The LDL modification hypothesis of atherogenesis: an update

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Abstract The accumulated evidence that oxidative modification of LDL plays an important role in the pathogenesis of atherosclerosis in animal models is very strong. The negative results in recent clinical studies have caused many to conclude that LDL oxidation may not be relevant in the human disease. Yet many of the lines of evidence that support the hypothesis have been demonstrated to apply also in humans. In this review, we briefly summarize the lines of evidence on which the hypothesis rests, its strengths, and its weaknesses.—Steinberg, D. **The LDL modification hypothesis of atherogenesis: an update. *J. Lipid Res.* 2009.** 50: **S376–S381.**

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The defining characteristic of the fatty streak, the first visible lesion of atherosclerosis, both in animals and in humans is the "foam cell." This cell, loaded with droplets rich in cholesteryl esters, is derived mainly from arterial wall macrophages, which originate from circulating monocytes that have penetrated into the subendothelial space. Smooth muscle cells and endothelial cells in lesions also can and do accumulate lipid droplets, but monocyte-derived macrophage foam cells predominate. This being the case, an understanding of just how arterial macrophages take up and store their load of cholesterol should shed light on the mechanisms that initiate atherogenesis.

ORIGINS OF THE OXIDATIVE MODIFICATION HYPOTHESIS

Beginning in 1979, Goldstein, Brown, and their collaborators [reviewed in (1)] decided to pursue this problem, studying the metabolism of macrophages in cell culture. They noted the following apparent paradox: in patients with homozygous familial hypercholesterolemia, even in those who express absolutely no functional LDL receptors, macrophage-derived foam cells nevertheless accumulate in the artery walls just as they do in hypercholesterolemic patients that have perfectly normal LDL receptors. The implication was that LDL must be somehow altered prior to its uptake by macrophages and then taken up, not by the native LDL receptor, but rather by some alternative macrophage receptor. Indeed, they found that the rate of uptake of native LDL by normal resident mouse peritoneal macrophages was very low even at very high LDL concentrations. There was very little increase in cell cholesterol content and certainly no generation of foam cells. They then tried modifying the LDL physically, chemically, or enzymatically, looking for some form of LDL that could turn macrophages into foam cells in vitro. Several modifications worked, but the most striking was chemical acetylation. Treatment of LDL with acetic anhydride yielded a form of LDL that bound to the macrophage specifically and with high affinity, was actively internalized, and led to intracellular cholesterol accumulation. They dubbed the putative receptor the acetyl-LDL receptor. That receptor was later cloned and characterized by Kodama et al. (2) in the laboratory of Monty Krieger. Because of its possible role in the LDL receptor-independent uptake of LDL and because of its broad ligand specificity, it was redesignated scavenger receptor A (SRA). However, acetyl-LDL itself is not a biological product and has never been found in vivo. The search for the biological ligand for the scavenger receptor continued.

In 1981, Henriksen et al. (3) discovered that native LDL simply incubated overnight with cultured endothelial cells was converted to a form (endothelial cell-modified LDL) that was recognized specifically and with high affinity by peritoneal macrophages. They proposed that this endothelium-induced modification of LDL might be the missing step that permits rapid LDL uptake and foam cell formation. Later studies showed that during its incubation with endothelial cells (and with a number of other cell

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Abbreviations: apo, apolipoprotein; M-CSF, macrophage-colony-stimulating factor; SRA, scavenger receptor A.

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types), LDL was undergoing an oxidative modification (4, 5). This was the genesis of the so-called oxidative modification hypothesis of atherogenesis. Certainly the hypothesis has been heuristic; PubMed lists over 5,000 papers published to date under "oxidized LDL" and over 2,200 indexed under "oxidized LDL and atherosclerosis," Over 1,000 in the latter category have been published in the past 5 years alone. So the hypothesis is very much alive. Studies in animal models of atherosclerosis continue to strongly support the hypothesis. However, a series of negative clinical trials using vitamin E or β-carotene have raised doubts about the relevance of the hypothesis to the human disease. In this review, we attempt to assess the strengths and weaknesses of the evidence for the hypothesis and suggest future directions for research.

