Evolving lipoprotein risk factors: lipoprotein(a) and oxidized low-density lipoprotein

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Cardiovascular disease is the leading cause of morbidity and mortality in Westernized populations. Evolving lipoprotein risk factors include LDL oxidation and lipoprotein(a) [lp(a)]. Several lines of evidence support a role for oxidatively modified LDL in atherogenesis and its in vivo existence. There are both direct and indirect measures of oxidative stress. The most relevant direct measure of lipid peroxidation is urinary F2 isoprostanes. The most common indirect measure of LDL oxidation is quantifying the lag phase of copper-catalyzed LDL oxidation by assaying conjugated diene formation. Lp(a) is increased in patients with cardiovascular and cerebrovascular disease. However, not all prospective studies have confirmed a positive relationship between Lp(a) and cardiovascular events. Lp(a) appears to present three major problems: standardization of the assay, establishing its role in atherogenesis, and the lack of an effective therapy that can substantially lower Lp(a) concentrations. Thus, at the present time, Lp(a) concentrations should not be recommended for the general population but be reserved for patients with coronary artery disease without established risk factors, young patients with coronary artery disease or cerebrovascular disease, or a family history of premature atherosclerosis and family members of an index patient with increased concentrations of Lp(a). Although both LDL oxidation and Lp(a) are evolving risk factors for cardiovascular disease, more data are needed before they become part of the established lipoprotein repertoire.

Atherosclerosis is the major cause of cardiovascular disease and death in Western civilizations (1). The atherosclerotic lesion typically comprises fatty streaks that later develop into fibrous plaques. The initial fatty streaks are characterized by the presence of lipid-laden foam cells, mainly macrophage in origin. Fatty streaks may regress or progress via a transitional lesion to fibrous plaques. The fibrous plaque represents the most characteristic lesion of advancing atherosclerosis. The fibrous plaque consists of a fibrous cap made up of smooth muscle cells and dense connective tissue, an underlying cellular layer, and a deeper necrotic core. The fibrous plaque may undergo changes such as calcification, necrosis, hemorrhage, ulceration, or thrombosis to form a complicated lesion. These complicated lesions produce the clinical manifestations of atherosclerosis.

Oxidized LDL

The most important risk factors for atherosclerosis include smoking, hypertension, dyslipidemia (increased concentrations of LDL and decreased concentrations of HDL), diabetes, aging, and a family history of premature atherosclerosis. The conventional risk factors such as dyslipidemia, hypertension, diabetes, and smoking cannot account for all the cases of coronary artery disease. Thus, there are other nonconventional risk factors that appear to be related to premature atherosclerosis. One plausible postsecretory modification of LDL that may render it more atherogenic is oxidation (2). In fact, increased LDL oxidation could explain premature atherosclerosis in the face of a lipoprotein profile within reference values.

The precise mechanism by which LDL promotes the development of the atherosclerotic lesion remains to be elucidated (2, 3). However, several lines of evidence suggest that oxidation of LDL is a key early step in atherogenesis. LDL can be oxidatively modified in a cell-free system by transition metals such as iron and copper and by all the major cells of the arterial wall, such as endothelial cells, smooth muscle cells, and monocyte macrophages (2, 3). Physiologically relevant mechanisms for LDL oxidation in vivo are yet to be established. Various studies have suggested that superoxide, myeloperoxidase, 15-lipoxygenase, peroxynitrite, and thiols can mediate LDL oxidation in different systems (2–4). Both glucose autoxidation and LDL glycation can also promote LDL oxidation. The oxidizability of LDL also depends on its size. Subjects with a predominance of small, dense LDL have a greater risk of coronary artery disease than subjects...
with a predominance of large, buoyant LDL (5). Studies from numerous laboratories have shown that small, dense LDL is more susceptible to oxidation (4, 6).

