Conferences and Reviews

Why Are Low-Density Lipoproteins Atherogenic?

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Low-density lipoproteins (LDLs) carry most of the cholesterol in human plasma, and high levels of LDL cholesterol clearly cause heart disease. In recent years, many scientists have focused on elucidating the pathophysiologic steps that lie between elevated levels of LDL in the plasma and atherosclerotic plaques in the arterial wall. A large number of scientific studies indicate that oxidation of LDL within the arterial wall may be an important early step in atherogenesis. The uptake of oxidized LDL by macrophages is a likely explanation for the formation of macrophage foam cells in early atherosclerotic lesions. In addition, oxidized LDL has many other potentially proatherogenic properties.

(Young SG, Parthasarathy S: Why are low-density lipoproteins atherogenic? West J Med 1994; 160:153-164)

Low-density lipoproteins (LDLs) are spherical particles consisting largely of cholesteryl ester, phospholipid, and a single large protein, apolipoprotein (apo) B-100. The LDL particles, which contain about two thirds of the cholesterol in healthy human plasma, are derived from the metabolism of the triglyceride-rich very-low-density lipoproteins (VLDLs). Genetic, pathologic, and epidemiologic studies, studies using animals, and dietary and drug intervention trials using human subjects have implicated high plasma levels of LDLs in the pathogenesis of atherosclerosis.[1]* In recent years, important advances have been made in understanding why these spherical lipid-protein particles are atherogenic. A pathologic hallmark of atherosclerosis is the accumulation of lipids within the macrophages of the arterial wall. There is little doubt that the lipids within the macrophage foam cells of atherosclerotic fatty-streak lesions are derived from the circulating lipoproteins. What are the steps that lead to the uptake of lipoproteins by cells in the arterial wall? Laboratory investigations have indicated that native LDL particles, such as those that circulate in the plasma, are taken up slowly by macrophages; thus, they probably have only a limited capacity to contribute to atherosclerotic foam cell formation.[2] Accumulating evidence, however, suggests that a variety of cell types within the arterial wall can oxidize LDLs and that oxidative modification alters the biologic properties of LDL, converting these lipoproteins into a form that is taken up avidly by macrophages.[3] Thus, LDL oxidation may be important in the development of foam cell lesions in the arterial wall. In addition, oxidized LDL has other properties that appear to be important in the pathogenesis of atherosclerosis.[3] In this brief review we will summarize some of the recent research indicating the role of oxidized LDLs in the pathogenesis of atherosclerosis.

*See also the editorial by A. Chait, MD, "Low-Density Lipoprotein and the Pathogenesis of Atherosclerosis," on pages 183-184 of this issue.

What Are Low-Density Lipoproteins, and Where Do They Come From?

Low-density lipoproteins have a diameter of about 22 nm and a hydrated density ranging from 1.019 to 1.063 grams per ml. The core of the LDL particle consists largely of cholesteryl ester, with a small amount of triglycerides. The surface coat of LDL consists of phospholipids—mainly phosphatidylcholine and sphingomyelin—free cholesterol, and a single molecule of a large protein, apo B-100.[4] The fatty acids of the LDL phospholipids reflect, to an extent, the fatty acids consumed in the diet.[5] The most abundant fatty acid in human LDL is the polyunsaturated fatty acid, linoleate (18:2).[5] Apolipoprotein B-100, which is 4,536 amino acids in length, has numerous sequences throughout its length that are thought to be important in lipid binding.[6] The apo B-100 polypeptide chain is thought to "circumnavigate" the LDL sphere,[7] and it has been speculated that numerous short hydrophobic segments of the apo B molecule "dive" in and out of the neutral lipid core of the particle.[8]

Low-density lipoproteins are the product of the metabolism of triglyceride-rich VLDLs, which are synthesized and secreted into the plasma by the liver.[4] The triglyceride core of the VLDL is hydrolyzed by lipopro-

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This work was supported by National Institutes of Health (NIH) grant 14197 (Arteriosclerosis SCOR) and NIH Project Grant 41633.

This article is a summary of a lecture given at the 1991 meeting of the American College of Cardiology in San Francisco, California, and updated in May 1993.

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ABBREVIATIONS USED IN TEXT

apo = apolipoprotein
HDL = high-density lipoprotein
LDL = low-density lipoprotein
VLDL = very-low-density lipoprotein
WHHL = Watanabe heritable hyperlipidemic [rabbits]

tein lipase, which is located along the capillary endothelium, principally in muscle and adipose tissue. As the triglyceride core of VLDL particles shrinks, the excess surface components of VLDL (including free cholesterol, several small apolipoproteins, and phospholipids) are transferred to the high-density lipoproteins (HDLs); at the same time, cholesteryl esters are transferred from the HDL to the VLDL remnant. Most of the cholesteryl ester in VLDL remnants is derived from the HDL. About half of VLDL particles are removed from the circulation in the liver by specific receptors on the hepatocyte cell membrane.[4] The remainder of VLDL is gradually metabolized in the circulation to smaller, denser, cholesteryl ester-rich particles of the LDL class. The final processing of VLDL remnants to LDL involves processing by another lipolytic enzyme, hepatic lipase. The residence time of LDL in the circulation is normally two to three days. About three fourths of LDL particles are removed by the liver and the remainder by extrahepatic tissues.[9] Most of the uptake of LDL by cells is mediated by a specific cellular receptor, the LDL receptor.[10] The apo B-100 on LDL particles is a ligand for the LDL receptor and mediates the binding and uptake of LDL by cells.[11]

Evidence That High Plasma Levels of Low-Density Lipoprotein Cause Atherosclerosis

Perhaps the best evidence that LDL is atherogenic comes from two monogenic inherited metabolic disorders that cause high plasma LDL levels. In familial hypercholesterolemia, the cellular LDL receptor is either absent or structurally altered so that it cannot bind and internalize LDL.[10] As a result of this genetic defect, LDL cannot be taken up by cells at a normal rate and therefore accumulates in the plasma. In familial defective apo B-100, a single amino acid substitution in apo B-100 interferes with the high-affinity binding of LDL–apo B-100 to the LDL receptor.[12] The metabolic consequences of this defect are virtually identical to those observed with LDL-receptor defects—an accumulation of LDL in the plasma. Affected heterozygotes for either genetic disorder have high LDL-cholesterol levels (> 4.90 mmol per liter [190 mg per dl]), tendon xanthomas, and premature coronary atherosclerosis.[10,13,14] Thus, two different gene defects, each having the primary effect of raising LDL levels, cause premature atherosclerosis.

In addition to genetic evidence implicating LDLs in the pathogenesis of atherosclerosis, many observational, epidemiologic, and cross-cultural studies have shown a strong association between elevated LDL levels and atherosclerotic heart disease.[14] Furthermore, experimental studies with nonhuman primates have demonstrated that cholesterol-rich and saturated fatty acid-rich diets (which elevate plasma LDL levels) result in foam cell-rich atherosclerotic lesions in the aorta and coronary arteries; stopping these atherogenic diets results in the reversal of atherosclerosis.[15-17] In humans, drug therapy specifically designed to lower LDL-cholesterol levels prevents the development of coronary heart disease in healthy hypercholesterolemic subjects[14,18,19] and improves coronary artery anatomy in those with already established atherosclerotic disease.[20-24] Thus, the evidence proving that elevated LDL levels are causally related to atherosclerosis is extremely strong and essentially incontrovertible.

Metabolic Changes in Low-Density Lipoproteins Produced by Chemical Modification

With the link between elevated LDL levels and atherosclerosis firmly established, many investigators of lipoprotein metabolism have shifted their efforts from determining whether or not LDLs are atherogenic to trying to understand *why* LDLs are atherogenic. An important insight into the mechanism of LDL atherogeneity was made by the laboratory of Brown and Goldstein.[2,25] They recognized, clearly articulated, and experimentally explored a seemingly paradoxical set of observations. The first observation was that elevated levels of LDL in the plasma led to the development of atherosclerotic fatty-streak lesions containing macrophage foam cells. The second observation was that in both humans and animals with genetic defects in the LDL receptor, typical atherosclerotic lesions develop that are rich in macrophage foam cells despite the absence of functional LDL receptors; this strongly implies that the LDL receptor does not mediate the uptake of lipoproteins into foam cells. The third observation was that normal monocyte-derived macrophages have an extremely limited capacity to take up and degrade freshly isolated LDL.

