Low Density Lipoprotein Oxidation and Its Pathobiological Significance*

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**Background**

The fact that low density lipoprotein (LDL) is extremely susceptible to oxidative damage has been known for some time (1, 2), but until quite recently this was primarily a nuisance for the student of lipoprotein metabolism. It now appears that oxidation of LDL plays a significant role in atherogenesis.

Beginning in the 1980s evidence began to accumulate that cholesterol accumulation in the developing atherosclerotic lesion was probably not due to the uptake of native LDL by way of the Brown/Goldstein LDL receptor but instead due to the uptake of some modified form of LDL (then still unidentified) by way of one or more alternative receptors (also then unidentified). This conclusion grew from two well accepted observations. First, patients and animals totally lacking the LDL receptor nevertheless accumulate cholesterol in foam cells much the same way as do patients and animals with normal LDL receptors; second, the two cell types in lesions that give rise to cholesterol-laden foam cells (the monocyte/macrophage and the smooth muscle cell) do not accumulate cholesterol in vitro even in the presence of very high concentrations of native LDL (3, 4). This paradox could be resolved if circulating LDL underwent some form of modification and if the modified form, rather than native LDL itself, then served as the ligand for delivery of cholesterol to developing foam cells. Acetylation of LDL in vitro generated a modified LDL that could induce cholesterol accumulation in macrophages (3). The uptake of this acetylated LDL was by way of a new receptor designated the acetyl LDL receptor (later cloned and renamed scavenger receptor A (SRA)) (5). SRA, unlike the LDL receptor, is not down-regulated when the cholesterol content of the cell increases. Thus, acetyl LDL could, in principle, account for foam cell formation. However, there was (and still is) no evidence that acetylation of LDL occurs to any extent in vitro. Another modified form of LDL emerged as a candidate when it was shown that simply incubating LDL overnight with a monolayer of arterial endothelial cells converted it to a form that was taken up much more rapidly by macrophages and capable of increasing their cellular cholesterol content (6–8). The uptake was specific and saturable, and it occurred in part by way of the acetyl LDL receptor. Incubation with smooth muscle cells could also modify LDL in much the same way (7, 8). This cell-mediated modification turned out to be, very simply, oxidative modification (9, 10). The addition of antioxidants to the culture medium completely blocked cell-induced modification, and the changes induced by the cells could be duplicated by incubating LDL in the presence of transition metals in the absence of cells. Thus, oxidative modification induced by cells appeared to be a biologically plausible modification of LDL that could account for foam cell formation and the initiation, or at least acceleration, of the atherosclerotic process.

**Additional Potentially Proatherogenic Properties of Oxidized LDL**

As already mentioned, the first property of oxidized LDL to be discovered is that it is recognized by the scavenger receptors and can therefore give rise to foam cells (7). Additional potentially proatherogenic properties became apparent soon thereafter, including the fact that OxLDL is itself a chemotactic factor for monocytes (20) and that it inhibits the motility of tissue macrophages (21). Oxidized LDL is cytotoxic for endothelial cells in culture (22); it inhibits the vasodilatation that is normally induced by NO (23); it is mitogenic for macrophages and smooth muscle cells (24, 25); it can stimulate the release of MCP-1 and M-CSF from endothelial cells (17, 18). OxLDL is immunogenic, and autoantibodies are commonly found both in animals and patients (26–28). Titers tend to be higher in patients with more rapidly progressive disease (27), but, paradoxically, immunization of rabbits with OxLDL to raise antibody titer actually inhibits lesion progression (29).

There are as many as 20 additional biological effects that have been described, but almost none of these has been evaluated in vivo. In any case, these examples will suffice to demonstrate that the oxidative modification of LDL leads to a possibly very large array of consequences above and beyond the generation of foam cells that could be important in atherogenesis. It is important also...
to stress that some of these biological effects can be exercised by minimally modified LDL. Many of these appear to be attributable to partially oxidized phospholipids that may mimic the effects of platelet-activating factor or of autacoids (30, 31). The immunogenicity of OxLDL appears also to be attributable in part to oxidized phospholipids, possibly complexed with protein or other lipids (32, 33). Before leaving this topic, it is worth noting, as discussed further below, that the oxidation of the lipoprotein matrix of a plasma membrane is somewhat analogous to the oxidation of an LDL particle. Just as oxidized LDL can exert a number of effects, including regulation of gene expression, oxidation of the plasma membrane of a cell may give rise to analogous biological effects that could be relevant during apoptosis or under conditions of high oxidative stress.