SUMMARY OF THE KEY LINES OF EVIDENCE SUPPORTING THE OXIDATIVE MODIFICATION HYPOTHESIS, TOGETHER WITH CAVEATS THEREOF

Many excellent reviews have dealt with the evidence linking oxidized LDL (OxLDL) to atherogenesis (6–13). Because of space limitations, it will not be possible to cite all the primary references and so the reader is asked to consult these reviews, especially for older references.

What are the major findings that form the basis for the oxidative modification hypothesis?

Monocyte/macrophages in culture take up OxLDL much more rapidly than they take up native LDL

As discussed above, they bind OxLDL with high affinity to specific plasma membrane receptors [including SRA, SRB (CD36), and lectin-like oxLDL receptor]. These receptors are not downregulated as the cholesterol content of the macrophage increases, allowing progressive accumulation of cholesterol to the point of foam cell generation.

Caveats. There are a number of alternative mechanisms for generation of foam cells that could also play a role, as we have discussed elsewhere (14). These include uptake of aggregated LDL (via phagocytosis and facilitated by the LDL receptor), β-VLDL (probably via the LDL receptor), and LDL immune complexes (via the F_c receptor). There is as yet little or no in vivo evidence to establish that these alternative pathways actually contribute to foam cell formation.

Recently, Kruth et al. (15) have shown that macrophages activated by incubation with phorbol-12-myristate-13-acetate or macrophage-colony-stimulating factor (M-CSF) can take up native LDL from the medium by macropinocytosis, a process that internalizes large volumes of surrounding medium together with whatever solutes they contain. If the concentration of native LDL is extremely high (about 2 mg/ml), the rate of uptake by this nonspecific pathway can be sufficient to cause cholesterol accumulation and foam cell formation (15, 16). Note that the concentration of LDL needed to induce foam cell formation (2 mg/ml) is 40-fold greater than the concentration of OxLDL needed (ca. 50 μg/ml). Exactly how the contents of the macropinosome are sorted and distributed to cellular compartments is still unclear, nor has it been shown that this pathway is actually operative in vivo. Clearly it deserves further investigation.

OxLDL is present in plasma and in atherosclerotic lesions, both in experimental animals and in humans (17); antibodies against OxLDL are present in normal animal and human plasma; and both OxLDL and antibodies against it are present at higher levels in the presence of atherosclerosis.

OxLDL exhibits a wide array of biological properties that would be expected to be proatherogenic

Many of these are effected by oxidized phospholipids within the OxLDL (11, 18, 19). These oxidized phospholipid products are also the principal epitopes recognized by autoantibodies to OxLDL and the major structural feature by which scavenger receptors recognize OxLDL as a ligand. Oxidized cholesterol esters also play a role.

a) OxLDL is cytotoxic for endothelial cells cultured in serum-free medium; *b*) It induces expression and release of monocyte chemoattractant protein-1 from endothelial cells; *c*) It is chemotactic for monocytes (but inhibits migration of macrophages); *d*) It induces endothelial expression of M-CSF; *e*) It increases collagen synthesis in smooth muscle cells; *f*) It inhibits lipopolysaccharide-induced expression of nuclear factor-κB; *g*) It induces apoptosis; *h*) It inhibits release and/or function of nitric oxide (vasospasm); *i*) It increases expression of vascular cell adhesion molecule-1; *j*) It increases tissue factor activity in endothelial cells (thrombosis); *k*) It induces a wide variety of proinflammatory cytokines in macrophages; and *l*) It is immunogenic, inducing an increase in circulating levels of antibodies against oxidation-specific epitopes (see review by J. L. Witztum in this issue).

These manifold effects of OxLDL have been mostly described in cell culture settings and in only a few instances have they been confirmed in the whole animal. The findings are compatible with and lend some support to the oxidative modification hypothesis, but until they are evaluated in intact animals they remain only suggestive. In vivo veritas.