It is generally believed that LDL oxidation occurs mainly in the arterial intima in microdomains sequestered from the antioxidant milieu (2, 3). Oxidation of LDL is a free radical-mediated process involving the peroxidative modification of the polyunsaturated fatty acids (PUFAs). LDL oxidation is initiated by reactive oxygen species that abstract a hydrogen atom from a double bond in PUFAs of surface phospholipids, followed by molecular rearrangement, leading to the formation of conjugated double bonds, called conjugated dienes. Initially, the rate of oxidation is suppressed by endogenous antioxidants, which produces the lag phase of oxidation (6). The lag phase is followed by a rapid propagation phase, which involves abstraction of another hydrogen atom from another PUFAs, leading to the formation of lipid peroxides. The propagation phase is followed by a decomposition phase, during which there is cleavage of double bonds, leading to the formation of aldehydes such as hydroxynonenal, hexanal, and malondialdehyde. Some of the salient characteristics of oxidized LDL include the following (3, 6, 7): reduction in PUFAs, increase in lipid peroxides, increased content of oxysterols, increased lyssolecithin content, increased negative charge on apolipoprotein B100, fragmentation of apolipoprotein B100, reduced uptake by the LDL receptor, and increased uptake by the scavenger receptor mechanism.

Oxidized LDL exerts several biological effects that may contribute to the progression of the atherosclerotic lesion. During the oxidation of LDL, initially minimally modified LDL (MM-LDL) is formed in the subendothelial space (4, 8). MM-LDL differs from oxidized LDL in that it has undergone only mild lipid peroxidation, but it is still recognized by the LDL receptor (4, 8). MM-LDL can induce leukocyte-endothelial cell adhesion and promote secretion of monocyte chemotactic protein-1 and macrophage colony-stimulating factor by the endothelium. This produces monocyte binding and migration into the subendothelial space, where macrophage colony-stimulating factor promotes differentiation into macrophages. Macrophages in turn further oxidize MM-LDL to oxidized LDL. Oxidized LDL is no longer recognized by the LDL receptor; instead it is taken up by the scavenger receptor system on the monocyte macrophages, which are not regulated by the intracellular cholesterol content. This produces appreciable cholesterol accumulation in macrophages, leading to foam cell formation (2). Oxidized LDL is a chemoattractant for monocytes and T lymphocytes and also inhibits macrophage motility, thereby promoting retention of macrophages in the arterial wall (2, 3). Oxidized LDL is cytotoxic and could promote endothelial dysfunction and the evolution of the fatty streak into a more advanced lesion. Oxidized LDL can also promote atherogenesis by stimulating expression of several other genes in the arterial wall such as interleukin-1. Interleukin-1 has been shown to induce smooth muscle cell proliferation, promote a procoagulant state, and stimulate leukocyte-endothelial cell adhesion (9). Oxidized LDL can adversely affect coagulation by stimulating tissue factor and plasminogen activator inhibitor-1 synthesis (2, 3, 7). In addition, oxidized LDL inhibits endothelium-derived relaxation factor-mediated vasodilation (2, 3, 7). Another atherogenic property of oxidized LDL is its immunogenicity (3). Malondialdehyde-modified LDL has been shown to stimulate formation of autoantibodies, and immune complexes of LDL aggregates are efficiently internalized by macrophages via Fc receptors, further promoting cholesterol accumulation.

