Oxidation of Low Density Lipoproteins Greatly Enhances Their Association with Lipoprotein Lipase Anchored to Endothelial Cell Matrix*

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Native and oxidized low density lipoprotein retention within arterial wall endothelial cell matrix (ECM) is an early event in the pathogenesis of atherosclerosis. Previously we showed lipoprotein lipase (LPL) addition to ECM enhanced the retention of apoB-containing lipoproteins. In the present studies we examined whether the oxidation of low density lipoprotein (LDL) increases its retention by LPL-containing ECM. Except where noted, 125I-labeled moderately oxidized LDL (ModOxLDL) was prepared by long term storage of 125I-LDL. Without LPL, 125I-ModOxLDL matrix binding was low and nonrepeatable. LPL preanchored to ECM resulted in 125I-ModOxLDL binding that was saturable and 20-fold greater than in the absence of LPL, with an association constant equal to 2.6 nM. Copper-oxidized LDL (Cu-OxLDL) was able to compete with 125I-ModOxLDL, whereas a 60-fold native LDL excess had no effect. Reconstituted apolipoprotein B from Cu-OxLDL also reduced 125I-ModOxLDL to LPL, whereas liposomes derived from the lipid extract of Cu-OxLDL had no effect on binding. These data suggest that the increased binding of oxidized LDL to LPL-ECM may be due to the exposure of novel apoB binding sites and not an oxidized lipid moiety. 125I-ModOxLDL binding was also not affected by either preincubation with a 300-fold molar excess of apoE-poor HDL or an 340-fold molar excess of Cu-Ox-HDL. In contrast, a 4-fold apoE-rich HDL excess (based on protein) totally inhibited 125I-ModOxLDL matrix retention. Positively charged peptides of polyarginine mimicked the effect of apoE-rich HDL in reducing the 125I-ModOxLDL retention; however, polylysine had no effect. We postulate that the oxidation of LDL may be a mechanism that enhances LDL retention by the ECM-bound LPL and that the protective effects of apoE-containing HDL may in part be due to its ability to block the retention of oxidized LDL in vivo.

Low density lipoprotein oxidation is believed to be an early event in the initiation and progression of atherosclerosis (1–3). The oxidation of LDL* confers many biological properties on the molecule that render the lipoprotein more atherogenic (4–6). Several in vitro biologic consequences of oxidized LDL (OxLDL) have been demonstrated including, increased monocyte adhesion to endothelial cells (7–10), monocyte chemotaxis (11), decreased macrophage motility and increased cytotoxicity (12–15). Studies imply that OxLDL promotes recruitment and retention of monocytes/macrophages in the subintima. Oxidized LDL (OxLDL) or its components have also been shown to stimulate the expression of several endothelial cell proteins including vascular cell adhesion molecule-1 (16) and other adhesion molecules (17), monocyte chemotactic protein-1 (18, 19), macrophage colony-stimulating factor (20). Some of these effects may result from the activation of transcription factors such as NF-kB (21). OxLDL is also avidly scavenged by macrophages (22–24) and leads to macrophage-derived foam cell formation (3, 22).

The process of LDL oxidation is not very well understood, but it appears to occur within the artery wall, facilitated by cellular lipoxigenase-dependent mechanisms or by nonenzymatic pathways. The phospholipid fatty acids of LDL are believed to be oxidatively modified first (25, 26), resulting in the formation of phospholipids containing a spectrum of peroxidized fatty acids. Some of these, through chemical decomposition and/or enzymic hydrolysis, are believed to generate reactive aldehydes, which covalently modify the lysine residues of apoB and cause the surface charge to become more negative (27). Due to the oxidative attack, apoB also undergoes deavage, which exposes neoepitopes on the protein (28). Although most of the biological effects of OxLDL are thought to result from oxidized lipid products, the increased scavenger receptor uptake of OxLDL is mediated by changes in surface protein charge (3). Another important step in the process of atherogenesis is believed to be the trapping of LDL within the subendothelial space. LDL retention increases its residence time within the artery wall and therefore its susceptibility to oxidative modification. In physiological ion and salt concentrations, very little native (unmodified) LDL binds to endothelial cell-derived matrix in vitro (29). Although lowering the salt concentration or adding divalent cations can increase LDL retention to ECM (30, 31), this does not appear to be physiologically relevant. However, under physiological conditions, the ECM avidly binds lipoprotein lipase (LPL), which in turn markedly enhances LDL binding (29). LPL is normally present in the artery wall and increases, due to macrophage and smooth muscle cell production, during atherogenesis (32, 33). The increased binding of LDL to LPL-containing ECM is mediated by positively charged clusters of arginine and lysine domains of apoB (34). Furthermore, purified or HDL-associated apolipoprotein E (apoE) is able to efficiently displace LDL from LPL-containing ECM by virtue of its positively charged domains (35). Arginine or lysine polymers were able to mimic the LDL displacement effects of apoE (34).