Severity of atherosclerosis in a number of animal models (rabbit, mouse, monkey, hamster) can be significantly ameliorated by treatment with a variety of antioxidant compounds

Effective antioxidants include probucol, probucol analogs, butylated-hydroxytoluene, *N,N'*-diphenylphenylenediamine, BO-653 (an analog of probucol), and vitamin E. The fact that these several compounds, quite different in structure and metabolism but sharing an ability to trap free radicals, have all been shown to act as inhibitors of atherosclerosis considerably strengthens the oxidative modification hypothesis. Further support comes from a study in which the protective effect of vitamin E was confirmed in apolipoprotein (apo) E-deficient mice and then shown to be almost abolished when the mice were also deficient in 12/15-lipoxygenase (20). The authors suggest therefore that the protection afforded by vitamin E and that af-

forded by targeting 12/15-lipoxygenase share a final common pathway.

Caveats. There are problems with the interpretation of some of these animal studies. For example, *N,N*′-diphenylphenylenediamine and butylated-hydroxytoluene have been used in only one study each. Results with vitamin E have yielded convincing results in mice but conflicting results in rabbits.

Probucol was the first antioxidant to be tested and it has been the most consistently effective agent. Recently, Stocker et al. (9) have raised the question of whether the antiatherosclerotic action of probucol is necessarily dependent on its antioxidant activity or whether it may be due to some other biological effects of the compound.

Gene targeting studies implicate a number of proteins thought to be involved in atherogenesis and LDL oxidation

Targeting of 12/15 lipoxygenase decreases the severity of atherosclerosis in three different mouse models: apoE-deficient mice (21, 22), LDL receptor-deficient mice (23), and mice deficient in the apoB mRNA editing enzyme (24). Much of the effect can be attributed to the lipoxygenase in macrophages, because bone marrow transplants from 12/15-lipoxygenase-deficient mice into apoE-deficient mice affords almost the same degree of protection from lesion formation as the global knockout (25). Furthermore, site-specific overexpression of 15-lipoxygenase in endothelium accelerates atherosclerosis in LDL receptor-deficient mice (26).

Targeting of scavenger receptors ameliorates atherosclerosis in mouse models. SRA, SRB (CD36), and lectin-like oxLDL receptor are the three best-studied receptors that recognize OxLDL. Together, SRA and CD36 account for almost 90% of macrophage uptake of OxLDL (27). In each case, knockouts in either apoE-deficient or LDL receptor-deficient mouse models have been shown to ameliorate the severity of atherosclerosis.

Targeting of paraoxonase-1, an enzyme that indirectly inhibits LDL oxidation (possibly by interfering with the ability of HDL to protect LDL against oxidation) enhanced lesion formation by 50% or more in apoE-deficient mice (28).

Targeting of some of the genes coding for proteins thought to mediate the atherogenic effects of OxLDL ameliorates atherogenesis. These include, e.g., MCP-1, M-CSF, and selectins P and E. However, these knockouts would likely affect atherogenesis no matter what the details of the underlying pathogenesis, because monocyte recruitment and retention must be involved in any arterial inflammatory disease. So the findings are compatible with the hypothesis but do not directly and specifically implicate OxLDL.

Caveats. Shen et al. (29) overexpressed the human 15-lipoxygenase gene specifically in the macrophages of cholesterol-fed rabbits and WHHL rabbits and observed an amelioration of atherosclerosis rather than the anticipated exacerbation. In contrast, macrophage-specific deficiency of 12/15-lipoxygenase in the apoE-deficient mouse ameliorates atherosclerosis (25). As pointed out by Kuhn and Chen (30), 12/15-lipoxygenase may have both pro- and antiinflammatory effects. Their results with overexpression in the rabbit and the results with lipoxygenase targeting in mouse models are not necessarily mutually contradictory. Some level of lipoxygenase may be mandatory for atherogenesis, while levels above normal may exert antiinflammatory effects. While this explanation seems to offer an attractive resolution for many of the discordant findings, it doesn't resolve the directly contradictory results on the consequence of global knockout of 12/15-lipoxygenase in the apoE-deficient mouse very recently reported by Merched et al. (31) in Lawrence Chan's laboratory. In their hands, knockout increased rather than decreased the extent of lesions. The reasons for the contradictory results remain to be determined.