The question that naturally arises from these observations is, What mechanism accounts for the uptake of LDL into macrophage foam cells in the arterial wall? As reported in a landmark article,[25] a chemical modification of LDL was found with acetic anhydride (which acetylates the ϵ amino group of lysine residues of apo B, thereby increasing the negative charge of LDL) that dramatically alters the intrinsic metabolic properties of the LDL. Unlike normal LDL, the acetylated LDL (acetyl LDL) could not bind to the LDL receptor on cultured fibroblasts. The acetyl LDL was, however, taken up and degraded by cultured monocytes or macrophages through the action of a cellular receptor—which Goldstein and co-workers designated the scavenger receptor—distinct from the LDL receptor. The uptake of acetyl LDL by cultured macrophages was saturable and extremely rapid; macrophage foam cells could be produced in cell culture overnight by incubating the cells with acetyl LDL. Unlike the LDL receptor, the activity of the scavenger receptor could not be inhibited by incubating the macrophages with sterols. The scavenger receptor also recognized a variety of other lig-

ands, including acetoacetylated LDL, malondialdehyde-modified LDL, polyinosinic acid, and other polyanionic compounds.[2] A cell membrane receptor that was capable of binding acetyl LDL—designated the acetyl-LDL receptor—has been purified and its primary structure determined.[26-29]

The biologic relevance of acetyl LDL produced by chemical modification of LDL was not clear initially because substantial amounts of acetylation of plasma proteins do not occur in vivo.[2] In 1981, however, Henriksen and associates reported that cultured endothelial cells have the capacity to biologically modify LDL in such a way that it could be taken up rapidly by cultured macrophages.[30] Like chemically acetylated LDL, the endothelial cell-modified LDL had an increased negative charge. Cell culture studies revealed that this modified LDL could compete with acetyl LDL for uptake by the acetyl-LDL receptor of macrophages, indicating that it binds to the same receptor.[30,31] The observation that endothelial cells could biologically modify LDL was of immediate interest because it posed a plausible hypothesis for how LDL might be taken up in vivo by macrophage foam cells in the arterial wall. Subsequent cell culture studies of LDL modification have shown that each principal cell type found in the arterial wall—endothelial cells, smooth muscle cells, macrophages, and fibroblasts—have the capacity to modify LDL so that these lipoproteins are taken up rapidly by cultured macrophages.[3,32] Furthermore, in situ hybridization and immunocytochemistry studies have shown that macrophages in the arterial atherosclerotic lesions (but not cells of nonlesioned regions of the artery wall) express the acetyl-LDL receptor—the receptor that recognizes biologically modified LDL.[3,33,34] In contrast, the arterial macrophage foam cells express little LDL receptor.[34] The acetyl-LDL receptor has also been identified in rabbit smooth muscle cells by the laboratory of Pitas.[35]

Nature of the Biologic Modification of Low-Density Lipoprotein by Cells

Studies at the University of California, San Diego,[30,36] and other laboratories[37] have shown that the biologic modification of LDL by cultured endothelial cells involves oxidation of the polyunsaturated fatty acids in the low-density lipids and that this modification reaction can be largely or completely inhibited by antioxidants such as butylated hydroxytoluene or vitamin E.[3,38] Oxidative modification of LDL by cultured cells is absolutely dependent on small concentrations of copper or iron and can be inhibited by divalent metal chelators such as edetate. The oxidation of LDL requires the free radical-mediated abstraction of a hydrogen atom of a polyunsaturated fatty acid, forming a fatty acid radical, that then can react with oxygen to form lipid hydroperoxides.[32] The initial peroxides or radicals can react with antioxidants contained within LDL, such as vitamin E, and thereby lose their ability to produce oxidation; an LDL particle that is sufficiently enriched in antioxidants is resistant to oxidative modification.[39] Once the antioxidants are depleted, however, the lipid hydroperoxides can be a source of further free radicals by ferric or cupric ion catalyzed reactions, leading to an acceleration of polyunsaturated fatty acid peroxidation. The speed and extent of fatty acid peroxidation is dramatically increased by phospholipase A_2 activity, which is intrinsic to the LDL particle and may be specified by some part of the apo B-100 molecule.[39,40] It seems likely that this phospholipase activity specifically acts on oxidized phospholipids at the surface of LDL, thereby liberating fatty acid hydroperoxides and allowing them to disperse throughout the LDL particle, accelerating the oxidation reaction. Inhibitors of phospholipase A_2 such as *p*-bromophenacyl bromide dramatically inhibit LDL oxidation.[40] The phospholipase activity within LDL is responsible for an increased content of lysolecithin in oxidized LDL. Similar to the use of cultured cells, LDLs can be oxidized in vitro simply by incubating them with low concentrations of cupric or ferric ions.[32]

The mechanism for initiating LDL oxidation by cultured cells is not understood in detail and requires further study. Some investigators think that the oxidation of polyunsaturated fatty acids is initiated by the secretion of superoxide anions, which are eventually capable of generating highly reactive hydroxyl radicals that can attack polyunsaturated fatty acids to form fatty acid hydroperoxides.[41] Other evidence suggests that superoxide anions may have only a secondary role in LDL oxidation.[42] Another possibility for the oxidation of LDL is that a cellular enzyme, 15-lipoxygenase, generates fatty acid hydroperoxides, which could be incorporated into the cell membrane, where they could interact with extracellular LDL, or could leave the cell, where they might interact directly with LDL.[32,42] In the presence of divalent metal ions, fatty acid hydroperoxides can lead to a rapid oxidation of LDL fatty acids. Sparrow and associates have shown that a purified soybean lipoxygenase can oxidize LDL effectively, particularly when a phospholipase A_2 is included in the reaction.[43] Cell-generated cysteine, through cellular cysteine recycling, has also been suggested to have an important role in the generation of extracellular free radicals.[44] At present, it is generally thought that different cell types may contribute to the oxidation of LDL by one or more mechanisms, and miscellaneous factors including cytokines and cell activation may influence the rate of cell-induced oxidation of LDL.

Oxidation results in a number of chemical changes, in both the lipid and protein moieties of LDL.[36] During the oxidation of LDL by cultured cells or by incubation with low concentrations of cupric ions, the apo B molecule becomes fragmented[36]; frequently little or no full-length apo B-100 can be detected in oxidized LDL preparations. The fragmentation is almost certainly the result of an oxidation reaction, since it cannot be blocked by various inhibitors of proteolysis.[45] In addition, several amino acids are susceptible to oxidation. In particular, there is a considerable loss of lysines, histidines, methionines, prolines, and cysteines. Oxidation of LDL also leads to a striking depletion of polyunsaturated fatty acids.[46] In contrast, monounsaturated fatty acids such as oleic acid (18:1) are

not as susceptible to oxidative degradation and may actually retard the oxidative modification of LDL when they are present in LDLs in sufficiently high concentrations.[47] (Resistance of oleic acid to oxidation offers an attractive scientific basis for the beneficial effects of a "Mediterranean" diet in protecting against coronary artery disease.[5]) Low-density lipoprotein sterols also undergo oxidation; the most abundant oxidized sterols are 7-ketocholesterol, 7-hydroxycholesterol, hydroperoxycholesterol, and 5,6-epoxycholesterol.[48-50]

The effects of oxysterols on cellular metabolism have not been studied in detail. It has been suggested that some of the oxidized cholesterol derivatives may affect the acyl-coenzyme A:cholesterol acyltransferase reaction, thus accounting for the decreased esterification of cholesterol when oxidized LDL is incubated with macrophages.[49,50] In addition, the peroxidation of the polyunsaturated fatty acids of LDL leads to the production of a large number of highly reactive ketones and aldehydes, including malondialdehyde, hexanal, and 4-hydroxynonenal.[46] Either the fatty acid hydroperoxides themselves[51] or the fatty acid breakdown products[3] covalently react with the ε amino groups of lysine residues in apo B; consequently, the lysine content of the apo B of oxidized LDL is considerably lower than is observed in the apo B of normal LDL.[52] Derivatization of the apo B lysines by-products of LDL oxidation almost certainly accounts for the increased negative charge of oxidized LDL and for the ability of oxidized LDL to bind to the scavenger receptor. Parthasarathy and colleagues have successfully solubilized the derivatized and fragmented apo B from delipidated oxidized LDL and have demonstrated that it competes with acetylated LDL for binding to the acetyl-LDL receptor.[53] Also, LDL modified in vitro with malondialdehyde[54,55] competes with acetyl LDL for uptake by the acetyl-LDL receptor. Strongly oxidized LDL preparations no longer have the capacity to bind to the LDL receptor, undoubtedly because of the extensive fragmentation and derivatization of apo B. A summary of the important chemical, physical, and intrinsic metabolic properties of oxidized LDL is shown in Table 1.

