Chapter 2

LIPOPROTEINS AND OXIDATION

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Introduction

Oxidation of lipoproteins, and in particular low density lipoprotein (LDL), has been implicated as a major factor in the initiation and progression of atherosclerosis[1].Over the last 25 years, research from many laboratories has elucidated multiple mechanisms through which oxidized LDL (OxLDL) is atherogenic. Oxidation of LDL in the vessel wall leads to an inflammatory cascade that activates many atherogenic pathways, including the unregulated uptake of OxLDL by scavenger receptors of monocyte-derived macrophages leading to foam cell formation. Accumulation of foam cells leads to fatty streak formation, the earliest visible atherosclerotic lesion. It consists primarily of cholesterol ester-laden cells, mostly derived from circulating monocytes that have penetrated through the endothelial layer, but also from modified smooth muscle cells. Foam cell necrosis and/or apoptosis and continued accumulation of oxidized lipids in the extracellular space eventually lead to atheroma formation. The complex interplay of oxidized lipids, inflammatory processes, endothelial dysfunction and platelet activation and thrombus ultimately lead to plaque progression and/or disruption leading to clinical events. Inflammatory cells play a central role throughout all these events, which results in atherosclerotic lesions having many features of a chronic inflammatory disease[2].

Palinski, Napoli and colleagues have established that fatty streaks may appear as early during the development of atherosclerosis as during human fetal life. In fact, they documented that OxLDL was present in aortas of fetuses whose mothers were hypercholesterolemic even prior to monocyte

entry into the vessel wall, suggesting that LDL oxidation is involved *a priori* in the recruitment of monocytes into the vessel wall[3,4]. They also showed that maternal hypercholesterolemia is an important factor in the progression of atherosclerosis of children, that, in the setting of maternal hypercholesterolemia in utero, there is altered gene expression that mediates subsequent atherosclerosis and that treatment of hypercholesterolemia with antioxidants or lipid lowering agents in pregnant animal models reduces progression of atherosclerosis in progeny[5,6]. These observations suggest that oxidation of LDL is one of the earliest atherogenic changes that mediate progression of atherosclerosis. More recent studies also suggest that OxLDL is intimately involved in the transition of stable atherosclerotic lesions to vulnerable plaques and plaque disruption, as will be discussed later.

Mechanisms of LDL oxidation

Each LDL particle contains approximately 700 molecules of phospholipids, 600 of free cholesterol, 1600 of cholesterol esters, 185 of triglycerides and 1 copy of apoprotein B-100, which in turn is made of 4536 amino acid residues. The protein and the lipid moieties of the LDL particle are both exquisitely sensitive to oxidation and may undergo oxidative damage. LDL in plasma is relatively stable but once it has been purified and isolated it begins to oxidize rapidly, unless a chelating agent such as EDTA, is present throughout the stages of preparation[7]. In particular, copper (5 μM) and other divalent cations are able to catalyze oxidative modification of LDL during overnight incubation, resulting in the modified LDL which becomes a ligand for the acetyl LDL receptor, leading to foam cell formation[8]. Copper-catalyzed oxidative modification of LDL results in degradation of as much as 40% of the phosphatidylcholine, present on the polyunsaturated fatty acids (PUFA) in the *sn*-2 position, which is then converted to lysophosphatidylcholine. In addition, 50-75% of the PUFA are destroyed by attacks at the double bonds[9,10]. Apoprotein B-100 is also altered by direct oxidative attack and conjugation of lipid aldehyde fragments generated from the polyunsaturated fatty acids with epsilon amino groups of lysine residues of apoprotein B-100 leads to generation of immunogenic and atherogenic oxidation-specific neoepitopes. The LDL particle becomes smaller, denser, in some cases as dense as HDL, and more negatively charged.

The term "OxLDL" does not imply one specific structure or homogenous molecular form, but a variety of lipid and protein modifications of LDL that are generated from lipid peroxidation that make it atherogenic[11]. Therefore, it is imperative that the conditions under which OxLDL is generated be well defined to allow comparison among studies. This is particularly relevant for

clinical studies where measures of circulating OxLDL are increasingly being performed. For this reason, in studies measuring circulating OxLDL we have previously suggested that the antibody used to quantitate OxLDL be used in the designation of OxLDL, to reduce confusion about what is being measured and allow comparisons between published studies. For example, a plasma measure of OxLDL that has been generated by our group, OxLDL-E06, denotes the measurement of oxidized phospholipid (OxPL) epitopes on LDL that is detected by the natural murine IgM autoantibody E06[12-15].

Nonenzymatic

Nonenzymatic oxidation catalyzed by Cu^{2+} is believed to depend upon the presence of lipid hydroperoxides in the LDL[16]. These hydroperoxides are degraded to peroxy and alkoxy radicals by Cu^{2+} and in turn, those radicals can initiate a cyclic chain reaction that can generate many more hydroperoxides. The fatty acid side chains of cholesterol esters and cholesterol's polycyclic sterol ring structure are susceptible to oxidative attack[16]. Generation of minimally modified LDL (mmLDL) can be initiated by incubation of LDL with Cu^{2+} for even a few hours, resulting in inflammatory and proatherogenic biological properties[17], prior to recognition by scavenger receptors[18,19].