5-Lipoxygenase has been implicated as proatherogenic, not through oxidation of LDL, but via its other proinflammatory effects exercised in part via LTB_4 (32). The enzyme and its products are present in lesions and pharmacologic inhibition can reduce lesion size (33). However, Cao et al. (34) reported that neither global knockout nor pharmacologic inhibition of 5-lipoxygenase affects atherogenesis in the apoE-deficient mouse.

Oxidative stress may be involved much more broadly in atherogenesis. Reactive oxygen species are produced in endothelium, in smooth muscle cells, and adventitia. A large body of evidence documents their potential importance in vasomotor activity, smooth muscle cell growth, expression of adhesion molecules, apoptosis, activation of metalloproteinases, and, of course, lipid oxidation (35). Any or all of these oxidation-related processes may accompany oxidation of LDL or occur independently of it.

Recent studies in Freeman's laboratory failed to confirm the ameliorating effect of CD36 and SRA knockouts cited above (36). The authors suggested that the contradictory results might be due to differences in genetic background. Other possible explanations have been discussed in detail by Witztum (37).

Clinical trial data

Trials in general populations. Most of the large clinical trials of antioxidants have been done using vitamin E or β-carotene. Meta-analysis of the data from these large studies (n = ca. 80,000) shows no benefit at all with regard to cardiovascular outcomes (38). There is no doubt that at the doses used (50–400 mg/d), vitamin E offered no protection against coronary heart disease in a general population. Do these disappointingly negative results mean that the oxidative modification hypothesis is irrelevant in the human disease? Not necessarily. As discussed elsewhere (39), vitamin E may be the wrong antioxidant in humans, the dosage may have been too low, treatment may have been started too late in life, or antioxidant treatment may be beneficial only in some subset of patients subject to unusual oxidative stress (see below). After all, the hypothesis is not that any antioxidant, at any dosage, in any individual will necessarily be effective. The hypothesis is

that oxidative modification plays a quantitatively significant role in pathogenesis. Of course, the implication is that one day an effective antioxidant intervention will be found that will slow the progress of the disease. It would be a mistake to ignore the strong scientific base of the hypothesis and assume prematurely that the human disease is categorically different from that in experimental animals, including the nonhuman primate (40).

The major exception in this category of trials in a general population was the very first trial, the Cambridge Heart Antioxidant Study. Stephens et al. (41) followed just over 2,000 men with angiographically established coronary heart disease randomized to placebo or to 400–800 units of vitamin E daily. There was a statistically significant 47% reduction in the primary combined end point of cardiovascular death or nonfatal myocardial infarction, mainly due to the latter. It is not clear why this study yielded a positive result in contrast to the several larger studies that followed. The dosage of vitamin E (400–800 units/d) was higher and the natural form of the vitamin rather than the racemic mixture was used. Otherwise the protocols were much the same.

A secondary prevention trial (3 year follow-up) using a succinylated analog of probucol (succinobucol) was reported recently. The drug (AGI-1067) had been shown to inhibit atherogenesis in animal models (42), but it failed to show any decrease in its primary composite end point of cardiovascular death, resuscitated cardiac arrest, nonfatal myocardial infarction, nonfatal stroke, hospitalization for unstable angina, or coronary revascularization above that seen with statin therapy alone (43). Data to evaluate antioxidant effect were not presented.

Trials in subpopulations. While vitamin E probably confers no benefit in general populations, there is some evidence that it may benefit patients under increased oxidative stress. For example, the SPACE trial was carried out in patients with end-stage renal disease undergoing hemodialysis, patients known to be under oxidative stress. This study randomized 196 patients to 800 mg/d vitamin E or placebo (44). Pooled vascular events were reduced by 54% and myocardial infarction by 70%, both at $P < 0.02$. An extensive literature establishes that patients in this category are subject to increased oxidative stress, in part due to prooxidant conditions accompanying dialysis and in part due to the disease itself. Another study in patients with end-stage renal failure using N-acetylcysteine as the antioxidant also showed a significant 40% decrease in the combined primary end point of cardiovascular events (45). N-acetylcysteine has also been shown to significantly suppress atherosclerotic lesion progression in uremic apoE-deficient mice (46).