The neutral lipid core and surface coat of LDL contain several lipid-soluble antioxidants, including β-carotene, α-tocopherol, γ-tocopherol, lycopene, ubiquinol, and other compounds. On a molar basis, however, LDL contains several hundredfold more molecules of polyunsaturated fatty acids than these natural antioxidants.[46,56,57] In vitro studies of the oxidation of LDL have demonstrated that these natural antioxidants protect LDL from fatty acid peroxidation for only a short period. Following a "lag time" during which the antioxidants are consumed, the destruction of polyunsaturated fatty acids proceeds rapidly, along with the fragmentation of apo B and the covalent modification of apo B molecules. There is heterogeneity in the lag time preceding LDL oxidation with LDL specimens from different persons,[58-60] as well as in the extent of oxidation of LDL specimens from different persons. This heterogeneity has been seen even when the oxidation reactions are carried out under standardized conditions,[38] apparently reflecting different antioxidant contents of different LDL specimens. The fatty acid composition of the LDL may also play an important role in the susceptibility of LDL to oxidation.[5,47]

TABLE 1.—*Selected Properties of Oxidized (or Endothelial Cell-Modified) Low-Density Lipoprotein (LDL)*

↑ Negative charge
↑ Density
↓ Uptake by the LDL receptor
↑ Uptake by scavenger receptors
↓ Vitamin E, β-carotene, and other antioxidants
↓ Polyunsaturated fatty acids
↓ Phosphatidylcholine and ↑ lysophosphatidylcholine
↑ Cholesterol oxidation products
↑ Hydroxy fatty acids and carbonyl compounds
Apo B-100 fragmented
Amino acids oxidized and histidine, lysine, and proline residues lost
↑ Lipid and protein fluorescence
↑ Tendency to form aggregates
↑ = increased, ↓ = decreased

Oxidation of LDL is not a simple chemical reaction, and oxidized LDL is not a simple, homogeneous substance. Many different experimental protocols for generating oxidized LDL have been reported, and the degree of oxidation of the lipids and the amount of derivatization of apo B vary widely with different oxidation protocols.[38] For example, only minimal physical and chemical changes related to oxidation are produced by a prolonged storage of LDL with oxygen or by incubation with low concentrations of copper ions.[61] Low-density lipoprotein with low degrees of oxidation, frequently called minimally modified LDL, has little apo B fragmentation, no loss of the capacity to bind to the LDL receptor, and no ability to bind to the scavenger receptor. Minimally modified LDL nevertheless has biologic properties that may be important in leading to the early development of atherosclerotic lesions (discussed later). On the other hand, profound physical, chemical, and metabolic alterations in LDL can be produced by incubating the LDL with higher concentrations of copper or with cultured cells.[32] Aside from the fact that different oxidized LDL preparations may differ in their propensity to undergo oxidation, various investigators have used different types of operational assays for assessing LDL oxidative changes.[38] When attempting to interpret the reported biologic effects of oxidized LDL, it is important to take into account these different oxidation protocols and experimental methods for assessing oxidation and the heterogeneity in the susceptibility of various LDL preparations to oxidation (even those prepared by the same laboratory under standardized conditions).[38]

Evidence That Oxidized Low-Density Lipoproteins Exist In Vivo

Laboratory studies have shown that it is difficult to oxidize LDL in the presence of either serum or plasma.[3] This finding is probably related to the presence of many

different antioxidant compounds in plasma; in addition to lipid-soluble antioxidants, other plasma components, including ascorbate, uric acid, albumin, bilirubin, transferrin, and ceruloplasmin, have antioxidant properties.[56] Because of these high concentrations of antioxidants, oxidation of LDL probably cannot occur to an appreciable extent within the plasma compartment. Cell-mediated oxidation could conceivably occur in a sequestered microenvironment within the arterial wall, where the concentration of antioxidants is lower.[3] Over the past five years, increasing evidence has indicated that the oxidation of LDL can and does occur within the arterial wall.

One of the main reasons to think that oxidation occurs within the arterial walls comes from the examination of lipoproteins extracted from lesioned areas of human arteries and the arteries of Watanabe rabbits with heritable hyperlipidemia (WHHL). (These rabbits have a structurally abnormal LDL receptor[62] and have pronounced hypercholesterolemia and accelerated atherosclerosis.[63]) Ylä-Herttuala and co-workers obtained fresh human aortas from organ donors and gently extracted and then concentrated the lipoproteins from the intima of atherosclerotic lesions.[64,65] The lipoproteins isolated from lesions of human aortas had many of the same chemical and physical properties as those of oxidized LDL prepared by incubating with cultured cells or with copper ion. These properties included an increased electrophoretic mobility (increased negative charge), fragmentation of apo B, increased density, increased lysolecithin content, and a decreased content of polyunsaturated fatty acids.[62,63] In addition, the lesion LDL had a markedly increased ability to be taken up and degraded by cultured macrophages, and this increased rate of uptake and degradation could be competed for by oxidized LDL and other ligands for the acetyl-LDL receptor. Hoff and O'Neil prepared LDL from lesions of human aortas obtained at autopsy and generally found physical and chemical alterations in the lesion LDL that were similar to those identified by Ylä-Herttuala and associates.[64-66] In addition to documenting that the LDL had an increased electrophoretic mobility and increased rate of uptake by the scavenger receptor, they found that the lesion LDL had increased fluorescence emission (420 to 450 nm, excitation at 360 nm), which is characteristic of Schiff-base adducts formed by the products of lipid oxidation and the lysines on apo B.[66]

There is also immunochemical evidence for LDL oxidation. Investigators at the University of California, San Diego, have shown that monoclonal and polyclonal antibodies can be developed that are highly specific for chemically modified lysine residues, including the chemical modifications of lysines that occur during LDL oxidation.[67,68] For example, they have developed antibodies against lysine residues that have been modified by either malondialdehyde or 4-hydroxynonenal, two aldehydes that are formed during the breakdown of polyunsaturated fatty acids. Both Ylä-Herttuala and colleagues and Hoff and O'Neil have used these immunochemical reagents to characterize the LDL isolated from atherosclerotic lesions.[64-66] Both groups found that this LDL increased in reactivity with antibodies specific for malondialdehyde-modified lysine residues. Further support for the existence of oxidation in the arterial wall has come from studies of Rosenfeld and co-workers on the properties of macrophages within atherosclerotic lesions.[69] They generated atherosclerotic lesions in the aortas of rabbits by balloon de-endothelialization followed by diet-induced hypercholesterolemia. They purified macrophage foam cells from the aortic lesion and demonstrated that these cells showed positive immunoreactivity to polyclonal and monoclonal antibodies directed against malondialdehyde- and 4-hydroxynonenal-modified LDL. These macrophages also degraded oxidized LDL by the acetyl-LDL receptor pathway. Furthermore, these cells had the capacity to oxidize LDL in vitro.[69]

Immunohistochemical studies have also provided support for the proposition that LDL is oxidized within atherosclerotic lesions. Haberland and colleagues prepared a monoclonal antibody specific for malondialdehyde-derivatized lysines and used it to look for evidence of malondialdehyde-modified proteins in atherosclerotic lesions of WHHL rabbits.[70] They found that this antibody strongly stained the extracellular matrix surrounding macrophage foam cells and that this staining colocalized with immunostaining with an apo B-100-specific monoclonal antibody. Subsequently, Palinski and associates also showed staining of rabbit arterial lesions by antibodies specific for oxidized LDL, malondialdehyde-modified LDL, or 4-hydroxynonenal-modified LDL.[68] Moreover, these antibodies stained both extracellular areas and macrophages. Colocalization of the epitopes for oxidized LDL and apo B was not always observed, in contrast to the results reported by Haberland and co-workers.[70]

The immunostaining observed with antibodies to malondialdehyde- and 4-hydroxynonenal-modified LDL proteins cannot be viewed as conclusive evidence for the presence of oxidized LDL in the lesions, as these antibodies recognize only derivatized lysine residues and would bind to modified lysine residues in *any* protein (not just apo B). These studies also do not show that oxidation of the lipoproteins occurred before the lipoproteins were taken up by the macrophages. Studies by Mitchinson's group at Cambridge (England) University have shown that macrophages can oxidize lipids intracellularly and generate "ceroids" that are thought to be oxidized lipid-protein complexes.[71] Despite these qualifications, the immunohistochemical studies provide strong circumstantial evidence for LDL oxidation in the arterial wall. Another finding that lends circumstantial support to LDL oxidation in vivo is that in both rabbits and humans, autoantibodies frequently develop against oxidized LDL.[68]

Ylä-Herttuala and co-workers recently used immunohistochemical studies and in situ hybridization studies to compare the distribution of antibodies to oxidized LDL (such as malondialdehyde-derivatized proteins) and several gene products thought to be important in LDL oxidation and the generation of foam cells.[34,72] They demonstrated that the proteins containing epitopes of oxidized LDL colocalize with the messenger RNA for 15-

lipoxygenase in arterial lesions of WHHL rabbits.[72] As described earlier, 15-lipoxygenase is a cellular enzyme that may have a role in initiating LDL oxidation.[42] In a follow-up study, they examined human atherosclerotic lesions obtained from organ donors and found that the immunostaining of lesions with antibodies to oxidized LDL colocalized with the messenger RNA for 15-lipoxygenase and that for the acetyl-LDL receptor.[34] The immunohistochemical and in situ hybridization studies (together with the studies on LDL extracted from arterial lesions) favor the proposition that oxidized LDL exists in the arterial wall. These histologic studies, however, provide only "snapshots" of different events in the arterial wall and cannot by themselves be used to determine whether the observed phenomena are mechanistically related or whether they represent important events in atherosclerosis. Nevertheless, they lend support to the hypothesis of the existence of LDL oxidation in vivo and a possible role for the scavenger receptor-mediated uptake of cell-oxidized LDL in the generation of early atherosclerotic lesions.