The Nature of “Oxidized LDL”

Originally, oxidized LDL was defined primarily in terms of its biological properties, notably the fact that it was no longer a ligand for the native LDL receptor but was a ligand for the acetyl LDL receptor and that its uptake by macrophages was therefore much more rapid, sufficient to cause cholesterol accumulation. This degree of oxidation could be effected by incubation overnight with cultured cells in the appropriate medium or by incubation with 5–10 μM Cu²⁺ for 8–16 h. Later studies showed that after much gentler oxidative modification (too little to alter its binding by the LDL receptor and yet not enough to make it a ligand for the acetyl LDL receptor) the oxidized LDL had different and potentially very important biological properties of other kinds, including the ability to stimulate the release from endothelial cells of MCP-1 and MCSP (17, 18). This minimally oxidized LDL, designated “MM-LDL,” is very different from LDL incubated overnight with copper ions, which may deserve to be redesignated as “maximally oxidized LDL.” Obviously there is potentially a continuous spectrum of degrees of oxidation and a great deal of molecular heterogeneity in what we call “oxidized LDL” (34). Even if oxidative conditions are controlled as precisely as possible, the product will still vary from experiment to experiment depending on the composition of the starting LDL. LDL particles rich in polyunsaturated fatty acids are more readily oxidized than are LDL particles enriched in saturated fatty acids or monounsaturated fatty acids (35). The content of vitamin E and other naturally occurring indigenous antioxidants will influence the susceptibility of LDL preparations to oxidation under any given set of conditions. The enormous complexity of the problem is evident when one considers that the average LDL particle contains about 700 molecules of phospholipids, 600 of free cholesterol, 1600 of cholesterol esters, 185 of triglycerides, and 1 of apolipoprotein B (apoB) containing 4536 amino acid residues! Both the lipids and the protein are subject to oxidation and both are indeed oxidized. Direct oxidative damage to proteins is discussed by Berlett and Stadtman the previous Minireview in this series (74), and almost certainly there is some direct apoB oxidation. Cholesterol is converted to oysterols, especially at the 7-position. The polyunsaturated fatty acids in cholesterol esters, phospholipids, and triglycerides are subject to free radical-initiated oxidation and can participate in chain reactions that amplify the extent of damage. A key feature of LDL oxidation is the breakdown of these polyunsaturated fatty acids to yield a broad array of smaller fragments, 3–9 carbons in length, including aldehydes and ketones that can be conjugated to other lipids (especially amino lipids) or to the apoB (36). For example, malondialdehyde (or other aldehydes) generated during oxidation can form Schiff bases with the e-amino groups of lysine residues and can go on to generate cross-links between lipid and protein or among lipid molecules. 4-Hydroxyen- nol and other α,β-unsaturated aldehydes can conjugate preferentially by Michael addition (37). During the oxidation of LDL to a form recognized by SRA, 40–50% of the reactive lysine e-amino groups become masked (38, 39). This may be a sufficient explanation of the shift in receptor specificity because treatment of LDL in vitro with acetic anhydride also generates a form of LDL recognized by SRA when 60% or more of the lysine amino groups are masked. This, then, explains what was somewhat perplexing at first, namely why oxidized LDL and acetyl LDL should have overlapping receptor-binding specificities. In part, at least, it would appear to be because both involve masking of lysine e-amino groups with consequent changes in protein charge and configuration.

