taken 24 h after the addition of macrophages: The LDL aggregates are gone and the macrophage shows abundant cytoplasmic cholesteryl ester droplets (*arrow*) characteristic of foam cells. *Bar*, 1 μm. Reprinted from Reference 98 with permission of the American Society for Biochemistry and Molecular Biology.

During a search for a mammalian SMase that might carry out this extracellular vessel-wall reaction, Schissel et al (84, 85) discovered a novel form of SMase called secretory SMase, or S-SMase. Although S-SMase is secreted by macrophages, it is secreted even more abundantly by endothelial cells (60). Thus, S-SMase could be present in prelesional arteries and therefore might explain very early lipoprotein aggregation (see above). In fact, Öörni et al (69) have shown that LDL aggregation/fusion mediated by SMase leads to more avid binding to human aortic proteoglycans, which suggests that SMase also may promote lipoprotein retention (98). Further evidence implicating a role for S-SMase in lipoprotein aggregation in atherosclerotic lesions includes its ability to hydrolyze and aggregate atherogenic lipoproteins (83), its increased secretion from endothelial cells treated with cytokines that have been implicated in atherogenesis (60), and its presence in atherosclerotic lesions by immunohistochemistry (S Marathe, G Kuriakose, KJ Williams, & I Tabas, submitted for publication). Finally, SMase-induced aggregation of LDL is inhibited by high-density lipoproteins and apolipoprotein A-I (S Schissel & I Tabas, unpublished observations; see also 39), which suggests a possible arterial wall–based mechanism to explain at least part of the antiatherogenic effect of these agents (cf 74, 79).

Aggregated Lipoproteins as Inducers of Macrophage Foam Cell Formation

As mentioned above, aggregated lipoproteins are among the most potent inducers of macrophage CE accumulation (28, 38, 94, 99, 110). This was clearly demonstrated using vortexed LDL (38) and phospholipase C–treated LDL (94). Moreover, vortexed aggregated LDL can induce CE loading of vascular smooth muscle cells in culture (51), which is another cellular event in atherosclerosis (78). Uptake of these forms of aggregated LDL by macrophages appears to involve phagocytosis, as indicated by susceptibility to cytochalasin D and by morphological criteria, and internalization was mediated by the LDL receptor (38, 94, 110). Recent, more detailed morphological studies (116) showed that vortexed aggregated LDL enters deeply convoluted surface-connected compartments of macrophages. Entry into the these compartments was dependent on an intact actin cytoskeleton but not on the LDL receptor (116). Intracellular degradation of the aggregated LDL was relatively slow and was, unlike entry into the surface compartments, dependent on the LDL receptor (116). These surface-connected compartments are reminiscent of cell-surface structures called STEMs (surface tubules for entry into macrophages), which mediate the interaction of macrophages with another atherogenic lipoprotein, β-VLDL (63). Acetylated LDL, another potent foam cell inducer, also demonstrates prolonged interaction with the macrophage cell surface (46, 113). Therefore, the surface-connected compartments utilized by aggregated LDL may be

involved in special cholesterol metabolic pathways that are critical to foam cell formation (97).

GLYCATION OF LDL

Diabetes is a well-known risk factor for atherosclerosis, and investigators have studied whether specific modifications of lipoproteins by glycation may contribute to this susceptibility. In particular, diabetics, as well as nondiabetics with renal failure, have increased levels of LDL modified by advanced glycation end products (AGEs) in plasma as well as in atherosclerotic lesions (55, 105). Several investigators have observed that LDL obtained from diabetics is recognized poorly by LDL receptors in cultured cells and has delayed clearance in vivo (12, 93). A possible mechanism for this effect may be the presence of AGEs in the area of the receptor-binding region of apolipoprotein B-100 (13, 106), which would block clearance by hepatocytes. Another potential atherogenic effect of glycated lipoproteins may be related to the fact that macrophages and endothelial cells possess receptors for AGEs, including class A scavenger receptors and the receptor for AGE (RAGE) (55, 105). Therefore, it is possible that the interaction of AGE-modified LDL with these receptors leads to cellular lipid loading (53, 57) and/or specific cellular responses, such as oxidant stress, that promote atherogenesis (55, 105). Additional effects of LDL glycation demonstrated in vitro include increased binding to arterial-wall proteoglycans (see above), increased susceptibility to oxidation, increased formation of LDL-containing immune complexes (see below), enhancement of thrombin-induced platelet aggregation, and antifibrinolytic effects on vascular endothelial cells (55, 114).