Several lines of evidence also exist to support the in vivo existence of oxidized LDL (2, 3, 7, 8). LDL extracted from human atherosclerotic lesions cross-reacts with antibodies to malondialdehyde-lysine conjugates and is processed more avidly by macrophages, leading to increased cholesterol esterification. Antibodies against epitopes on oxidized LDL recognize material from atherosclerotic lesions, but not from nondiseased arteries. Antibodies to epitopes on oxidized LDL have also been demonstrated in patients with diabetes, chronic renal failure, and coronary atherosclerosis (2, 7). However, some investigators have failed to confirm this (10, 11). Also, the presence of these autoantibodies to oxidized LDL has been positively correlated with the progression of atherosclerosis manifested by carotid artery stenosis. The oxidative susceptibility of LDL (lag phase) has been shown to correlate with the severity of coronary atherosclerosis, as evaluated by angiography. Finally, the most persuasive evidence comes from the studies with antioxidants such as α-tocopherol, probucol, butylated hydroxytoluene, and diphenyl phenylenediamine, which have been shown to decrease LDL oxidation and atherosclerosis lesion progression in various animal models of atherosclerosis (3, 7). Although butylated hydroxytoluene and diphenyl phenylenediamine are effective in the prevention of atherosclerosis in animals, toxicity limits their utility in humans (7). Thus, supplementation with antioxidant nutrients may be a better approach in the prevention of atherosclerosis. Doses of vitamins E and C in excess of the recommended dietary allowances are well-tolerated (7, 12). Much of the antioxidant nutrient research with LDL oxidation has been done with α-tocopherol (6, 12). α-Tocopherol is the most prevalent and biologically active form of vitamin E. It is the predominant lipophilic antioxidant in tissue and LDL. In LDL, the concentration of α-tocopherol is 6 mol/mol of LDL (6). α-Tocopherol traps peroxyl free radicals and thus acts as a chain-breaking antioxidant. α-Tocopherol inhibits LDL oxidation in vitro (6). Numerous human studies have shown that α-tocopherol supplementation reduced LDL oxidizability in healthy subjects.
The 8 epi F2 isoprostane has potent vasoconstrictor activity measured by gas chromatography–mass spectrometry. These stable products of protein oxidation are measured by mass spectrometry and are inhaled volatile hydrocarbons. As discussed previously, breath volatile hydrocarbons such as pentane are tedious to measure in the clinical laboratory and very sensitive to extraneous factors. With regard to antibodies to oxidized LDL, the major problem with this assay is that different laboratories use different degrees of LDL oxidation as antigen; thus there is much variability in the reporting of autoantibodies to oxidized LDL in different studies. F2 isoprostanes are prostaglandin-like compounds formed in vivo from free radical-catalyzed peroxidation of arachidonic acid. They are generally measured by gas chromatography–mass spectrometry. The 8 epi F2 isoprostane has potent vasoconstrictor activity and also modulates platelet aggregation. F2 isoprostanes can be detected both in body tissues and biological fluids such as plasma and urine. Increased concentrations have been detected in oxidized LDL and also in patients with diabetes, hypercholesterolemia, and in smokers. Recently, immunoassays for measurement of isoprostanes have become available, and it will be interesting to determine the correlation between these immunoassays and gas chromatography–mass spectrometry in different syndromes of increased oxidative stress. Stable products of protein oxidation also appear to be a valid measure of increased total body oxidative stress. Products of the myeloperoxidase pathway include 3-chlorotyrosine and dityrosine, whereas products of the reactive nitrogen pathway include 3-nitrotyrosine. These stable products of protein oxidation are measured by mass spectrometry and are increased in human atherosclerotic tissue. Although these newer direct measures of oxidative stress, such as chlorotyrosine, nitrotyrosine, and isoprostanes, offer great promise, much additional work is required to validate their clinical utility. Of the indirect measures, LDL can be rapidly isolated and subjected to an oxidative stress in vitro. From this could be computed the lag phase and oxidation rate, allowing an indirect measure of LDL oxidation. Assays that can be used include formation of conjugated dienes, lipid peroxides, electrophoretic mobility, and increased fluorescence. Thus, although there are numerous assays for the measurement of LDL oxidation, it appears that the most relevant, direct assay with regard to lipid peroxidation is measurement of F2 isoprostanes either in urine or blood. The measurement of LDL oxidative susceptibility by the formation of conjugated dienes is the most commonly used assay.

Thus, the LDL oxidation hypothesis remains a very plausible hypothesis to explain atherogenesis; what is very exciting is that dietary factors such as antioxidants could become an additional cost-effective member of the antiatherosclerotic regimen. In the near future, the clinical laboratory will need to provide an assessment of antioxidant status and some measure of oxidative stress, either direct or indirect. There is an urgent need to standardize assays for measuring LDL oxidation. Sadly, no organization to date has undertaken the challenge to standardize assays for LDL oxidation. These assays will have their greatest use in young patients with premature atherosclerosis without established risk factors, diabetics, hypertensives, smokers, and subjects with a family history of premature atherosclerosis.