Oxidation of LDL in Vivo

As mentioned above, modification of LDL by endothelial cells in vitro can be completely prevented by the addition of antioxidants such as vitamin E or for short periods of time. These antioxidants are almost certainly inhibited also by the addition of as little as 5 or 10% fetal calf serum. How, then, can LDL undergo oxidative modification in vivo? Even in the extracellular fluid one would guess that the concentrations of antioxidants (proteins, vitamin C, uric acid, etc.) would be amply to inhibit cell-induced oxidative modification. Logic to the contrary notwithstanding, it does get oxidized. 1) The lipoprotein fraction gently extracted from atherosclerotic lesions (both rabbit and human) contains oxidized LDL, identified both by its physical properties and by its recognition by scavenger receptors (40); 2) immunohistochemistry using antibodies generated against oxidized LDL demonstrates the presence of oxidized LDL (or antigens very similar to it) in arterial lesions but not in normal artery (41); 3) both in animals and in humans autoantibodies that react with oxidized LDL have been demonstrated in the serum (41); 4) administration of antioxidants that can prevent oxidative modification of LDL slows the progression of atherosclerosis in several experimental animal models, as discussed in more detail below.

How and where does this oxidation of LDL take place? That is really not known, but it seems it must occur in sequestered microenvironments in which the LDL is no longer protected by those antioxidant components that so effectively protect it in whole plasma or in extracellular fluid. When macrophages (and perhaps other cell types as well) adhere to a substratum they behave a bit like the tentacles of an octopus, i.e. microdomains of the cell membrane attach themselves to the dish in a circular pattern creating microenvironments from which large molecules are excluded (42). These “pockets” are so minute that the composition of the fluid in them can be very rapidly changed by virtue of transport across the cell membrane making up the “cap” of the microenvironment. This might, for example, sharply reduce the levels of antioxidants. If cells in vivo adhere to connective tissue substrata (or to neighboring cells) in a similar fashion, this postulated mechanism might explain oxidation of LDL in vivo, but there is to date no experimental evidence one way or the other.

Heavily acetylated LDL and heavily oxidized LDL injected intravenously disappear from the plasma compartment with a very short half-life, only a matter of minutes in the rat or in the rabbit. This largely reflects extremely rapid uptake into hepatic Kupffer cells and sinusoidal endothelial cells (43). These cells express the acetyl LDL receptor and probably other receptors for OxLDL and are highly efficient in sweeping it out of the plasma. Consequently one would not expect to find heavily oxidized LDL in the plasma at any significant concentration because it would have to be generated at an implausibly high rate. On the other hand, because MM-LDL is not a ligand for the scavenger receptors, it would probably have a half-life not much different from that of native LDL and could build up in the plasma compartment. Similarly, LDL in which a small percentage of lysine e-amino groups have been masked (but not enough to make it a ligand for the scavenger receptors) might have a half-life even longer than that of native LDL and could, again, build up.

Enzymes and Tissues Involved in LDL Oxidation in Vivo

Studies in cell culture have identified a number of enzyme systems that could in principle play a role in the oxidation of LDL. These include NADPH oxidase, 15-lipoxygenase, NADPH leukocyte oxidase, and the mitochondrial electron transport system, and others. Which of these contribute to LDL oxidation in vivo and to what extent is still uncertain, but analysis of products isolated from atherosclerotic lesions strongly supports the involvement of lipoxygenases (44, 45) and of myeloperoxidase (46). Again cell culture studies identify many cell types capable of oxidizing LDL including endothelial cells, smooth muscle cells, monocytes, macrophages, fibroblasts, neutrophils, and others. Since oxidized LDL is present in arterial lesions at significant concentrations, it seems reasonable to assume that the cells characteristic of those lesions, i.e. endothelial cells,
Macrophages, and smooth muscle cells, are involved in its oxidation. However, there is no convincing in vivo evidence to implicate one or another of these as more important than the others.