Atherogenic Effects of Oxidized Low-Density Lipoprotein

As outlined, the internalization of oxidized LDL by scavenger receptors of arterial wall macrophages is a plausible mechanism for the formation of macrophage foam cells. Recent studies have indicated that oxidized LDL may be "double trouble" for macrophages. Not only is it taken up avidly by the scavenger receptor, but it also tends to accumulate within cells because of a defect in metabolizing oxidized LDL. Sparrow and colleagues showed that oxidized LDL accumulates within cells, in contrast to acetyl LDL.[73] Lougheed and associates recently demonstrated that the derivatization of apo B by lipid peroxidation products results in extremely inefficient digestion of the modified apo B by lysosomal proteases.[74] Inefficient degradation of oxidized LDL could be an important factor contributing to foam cell development because it could retard the efflux of ingested lipids. Furthermore, this finding could help to understand earlier reports[69] that oxidatively modified LDL can be demonstrated in macrophages by immunohistochemical techniques using various antibodies specific for derivatized lysine residues.

Aside from participating in foam cell development, oxidized LDL has many other properties that could play a role in atherosclerosis. One of the biologic effects of oxidized LDL is its cytotoxic effect on cultured endothelial cells. This effect was reported by Henriksen and colleagues and by Chisolm and co-workers.[37,75] Both groups observed that LDL that was incubated with cultured cells under conditions permitting oxidation became toxic to the cells. This work was extended by Chisolm and colleagues in recent years, and it now appears that the cytotoxicity of oxidized LDL is influenced by the phase of the cell cycle.[76] They have shown that cells in the proliferative phase are affected by oxidized LDL to a greater extent than are confluent, resting cells. Cytochalasin B, a blocker of cell proliferation and migration, as well as irradiation (which blocks cell proliferation), prevents the cytotoxic effects of oxidized LDL. Similarly, the treatment of cells with hydroxyurea to prevent them from entering the S phase of growth blocked the toxicity of oxidized LDL, indicating that cell toxicity is selective to cells in the S phase. Neither the active cytotoxin nor the precise mechanism(s) by which the toxicity of oxidized LDL is effected is clear at present. Antioxidants seem to ameliorate the cytotoxic effect of already oxidized LDL on cultured cells. For example, cells previously exposed to antioxidants appear to be protected against the cytotoxicity of oxidized LDL.[77] Whatever the exact mechanism, damage to the arterial endothelia by oxidized LDL could contribute to the development of atherosclerosis by increasing the flux of lipoproteins into the arterial intima, facilitating the entry of leukocytes into the intima, or by promoting thrombosis and platelet aggregation.

The effects of oxidized LDL may also explain why monocytes, the progenitor cells of tissue macrophages, enter the artery and why the macrophages become entrapped in the atherosclerotic artery. Quinn and associates have established that the lipid components of oxidized LDL contain chemotactic factors for circulating monocytes, possibly explaining the initial recruitment of monocytes into the arterial wall.[78] Furthermore, they have shown that the lipid components of oxidized LDL have the capacity to inhibit the migration of resident macrophages in culture, perhaps explaining why macrophages become trapped in the intima. Quinn and co-workers demonstrated that lysolecithin (generated by phospholipase A_2 during LDL oxidation) is responsible for the monocyte chemotactic activity of LDL.[79] These early studies were important because they stimulated enormous interest in elucidating new mechanisms (aside from foam cell formation) whereby oxidized LDL might contribute to atherogenesis. Other investigators have since established many other potentially proatherogenic properties of oxidized LDL (Table 2 and Figure 1). It is noteworthy that many of the effects of oxidized LDL are mediated by its lipid components.

Lysolecithin, a cytolytic lipid found in oxidized LDL, possesses several potent biologic properties. In addition to its monocyte chemotactic activity, it is chemotactic to T lymphocytes.[80] T lymphocytes, which are present in atherosclerotic lesions, together with monocytes-macrophages, produce various cytokines (such as interleukin-4 and gamma interferon) that have profound effects on monocyte-macrophage functions, including the induction and regulation of 15-lipoxygenase and the activity of the scavenger receptor. In contrast, B lymphocytes and neutrophils do not respond to chemotactic stimulus by lysolecithin. Lysolecithin also induces adhesion molecules on endothelial surfaces. Kume and colleagues reported that lysophosphatidylcholine induced the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in cultured endothelial cells, a property mimicked by several other metabolizable lysophospholipids.[81] These adhesion molecules support the binding of

TABLE 2.—*Potentially Proatherogenic Effects of Oxidized Low-Density Lipoprotein (LDL)*

Biologic Effect	*Responsible Component of Oxidized LDL*
Increased uptake by macrophages	Modified protein
Monocyte chemotaxis	Lysophosphatidylcholine
Inhibited macrophage chemotaxis	Unknown lipid
Cellular cytotoxicity	Lipid, possibly an oxysterol
Inhibited endothelium-dependent relaxation	Lysophosphatidylcholine
T-lymphocyte chemotaxis	Lysophosphatidylcholine
Increased monocyte adhesion to endothelium	Unknown lipid
Induced granulocyte and macrophage colony-stimulating factors	Unknown lipid
Induced monocyte chemotactic protein	Unknown lipid
Increased interleukin-1 production	9-HODE, 13-HODE, modified protein
Increased antigenicity	Modified protein
Increased smooth muscle cell proliferation	Unknown components
Inhibited endothelial migration	Unknown lipid

9-HODE = 9-hydroxyoctadecadienoic acid, 13-HODE = 13-hydroxyoctadecadienoic acid

various leukocytes, including monocytes, to endothelial cells.

A potentially extremely important property of oxidized LDL is its ability to affect vasomotor properties of blood vessels. Many studies have documented pronounced vasomotor abnormalities accompanying atherosclerosis and hypercholesterolemia, including impaired responsiveness to vasodilators and augmented responses to vasoconstrictors.[82] Vascular endothelial cells synthesize endothelium-derived relaxing factor, which has properties similar (if not identical) to nitric oxide. This factor diffuses from endothelial cells and relaxes smooth muscle cells. Kugiyama and co-workers have shown that arteries exposed to oxidized LDL show an unresponsiveness to endothelium-derived vasodilators that is similar to that observed in atherosclerotic vessels.[83] They presented evidence that this defect was due to the lysolecithin in oxidized LDL. Chin and associates presented convincing evidence that a lipid component in oxidized LDL inactivates nitric oxide.[84] In addition to its effects on vascular tone, reducing levels of endothelium-derived relaxing factor may actually increase the development of atherosclerotic lesions in rabbits.[85] Oxidized LDL has also been reported to induce the expression of endothelin from human and porcine endothelium.[86] Endothelin is a polypeptide vasoconstrictor that could contribute to vasospastic events in atherosclerotic vascular disease.

The groups of Berliner and Fogelman at the University of California, Los Angeles, have extensively analyzed the biologic properties of minimally modified LDL.[61] These LDLs have only small amounts of oxidized lipids, little fragmentation of apo B, and insufficient apo B derivatization for recognition by the scavenger receptor; nevertheless, they may have extremely important proatherogenic properties. The treatment of cultured endothelial cells with minimally modified LDL causes a notable increase in the endothelial cell production of a chemotactic factor for monocytes and also results in an increase in the binding of monocytes to endothelial cells.[61] The activity responsible for these actions was located in the polar lipid fraction of the LDL. Subsequently, the same investigators have shown that the monocyte chemotactic factor produced by endothelial cells in response to minimally modified LDL is monocyte chemotactic protein 1.[87] They also showed that minimally modified LDLs cause a pronounced and rapid induction of various monocyte and granulocyte growth factors known to affect the migration, metabolism, survival, growth, and differentiation of monocytes-macrophages and endothelial cells.[88] Those growth factors could be important in the initiation and growth of early atherosclerotic lesions. The specific lipid components of oxidized LDL responsible for inducing monocyte chemotactic protein 1 and the monocyte-granulocyte growth factors are not yet known. In addition to these effects, minimally modified LDLs dramatically induce tissue factor production by endothelial cells.[89] This increased tissue factor production could be important in promoting thrombosis in atherosclerotic lesions.