Cell-mediated and enzymatic

Due to the presence of abundant antioxidant defenses present in plasma, it is believed that most of the oxidation of LDL occurs in the vessel wall rather than in plasma. In fact, although oxidation-specific epitopes can be present on circulating LDL (i.e. OxLDL-E06), fully oxidized LDL, i.e. the type that is generated in vitro by prolonged exposure to copper, is not present in any significant amounts in plasma. In fact, injection of fully oxidized LDL in animal models results in the rapid elimination from plasma within minutes[20-22]. Incubation of LDL with all cells that are found in atherosclerotic lesions, such as endothelial cells, smooth muscle cells and monocyte/macrophages, and also neutrophils and fibroblasts, accelerates *in vitro* oxidative modification. LDL can also be oxidized at sites of inflammation[23].

In addition to cell mediated oxidation, a number of different enzyme systems such as lipoxygenases[24-28], and phagocyte (i.e. macrophage)-derived myeloperoxidase (MPO)[29], NADPH oxidase[30], inducible nitric oxide synthase and other peroxidases[31], potentially contribute to the oxidation of LDL. Macrophages and/or other phagocytes, which express these enzymes as mechanisms for generating antimicrobial reactive oxygen species essential for native immunity[32], likely amplify oxidative reactions in macrophage-rich areas of atherosclerotic lesions.

Convincing data for a role of the enzyme 12/15-lipoxygenase (LO) in enhancing in vivo oxidation and accelerating murine atherogenesis has recently been demonstrated[33-35]. 12-15 LO "seeds" LDL in tissue fluid, resulting in initiation of lipid peroxidation by hydroperoxides, generation of proinflammatory OxLDL and subsequent enhanced uptake by macrophages[36]. Atherosclerotic lesions of rabbits and humans, but not normal arteries, contain mRNA and protein of 15-LO (the homologous enzyme in rabbits and humans)[37], and stereospecific products of the LO reaction can be found in atherosclerotic lesions, consistent with enzymatic oxidation[38,39]. Additional evidence to support this hypothesis includes the observations that incubation of LDL with isolated soybean LO leads to oxidation of LDL[24]; that inhibitors of macrophage 12/15-LO decrease the ability of macrophages to initiate oxidation of LDL[28], and that LDL incubated with fibroblasts transfected with LO become "seeded" with fatty acid hydroperoxides, which can then propagate lipid peroxidation under the proper conditions[25,40]. Also, treatment of hypercholesterolemic rabbits with specific inhibitors of 15-LO reduces the progression of atherosclerosis[41,42].

However, the most convincing evidence for the in vivo role of 12/15 LO, at least in murine atherogenesis, has been provided by showing that crossing 12/15-LO deficient mice into apoE-deficient mice caused an ~50% reduction in the extent of atherosclerotic lesions, despite similar blood lipid profiles in both groups[33-35]. Urinary and plasma levels of F_2-isoprostanes, non-enzymatic breakdown products resulting from lipid peroxidation of arachidonic acid, and OxLDL autoantibodies were also reduced and both highly correlated with plaque burden and with each other. In another study, 12/15-LO deficient mice crossed into $LDLR^{-/-}$ mice also had reduction in the extent of atherosclerosis[43]. Conversely, overexpression of 15-LO in endothelial cells led to an enhancement of atherosclerosis in LDLR-negative mice[44] and 12/15-LO overexpression in C57BL/6J mice was shown to mediate monocyte/endothelial cell interactions in the vessel wall at least in part through molecular regulation of expression of endothelial adhesion molecules[45]. Additional studies have shown that combined paraoxonase/apoE-deficient knockout mice have enhanced LDL oxidation, detected by enhanced clearance of intravenously injected LDL and faster generation of plasma levels of circulating OxLDL and immune complexes, and attendant enhanced atherosclerosis[46]. Although it is possible that 12/15-LO affected atherogenesis by other mechanisms, these studies lend strong support to the concept that a major mechanism by which 12/15-LO deficiency decreased atherosclerosis was by decreasing the extent of lipid peroxidation, and specifically, the generation of OxLDL. However, not all data are consistent in this regard and it is possible that species differences may exist as exemplified by a study showing that macrophage-specific

overexpression of 15-LO led to protection against atherosclerosis in cholesterol fed-rabbits[47,48]. The reasons for these differences are unclear but the 12/15-LO deletion in the mouse studies was global, while the studies with 15-LO overexpression in rabbits were tissue-specific. Similarly, conflicting results have been observed for the contributions of endothelial and inducible nitric oxide synthases to the development of atherosclerosis in mouse models[49-52]. It is possible that mechanisms responsible for LDL oxidation differ between humans and animal models.

It is also likely that in vivo there are many mechanisms beside LO by which LDL is oxidized within the artery wall[53,54]. For example, MPO is a heme enzyme secreted by neutrophils and monocytes that generates a number of oxidants, including hypochlorous acid (HOCl), which can initiate lipid oxidation and peroxidation. MPO has been identified in human atherosclerotic lesions and is of particular interest because lipid modifications found in human atherosclerosis bear similarities to hypochlorous acid-mediated derivation of lipoprotein constituents in vitro[55]. Interestingly, a recent study showed that a single initial measurement of plasma myeloperoxidase levels in patients presenting to the emergency room with chest pain and negative troponin T levels independently predicted in-hospital risk of myocardial infarction (MI), as well as the risk of major adverse cardiac events at 30-day and 6-month periods[56]. However, in bone marrow transplantation experiments in which $LDLR^{-/-}$mice received MPO-deficient bone marrow progenitor cells, larger lesions were observed than in $LDLR^{-/-}$ mice transplanted with wild type progenitor cells. Similar results were seen when MPO-deficient mice were crossed into $LDLR^{-/-}$ mice. However, there was no evidence for the presence of MPO in murine lesions and the types of MPO-dependent oxidation products found in human lesions were not present in murine lesions[57], suggesting that MPO could not be directly related to lesion formation in mice.

