Antioxidants and Atherosclerosis
A Current Assessment
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The purpose of this paper is to review briefly the strengths and weaknesses of the oxidative
modification hypothesis of atherogenesis. As with any new hypothesis, there is danger that
the supporting evidence, even if limited, will generate enthusiasm that runs too far and too fast. Because the hypothesis has not yet been falsified, it deserves further study. It is hoped that it will stand up to closer inspection and critical evaluation. At this time, however, it remains a hypothesis that rests primarily on in vitro data, a few in vivo rabbit studies and some epidemiological correlational data. Before a clinical trial can be rationally planned, we need to learn more about the mechanisms involved, possibly critical species differences, and the pros and cons of the several potential interventions available to test the oxidative modification hypothesis. It would be unfortunate if the first tests of the hypothesis were done prematurely, using inadequate intervention modalities and resulting in a false negative result. Clinical trials are expensive and time consuming; they should only be undertaken when the science base is sufficiently strong.

Oxidative Modification Hypothesis
of Atherogenesis

Because the rationale for the oxidative modification hypothesis has been reviewed elsewhere, we will limit our discussion to a brief outline of the hypothesis (Figure 1) and the evidence that supports it.

Monocyte/macrophages express the low density lipoprotein (LDL) receptor, but the rate at which they take up native LDL is evidently insufficient to generate foam cells, as first noted by Goldstein et al. This led to the prediction that there must be some

modified form of LDL that was the actual ligand taken up by monocyte/macrophages and generating cholesterol-laden foam cells. LDL treated with acetic anhydride (acetyl LDL) was taken up much more rapidly than native LDL, and its uptake was specific and saturable. The specific receptor for acetyl LDL has now been cloned and characterized. Other chemical modifications that mask the epsilon amino groups of lysine residues in LDL also converted it to a form recognized by this receptor. However, there was no evidence that these chemical modifications occurred in vivo. Henriksen et al then showed that incubation of LDL with cultured endothelial cells or smooth muscle cells converted the LDL to a radically altered form that was taken up much more rapidly by macrophages in culture than native LDL and that this uptake could lead to foam cell formation. Moreover, the uptake occurred in part by the acetyl LDL receptor. Subsequent studies showed that the modification was basically an oxidative damage to the LDL. Oxidized LDL is potentially more atherogenic than native LDL in several additional ways: 1) It is chemotactic for circulating monocytes. 2) It is an inhibitor of the motility of resident macrophages. 3) It is cytotoxic for cells in culture. 4) It can stimulate release of a chemotactic factor from endothelial cells in culture. 5) It can stimulate the release of a colony stimulating factor and of a monocyte chemotactic factor from endothelial cells in culture.

More recently, evidence has been presented that oxidative modification of LDL does indeed occur in vivo. These lines of evidence include the following: 1) Treatment of LDL receptor-deficient rabbits (WHHL rabbits) with probucol inhibits the progression of atherosclerotic lesions. Probucol was shown in 1986 to be a very effective antioxidant and to protect LDL against oxidative modification in vitro. In the studies of Carew, Schwenke and Steinberg a control group of rabbits was treated with an unrelated cholesterol-lowering agent, lovastatin, which is not an antioxidant. The dosage was adjusted so that there was no statistically significant difference in the cholesterol levels in the two groups. Despite this, the group treated with the antioxidant, probucol, had much less extensive lesions. Furthermore, in the probucol-treated group, but not in the lovastatin-treated group, the rate at which injected labeled native LDL was taken up in macrophage-rich
lesions was reduced, whereas there was no inhibition of its uptake and degradation in normal sections of the artery. Kita et al.\(^18\) independently observed an antiatherogenic effect of probucol in WHHL rabbits, but did not study LDL metabolism by the artery wall. Although these data are impressive and represent the first in vivo evidence supporting the antiatherogenic potential of an antioxidant, it should be pointed out that there are additional mechanisms that could contribute to the antiatherogenic effect. For example, probucol has been shown to increase the plasma activity of cholesteryl ester transfer protein,\(^20\) thus increasing the rate of transfer of cholesteryl esters from HDL into lower-density lipoprotein fractions. Work by Ku et al.\(^21\) shows that probucol treatment inhibits the release of interleukin-1. Finally, there are studies\(^22\) suggesting that probucol interferes with the ability of macrophages to take up and degrade lipoproteins, although others\(^17,23\) have not been able to confirm that result. Therefore, even what is generally regarded as the best evidence for a protective effect of an antioxidant is less than definitive. 2) Antibodies against oxidized LDL or so-called “models” of oxidized LDL, such as malondialdehyde-conjugated LDL or 4-hydroxynonenal-conjugated LDL, show positive reactivity in atherosclerotic lesions, but not in normal arteries.\(^24-27\) 3) Using gentle extraction procedures, it is possible to isolate and characterize LDL derived from atherosclerotic lesions.\(^25,28\) This LDL includes oxidized LDL, characterized in terms of its physical and biological properties and also by Western blotting using antibodies against oxidized LDL or analogues thereof. 4) Autoantibodies against oxidized LDL have been demonstrated in the plasma of patients and of Watanabe and New Zealand White rabbits.\(^25,29\)

In summary, there is certainly sufficient evidence to warrant further study of the oxidative modification hypothesis, but evidence in vivo is thus far limited.