While oxidation of LDL in the artery wall has received the most attention, it seems very likely that oxidation of LDL takes place at many other sites, perhaps at all sites of inflammation. Because of increased vascular permeability at sites of inflammation, the concentration of LDL in the inflammatory fluid would be higher than it is in normal extracellular fluid. Because of the infiltration by neutrophils and monocyte/macrophages the conditions for LDL oxidation at inflammatory sites would be propitious. However, LDL oxidation at peripheral sites would not have the same significance as oxidation of LDL in the artery wall unless the LDL oxidized at peripheral sites reenters the bloodstream and is subsequently delivered to the artery. If LDL in the periphery were to undergo limited oxidation before reentering the blood it would have a prolonged half-life, as discussed above, and it could then be taken up into developing arterial lesions. Being already partially oxidized, this LDL might make an unusually large contribution to the further progression of the lesion. Immunochemical studies have provided evidence for the presence of oxidized LDL (or at least of antigens closely related to it) at sites of inflammation (47). The functional significance of this remains to be explored.

Antioxidant Inhibition of Atherogenesis in Experimental Models

If oxidative modification of LDL plays a significant role in atherogenesis, its inhibition by an appropriate antioxidant should slow the progression of the disease. Indeed this has now been demonstrated in several different animal models (the LDL receptor-deficient rabbit, the cholesterol-fed New Zealand White rabbit, the cholesterol-fed hamster, the cholesterol-fed cynomolgus monkey, the LDL receptor-deficient mouse, and the apoprotein E-deficient mouse) and using one of several different antioxidants (probucol, butylated hydroxytoluene, diphenylphenylenediamine, and vitamin E) (reviewed in ref. 12 for review and specific citations). A total of 23 studies has been reported of which 16 were strongly positive (more than 50% inhibition of the rate of progression), 2 were borderline, and 5 negative. An important question to be asked is whether the antioxidants exerted their inhibitory effect on lesion progression only because of their antioxidant properties or, possibly, because of additional biological properties. This is the same kind of problem that arises with the use of any inhibitor in biology. In fact the first antioxidant tested, probucol, does indeed have additional biological properties that might be relevant (48), including the ability to inhibit interleukin-1 release and to increase expression of cholesterol ester transfer protein. However, the fact that two antioxidants as structurally diverse as probucol and diphenylphenylenediamine share the ability to inhibit atherogenesis suggests that the effect is attributable primarily to their shared antioxidant properties. Further evidence that the effect depends upon antioxidant activity comes from the rough parallelism observed in some studies between the effectiveness of these compounds in protecting circulating LDL from oxidation in an ex vivo test system and their effectiveness in inhibiting atherogenesis (49).

At this time there is insufficient evidence, however, to allow a confident prediction of the anti-atherosclerotic effectiveness of a compound from its antioxidant effectiveness ex vivo. It appears that some rather high threshold of antioxidant effect must be reached before any anti-atherosclerotic effect is evident (49, 50). Even a 4-fold prolongation of conjugated diene lag time (a commonly used measure of the resistance of LDL to oxidation) may still be inadequate. Yet many clinical correlations are being accepted as meaningful when the diene conjugation lag time is increased as little as 30%!

Receptors for OxLDL and Their Evolutionary Raison d’Etre

OxLDL is bound and internalized by at least two and possibly three different macrophage receptors. Because these receptors tend to have a much broader ligand specificity than previously studied receptors they have been designated “scavenger receptors” or “multiligand” receptors (51, 52). The best studied example is the original acetyl LDL receptor, now redesignated scavenger receptor A, which occurs in two differentially spliced forms (SRAI and SRAII) that have very similar ligand binding specificity. Unlabeled acetyl LDL can only inhibit some 40–60% of the binding of OxLDL to mouse peritoneal macrophages (7, 8), and macrophages from mice in which SRA has been targeted show only a partial defect in OxLDL binding, implying that additional receptors are involved (53). Recent evidence shows that SRA can also play a role in the adherence of macrophages to plastic surfaces (54) and to glycosylated collagen (55).

The pathogenetic role of SRA in atherogenesis has now been demonstrated by crossing SRA-targeted mice with apoprotein E-targeted mice and finding a highly significant 58% reduction in lesion severity (53). Since OxLDL is one of the major naturally occurring ligands for SRA, these findings further support the oxidative modification hypothesis of atherogenesis.