The leukocyte 5-LO has recently been identified as a significant modifier of susceptibility to atherosclerosis in inbred strains of mice[58,59]. This observation may be linked to LDL modification, but a direct association has not yet been established.

Macrophage foam cell formation

Oxidative modification of LDL

The most compelling evidence of the primacy of LDL in contributing to the pathogenesis of atherosclerosis is demonstrated by the premature atherosclerosis seen in homozygous familial hypercholesterolemia (HFH)[60]. Patients with HFH, with a prevalence of 1:1,000,000, develop plasma LDL cholesterol levels of 500-1000 mg/dl and have been documented to have MIs as early as 18 months of age and usually succumb to various manifestations

of ischemic cardiovascular disease by the third decade of life. HFH, estimated to be present in 1:500 individuals, results in LDL cholesterol levels of 200-350 mg/dl, and greatly accelerated, clinically relevant, atherosclerosis as well. Before the statin era, ~5% of males had an MI by age 30 years; 25% had died of MI by age 50 years; and 50% had died by age 60 years compared to only 10% of non affected male siblings. Even 30% of FH females will have coronary artery disease by age 60[61]. This monogenic disorder manifests its phenotypic expression through one of >600 reported mutations within the LDL receptor (LDLR) gene, a membrane protein to which LDL binds with high affinity and which leads to its internalization and degradation within the cell, that reduces LDLR number and/or activity[62].

Paradoxically, patients with HFH, despite the absence of LDLR, develop excessive numbers of foam cells, manifested by the accumulation of cholesterol in subcutaneous and tendon xanthomas and in arterial lesions. Their circulating LDL, while it does show some relatively minor differences in structure from that of normal LDL, behaves metabolically like LDL from normal subjects[63]. In addition, incubation of monocyte/macrophages with very high concentrations of native LDL *in vitro* does not lead to accumulation of cholesterol[64]. The underlying mechanisms mediating macrophage foam cell formation in the absence of LDLR were clarified in 1979 by the landmark studies of Brown and Goldstein when they discovered the "acetyl LDL receptor"[64]. They demonstrated that treatment of LDL *in vitro* with acetic anhydride leading to modification of a significant fraction of the lysine residues of apoB, generated a modified form of LDL, acetyl-LDL, that was taken up much more rapidly than native LDL by a specific, saturable receptor by cultured macrophages (*Figure 1*).

Unlike the LDL receptor which downregulates as the cell cholesterol content increases, the acetyl LDL receptor, which is expressed at normal levels in patients with LDLR deficiency, is not downregulated and is fully active even as the cell cholesterol content increases markedly. Kodama et al[65] subsequently cloned and sequenced the acetyl LDL receptor which was redesignated the scavenger receptor, type A, or SR-A. In vitro, chemical acetoacetylation or conjugation of LDL with malondialdehyde (MDA) or via aggregated platelets releasing MDA generates MDA-LDL, which is recognized by SR-A[66,67]. However, the concentrations of MDA needed to generate MDA-LDL in vitro seem to be significantly higher than could be achieved in vivo, although MDA-LDL epitopes are abundant in atherosclerotic lesions[68-70].

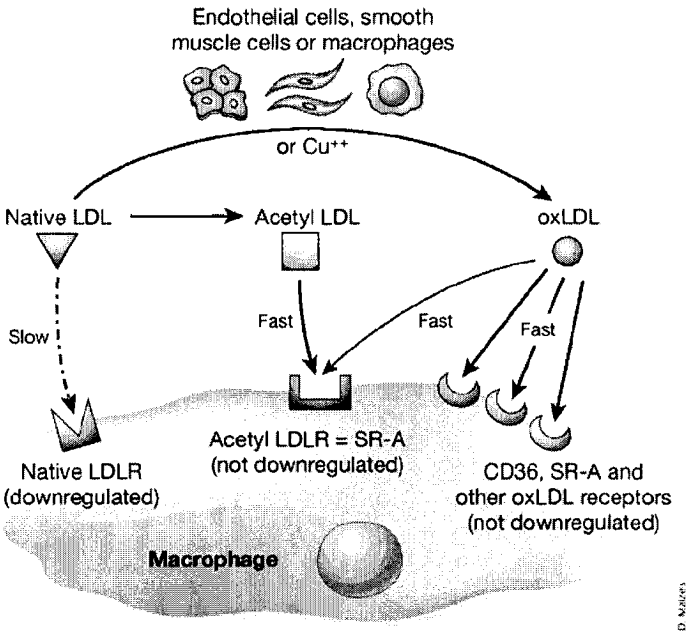

Figure 1. **Mechanisms of OxLDL uptake by monocytes.** Native LDL cannot induce foam-cell formation because uptake is slow and because the LDL receptor downregulates. Either acetyl LDL or OxLDL can induce cholesterol accumulation in macrophages resulting in foam-cell formation because uptake is rapid and the scavenger receptors do not downregulate in response to an increase in cellular cholesterol. Reproduced with permission from Steinberg.[1]