Levy (47) has shown that the haptoglobin 2-2 genotype is associated with inferior antioxidant protection. In diabetics, this haptoglobin genotype is associated with a 2-5-fold increased risk for cardiovascular disease. His group has recently reported a trial of vitamin E (400 U/d versus placebo) in a group of 1,434 diabetic patients with the haptoglobin 2-2 genotype (48). The study was terminated at approximately 500 days, because the first interim analysis showed highly significant protection in the treated group. The primary composite end point of cardiovascular death, nonfatal myocardial infarction, or stroke was reduced by 47% in the treated group ($P = 0.01$). In a later study, again in a cohort of diabetics with the haptoglobin 2-2 genotype, it was shown that adding vitamin E to statin therapy reduced risk by over 60% compared with statin-only treatment (49). These are striking results suggesting that oxidative modification does indeed occur in the human disease but that the benefit of vitamin E (and perhaps the other natural antioxidants) is only apparent in populations under unusual oxidative stress. Diabetic patients with the 2-2 haptoglobin phenotype only represent a few percent of the population, but with these encouraging data in hand, it will be important to look for other subgroups that may benefit and to look for other antioxidant interventions that might be more generally effective.

The benefits of antioxidant treatment in these subgroups should be further tested as a means of preventing vacular events in these types of patients and others that may be uncovered by further research. In any case, these studies provide evidence that oxidative stress may indeed be involved in the human disease and that alternative approaches to antioxidant therapy in the general population may be discovered: different antioxidant agents, different doses, different treatment schedules (including treatment earlier in life).

FUTURE DIRECTIONS

How are we going to resolve some of the conflicting data reviewed above? First, it is essential to go back to studies of the animal models in which antioxidant treatment works and ask hard questions about the detailed mechanisms involved. If oxidation of LDL is an obligatory step in pathogenesis, where and how does it come about? Which enzyme system(s) are responsible? The sometimes contradictory results of gene targeting experiments in mouse models need to be rationalized. The fate of OxLDL in vivo needs to be studied, and the in vivo relevance of the biological effects observed in vitro needs to be explored. Once some of these fundamental questions are answered, we will have a basis for choosing the best antioxidant intervention regimen. Does LDL oxidation occur at all intravascularly? At or under the endothelium? Is it possible that OxLDL found in plasma represents in part LDL that has acquired oxidized lipids, especially oxidized phospholipids, from apoptotic cell membranes by an exchange process? Does macrophage uptake of OxLDL protect (at least initially) by sequestering OxLDL, a potentially cytotoxic substance? Can oxidation take place at other than vascular sites? Can we devise a test for the rate at which LDL oxidation is occurring, a test that could be used to evaluate effectiveness of a potential antioxidant intervention in vivo? Chain-breaking antioxidants like vitamin E may not be the right antioxidants in humans. Once we have the answers to questions like these, we should be able to design more meaningful clinical trials based on solid basic science. Second, the al-

ternative pathways for generating foam cells should be intensively explored in animal models. For example, if LDL could be altered somehow so as to reduce its tendency to aggregate, would that ameliorate atherosclerosis? Do knockouts or inhibitors that interfere with macropinocytosis ameliorate atherosclerosis?

Finally, we must keep an open mind about the still imperfectly understood pathogenesis of this disease. It may very well be that multiple pathways are involved and to different extents at different stages of the disease. For example, oxidation of LDL could play a role in initiation in the generation of foam cells but become less important in the later stages of plaque evolution. Perhaps clinical studies need to be done in younger people, inhibiting at the fatty streak stage. At present that would be difficult to do, but with improvements in noninvasive imaging techniques, it should be possible in the near future.

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