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The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, endothelial cell matrix; HDL, high density lipoprotein; LPL, lipoprotein lipase; ModOxLDL, moderately oxidized LDL; Cu-OxLDL, copper-oxidized LDL.
The binding characteristics of OxLDL to ECM are largely unknown. Because the association of OxLDL to the arterial subendothelial matrix is a potentially pivotal step in the initiation and progression of atherosclerosis, we explored the requirements for its binding and potential physiologic competitors. To exclude interference in the binding studies from enzymes typically used for enzymatic oxidation of LDL (lipooxygenase/ phospholipase), we utilized OxLDL prepared by long term storage or by Cu²⁺ oxidation. The OxLDL, used in these studies, formed during long term storage in the absence of EDTA, was analyzed by the standard measures for the determination of the extent of oxidation (content of thiobarbituric acid-reactive material, free amino group, fluorescence (excitation: 360 nm; emission: 430 nm), and migration on agarose gels. Because these particles were intermediate in native and maximally oxidized LDL for all the aforementioned assays, we used the term moderately oxidized (ModOxLDL) to describe these LDLs. We found that ModOxLDL's bound to the LPL with greater affinity than LDL, that the increased binding was due to oxidative modification of the protein moiety and not lipid, and that apoE-rich HDL and polyarginine effectively blocked the interaction between OxLDL and LPL anchored to the ECM.

MATERIALS AND METHODS

Preparation of Lipoproteins—LDL (d = 1.019–1.063 g/ml) and HDL (d = 1.063–1.22 g/ml) were isolated by sequential flotation ultracentrifugation (36) of plasma obtained from normolipidemic human volunteers. LDL was radioiodinated by the iodine monochloride method of Parthasarathy et al. (37) as modified by Bilheimer (38). The specific activities of the 125I-LDL preparations ranged from 360 to 600 cpm/ng. LDL was radioiodinated by the iodine monochloride method of Parthasarathy et al. (37) as modified by Bilheimer (38). The specific activities of the 125I-LDL preparations ranged from 360 to 600 cpm/ng. The copper sulfate was then removed utilizing Centriflo 50 concentrator. Aortic Endothelial Cell Culture and OxLDL Retention Studies—Porcine aortic endothelial cells were isolated and cultured to confluence, and subendothelial cell matrix was prepared as described previously (35). OxLDL and native LDL matrix retention studies were performed in both the presence and absence of LPL. OxLDL was prepared from fresh unpasteurized milk using Affi-Gel heparin chromatography (Bio-Rad) essentially as described by Rifici et al. (43). Bovine Milk LPL Purification—Bovine milk LPL was purified from fresh unpasteurized milk using Affi-Gel heparin chromatography (Bio-Rad) essentially as described by Rifici et al. (43).

Aortic Endothelial Cell Culture and OxLDL Retention Studies—Porcine aortic endothelial cells were isolated and cultured to confluence, and subendothelial cell matrix was prepared as described previously (35). OxLDL and native LDL matrix retention studies were performed in both the presence and absence of LPL. OxLDL was prepared from fresh unpasteurized milk using Affi-Gel heparin chromatography (Bio-Rad) essentially as described by Rifici et al. (43).