Further evidence for a potential clinically relevant mechanism of foam cell formation was provided by Steinberg and colleagues in 1981 when they showed that overnight incubation of LDL with a cultured monolayer of endothelial cells[71], vascular smooth muscle cells[72] or with peritoneal macrophages[73,74] in a medium rich in metal ions generated modified LDL with a marked increase in the rate of uptake and degradation by mouse peritoneal macrophages. The binding and uptake of the modified LDL was competitively inhibited by unlabeled acetyl LDL (~60%) indicating that a large part of the uptake was by way of SR-A and implying at the same time that additional receptors must be involved. Subsequent studies showed that these changes could be reproduced by simply incubating the LDL with copper to catalyze nonenzymatic oxidation and that all of these cell-induced changes can be blocked by adding vitamin E to the medium[8]. Morel et al[75]

also observed that oxidatively modified LDL was cytotoxic for cultured endothelial cells and that antioxidants prevented generation of that cytotoxicity.

Alternative ways to account for foam cell formation

There are a number of potential alternative or complementary mechanisms by which foam cells might be generated, but these have not been as extensively studied nor as well documented as oxidative modification.

βVLDL and other lipoproteins rich in apoprotein E

βVLDL is a minor component of normal plasma and has a density like that of VLDL but beta electrophoretic mobility similar to LDL. βVLDL is enriched in apoprotein E, binds with high affinity to the LDL receptor on macrophages in vitro[76] and is taken up at a sufficiently rapid rate to increase the macrophage cholesterol content[77]. Uptake of βVLDL may occur via other receptors as well, such as LRP, perhaps assisted by binding of lipoprotein lipase (LPL)[78].

Aggregated LDL

Aggregates of LDL, generated by vigorous mixing resulting in denaturation of LDL, results in avid uptake of aggregated LDL via the native LDLR through phagocytosis, rather than endocytosis, which may result in accumulation of intracellular cholesterol in macrophages[79]. Large aggregates of LDL in the matrix of the rabbit arterial intima soon after an intravenous injection of a large single bolus of LDL can be noted[80], but whether this occurs spontaneously in vivo is unclear.

LDL-autoantibody immune complexes

Complexes of LDL or of aggregated LDL with IgG antibodies are taken up by macrophages at a markedly increased rate[81,82]. This is partly because the complex can now be taken up both by way of the LDL receptor and by way of the Fc receptor and perhaps partly because the LDL is further aggregated in the presence of a sufficient concentration of antibody. Increased levels of apoB-immune complexes have been recently documented in patients with acute coronary syndromes (ACS)[14] and following percutaneous coronary intervention (PCI)[15] and decreased levels following treatment of ACS patients with atorvastatin[83].

Complex formation between LDL and proteoglycans

LDL binds tightly to certain forms of proteoglycans, such as dextran sulfate[84,85], and after binding appears to be more susceptible to subsequent oxidative modification[86].

Enzymatically-modified LDL

Several enzyme mediated modifications of LDL (E-LDL), such as sphingomyelinase[87] and trypsin/cholesterol esterase[88,89] and cathepsin H,[90] have documented enhanced uptake by macrophages as well as C-reactive protein binding of E-LDL leading to complement activation[91].

Macrophage scavenger receptors

Macrophages express a variety of scavenger receptors, such as SR-A, CD36, SR-BI, CD68 and scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX), that mediate binding and uptake of OxLDL[92-94] (*Figure 1*). As a class, scavenger receptors tend to recognize polyanionic macromolecules and have been proposed to play physiologic roles in the recognition and clearance of pathogens, such as gram positive and negative bacteria[95-97], and apoptotic cells[98-101]. For example, mice generated with SR-A deletion were found to be more susceptible to infections[102]. Since atherosclerosis does not exert any evolutionary pressure, it is unlikely that scavenger receptors evolved as a mechanism for clearing OxLDL. In fact these receptors are found in lower mammals and, at least functionally, as far back as Drosophila[103]. CD36 has also been demonstrated to function as a fatty acid transport protein in adipose tissue and muscle[93], while SR-BI mediates selective uptake of HDL cholesterol esters in liver and steroidogenic tissues[104-106].

Studies in SR-A knockout mice comparing OxLDL binding and internalization by macrophages show that 20-30% of OxLDL uptake is attributable to SR-A[102]. In patients with total deficiency of CD36, monocyte/macrophage uptake of OxLDL is approximately 50% of total compared to patients with normal monocyte/macrophages[107]. Gene deletion and bone marrow transplantation experiments suggest that SR-A and CD36 knockouts result in a significant reduction in the progression of atherosclerosis[102,108,109], implying important quantitative roles of scavenger receptors in mediating atherogenesis. In contrast, studies of the SR-BI gene indicate that it plays an anti-atherogenic role[110,111], as it may facilitate reverse cholesterol transport by HDL by ABCA1-mediated cholesterol efflux in macrophages[112]. Macrophages from mice with combined SR-A and CD36 deficiency show a 75% decrease in uptake of OxLDL in vitro[113]. Interestingly, SR-BI/apo E double knockout mice exhibit severe atherosclerosis with evidence of plaque rupture and acute MI as early as six

weeks of life, complications that are rare in other murine models of atherosclerosis this early in life[110].