LDL-CONTAINING IMMUNE COMPLEXES

Autoantibodies against both oxidized LDL and glycated LDL (see above) as well as circulating anti-LDL–LDL immune complexes have been found in human plasma, particularly in patients with vascular disease; as expected, antiglycated LDL antibodies are found primarily in diabetic patients (71, 96, 109). The potential significance of these observations is that LDL-containing immune complexes, perhaps facilitated by erythrocyte absorption, can induce macrophage foam cell formation in vitro (54), evidently via internalization by macrophage Fc gamma R1 receptors (52). According to Huang et al (33), internalization of LDL-containing immune complexes leads to a paradoxical stimulation of transcription of the gene for the LDL receptor, although the cellular mechanism of this surprising effect remains to be elucidated. LDL-containing immune complexes also stimulate release of interleukin-1β and

tumor necrosis factor-α, as well as a respiratory burst in cultured macrophages, events that may promote atherogenesis (104). It must be noted, however, that immunization of rabbits with LDL and oxidized LDL to generate high levels of anti-lipoprotein antibodies decreased the development of atherosclerotic lesions (2, 70). Thus, in vivo, either the potential proatherogenic affects of LDL-containing immune complexes are overcome by their antiatherogenic effects (e.g. increased lipoprotein clearance) or the "natural" formation of these immune complexes in patients with vascular disease is fundamentally different from their formation as a result of immunization (e.g. "natural" formation may occur locally in the arterial wall, which may be mostly proatherogenic).

LDL-PROTEOGLYCAN COMPLEXES

LDL binds to arterial-wall proteoglycans of the subendothelial matrix in vivo (see above), and LDL can form complexes with aortic proteoglycans in vitro (34, 81, 103, 112). These LDL-proteoglycan complexes are internalized by cultured macrophages and smooth muscle cells and lead to foam cell formation (34, 81, 102, 103, 112), although one study showed that much of the internalized material was sequestered in lysosomes (81). Macrophage uptake of the complexes can be blocked partially by acetyl-LDL but not by polyanionic compounds that are known to block uptake by the class A scavenger receptors (103, 112). The enhanced uptake of LDL-proteoglycan complexes is not dependent on LDL aggregation but rather may involve a change in the surface of LDL (34). Of note, the stimulation of cholesteryl ester synthesis by LDL-proteoglycan complexes in smooth muscle cells required coculturing with macrophages (102). Although these in vitro studies are interesting, major questions about the significance and mechanisms related to this model remain, including whether LDL-proteoglycan complexes are internalized by macrophages in the arterial wall and the nature of the receptor that mediates the uptake of the complexes.

LDL-DERIVED FREE CHOLESTEROL-RICH LIPOSOMES

Free cholesterol-rich liposomes are present in atherosclerotic lesions of both humans and experimental animals (14, 27, 61). It is hypothesized that these liposomes are derived from lesional LDL, although secretion of liposomes from foam cells (45) or generation from the surface of chylomicrons and VLDL during lipolysis (16) are other possible mechanisms. The best-studied in-vitro manipulation that leads to the formation of free cholesterol-rich liposomes from LDL is treatment of proteolyzed or oxidized LDL with cholesteryl ester

hydrolase (6, 15). In lesions, it is possible that proteases (43) and cholesterol esterase (47, 48) are secreted by cells, or these enzymes may be released from the lysosomes of necrotic cells (5). It is interesting that lesional lipid droplets that have the properties of the above-described free cholesterol-rich liposomes can activate the alternative complement pathway in vitro (87); note that there is some evidence that the complement pathway is active in atherosclerosis (87). LDL treated in vitro with trypsin, cholesterol esterase, and neuraminidase resembles lesional lipid droplets in terms of both morphology and complement-activating activity (6); this enzyme-modified LDL also can enter macrophages by class A scavenger receptors and cause foam cell formation (6). Monoclonal antibodies that recognize enzyme-modified LDL and lesional complement-activating liposomes, but not native or oxidized LDL, stain human early coronary artery lesions in the vicinity of C5b-9 deposits (100).

CONCLUSIONS AND PERSPECTIVE

Atherosclerosis is the underlying etiology of the most fatal diseases in the industrialized world, myocardial infarction and stroke (10). Given the aging of the world population and the increasing ingestion of saturated fats by societies with previously low-fat diets, this situation is likely to worsen in the next 50 years (10). To help combat this major global health problem, investigators have successfully developed drugs that lower plasma LDL levels and decrease the incidence of vascular disease and death (23). What is striking, however, is the large percentage of treated patients who remain at risk for vascular disease despite marked LDL lowering (58). In fact, all the currently known risk factors for cardiovascular disease fall far short in explaining the difference in risks among individuals (17). These findings strongly suggest that unknown risk factors still need to be identified and then treated in order to substantially decrease the currently high incidence of vascular disease. Past and recent work in support of the response-to-retention hypothesis (107, 108) implicate arterial-wall molecules that promote either the subendothelial retention of lipoproteins or the response to these retained lipoproteins as likely candidates for these "unknown" risk factors. Among such molecules are those that promote atherogenic lipoprotein modifications, particularly modifications that convert plasma lipoproteins into particles that can induce macrophage CE loading or foam cell formation. Clearly, lipoprotein oxidation occurs in lesions and appears to have some role in certain atherogenic events. As discussed in this review, however, foam cell formation per se and other atherogenic events may be best explained, or at least additionally explained, by other modifications, including lipoprotein aggregation, lipoprotein glycation, lipoprotein-containing immune complex formation, lipoprotein-proteoglycan complex formation, and lipoprotein-derived liposome

formation. In the context of the substantial work that remains to be done in combating atherosclerotic vascular disease, the determination of which of these modifications are important in atherogenesis in vivo, the identification of molecules responsible for such modifications, and the eventual development of therapeutic strategies to prevent or reverse the modifications represent worthy goals in heart disease research.

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