Several other chemical components of oxidized LDL have been suggested to have potent biologic properties. For example, 9- and 13-hydroxyoctadecadienoic acid, oxidized fatty acid derivatives present in oxidized LDL, elicit interleukin-1 secretion from resting mouse peritoneal macrophages.[90] Interleukin-1 is a cytokine produced by monocytes-macrophages in response to various immune and inflammatory events. It can influence the pathogenesis of atherosclerosis by inducing vascular smooth muscle proliferation and promoting the adherence of

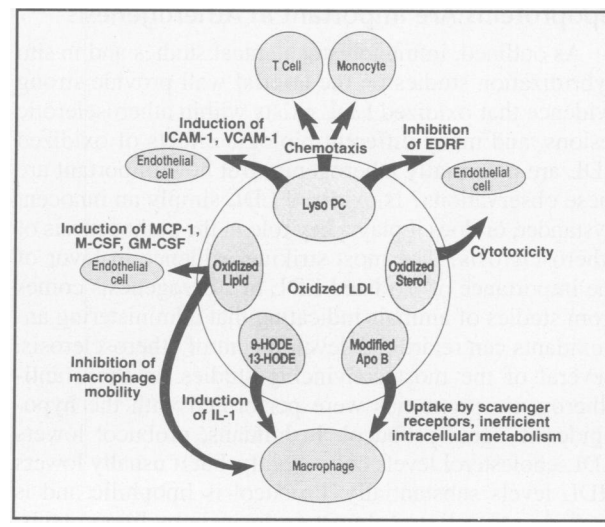

Figure 1.—Mechanisms are shown whereby various components of oxidized low-density lipoprotein (LDL) might contribute to atherogenesis. Apo B = apolipoprotein B, EDRF = endothelium-derived relaxing factor, GM-CSF = granulocyte and macrophage colony-stimulating factor, 9-HODE = 9-hydroxyoctadecadienoic acid, 13-HODE = 13-hydroxyoctadecadienoic acid, ICAM-1 = intercellular adhesion molecule-1, IL-1 = interleukin-1, Lyso PC = lysophosphatidylcholine, MCP-1 = monocyte chemotactic protein 1, M-CSF = monocyte colony-stimulating factor, VCAM-1 = vascular cell adhesion molecule-1

leukocytes to the endothelium. Recent results suggest that the protein component of oxidized LDL can induce the release of interleukin-1 from foam cells isolated from the atherosclerotic lesions of cholesterol-fed rabbits (B. A. Lipton, S. Parthasarathy, V. A. Ord, S. K. Clinton, P. Libby, M. E. Rosenfeld, "Oxidized Low-Density Lipoprotein Stimulates Interleukin-1 Production by Rabbit Arterial Foam Cells," April 1993, unpublished manuscript). Acetyl LDL does not share this property.

In addition to those effects, oxidized LDL has several other proatherogenic effects. For example, recent studies of Murugesan and colleagues show that oxidized LDL, presumably a lipid peroxide, inhibits the migration of endothelial cells.[91] These authors point out that such an effect, if it persists in vivo, could compromise the response of the endothelium to wound healing after injury.

The effects of oxidized LDL on gene expression and on cellular metabolism are being studied intently in many laboratories, and new reports appear monthly. Despite the excitement generated by this research, caution should be exercised in interpreting the reported biologic effects of oxidized LDL, because many of the in vitro cell culture experiments may not accurately reflect in vivo conditions in the arterial wall. We also emphasize that some effects of oxidized LDL may not be atherogenic. For example, it has been suggested that oxidized LDL increases prostacyclin production by cultured cells.[92] It also suppresses the synthesis of platelet-derived growth factor-β[93] and tumor necrosis factor-α synthesis[94] by endothelial cells and macrophages. Whether these phenomena are atherogenic is not yet clear.

Evidence That Oxidized Low-Density Lipoproteins Are Important in Atherogenesis

As outlined, immunohistochemical studies and in situ hybridization studies on the arterial wall provide strong evidence that oxidized LDL exists within atherosclerotic lesions, and many different biologic effects of oxidized LDL are potentially atherogenic. But how important are these observations? Is oxidized LDL simply an innocent bystander, or does it play a key role in the pathogenesis of atherosclerosis? The most striking evidence in favor of the importance of oxidized LDL in atherogenesis comes from studies of animals indicating that administering antioxidants can retard the development of atherosclerosis. Several of the most convincing studies on these antiatherogenic properties were performed with the hypolipidemic agent probucol. In humans, probucol lowers LDL-cholesterol levels only slightly, but it usually lowers HDL levels substantially. Probucol is lipophilic and is carried in the plasma almost exclusively by lipoproteins. It is also a powerful antioxidant—even more powerful than vitamin E. Low-density lipoprotein prepared from the plasma of human subjects taking therapeutic doses of probucol is highly resistant to oxidative modification by cultured endothelial cells when compared with control LDL preparations and consequently has considerably reduced uptake by cultured macrophages.[95] Carew and coworkers tested whether probucol would reduce atherosclerosis in WHHL rabbits.[96] Their study included a group of probucol-treated animals and two control groups. One control group was given no drug and the other control group was given small doses of lovastatin titrated carefully to maintain cholesterol levels at the same level as those in the probucol-treated animals. After seven months, they observed an approximately 50% reduction in the surface area of lesions in the probucol-treated animals compared with those of the cholesterol-matched, lovastatin-treated controls. Furthermore, using sophisticated arterial wall metabolism studies that involved radiolabeled LDL tracers, they documented that in the probucol-treated group, the uptake and degradation of LDL within the lesioned areas of the aorta were reduced compared with those in the lovastatin-treated group. Virtually simultaneously, Kita and colleagues performed similar studies on the effect of probucol on atherosclerosis in WHHL rabbits, and they found essentially identical results—a major reduction in atherosclerotic lesions.[97] Since these two studies, the antiatherogenic properties of probucol have been established by various laboratories both in WHHL rabbits and in cholesterol-fed rabbits.[98]

The efficacy of antioxidants in cholesterol-fed animals, particularly in rabbits, in which the predominant cholesterol-carrying lipoprotein is β-VLDL, raises an interesting issue. The β-VLDLs are large, cholesterol-rich lipoproteins that contain a substantial amount of apo E in addition to apo B. These lipoproteins are taken up effectively by macrophages by an apo E-mediated pathway, a situation that can lead to massive cholesterol accumulation without any requirement for oxidative modification. The effectiveness of antioxidants in retarding atherosclerosis in cholesterol-fed rabbits suggests the possibility that the oxidation of β-VLDL plays a role in inducing atherosclerosis in this experimental model. In vitro studies suggest that β-VLDLs undergo oxidative modification similar to that observed with LDL and that the modified β-VLDLs are taken up by macrophages by the scavenger receptor. Oxidation of β-VLDL in vivo offers a possible explanation for a portion of the foam cell formation and for some of the other pathologic features of atherosclerosis (such as the attraction of T lymphocytes and macrophages into the lesions).

In addition to its antioxidant properties, probucol also affects LDL- and HDL-cholesterol levels, inhibits the release of interleukin 1 from macrophages, and increases the transfer of cholesteryl esters from HDL to LDL. Consequently, the antiatherogenic properties of probucol may not be due solely to its antioxidant properties. To examine this issue further, scientists at Marion Merrell Dow Inc (Kansas City, Missouri) have developed and characterized several chemical derivatives of probucol that retain antioxidant and lipophilic properties but have minimal or no effects on lipoprotein metabolism.[99,100] These analogues retained the ability to inhibit atherosclerosis in WHHL rabbits, implying that the antioxidant properties of probucol may play a key role in inhibiting atherogenesis. The notion that probucol's antioxidant effects are important

is supported by the fact that other antioxidants, including butylated hydroxytoluene[101] and *N,N'*-diphenyl-1,4-phenylenediamene,[102] have antiatherosclerotic effects in animal models. Sparrow and associates showed that *N,N'*-diphenyl-1,4-phenylenediamene effectively inhibited LDL oxidation in vitro at low concentrations and found that it reduced the incidence of aortic lesions in cholesterol-fed rabbits by 50% to 70% without affecting total plasma cholesterol levels.[102]

Another major reason to think that oxidized LDL may be important in atherogenesis is that epidemiologic studies have indicated that plasma levels of natural antioxidants are inversely proportional to the risk of ischemic heart disease. Gey and co-workers ascertained levels of vitamin E and other plasma antioxidants in 16 different European populations that differed sixfold in mortality from ischemic heart disease.[103] Among 12 populations with similar cholesterol levels (clustered around "normal" levels—5.70 to 6.20 mmol per liter [220 to 240 mg per dl]), the blood pressure readings and the serum cholesterol levels were not predictive of ischemic heart disease mortality. A striking inverse correlation was found, however, between heart disease mortality and vitamin E levels. Inverse relationships also were observed between heart disease risk and two other antioxidants, vitamins A and C, although the relationships were considerably weaker. The inverse relationship of ischemic heart disease risk and antioxidants remained significant after adjustment for other classical risk factors—smoking, blood pressure, and cholesterol level.