Manipulation of scavenger receptor number and activity may have theoretical clinical applications. However, this is tempered by the realization that these receptors are involved in multiple beneficial functions unrelated to atherogenesis, such as clearing microorganisms and apoptotic cells. For example, mice generated with SR-A deletion were found to be more susceptible to infections[102]. Therefore, although atherosclerosis may be ameliorated, additional infectious and proliferative lesions may develop.

Cholesterol homeostasis and foam cell formation

Macrophages possess mechanisms for preserving intracellular cholesterol homeostasis via either ABCA1-mediated transport of unesterified cholesterol and phospholipids to nascent HDL and/or conversion of cholesterol to cholesteryl esters[114]. These pathways appear to be overwhelmed in the setting of atherosclerosis through scavenger receptor-mediated uptake of modified lipoproteins resulting in foam cell formation (*Figure 2*). Cholesterol esterification is carried out by acyl coenzyme A: acylcholesterol transferase (ACAT)[115]. Under conditions in which cholesterol efflux pathways become saturated, cholesterol esterification seems to be a protective response to excess free cholesterol, which can be toxic[116]. Disposal of excess cholesterol can be achieved either by delivery to extracellular acceptors, such as lipid-poor apo AI, or by conversion to more soluble forms. Recent studies indicate that members of the ABC family of transport proteins, including ABCA1, also play an important role in the mechanism by which cells transfer excess cholesterol to HDL acceptors. Loss of ABCA1 results in Tangier disease, a condition in which patients have extremely low levels of circulating HDL and massive accumulation of cholesterol in macrophage-rich organs[117-120], and an apparent increased risk of atherosclerosis[114].

Roles of PPARs and LXRs in regulating scavenger receptor activity and cholesterol homeostasis

Peroxisome proliferators activated receptors (PPARs), PPARα, PPARγ and PPARδ, are members of the nuclear receptor superfamily of ligand activated transcription factors[121].The endogenous ligands that regulate PPAR activity remain poorly characterized but are presumed to include fatty acids and their metabolites[122,123]. The prostaglandin 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 and lipoxygenase products including 12 HETE and 13 HODE present in OxLDL, have been suggested to be endogenous ligands for PPARγ in macrophages[124-127]. Fibrates and thiazolidinediones are synthetic ligands for PPARα[128-130] and PPARγ[131], respectively. PPARγ is highly

expressed in macrophages and foam cells of atherosclerotic lesions and several lines of evidence suggest that PPARγ agonists can exert both atherogenic and antiatherogenic effects on patterns of gene expression[127,132-134]

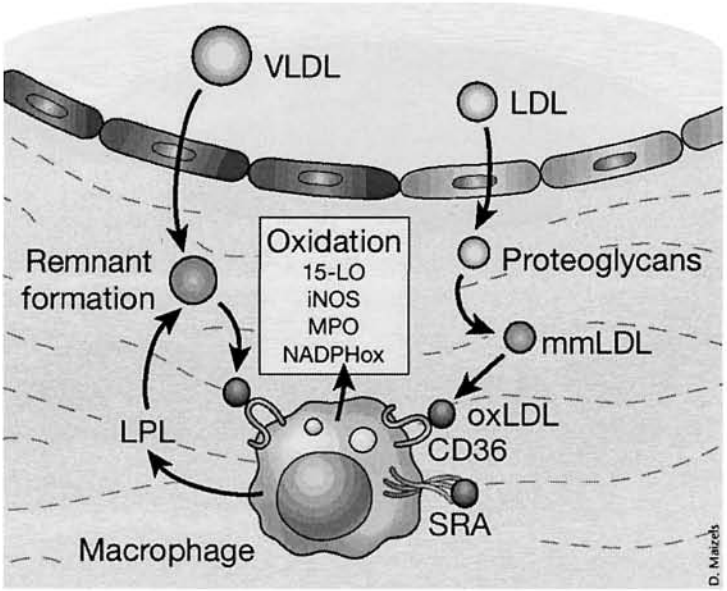

Figure 2. **Mechanisms contributing to foam cell formation.** LDL penetrates into the artery wall where it is trapped after adhering to proteoglycans. It is then highly susceptible to oxidation by enzymes such as lipoxygenases, MPO and iNOS. VLDL particles are subject to modification by lipoprotein lipase. The resulting remnant particles are also subject to trapping by proteoglycans, oxidative modification and uptake by macrophages. mmLDL, minimally modified LDL; SR-A, scavenger receptor class A. Reproduced with permission from Li et al.[206]

PPARγ stimulates expression of the scavenger receptor CD36[134]. PPARγ agonists also inhibit the program of macrophage activation in response to inflammatory mediators such as interferon γ and lipopolysaccharide[133, 135,136]. In addition, PPARα and PPARγ have been reported to induce the expression of Liver X receptor α (LXRα), suggesting that PPARs may exert anti-atherogenic effects through secondary activation of LXR target genes[137,138]. LXRs induce the expression of ABC transporters that have been linked to cholesterol efflux[139], serve as an acceptor of cholesterol transported by ABCA1-dependent processes[140], and induce synthesis of

fatty acids that are preferential substrates of ACAT in cholesterol esterification reactions[141]. Thus, these genes act in concert to reduce free cholesterol levels and protect macrophages from its cytotoxic effects. The use of LXR agonists indicate that LXRs exert anti-atherogenic effects in mouse models of atherosclerosis[142,143]. The net effects of TZDs in mouse models of atherosclerosis seem to be protective[142,144-146]. In addition, LDLR$^{-/-}$ mice transplanted with PPARγ$^{-/-}$ bone marrow progenitor cells develop more extensive atherosclerosis than animals transplanted with wild-type bone marrow, demonstrating a protective role of PPARγ in monocyte-derived macrophages[137].