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**FIGURE 1.** A schematic outline of the oxidative modification hypothesis, showing the several ways in which oxidized low density lipoprotein (LDL) is potentially more atherogenic than native LDL. Monocytes, the major precursor for foam cells in the fatty streak, are shown adhering to the endothelium and then penetrating to the subendothelial space. Oxidized LDL can directly stimulate this by virtue of its lysolceithin content, and lightly oxidized LDL (MM-LDL) can stimulate indirectly by increasing the release of MCP-1 from endothelial cells. Oxidized LDL is a ligand for the scavenger receptor that is expressed as the monocyte differentiates to a tissue macrophage, and this leads to the accumulation of lipids in the developing foam cells. This monocyte/macrophage differentiation can be facilitated by the release of macrophage-colony stimulating factor (M-CSF) from endothelial cells under the influence of MM-LDL. Finally, oxidized LDL can induce endothelial damage and thus facilitate the atherogenic process by allowing entry of elements from the blood and by allowing adherence of platelets. Additional properties of oxidized LDL not shown here that may make it more atherogenic are the fact that it is immunogenic and the fact that it interferes with response of arteries to endothelial-derived relaxation factor (EDRF).
Earlier studies showed the ability of probucol to inhibit atherogenesis in cholesterol-fed rabbits or in monkeys, but in those studies the hypocholesterolemic effect of probucol may have been a sufficient basis for the observed effects on atherosclerosis (i.e., its effects as an antioxidant were not uniquely tested). Recent studies by Stein et al failed to demonstrate an antiatherogenic effect of probucol in cholesterol-fed rabbits, whereas Daugherty et al showed a striking inhibition. A recent study by Björkhem and coworkers showed that butylated hydroxytoluene, a close chemical homologue of probucol and an equally effective antioxidant, also slows the progression of atherosclerosis in cholesterol-fed rabbits.

Evidence in Humans

Three clinics have reported regression of cutaneous and tendinous xanthomata with probucol treatment out of proportion to the degree of lowering of the plasma LDL level. Most of these observations were made in patients with heterozygous or homozygous familial hypercholesterolemia. In the paper by Yamamoto et al an attempt at quantification was made by xerography of the Achilles tendon as a function of time, but for the most part the studies lack quantification and are difficult to evaluate.

There are no reported clinical trial data on the effectiveness of probucol treatment in slowing the progression of atherosclerosis. Such a trial is now in progress in Sweden, using femoral angiography to assess progression or regression of lesions. Unfortunately, the design of this study is such that it will probably not answer the question of whether the antioxidant effect of probucol contributes to any observed beneficial effect. Only subjects who showed at least a 10% further drop in cholesterol level when started on probucol were eligible for randomization. Consequently, there will be at least a 10% difference in cholesterol level between the probucol-treated group and the group not receiving probucol. Therefore, even if there is an apparent benefit in terms of progression, it will not be possible to ascribe that benefit to the cholesterol-lowering effect, to the antioxidant effect, or to both. Ultimately, if one wants to specifically assess the antioxidant property of probucol as it relates to atherogenesis, a design analogous to that used by Carew, Schwenke and Steinberg in studies of Watanabe rabbits will have to be used.

There is a sizable literature on the use of antioxidants in the clinic, but almost without exception these papers are anecdotal or do not have the kind of careful, double-blind design that is needed to firmly establish the effectiveness of a therapy. The first double-blind study is in progress (the Harvard Physicians’ Heart Study), and a preliminary report indicates a significant protective effect with β-carotene supplementation.

Also worthy of note is a growing body of epidemiologic evidence showing a negative correlation between levels of antioxidants and risk of coronary heart disease. But of course epidemiologic correlations cannot establish causal relationships; they can only suggest experiments.

Do We Have a Sound Basis for Planning a Clinical Trial of “Antioxidant Therapy”?