The B class of scavenger receptors includes CD36 (56) and SR-B1 (57). Finally, a receptor with scavenger receptor-like activity has been cloned from Drosophila (58), and it has been designated as the first member of a new class of scavenger receptors, SRC.

Recent studies have shown that macrofascialin and its human homologue, CD68, can bind OxLDL in ligand blots and that antibodies against CD68 can partially inhibit the binding and uptake of OxLDL by a human monocyte-derived cell line, the THP-1 cell line (59, 60). However, only a very small fraction of macrofascialin or of CD68 is expressed on the plasma membrane, and their importance in the uptake of OxLDL by normal monocytes/macrophages remains to be further evaluated.

As mentioned above, receptors with at least some of the properties of SRA and SRB can be found all the way back to Drosophila (58). Why have these receptors persisted in evolution? Surely it can have nothing to do with any role they play in atherogenesis. Atherosclerosis is almost exclusively a human disease, and in any case, its clinical effects occur after the preclinical period is over so there cannot be any selective genetic pressure (positive or negative). We have suggested that oxidative damage to a cell membrane macromolecule mimics the action of a ligand, thereby attracting a scavenger receptor (55). This oxidative damage may generate lipid-protein products similar to those found also in oxidatively damaged LDL (61). Indeed, the binding of oxidatively damaged red blood cells to macrophages is competitively inhibited by OxLDL but, interestingly, not by acetyl LDL. The binding of apoptotic thymocytes to macrophages is also inhibited by oxidized LDL (62), a finding compatible with the hypothesis. The two best studied receptors for oxidized LDL, SRA and CD36, also bind apoptotic cells. The role of CD36 in this respect has been extensively studied (63) and appears to involve cooperative interaction with αvβ3 and thrombospondin. Recent studies show that peritoneal macrophages from mice in which SRA has been “knocked out” show a deficit in the phagocytosis of apoptotic thymocytes (64). Thus it may be that as we search for receptors that bind oxidatively damaged LDL we are at the same time on the trail of receptors whose primary function is to recognize damaged (apoptotic) cells.

Is OxLDL Relevant to the Human Disease?

The basic pathobiology of experimental atherosclerosis appears to be very much the same as that of the human disease, suggesting that antioxidants should work in humans. Furthermore, epidemiologic studies have repeatedly shown a negative correlation between levels of dietary intake or plasma levels of antioxidant vitamins, on the one hand, and risk of coronary heart disease, on the other (65). On the other hand, the time scale over which lesions develop in animal models is very short (weeks or months) compared with the time scale over which human lesions evolve (decades). Also, the degree of antioxidant protection we can achieve in humans may be less than that achieved in animal studies.

Several clinical trials on effects of β-carotene have primarily to test its possible efficacy in preventing cancer, have also recorded cardiovascular events (66–68). All of them have been negative with respect to effects on either cancer or cardiovascular disease. Unfortunately, it was not recognized until recently that β-carotene is actually relatively ineffective in protecting LDL (much less effective than vitamin E). Carefully conducted trials in human subjects show that supplementation even with very large doses of β-carotene (doses sufficient to increase the β-carotene concentration in the LDL fraction severalfold) fails to protect the circulating LDL against oxidation ex vivo (69, 70). β-Carotene is an effective
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quencher of singlet oxygen, but it is much less effective as a chain-
interrupting antioxidant. To the extent that protection of circu-
lating LDL is a rough index of efficacy, these β-carotene trials should
not be considered appropriate tests of the oxidative modification
hypothesis.

Vitamin E, on the other hand, is very effective in protecting circu-
lating LDL against oxidation *ex vivo* (69, 71, 72). The degree of pro-
tection is a function of the extent to which the vitamin E content
of the circulating LDL is increased, and doses of 400–800 IU
per day in a placebo-controlled, double-blind trial in
patients with established coronary heart disease (70). Those
randomized to vitamin E showed 47% fewer nonfatal myocardial in-
farctions and cardiovascular deaths (the primary end point) than
the control group, and the result was significant at the p = 0.001
level. Additional trials are in progress. Decisions about the use
of antioxidants in human atherosclerosis should be deferred until
new data become available.

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