Properties of oxidized LDL that make it potentially more atherogenic than native LDL

A partial list of biological properties of OxLDL that may make it more atherogenic than native LDL is shown in *Table I.* The four most important properties are described below in detail.

Table 1. **Potential mechanisms by which oxidized LDL may influence atherogenesis**

- OxLDL has enhanced uptake by macrophages leading to foam cell formation
- Products of OxLDL are chemotactic for monocytes and T-cells and inhibit the motility of tissue macrophages
- Products of OxLDL are cytotoxic, in part due to oxidized sterols, and can induce apoptosis
- OxLDL, or products, are mitogenic for smooth muscle cells and macrophages
- OxLDL, or products, can alter gene expression of vascular cells, e.g. induction of MCP-1, colony-stimulating factors, IL-1 and expression of adhesion molecules
- OxLDL, or products, can increase expression of macrophage scavenger receptors, thereby enhancing its own uptake
- OxLDL, or products, can induce proinflammatory genes, e.g. hemoxygenase, SAA and ceruloplasmin
- OxLDL can induce expression and activate PPARγ, thereby influencing many gene functions
- OxLDL is immunogenic and elicits autoantibody formation and activated T-cells
- Oxidation renders LDL more susceptible to aggregation, which independently leads to enhanced uptake. Similarly, OxLDL is a better substrate for sphyingomyelinase, which also aggregates LDL
- OxLDL may enhance procoagulant pathways, e.g. by induction of tissue factor and platelet aggregation
- Products of OxLDL can aversely impact arterial vasomotor properties
- OxLDL is involved in acute coronary syndromes and may potentially lead to plaque disruption

Modified from Tsimikas et al[207]

The ability to induce foam cell formation from monocyte/macrophages and smooth muscle cells

As discussed above, the unregulated uptake of OxLDL by macrophage scavenger receptors causing cholesterol accumulation and foam cell formation was the first observation that suggested this mechanism of the potential importance of OxLDL in atherogenesis.

Recruitment of monocytes from the circulation into the artery wall

In fetal atherosclerosis, OxLDL is present in the vessel wall prior to monocyte recruitment[3]. OxLDL is a chemoattractant for monocytes[147] and T-lymphocytes[148], which are the major cell types that are found in atherosclerotic lesions[149]. OxLDL also inhibits the motility of tissue macrophages[150] which may prevent macrophages from exiting atherosclerotic lesions. MmLDL, which is still recognized by the LDL receptor, stimulates release of monocyte chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) from human aortic endothelial cells which can induce recruitment of monocytes into the vessel wall. Many of these biological effects of mmLDL are attributable to oxidized phospholipids, which exist at concentrations that would be biologically active in vivo[151,152]. Lysophosphatidylcholine, a major component of more extensively OxLDL[8], can induce the expression of adhesion molecules and thus contribute to monocyte recruitment[153].

Cytotoxicity

Several laboratories independently showed that endothelial cells or smooth muscle cells incubated in the presence of LDL showed signs of toxicity going on to cell death in 24 to 48 hours[154-156]. This was attributed primarily to conversion of LDL in the medium to OxLDL and the addition of antioxidants or whole serum completely prevented the cytotoxicity. It is not clear if such concentrations may occur in vivo, but the potential for endothelia dysfunction due to endothelial cell toxicity is obviously present.

Inhibition of vasodilatation in response to nitric oxide

Arteries exposed to OxLDL in vitro show an endothelium-dependent vasodilator impairment[157]. Clinical studies show that treatment of patients with coronary artery disease with statins and/or antioxidant compounds can improve endothelial-dependent coronary vasomotion[158,159]. Several studies have shown that circulating OxLDL levels have been associated with endothelial dysfunction[160]. For example, Tamai et al showed that a single session of LDL apheresis significantly increased brachial artery acetylcholine-induced flow mediated dilatation (FMD) within four hours. Interestingly, the best predictors of improvement in FMD were reduction in

plasma levels of OxLDL and increased production in nitrate/nitrites[161]. Similarly, the extent of susceptibility of plasma LDL to oxidation[162] and the presence of elevated plasma OxLDL-E06, have been strongly correlated with coronary endothelial dysfunction in a statin regression study[160]. In addition, plasma levels of OxLDL-E06[163] and OxLDL measured by another independent assay[164] have been shown to correlate with coronary endothelial dysfunction in heart transplant recipients.

Evidence that oxidation of LDL takes place in vivo

Because oxidation of LDL is relatively easily prevented if kept in the presence of 5% serum, albumin or several different antioxidants, it suggested that the normal components of extracellular fluid were adequate to provide antioxidant protection under ordinary circumstances[165]. However, there are now many lines of evidence that oxidation of lipoproteins does occur *in vivo* and that this process is quantitatively important[166].