**Lipoprotein(a)**

Lipoprotein (a) [Lp(a)] is an additional evolving lipoprotein risk factor. It is distinguished from LDL in that, in Lp (a), attached to the LDL particle is another apoprotein, termed apoprotein(a) [apo(a)]. This is linked to the LDL particle by a disulfide linkage. This apo(a) is a highly glycosylated protein. Analysis of apo(a) cDNA reveals close homology with plasminogen, an important component of fibrinolysis, and both apo(a) and plasminogen appear to derive from the plasminogen gene superfamily. Both genes are located on chromosome 6. apo(a) consists of multiple tandem repeats of the Kringle 4 of plasminogen and one repeat of Kringle 5. These Kringles have close homology but are not identical to the corresponding ones in plasminogen. It also has the protease domain, but unlike plasminogen, it is devoid of enzyme activity. There appear to be 10 types of the Kringle 4 domain, and much of the variability reside in the Type 2 Kringle 4 domain. apo(a) is highly polymorphic in size and has >35 alleles present at the apo(a) locus. Different individuals can have between 3 and 40 Type 2 Kringle 4 repeats. There is an inverse correlation between the size of the apo(a) isoforms and Lp(a) concentrations: the largest isoforms are associated with the lowest concentration. Although the intraindividual variability in Lp(a) is small (15%), the interindividual variability is large (1000-fold). Plasma concentrations appear to be highly heritable. The distribution of Lp(a) concentration varies in different population groups. In Caucasian populations, Lp(a) values are skewed to lower concentrations; however, in African-Americans, Lp(a) values have a less skewed distribution, with high median values. The apo(a) gene is the primary determinant of Lp(a) concentrations. The LDL receptor does not appear to have a crucial role in the clearance of Lp(a). This is underscored by the fact that the β-hydroxy-β-methylglutarylcoenzyme A-reductase inhibitors, which produce a
substantial up-regulation of the LDL receptor, have no appreciable effect on Lp(a) concentrations (23).

Lp(a) concentrations are increased in some patients with coronary artery disease and cerebrovascular disease. In addition, Lp(a) concentrations appear to be increased in patients with end-stage renal disease and the nephrotic syndrome (17–19). Factors that do not appear to markedly affect Lp(a) include gender, age, weight, moderate exercise, and most lipid-lowering medications (19–22). The only exception is postmenopausal women, in whom concentrations are increased (24). The effect of omega-3 fatty acids, hormones, and other drugs that lower Lp(a) are discussed later under treatment. With regard to diabetes and familial hypercholesterolemia, conflicting data has been reported with respect to increased concentrations of Lp(a) (20–22).

Lp(a) could promote atherogenesis via several mechanisms (20–22, 25). Because it has LDL as its component, it could promote cholesterol delivery into the artery wall. Lp(a) that is oxidized is avidly taken up by the scavenger receptor pathway. Lp(a) has been shown to stimulate smooth muscle cell proliferation. This appears to be mediated via inhibition of plasmin generation and activation of transforming growth factor-β. In addition, because it has apo(a), this could potentially contribute to thrombogenesis. Lp(a) in vitro competes with binding of plasminogen to fibrin, fibrinogen, and the plasminogen receptor. Investigators have reported that homocysteine increases the affinity of Lp(a) to fibrin (25). In addition, Lp(a) has been demonstrated in atherosclerotic plaques. Despite the compelling in vitro evidence for procoagulant activity for Lp(a), studies in humans have provided little support for such effects in vivo (26).

Numerous case control studies have now demonstrated that Lp(a) concentrations are increased in patients with established coronary artery disease (19–22, 27). However, high Lp(a) does not appear to be associated with an increased risk for coronary artery disease in subjects over the age of 65 with coronary artery disease and is not proportional to the risk for coronary artery disease in African-Americans, despite being in higher concentrations than in Caucasians (20, 22, 27, 28). In addition, prospective studies have revealed conflicting results (20–22, 27–29). Some, such as the Lipid Research Clinic Study (30) and the Gottingen Risk Incidence and Prevalence Study (31), have shown a positive relationship between Lp(a) and coronary artery disease. However, among others, the Helsinki Heart Study (32) and the Physicians’ Health Study (33) have failed to show a relationship between plasma Lp(a) and coronary artery disease. Conflicting results could be explained by the following factors: condition of the samples, the methodology used for assaying Lp(a), apo(a) isoform distribution, sample size, and duration of follow-up (20, 22, 28). Future studies examining the relationship between Lp(a) and coronary artery disease should include a larger sample size that adequately represents apo(a) isoforms to avoid possible selection bias. Isoforms should be determined by state-of-the-art methods; antibodies used in the assay should be characterized and the effect of isoforms size documented to correctly interpret results.