Analytical Methods—Lipoprotein apolipoprotein content was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Novex, San Diego, CA). Protein concentrations were determined using the method of Lowry et al. (45), employing BSA as a standard.
LDL Oxidation Enhances Binding to LPL Matrix

Fig. 2. Agarose gel electrophoresis of Cu²⁺-oxidized LDL. LDL was oxidized to varying degrees by the addition of copper sulfate as described under "Materials and Methods." Lane 1, native LDL; lane 2, 125I-ModOxLDL (prepared from long term storage); lanes 3–7, LDL incubated for 12 h at 37°C in the presence of 2, 4, 6, 8, and 10 μM copper sulfate, respectively.

RESULTS

LPL Increases OxLDL Binding to ECM—125I-ModOxLDL retention by the ECM was examined in the absence or presence of LPL (Fig. 1A). Without LPL, 125I-ModOxLDL binding to ECM was low and nonsaturable. With LPL (8 μg/ml) 125I-ModOxLDL binding to ECM increased 25-fold, achieving saturation at 12.5 μg/ml (approximately 200 ng of apoB bound per well), which corresponds to approximately 1.25 mol of 125I-ModOxLDL binding per mol of LPL. Scatchard analysis estimated an association constant of 2.6 nM (Fig. 1A, inset). In a parallel experiment, 125I-LDL (Fig. 1B) also bound with high affinity in the presence of LPL; however, the association constant was one-fourth that of OxLDL. The amount of LDL retained corresponds to approximately 0.5 mol of LDL/mmol of LPL. A subsaturating level of 125I-ModOxLDL (3.125 μg/ml) was chosen for the remaining studies.

Specificity of OxLDL Binding to ECM —The specificity of ModOxLDL binding to LPL-containing ECM was examined by competition experiments utilizing native LDL, native LDL that was vortexed to promote aggregation, LDL that was oxidized with copper to varying degrees (Fig. 2), or the lipid extract or reconstituted protein from Cu-OxLDL. A 60-fold excess of native LDL or vortexed native LDL was ineffective in blocking the retention of 125I-ModOxLDL (Fig. 3). Conversely, Cu-OxLDL was able to completely block 125I-ModOxLDL binding to ECM (Fig. 4). Using LDL preparations oxidized to various degrees with copper as shown in Fig. 2, competition was shown to be dependent on both the concentration of Cu-OxLDL and the extent of copper oxidation. When 125I-ModOxLDL (12.5 μg/ml) was incubated in the presence of 12.5 μg/ml of Cu-OxLDL or reconstituted apoB from the same Cu-OxLDL there was a 50 and 25% decrease in binding, respectively (Fig. 4B). Lipid vesicles derived from the lipid extract of the Cu-OxLDL, at concentrations up to 25 μg/ml, had no effect on 125I-ModOxLDL retention by LPL-containing ECM (Fig. 4B). A 4-fold excess of Cu-OxLDL was also able to abolish the interaction of 125I-LDL (12.5 μg/ml) with LPL bound to the ECM (Fig. 5). These studies demonstrate that the major factor influencing the enhanced binding of ModOxLDL to LPL-ECM is the oxidation of the apoB protein.

Role of Exchangeable Lipids in the Increased Retention of OxLDL —To further demonstrate that the lipid moiety of oxidized LDL is not responsible for its increased affinity to LPL bound to matrix, 125I-ModOxLDL was incubated with a 16-fold excess (based on phospholipid, or approximately 300 HDL particles/LDL) of apoE-poor HDL for 24 h at 37°C to exchange the oxidized lipids and then reexamined for 125I-ModOxLDL binding. The binding characteristics of 125I-ModOxLDL to the LPL-containing ECM were essentially identical whether apoE-poor HDL was present during the preincubation or added just prior to the matrix binding experiment (Fig. 6). These results suggest that an exchangeable lipid on OxLDL is not responsible for its increased affinity to LPL-containing ECM. To determine if the oxidized effects observed were lipoprotein class-specific, we next copper-oxidized apoE-poor HDL (Cu-OxHDL) and used it to compete with 125I-ModOxLDL. Up to a 60-fold excess (based on protein, or approximately 340 HDL particles/LDL) of Cu-OxHDL had little effect on 125I-ModOxLDL binding (Fig. 7).