1) When LDL undergoes oxidative modification, a variety of oxidative neoepitopes that have been termed "oxidation-specific" epitopes by Witztum's group[68,69], are generated through the generation of highly reactive small carbon fragments of PUFA that may react with both the lipid and protein portions of autologous LDL. For example, two common epitopes are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which may form adducts with adjacent epsilon-amino groups of lysine residues leading to the generation of MDA-lysine adducts or Schiff base adducts and Michael-type adducts with lysine residues, respectively[69]. Many other similar modifications can be generated, yielding both lipid-protein and lipid-lipid adducts. Even subtle modification of LDL such as the nonenzymatic glycation of apoB results in adduct formation that renders autologous LDL immunogenic[167]. In order to develop antibodies that would recognize these epitopes, Witztum and colleagues prepared model OxLDLs from homologous LDL, such as MDA-LDL and 4-HNE-LDL, and used these to immunize mice to generate "oxidation-specific" murine monoclonal antibodies[68,168]. All of these antibodies immunostained such epitopes in atherosclerotic lesions in rabbits, non-human primates, and humans, but not in normal arterial tissue. Other investigators have developed similar antibodies that immunostain atherosclerotic lesions in a similar manner[169,170].

2) LDL, gently extracted from atherosclerotic tissue of rabbits and humans, shows all the physical, biological, and immunologic properties observed with LDL oxidized *in vitro*[171]. Of particular importance was the demonstration that LDL particles isolated from fatty streak lesions had

enhanced uptake by macrophage scavenger receptors and that this uptake could be competed for by *in vitro* OxLDL.

3) Oxidized lipids, including oxidized sterols, are routinely demonstrable in atherosclerotic tissue, but not in normal aortic tissue[38,39,172-174].

4) Numerous reports now document that circulating LDL displays chemical indices of early stages of oxidation[175,176] and oxidation-specific epitopes can be demonstrated in LDL particles by antibody-based immunochemical techniques[14,15,177-180].

5) Minimal modifications of autologous LDL render it immunogenic. It has been demonstrated that autoantibodies to a variety of epitopes of OxLDL can be found in sera of experimental animal models and humans with atherosclerosis[168,171,178,181]. For example, titers of autoantibodies to epitopes of OxLDL correlated significantly with the extent of atherosclerosis in apoE$^{-/-}$ and LDLR$^{-/-}$ mice[181] as well as with the presence and quantity of OxLDL in the vessel wall[182]. Furthermore the titers of such autoantibodies are related to the presence and/or the rate of progression of disease in animal models[34,182,183].

6) OxLDL autoantibodies and OxLDL-immune complexes are found in atherosclerotic lesions of mice, rabbits and humans[184,185]. Autoantibody titer to MDA-LDL was highly significant predictor of the progression of carotid intimal-medial-thickness in a group of middle-aged Finnish males[186] and in a recent Swedish cohort[187].There have been now a large number of studies in humans suggesting that the titer of antibodies to epitopes of OxLDL are associated with various clinical manifestations of atherosclerosis or with traditional risk factors for atherosclerosis such as hypertension, diabetes and smoking[188].

7) Oxidized LDL is present in the earliest human fetal lesions even before the presence of monocyte/macrophages[189].

8) Non-invasive imaging with radiolabeled murine and human antibodies show the physical presence of OxLDL in the vessel wall in vivo[70,190-192].

The role of OxLDL in human cardiovascular disease

It was first documented in 1989 by Palinski et al[168] and Ylä-Hertualla et al[171] that OxLDL exists in vivo in both animal and human atherosclerotic lesions. More recently, Nishi et al[180] documented that vulnerable carotid plaques from humans are greatly enriched in OxLDL and that plaque content of OxLDL was 70 times the plasma concentration. Statins have recently been shown to reduce the vessel wall content of OxLDL as well as the plasma levels of circulating OxLDL[83]. For example, in humans pretreated with pravastatin for three months prior to carotid endarterectomy, significantly reduced OxLDL immunostaining was noted in carotid

specimens stained with the monoclonal antibody NA59[68,69], which recognizes 4-hydroxynonenal epitopes of OxLDL[193].

Autoantibodies to OxLDL were first documented in animals and patients by Witztum's laboratory in 1989[168,171]. Many human studies have been published subsequently, not all of which are consistent, showing associations with various manifestations of atherosclerosis in patients. The reasons for these inconsistencies are multiple and varied[188] and measurement of autoantibodies to OxLDL, although interesting from a pathophysiological perspective, has not thus far provided additional clinical value above and beyond traditional risk factors.