The measurement of Lp(a) is problematic with respect to assay accuracy (19, 22, 34). Because Lp(a) has two proteins, apo B100 and apo(a) (which has homology to plasminogen), antibodies raised against Lp(a) interacting with either one of these proteins could cause major cross-reactivity. Also, the concentration of Lp(a) is dependent on its isoforms, and the fact that apo(a) has multiple repeats of Kringle 4 Type 2 (3–40) contributes further to the problem (20). Although it is ideal to have similar isoforms of assay calibrator and samples, the effect of dissimilar isoforms is dependent on assay configuration and how results are reported. Numerous methods have been reported to measure Lp(a). These include radioimmunoassay, ELISA, immunoturbidimetry, and immunonephelometry. However, it appears that the most common assay for measurement of Lp(a) at present is ELISA. An additional assay that has been reported to overcome the isoform problems is to assay the cholesterol content instead of measuring the protein. After binding of Lp(a) to lectin affinity columns, it is then eluted, and cholesterol is measured in the Lp(a) fraction (35). However, further studies are needed to validate this assay for clinical purposes.

Recently, Marcovina et al. (36) reported on a comparison of three methodologies to measure Lp(a). In all three assays, the capture antibody was a monoclonal antibody to apo(a) Kringle 4. However, the three assays differed in the detection antibody. This group used three different detection antibodies: monoclonal antibody a-5, monoclonal antibody a-40, and a polyclonal antibody to apo B. Monoclonal antibody a-5 recognizes the apo(a) Kringle 4 Type 2 repeats and thus is sensitive to apo(a) heterogeneity. The polyclonal antibody against the apo B component of Lp(a) is not sensitive to variability in size of apo(a). The remaining antibody (a-40) is a monoclonal antibody to apo(a) that does not recognize the Kringle 4 Type 2 repeats. In this study, they showed that the assays using apo B and monoclonal antibody a-40 as detecting antibodies provided the same Lp(a) values in samples with different apo(a) sizes. However, with monoclonal antibody a-5, the results were overestimated in samples with large apo(a) sizes and underestimated in samples with smaller apo(a) sizes.

It is imperative that, as previously achieved successfully for apolipoprotein A1 and apolipoprotein B, standardization for Lp(a) be undertaken. Numerous criteria need to be met, including selection and characterization of the antibodies and optimization of the assay format, selection of apo(a) polymorphs in a primary standard, reference material and assay calibrators, selection of a validated method for isolation of Lp(a) to be used as a primary standard, development of suitable reference material, uniform unit of expression of apo(a) values, and
development of a reference method (20, 21). This challenge has now been undertaken by a working group of the International Federation for Clinical Chemistry with the hope of providing a secondary reference preparation of Lp(a) shortly for use by diagnostic manufacturers in calibrating Lp(a) immunoassays (21).

Thus, it appears that Lp(a) poses three major problems: standardization of the assay, the establishment of its role in atherogenesis, and effective therapy that can substantially lower Lp(a) concentrations. The question that arises is: Should Lp(a) become part of the routine lipoprotein repertoire? Until further studies have established its place and effective therapies have evolved, Lp(a) should not be part of the routine lipoprotein repertoire. In addition, it is important to bear in mind that no intervention studies have demonstrated that lowering plasma concentrations of Lp(a) produces fewer cardiac events. Thus, screening is not recommended for the general population. Lp(a) should be reserved for the following subgroups: patients with coronary artery disease without established risk factors, young patients with coronary artery disease or cerebrovascular disease, or a family history of premature atherosclerosis and a family member of index patients with increased concentrations of Lp(a). There is an urgent need to conduct further prospective studies using reliable assays to determine the relationship between plasma Lp(a) and coronary events. In addition, there is an urgent need to develop drugs that selectively and efficaciously lower Lp(a) concentrations. At present, Lp(a) concentrations can be lowered with apheresis, niacin, certain anabolic steroids (danazol and stanozolol), estrogen replacement therapy, and omega-3 fatty acids (19, 22, 23, 28, 37). It is difficult to normalize Lp(a) concentrations except with apheresis; however, this decrease is not sustained.

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References
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