If we were asked to plan a clinical intervention trial today, what would we recommend? Which antioxidant should we use? And at what dosage? Unfortunately, we are not really in a position to answer these questions. We need to know more about the mechanisms involved. For example, is antioxidant protection limited to the protection conferred by the presence of the antioxidant in the LDL molecule itself? Or does the entry of the antioxidant into cells play a role? For example, it appears that probucol can act on monocytes to inhibit their ability to release interleukin-1. Recently Parthasarathy has used a water-soluble analogue of probucol to increase intracellular concentrations and found that cells loaded with probucol have a sharply reduced capacity to oxidatively modify LDL. In other words, the protection afforded in the earlier studies by Carew et al and by Kita et al may have been due not only to the presence of the probucol in LDL but also to some additional effect of probucol on cellular metabolism. If that is true, then the methods currently being used in many laboratories to compare candidate compounds for use in a clinical trial may yield misleading results. Most investigators simply measure the extent to which LDL containing the antioxidant is protected against this or that oxidative stress in vitro. On the other hand, it may develop that compounds have significant but variable additional effects because of their uptake into cells. Such effects would not be measured by the methods currently in use.

How should we assess the degree of protection afforded even in the in vitro systems? These systems are crudely defined at the moment and they may or may not reflect appropriately the conditions in the wall of the artery, which are the conditions that really matter. For example, a number of investigators are using the lag time during oxidation of LDL in vitro as an indicator of the extent of antioxidant protection afforded. Is that the only relevant parameter? What about the rate of and the extent of diene conjugation? The latter can be strongly influenced by the fatty acid composition of the LDL. Recent studies by Parthasarathy have shown that feeding rabbits a diet highly enriched in oleic acid leads to the formation of LDL molecules that are remarkably resistant to oxidative modification, not because of a difference in antioxidant content but because of the difference in fatty acid composition.

Another example will illustrate how far we are from fully understanding this system. We are not even sure whether the oxidation of LDL is due to the release of superoxide anion from the cells or the action of cellular lipooxygenases or some combination of the two. The in vitro systems we use have the LDL in free solution and depend upon the presence
of transition metal ions in the medium. In the subendothelial space the oxidation may occur in a sequestered "micro-environment" and may be based on quite different mechanisms.

Another example will illustrate the gap in our knowledge. We do not know whether the generation of cytotoxic fatty acid degradation products in LDL is more important in the atherogenic process perhaps than the conversion of the LDL to a form that can generate foam cells. If it is, then we should be measuring that property of oxidized LDL rather than simply measuring thiobarbituric acid-reactive substances or rates of uptake of modified LDL by macrophages.

Another issue that needs to be given careful thought is the age of subjects to be chosen for a clinical trial. The oxidation modification hypothesis originally was concerned primarily with the conversion of LDL to a form recognized by the scavenger receptor(s). If this were the major difference between native LDL and oxidized LDL, then oxidative modification might be relevant only to the generation of the fatty streak lesions and not necessarily relevant to the evolution of that lesion to the more complicated and more clinically significant lesions. On the other hand, there is considerable evidence that the fatty streak is the precursor of most of the advanced, clinically significant lesions, both in animals and in man. If we could defer the evolution of the fatty streak lesions we would presumably decrease the number of mature, threatening lesions that develop from them. Moreover, even the more advanced lesions seem to have at their edges an infiltration of foam cells and evidence of continuing inflammatory events that signify a growing lesion. To the extent that this is true, inhibition of oxidative modification may influence the further development even of more advanced lesions. Finally, we should take note that the cytotoxicity of oxidized LDL could contribute to the inflammatory process and, specifically, stimulate the release of growth factors that cause smooth muscle cells to proliferate, lay down connective tissue matrix, and produce the space-occupying lesion. Oxidized LDL itself, or macrophages that have taken it up and subsequently died, could cause ulceration and endothelial damage and, thus, precipitate terminal thrombosis. These are, of course, only speculations. We badly need to know more about just where and how oxidized LDL makes its impact on the natural history of the atherosclerotic lesion before we can plan a clinical trial wisely.

Still another issue has arisen recently. The fatty acid composition of the diet can have major effects on the ease with which LDL undergoes oxidative modification. Therefore, the nature of the diet may be an important variable in any study designed to test the efficacy of an antioxidant. If the fatty acid composition of the diet is not controlled, then the correlations with antioxidant levels may be lost. Furthermore, it may be wise to take advantage of these effects of fatty acid composition and design a trial with a diet containing a low content of polyunsaturated fatty acids and a high content of monounsaturated fatty acids. One could even protect the LDL doubly by increasing the antioxidant content of the LDL and by designing the diet to generate LDL that is intrinsically resistant to oxidation.

Summary

Oxidative modification of LDL may very well play a significant role in atherogenesis. At some point this hypothesis will have to be tested critically in a clinical setting. At this time, however, our knowledge is incomplete, and it is not really possible to make fully informed decisions about how such a trial should be conducted: that is, with which antioxidant, in which population, for how long, etc. It would be best to await results of further basic studies in animal models and the results of pilot studies in humans before launching what will necessarily be a very expensive and time-consuming project. It would be especially unfortunate if such a study were conducted in a less than optimal manner and yielded a negative or an inconclusive result. That might make it more difficult to obtain the necessary enthusiasm and financial support at a later time for a more appropriate clinical trial.

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