Stampfer and colleagues recently reported on a prospective study of the relationship between antioxidant vitamin intake and ischemic heart disease in more than 87,000 female nurses.[104] They found a significant (~35%) reduction in coronary heart disease risk in women with the highest quintile of vitamin use versus women with the lowest quintile of vitamin use, and this finding remained significant after adjustment for age, smoking, and other heart disease risk factors. The apparent benefit of vitamin E was largely confined to those who consumed vitamin E supplements for more than two years. Similarly, Rimm and associates assessed vitamin intake in more than 39,000 healthy male health care professionals.[105] During four years of follow-up, 667 cases of coronary artery disease occurred. For men consuming more than 60 IU per day of vitamin E, the risk of coronary disease was reduced by approximately 35%. No decrease in coronary risk was noted in men who had reported a high intake of vitamin C. Riemersma and co-workers reported on a case-control study that supported an inverse relationship between the plasma vitamin E level and the incidence of angina pectoris.[106] In this study, they measured vitamin E levels of 110 patients with angina and 394 control subjects. Patients with angina had significantly lower vitamin E levels, even after adjustment for smoking habits, age, lipid levels, weight, and blood pressure.

No large, prospective, randomized trials have tested whether administering antioxidant vitamins would reduce the incidence of atherosclerosis in humans. The best prospective evidence to date comes from a preliminary report from the Harvard Physicians Health Study that was originally designed to test the hypothesis that β-carotene might reduce cancer rates.[107] This study enrolled 333 male physicians with angina pectoris, coronary revascularization, or both. Those men assigned to receive β-carotene, 50 mg on alternate days, had a 44% reduction in major coronary events ($P < .05$) and a 49% reduction in all major vascular events ($P < .02$) after adjustment for age. Controlling for aspirin use and other major risk factors did not affect these findings. This randomized trial is continuing, and data on the primary prevention of heart disease with β-carotene should become available in a few years. Another large-scale trial testing vitamin E and β-carotene use in the primary prevention of heart disease has recently been funded by the National Institutes of Health.[107]

Several other intriguing studies in humans provide suggestive evidence for the importance of oxidation in atherogenesis. Salonen and associates analyzed whether the titer of antibodies directed against epitopes in oxidized LDL have any predictive value in the progression of carotid atherosclerosis in Finnish men.[108] They compared antibody titers against malondialdehyde-modified LDL in 30 men with accelerated progression of carotid atherosclerosis and in 30 controls without progression. Those with accelerated progression had a significantly higher antibody titer than controls, and the titer of antibodies remained significant in a multifactorial logistic model that considered many other atherosclerotic risk factors. This preliminary study suggests that the titer of antibodies against oxidized LDL might be correlated with the activity of the atherosclerotic disease process. These findings need confirmation, especially because the same investigators did not find any difference between cases and controls with respect to titers of other oxidized LDL autoantibodies.[108] If the results of these immunochemical studies are borne out by future studies, they will suggest that an immune response triggered by oxidized LDL may be important in atherogenesis.

Another recently published chemical study lending support to the pathogenetic importance of oxidized LDL analyzed the length of "lag phase" preceding copper ion-induced LDL oxidation in a group of patients who had had myocardial infarction and had undergone coronary angiography.[58] The authors found an inverse relationship between the lag phase and quantitative estimates of global coronary atherosclerosis, supporting the notion that the quantity of natural antioxidants in LDL has a clinically important effect on atherosclerosis.

Where We Stand With Oxidized Low-Density Lipoprotein

Considerable evidence has now accumulated that the oxidation of LDL in vivo plays an important role in atherogenesis. The story began with cell culture observations indicating that the oxidation of LDL could promote its uptake by macrophages and was toxic to endothelial cells. But in recent years, laboratory studies have focused

on several new mechanisms whereby LDL oxidation, even to minimal degrees, might be atherogenic. Studies of humans and animals have demonstrated the existence of oxidized LDL in atherosclerotic lesions. Studies of animals showing the antiatherogenic effects of several different antioxidants have strongly suggested that the oxidation of LDL plays a key role in atherogenesis. Epidemiologic studies showing an inverse relationship between antioxidant vitamin levels and heart disease have also lent credence to the hypothesis that oxidized LDL is important in atherogenesis.

The emergence of oxidized LDL as a potentially important "player" in atherosclerosis is exciting for both basic scientists and clinicians. For basic scientists, the oxidized LDL explanation has begun to shed light on the "black box" between high levels of LDL in the blood and the development of atheromas in the artery wall. Illuminating the pathway between high cholesterol levels and atherosclerosis has posed many new questions for investigators of atherosclerosis. For example, the topic of precisely which cellular chemical reactions are responsible for the initiation of LDL oxidation in the arterial wall is currently an active area of research. Further research into the mechanism of LDL oxidation may uncover new targets for the drug treatment of atherosclerosis. If 15-lipoxygenase turns out to be the most important enzyme in the initiation of LDL oxidation in the arterial wall, then searches for potent and safe 15-lipoxygenase inhibitors would be in order.[38] Similarly, which natural antioxidants are most effective is a hot topic of research. If future research indicates that ubiquinol-10 is the most effective natural antioxidant, as some have suggested,[109] then intervention studies with this agent should be done. The possible importance of oxidized LDL has led many scientists to focus their efforts on understanding the biology of specific gene products, such as the acetyl-LDL receptor and 15-lipoxygenase.[38] Over the next three or four years, new animal models will likely be created that lack these gene products or overexpress the gene products in the arterial wall. Study of these animals will undoubtedly help to clarify the importance of these gene products in the generation of oxidized LDL and macrophage foam cells.

From the point of view of clinicians, the recent studies on oxidized LDL hold out the promise of a relatively quick payoff. It has been less than a decade since oxidized LDL was proposed as having a possible role in atherosclerosis, yet primary prevention trials are already underway, and angiographic trials to assess the effect of natural antioxidants on coronary anatomy will begin soon.[107] If natural antioxidants are effective in humans, a new approach to the treatment of heart disease will have been established, and physicians would then have to be concerned with the concentration of LDL in their patients' plasma as well as protecting their patients' LDL from oxidation within the artery wall.

Oxidized LDLs have recently gained a lot of attention in the lay literature, and a large percentage of the United States population now consumes antioxidant vitamins with the hope of preventing heart disease. Should physicians "jump the gun" on the scientific trials and begin to recommend antioxidant vitamins or drugs to their patients? Steinberg has recently argued articulately and persuasively that we should await large, prospective, long-term, double-blind clinical trials in humans before advocating vitamin E supplements to our patients.[110] We agree with this point of view. Although the oxidized LDL hypothesis is tantalizing, we should insist on solid clinical trial evidence before making public health policy.

REFERENCES

1. Consensus Conference: Lowering blood cholesterol to prevent heart disease. JAMA 1985; 253:2080-2086

2. Brown MS, Goldstein JL: Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. Annu Rev Biochem 1983; 52:223-261

3. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol–Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 1989; 320:915-924

4. Havel RJ, Kane JP: Structure and metabolism of plasma lipoproteins, *In* Scriver CR, Beaudet AL, Sly WS, Valle D (Eds): The Metabolic Basis of Inherited Disease, 6th edition. New York, NY, McGraw-Hill, 1989, pp 1129-1138

5. Reaven P, Parthasarathy S, Grasse BJ, et al: Feasibility of using an oleate-rich diet to reduce the susceptibility of low-density lipoprotein to oxidative modification in humans. Am J Clin Nutr 1991; 54:701-706

6. Young SG: Recent progress in understanding apolipoprotein B. Circulation 1990; 82:1574-1594

7. Chatterton JE, Phillips ML, Curtiss LK, Milne RW, Marcel YL, Schumaker VN: Mapping apolipoprotein B on the low density lipoprotein surface by immunoelectron microscopy. J Biol Chem 1991; 266:5955-5962

8. Yang CY, Gu ZW, Weng SA, et al: Structure of apolipoprotein B-100 of human low density lipoproteins. Arteriosclerosis 1989; 9:96-108

9. Steinberg D: Lipoproteins and atherosclerosis–A look back and a look ahead. Arteriosclerosis 1983; 3:283-301

10. Brown MS, Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. Science 1986; 232:34-47

11. Milne R, Théolis R Jr, Maurice R, et al: The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. J Biol Chem 1989; 264:19754-19760

12. Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJ: Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. Proc Natl Acad Sci USA 1989; 86:587-591

13. Tybjaerg-Hansen A, Gallagher J, Vincent J, et al: Familial defective apolipoprotein B-100: Detection in the United Kingdom and Scandinavia, and clinical characteristics of ten cases. Atherosclerosis 1990; 80:235-242

14. Tyroler HA: Review of lipid-lowering clinical trials in relation to observational epidemiologic studies. Circulation 1987; 76:515-522