Effect of Polyarginine and Polylysine on 125I-ModOxLDL Binding to ECM —Previously studies from our laboratory demonstrated that apoE- and apoE-rich HDL blocked LDL retention by LPL anchored to ECM. We postulated that this effect was due to the arginine-rich regions contained within apoE competing with native LDL apoB for binding (35). To determine whether apoE-rich HDL could similarly compete with OxLDL, 125I-ModOxLDL (3.125 μg/ml) was coincubated with an increasing concentration of apoE-rich HDL (3.125–200 μg/ml) and assessed for binding to an LPL-treated ECM (Fig. 8A). ApoE-rich HDL blocked the retention of 125I-ModOxLDL to LPL-ECM in a concentration-dependent fashion, blocking greater than 90% of binding at 12.5 μg/ml. This level of competition was similar to that observed for LDL in our prior studies (35). ApoE-poor HDL at 400 μg/ml had no effect on retention (Fig. 8B). These results suggest that apoE competes with apoB-containing lipoproteins for binding to ECM-bound lipase, whether the lipoprotein is native or oxidatively modified. These experiments also yielded further evidence that the protein moiety of OxLDL is most likely responsible for its increased retention.

Effect of Polyarginine and Polylysine on 125I-ModOxLDL Retention —To further ascertain the role of the oxidized protein in the enhanced retention of OxLDL, we investigated the role of the positively charged polypeptides polyarginine and polylysine for their ability to block 125I-ModOxLDL retention (Fig. 9A). Polyarginine blocked 125I-ModOxLDL retention in a concentration-dependent manner, reducing binding by 50% at 12.5 μg/ml, nearly 100% inhibition at 100 μg/ml (Fig. 9B). This was similar to what we observed for competition with native LDL (34). However, polylysine at a concentration up to 100 μg/ml had no effect on binding (Fig. 9A). This is in contrast to the previously demonstrated ability of polylysine to compete with native LDL for LPL bound to ECM (34). The data suggest that...
the apoB arginine residues may play an important role in OxLDL retention.

**DISCUSSION**

The enhanced binding of native LDL to ECM is facilitated through an LPL bridge formed between matrix glycosaminoglycans and the lipoprotein (35). The retention of LDL did not require an enzymatically active protein, since inactivated LPL (overnight incubation at 25 °C) adsorbed to a microfilter plate was able to enhance LDL retention in an LPL concentration-dependent manner. However, the binding of LPL to the cell proteoglycans does require the enzyme to be active (46). We previously observed that apoE-rich HDL and apoE mimetics (polyarginine or polylysine) were effective competitors of native LDL bound to LPL-ECM and that this interaction did not release LPL from the matrix (34). In the subendothelium of atherosclerotic plaques epitopes of oxidized apoB have been found (47–49), suggesting either their localized subendothelium production or rapid sequestration from plasma. In this regard, in the current studies, we investigated the comparative binding affinity of native LDL and ModOxLDL to ECM alone and in the presence of LPL and the ability of apoE-rich and apoE-poor HDL and apoE mimetics to reduce this retention.

In the current studies, an experimental model of the subendothelial matrix was utilized to investigate the binding characteristics of 125I-ModOxLDL to the ECM. We found the binding of OxLDL to ECM alone was nonsaturating and of low affinity. In contrast, LPL greatly enhanced the saturable and high affinity binding of this modified lipoprotein. Compared with native LDL, OxLDL-bound ECM anchored LPL with a 4-fold greater affinity. The increased affinity was caused exclusively by the modification of LDL apoB and not the lipid. The affinity of OxLDL binding to LPL bound to ECM was dependent on the extent of LDL oxidation. Neither native LDL, aggregated LDL, nor apoE-poor HDL could effectively compete with 125I-ModOxLDL for ECM-bound LPL; in contrast, apoE-rich HDL efficiently blocked this interaction. Polyarginine mimicked the effects observed with apoE-rich HDL; however, polylysine was unexpectedly ineffective in competing with 125I-ModOxLDL for ECM-bound LPL.