The ability to measure circulating OxLDL, however, has opened a new arena in studying the role of OxLDL in human disease. Three laboratories have developed antibody-based assays for measuring circulating early forms of OxLDL[12,194,195]. Recent studies have shown that increased levels of circulating OxLDL are found in plasma of patients with coronary[196,197] and carotid artery disease[198] and in ACS[14,179,199]. For example, circulating OxLDL measured on isolated LDL by monoclonal antibody DLH3 correlated well with the presence of OxLDL in coronary atherectomy specimens and appeared to differentiate the severity of the underlying clinical presentation[179]. Increased levels of OxLDL are also associated with increased carotid intima-media thickness[198]. In addition, plasma levels of OxLDL have been shown to correlate with coronary[160] and brachial reactivity[161] following treatment with lovastatin or LDL apheresis, respectively. Recent studies have also shown an association between plasma OxLDL levels and acute cerebral infarction[200] and strong immunostaining for MDA-LDL has been reported in brain tissue of patients with Alzheimer's disease[201]. We recently documented that OxLDL-E06 strongly reflected the presence of ACS in a prospective study with a seven-month follow-up period, showing a characteristic rise and fall in levels. In addition, we documented a strong correlation between OxLDL-E06 and Lp(a) with a correlation of 0.91. Subsequently, we measured serial levels of OxLDL-E06 in patients undergoing uncomplicated PCI for stable angina and showed a 36% and 64% rise post procedure in OxLDL-E06 and Lp(a) levels, respectively (*Figure 3*)[15]. Interestingly, the oxidized phospholipids (OxPL) measured by antibody E06 were present equally on Lp(a) and other apoB lipoproteins post PCI, but subsequently were all transferred to Lp(a) by six hours, suggesting that Lp(a) may act as a sink for OxPL and may mediate their transport and clearance. These observations support the hypothesis that OxPL are present in disrupted plaques and are released into the circulation by PCI, where they are bound by apoB-containing lipoproteins, and preferentially by Lp(a). Furthermore, they define a novel relationship between OxPL and Lp(a) and suggest new insights into the role of Lp(a) in

normal physiology as well as in atherogenesis. We have proposed that Lp(a) may in fact contribute to a protective immune response by binding OxPL, similar to binding of OxPL by C-reactive protein[202], as it is highly enriched in PAF-acetylhydrolase which may serve to detoxify OxPL by cleaving the oxidized sn-2 fatty acid. On the other hand, its atherogenicity may rise from the fact that when plasma levels of Lp(a) are elevated, an enhanced number of Lp(a) particles would enter the vessel wall where Lp(a) is preferentially bound to the extra-cellular matrix and with its an enhanced content of OxPL, Lp(a) would have profound pro-inflammatory properties[18].

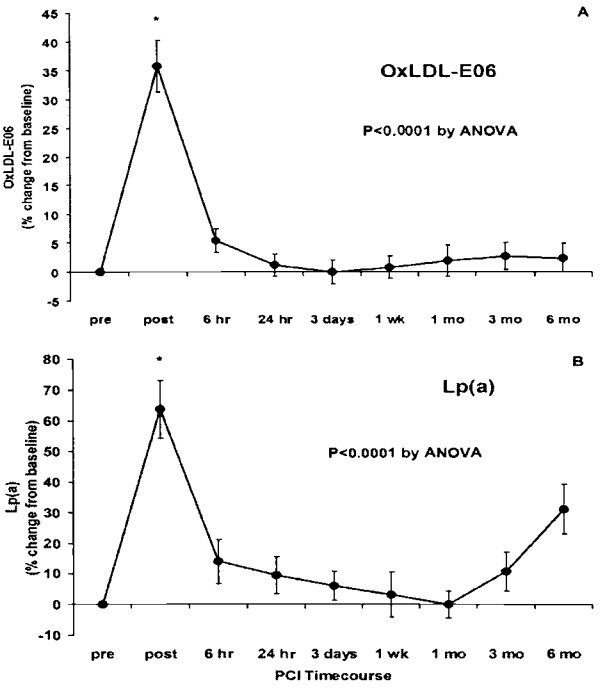

Figure 3. **Mean percent change from pre-PCI levels in OxLDL-E06 (A) and Lp(a) (B) levels following PCI.** *=P<0.001 compared to other timepoints. Reproduced with permission from Tsimikas et al.[15]

The role of OxLDL in response to treatment has not been defined. Iuliano et al[203] have shown that radiolabeled LDL injected into patients with carotid plaques undergoing endarterectomy accumulates in macrophages within these plaques and its uptake is markedly decreased by treatment with vitamin E (900 mg/day) for four weeks. Our group has also recently shown that high-dose atorvastatin (80 mg/day) significantly reduced total plasma

levels of OxLDL-E06 (total apoB-OxPL, i.e. OxPL associated with all circulating apoB-100 particles) in patients with ACS in the Myocardial Ischemia Reduction with Aggressive Lipid Lowering therapy (MIRACL) study[83]. Interestingly, in that study, there was enrichment of OxPL on a smaller pool of apoB-100 particles (i.e. increased OxPL/apoB ratio), in parallel with strikingly similar increases in Lp(a), suggesting binding by Lp(a). Additional data has also shown that most of the OxPL epitopes in plasma are associated with Lp(a)[14,15,204]. Unpublished data from our laboratory and our collaborators in monkeys, rabbits, and $LDLR^{-/-}$ mice undergoing dietary regression show similar changes in OxLDL-E06 (i.e. marked reduction in total apoB-OxPL but increased in OxPL/apoB in the remaining apoB particles at steady state) in conjunction with significantly reduced OxPL epitopes in the vessel wall. These data support the hypothesis that statins or aggressive lipid lowering promote mobilization and clearance of pro-inflammatory OxPL from the vessel wall and circulation, respectively, which may contribute to a rapid reduction in ischemic events. This is also supported by a recent study showing increases in both Lp(a) and OxPL/apoB levels in patients placed on low fat diets, a condition in which one might postulate efflux of OxPL out of the vessel wall[205].

Future studies will determine if OxLDL plasma measurements provide independent diagnostic or prognostic information above and beyond any easily measured lipoprotein parameters.

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