15. Clarkson TB, Bond MG, Bullock BC, McLaughlin KJ, Sawyer JK: A study of atherosclerosis regression in *Macaca mulatta*—V. Changes in abdominal aorta and carotid and coronary arteries from animals with atherosclerosis induced for 38 months and then regressed for 24 or 48 months at plasma cholesterol concentrations of 300 or 200 mg/dl. Exp Mol Pathol 1984; 41:96-118

16. Small DM, Bond MG, Waugh D, Prack M, Sawyer JK: Physicochemical and histological changes in the arterial wall of nonhuman primates during progression and regression of atherosclerosis. J Clin Invest 1984; 73:1590-1605

17. St Clair RW: Atherosclerosis regression in animal models: Current concepts of cellular and biochemical mechanisms. Prog Cardiovasc Dis 1983; 26:109-132

18. Lipid Research Clinics Program: The Lipid Research Clinics Coronary Primary Prevention Trial results–I. Reduction in incidence of coronary heart disease. JAMA 1984; 251:351-364

19. Frick MH, Elo O, Haapa K, et al: Helsinki Heart Study: Primary-prevention trial with gemfibrozil in middle-aged men with dyslipidemia–Safety of treatment, changes in risk factors, and incidence of coronary heart disease. N Engl J Med 1987; 317:1237-1245

20. Rossouw JE, Lewis B, Rifkind BM: The value of lowering cholesterol after myocardial infarction. N Engl J Med 1990; 323:1112-1119

21. Kane JP, Malloy MJ, Ports TA, Phillips NR, Diehl JC, Havel RJ: Regression of coronary atherosclerosis during treatment of familial hypercholesterolemia with combined drug regimens. JAMA 1990; 264:3007-3012

22. Brown G, Albers JJ, Fisher LD, et al: Regression of coronary artery disease as a result of intensive lipid-lowering therapy in men with high levels of apolipoprotein B. N Engl J Med 1990; 323:1289-1298

23. Buchwald H, Varco RL, Matts JP, et al: Effect of partial ileal bypass surgery on mortality and morbidity from coronary heart disease in patients with hypercholesterolemia–Report of the Program on the Surgical Control of the Hyperlipidemias (POSCH). N Engl J Med 1990; 323:946-955

24. Cashin-Hemphill L, Mack WJ, Pogoda JM, Sanmarco ME, Azen SP, Blankenhorn DH: Beneficial effects of colestipol-niacin on coronary atherosclerosis—A 4-year follow-up. JAMA 1990; 264:3013-3017

25. Goldstein JL, Ho YK, Basu SK, Brown MS: Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci USA 1979; 76:333-337

26. Kodama T, Reddy P, Kishimoto C, Krieger M: Purification and characterization of a bovine acetyl low density lipoprotein receptor. Proc Natl Acad Sci USA 1988; 85:9238-9242

27. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M: Type I macrophage scavenger receptor contains α-helical and collagen-like coiled coils. Nature 1990; 343:531-535

28. Freeman M, Ashkenas J, Rees DJG, et al: An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors. Proc Natl Acad Sci USA 1990; 87:8810-8814

29. Brown MS, Goldstein JL: Scavenging for receptors. Nature 1990; 343: 508-509

30. Henriksen T, Mahoney EM, Steinberg D: Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. Proc Natl Acad Sci USA 1981; 78:6499-6503

31. Freeman M, Ekkel Y, Rohrer L, et al: Expression of type I and type II bovine scavenger receptors in Chinese hamster ovary cells: Lipid droplet accumulation and nonreciprocal cross competition by acetylated and oxidized low density lipoprotein. Proc Natl Acad Sci USA 1991; 88:4931-4935

32. Parthasarathy S, Fong LG, Quinn MT, Steinberg D: Oxidative modification of LDL: Comparison between cell-mediated and copper-mediated modification. Eur Heart J 1990; 11(suppl E):83-87

33. Matsumoto A, Naito M, Itakura H, et al: Human macrophage scavenger receptors: Primary structure, expression, and localization in atherosclerotic lesions. Proc Natl Acad Sci USA 1990; 87:9133-9137

34. Ylä-Herttuala S, Rosenfeld ME, Parthasarathy S, et al: Gene expression in macrophage-rich human atherosclerotic lesions—15-Lipoxygenase and acetyl low density lipoprotein receptor messenger RNA colocalize with oxidation specific lipid-protein adducts. J Clin Invest 1991; 87:1146-1152

35. Pitas RE: Expression of the acetyl low density lipoprotein receptor by rabbit fibroblasts and smooth muscle cells—Up-regulation by phorbol esters. J Biol Chem 1990; 265:12722-12727

36. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL, Steinberg D: Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc Natl Acad Sci USA 1984; 81:3883-3887

37. Morel DW, DiCorleto PE, Chisolm GM: Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. Arteriosclerosis 1984; 4:357-364

38. Witztum JL, Steinberg D: Role of oxidized low density lipoprotein in atherogenesis. J Clin Invest 1991; 88:1785-1792

39. Parthasarathy S, Barnett J: Phospholipase A_2 activity of low density lipoprotein: Evidence for an intrinsic phospholipase A_2 activity of apoprotein B-100. Proc Natl Acad Sci USA 1990; 87:9741-9745

40. Parthasarathy S, Steinbrecher UP, Barnett J, Witztum JL, Steinberg D: Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. Proc Natl Acad Sci USA 1985; 82:3000-3004

41. Hiramatsu K, Rosen H, Heinecke JW, Wolfbauer G, Chait A: Superoxide initiates oxidation of low density lipoprotein by human monocytes. Arteriosclerosis 1987; 7:55-60

42. Parthasarathy S, Wieland E, Steinberg D: A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. Proc Natl Acad Sci USA 1989; 86:1046-1050

43. Sparrow CP, Parthasarathy S, Steinberg D: Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. J Lipid Res 1988; 29:745-753

44. Heinecke JW, Rosen H, Suzuki LA, Chait A: The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem 1987; 262:10098-10103

45. Fong LG, Parthasarathy S, Witztum JL, Steinberg D: Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. J Lipid Res 1987; 28:1466-1477

46. Esterbauer H, Jürgens G, Quehenberger O, Koller E: Autoxidation of human low density lipoprotein: Loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. J Lipid Res 1987; 28:495-509

47. Parthasarathy S, Khoo JC, Miller E, Barnett J, Witztum JL, Steinberg D: Low density lipoprotein rich in oleic acid is protected against oxidative modification: Implications for dietary prevention of atherosclerosis. Proc Natl Acad Sci USA 1990; 87:3894-3898

48. Parthasarathy S, Rankin SM: Role of oxidized low density lipoprotein in atherogenesis. Prog Lipid Res 1992; 31:127-143

49. Zhang H, Basra HJK, Steinbrecher UP: Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. J Lipid Res 1990; 31:1361-1369

50. Jialal I, Chait A: Differences in the metabolism of oxidatively modified low density lipoprotein and acetylated low density lipoprotein by human endothelial cells: Inhibition of cholesterol esterification by oxidatively modified low density lipoprotein. J Lipid Res 1989; 30:1561-1568

51. Fruebis J, Parthasarathy S, Steinberg D: Evidence for a concerted reaction between lipid hydroperoxides and polypeptides. Proc Natl Acad Sci USA 1992; 89:10588-10592

52. Steinbrecher UP: Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J Biol Chem 1987; 262:3603-3608

53. Parthasarathy S, Fong L, Otero D, Steinberg D: Recognition of solubilized apoproteins from delipidated, oxidized low density lipoprotein (LDL) by the acetyl-LDL receptor. Proc Natl Acad Sci USA 1987; 84:537-540

54. Shechter I, Fogelman AM, Haberland ME, Seager J, Hokom M, Edwards PA: The metabolism of native and malondialdehyde-altered low density lipoproteins by human monocyte-macrophages. J Lipid Res 1981; 22:63-71

55. Haberland ME, Fless GM, Scanu AM, Fogelman AM: Malondialdehyde modification of lipoprotein(a) produces avid uptake by human monocyte-macrophages. J Biol Chem 1992; 267:4143-4151

56. Esterbauer H, Puhl H, Dieber-Rotheneder M, Waeg G, Rabl H: Effect of antioxidants on oxidative modification of LDL. Ann Med 1991; 23:573-581

57. Esterbauer H, Striegl G, Puhl H, et al: The role of vitamin E and carotenoids in preventing oxidation of low density lipoproteins. Ann NY Acad Sci 1989; 570:254-267

58. Regnström J, Nilsson J, Tornvall P, Landou C, Hamsten A: Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. Lancet 1992; 339:1183-1186

59. Jialal I, Freeman DA, Grundy SM: Varying susceptibility of different low density lipoproteins to oxidative modification. Arterioscler Thromb 1991; 11:482-488

60. Lavy A, Brook GJ, Dankner G, Amotz AB, Aviram M: Enhanced in vitro oxidation of plasma lipoproteins derived from hypercholesterolemic patients. Metabolism 1991; 40:794-799