Four separate experiments suggest that the binding interaction between LPL bound to matrix and OxLDL is mediated by modified apoB rather than oxidized lipid. First, the isolated, reconstituted apoprotein moiety of Cu-OxLDL decreased the interaction of 125I-ModOxLDL with LPL-ECM, whereas the lipid portion had no effect. Preincubation with excess apoE-poor HDL, to remove any oxidized lipids, did not diminish matrix binding. Oxidized apoE-poor HDL also did not compete with OxLDL for binding. Last, both apoE-rich HDL and polyarginine effectively competed with OxLDL. Prior studies (31) have also shown that lyso-PC, a component of oxidized LDL, did not interfere with the retention of LDL with LPL-containing matrix. Collectively these data suggest that it is the oxida-
tion of apoB that causes the enhanced binding.

The experiments involving polyarginine and polylysine may help explain an apparent discrepancy between the present studies and our previous studies with nonoxidatively modified LDL. Native LDL modified by acetylation (modification of lysine residues) (50) and cyclohexanedione (modification of arginine residues) (51) do not block LDL binding to LPL-matrix as efficiently as native LDL (34). However, in the present study,
we show that oxidation of LDL, which has been shown to modify the lysine residues, greatly enhances its association with LPL and can completely block LDL retention. Why does OxLDL bind with higher affinity to LPL-containing ECM, and why is polylysine ineffective in blocking OxLDL retention? This apparent paradox may be explained with the process of LDL oxidation. Oxidation of apoB leads to its fragmentation (28,52,53), which may expose novel sites and also confer conformational changes. This, in turn, may result in a higher affinity binding of OxLDL to LPL-ECM. Oxidation also modifies the lysine residues on apoB. The lysine modification would decrease its role in the binding to LPL, which may account for the inability of polylysine to inhibit the OxLDL binding. Further experiments are necessary to delineate the precise mechanisms of these effects.

The ability of apoE-rich HDL to compete with oxidized LDL increases our understanding of the importance of this lipoprotein fraction and in particular apoE. Studies in apoE transgenic and apoE-deficient mice demonstrate the antiatherogenic properties of apoE. Mice, which are normally atherosclerosis-resistant, become very prone to atherosclerosis when they are made apoE-deficient by gene targeting (54, 55). Interestingly, these mice present with both circulating oxidized LDL and antibodies to epitopes of oxidized apoB (49). Recently, Shimano et al. created transgenic mice overexpressing apoE in the arterial wall (56). When these mice were fed an atherogenic diet, significant decreases in fatty streak formation as compared with nontransgenic controls was observed. Therefore, in these models, the excess of apoE within the lesions may impede the retention of OxLDL and thereby inhibit the progression of atherosclerosis.

Epitopes of oxidized apoB have been demonstrated in the extracellular matrix of lesion areas in animals and humans using antibodies to malondialdehyde-modified LDL (47–49, 57). Furthermore, OxLDL has been shown in the plasma of subjects with severe atherosclerosis and diabetes (28, 58–61). Based on the studies presented here, it is possible that when circulating OxLDL infiltrates the artery wall it is captured by matrix-bound LPL. Alternatively, native LDL may first bind to the matrix-bound LPL, and then be oxidized by cells within the arterial wall, thereby resulting in higher affinity binding. In this regard, it has been shown in vitro that the binding of LDL to matrix proteoglycans increases the lipoprotein’s susceptibility to oxidation (62). In either scenario, OxLDL may be selectively trapped within the artery wall by such interactions as those presented here. The accumulation of native and CuOx-LDL have been studied in vivo in balloon catheter-deendothelialized rabbit aorta (63). The accumulation of native and Ox-LDL in areas of regenerating endothelium were similar. However, in areas that were still de-endothelialized, OxLDL was retained 2.9-fold more than native LDL, despite its more rapid clearance from plasma. Additionally, the extent of lysine modification of ModOxLDL in these studies is sufficient to be recognized by the scavenger receptor (64). Thus, oxidation may enhance retention in the arterial wall by both increased binding to LPL and uptake through the scavenger receptor, markedly increasing its ability to promote various steps in the pathogenesis of atherosclerosis.

In conclusion, we have demonstrated that oxidized LDL has a higher affinity to LPL anchored to subendothelial matrix than native LDL and that the increased retention is due to the oxidation of apoB, not the lipids. Furthermore, unlike native LDL, it is mainly the arginine residues, not the lysines, that play an important role in this retention. And finally, apoE-rich HDL has been shown to be as effective in blocking the retention of OxLDL, strengthening its role as an antiatherogenic agent.

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