61. Berliner JA, Territo MC, Sevanian A, et al: Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. J Clin Invest 1990; 85:1260-1266

62. Yamamoto T, Bishop RW, Brown MS, Goldstein JL, Russell DW: Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbit. Science 1986; 232:1230-1237

63. Goldstein JL, Kita T, Brown MS: Defective lipoprotein receptors and atherosclerosis—Lessons from an animal counterpart of familial hypercholesterolemia. N Engl J Med 1983; 309:288-296

64. Ylä-Herttuala S, Palinski W, Rosenfeld ME, et al: Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. J Clin Invest 1989; 84:1086-1095

65. Ylä-Herttuala S: Macrophages and oxidized low density lipoproteins in the pathogenesis of atherosclerosis. Ann Med 1991; 23:561-567

66. Hoff HF, O'Neil J: Lesion-derived low density lipoprotein and oxidized low density lipoprotein share a lability for aggregation, leading to enhanced macrophage degradation. Arterioscler Thromb 1991; 11:1209-1222

67. Steinbrecher UP, Fisher M, Witztum JL, Curtiss LK: Immunogenicity of homologous low density lipoprotein after methylation, ethylation, acetylation, or carbamylation: Generation of antibodies specific for derivatized lysine. J Lipid Res 1984; 25:1109-1116

68. Palinski W, Rosenfeld ME, Ylä-Herttuala S, et al: Low density lipoprotein undergoes oxidative modification in vivo. Proc Natl Acad Sci USA 1989; 86:1372-1376

69. Rosenfeld ME, Khoo JC, Miller E, Parthasarathy S, Palinski W, Witztum JL: Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of low-density lipoproteins, and contain oxidation-specific lipid-protein adducts. J Clin Invest 1991; 87:90-99

70. Haberland ME, Fong D, Cheng L: Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits. Science 1988; 241:215-218

71. Mitchinson MJ, Hothersall DC, Brooks PN, De Burbure CY: The distribution of ceroid in human atherosclerosis. J Pathol 1985; 145:177-183

72. Ylä-Herttuala S, Rosenfeld ME, Parthasarathy S, et al: Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. Proc Natl Acad Sci USA 1990; 87:6959-6963

73. Sparrow CP, Parthasarathy S, Steinberg D: A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. J Biol Chem 1989; 264:2599-2604

74. Lougheed M, Zhang H, Steinbrecher UP: Oxidized low density lipoprotein is resistant to cathepsins and accumulates within macrophages. J Biol Chem 1991; 266:14519-14525

75. Henriksen T, Evensen SA, Carlander B: Injury to human endothelial cells in culture induced by low density lipoproteins. Scand J Clin Lab Invest 1979; 39:361-368

76. Kosugi K, Morel DW, diCorleto PE, Chisolm GM: Toxicity of oxidized low-density lipoprotein to cultured fibroblasts is selective for S phase of the cell cycle. J Cell Physiol 1987; 130:311-320

77. Kuzuya M, Naito M, Funaki C, Hayashi T, Asai K, Kuzuya F: Probucol prevents oxidative injury to endothelial cells. J Lipid Res 1991; 32:197-204

78. Quinn MT, Parthasarathy S, Steinberg D: Endothelial cell-derived chemotactic activity for mouse peritoneal macrophages and the effects of modified forms of low density lipoprotein. Proc Natl Acad Sci USA 1985; 82:5949-5953

79. Quinn MT, Parthasarathy S, Fong LG, Steinberg D: Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proc Natl Acad Sci USA 1987; 84:2995-2998

80. McMurray HP, Parthasarathy S, Steinberg D: Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. J Clin Invest 1993; 92:1004-1008

81. Kume N, Cybulsky MI, Gimbrone MA Jr: Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 1992; 90:1138-1144

82. Bossaller C, Habib GB, Yamamoto H, Williams C, Wells S, Henry PD: Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5′-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. J Clin Invest 1987; 79:170-174

83. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD: Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. Nature 1990; 344:160-162

84. Chin JH, Azhar S, Hoffman BB: Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. J Clin Invest 1992; 89:10-18

85. Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, Billingham ME: Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest 1992; 90:1168-1172

86. Boulanger CM, Tanner FC, Béa ML, Hahn AWA, Werner A, Lüscher TF: Oxidized low density lipoproteins induce mRNA expression and release of endothelin from human and porcine endothelium. Circ Res 1992; 70:1191-1197

87. Cushing SD, Berliner JA, Valente AJ, et al: Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. Proc Natl Acad Sci USA 1990; 87:5134-5138

88. Rajavashisth TB, Andalibi A, Territo MC, et al: Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. Nature 1990; 344:254-257

89. Drake TA, Hannani K, Fei H, Lavi S, Berliner JA: Minimally oxidized low-density lipoprotein induces tissue factor expression in cultured human endothelial cells. Am J Pathol 1991; 138:601-607

90. Ku G, Thomas CE, Akeson AL, Jackson RL: Induction of interleukin 1β expression from human peripheral blood monocyte-derived macrophages by 9-hydroxyoctadecadienoic acid. J Biol Chem 1992; 267:14183-14188

91. Murugesan G, Chisolm GM, Fox PL: Oxidized low density lipoprotein inhibits the migration of aortic endothelial cells in vitro. J Cell Biol 1993; 120:1011-1019

92. Triau JE, Meydani SN, Schaefer EJ: Oxidized low density lipoprotein stimulates prostacyclin production by adult human vascular endothelial cells. Arteriosclerosis 1988; 8:810-818

93. Malden LT, Chait A, Raines EW, Ross R: The influence of oxidatively modified low density lipoproteins on expression of platelet-derived growth factor by human monocyte-derived macrophages. J Biol Chem 1991; 266:13901-13907

94. Hamilton TA, Ma G, Chisolm GM: Oxidized low density lipoprotein suppresses the expression of tumor necrosis factor-α mRNA in stimulated murine peritoneal macrophages. J Immunol 1990; 144:2343-2350

95. Parthasarathy S, Young SG, Witztum JL, Pittman RC, Steinberg D: Probucol inhibits oxidative modification of low density lipoprotein. J Clin Invest 1986; 77:641-644

96. Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: Evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA 1987; 84:7725-7729

97. Kita T, Nagano Y, Yokode M, et al: Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA 1987; 84:5928-5931

98. Chisolm GM III: Antioxidants and atherosclerosis: A current assessment. Clin Cardiol 1991; 14:I-25-I-30

99. Mao SJT, Yates MT, Parker RA, Chi EM, Jackson RL: Attenuation of atherosclerosis in a modified strain of hypercholesterolemic Watanabe rabbits with use of a probucol analogue (MDL 29,311) that does not lower serum cholesterol. Arterioscler Thromb 1991; 11:1266-1275

100. Mao SJT, Yates MT, Rechtin AE, Jackson RL, Van Sickle WA: Antioxidant activity of probucol and its analogues in hypercholesterolemic Watanabe rabbits. J Med Chem 1991; 34:298-302

101. Björkhem I, Henriksson-Freyschuss A, Breuer O, Diczfalusy U, Berglund L, Henriksson P: The antioxidant butylated hydroxytoluene protects against atherosclerosis. Arterioscler Thromb 1991; 11:15-22

102. Sparrow CP, Doebber TW, Olszewski J, Wu MS, Ventre J, Stevens KA, Chao Y: Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant *N,N′*-diphenyl-phenylenediamine. J Clin Invest 1992; 89:1885-1891

103. Gey KF, Puska P, Jordan P, Moser UK: Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. Am J Clin Nutr 1991; 53:326S-334S

104. Stampfer MJ, Hennekens CH, Manson JE, Colditz GA, Rosner B, Willett WC: Vitamin E consumption and the risk of coronary disease in women. N Engl J Med 1993; 328:1444-1449

105. Rimm EB, Stampfer MJ, Ascherio A, Giovannucci E, Colditz GA, Willett WC: Vitamin E consumption and the risk of coronary heart disease in men. N Engl J Med 1993; 328:1450-1456

106. Riemersma RA, Wood DA, Macintyre CCA, Elton RA, Gey KF, Oliver MF: Risk of angina pectoris and plasma concentrations of vitamins A, C, and E and carotene. Lancet 1991; 337:1-5

107. Steinberg D: Antioxidants in the prevention of human atherosclerosis—Summary of the Proceedings of a National Heart, Lung, and Blood Institute Workshop: September 5-6, 1991, Bethesda, Maryland. Circulation 1992; 85:2338-2344

108. Salonen JT, Ylä-Herttuala S, Yamamoto R, et al: Autoantibody against oxidised LDL and progression of carotid atherosclerosis. Lancet 1992; 339:883-887

109. Stocker R, Bowry VW, Frei B: Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol. Proc Natl Acad Sci USA 1991; 88:1646-1650

110. Steinberg D: Antioxidant vitamins and coronary heart disease. N Engl J Med 1993; 328:1487-1489