Low-density lipoprotein oxidation

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Free radical mediated oxidation of low-density lipoproteins (LDL), which has been extensively studied in the last two decades, plays a central role in the development of the atherosclerotic plaque. Oxidation involves the lipid moiety of LDL in a chain reaction mechanism. In the initial phase, free radicals preferentially attack highly oxidizable polyunsaturated fatty acids. Subsequent recruitment of other molecules includes cholesterol and phospholipids. The process of oxidation is counteracted by antioxidants present in LDL. By-products formed during oxidation of LDL lipids, which may have biological activity, react with amino acid residues of the LDL protein backbone with the consequent modification of chemical and immunological properties responsible for cellular receptor shift. Oxidation-altered apolipoprotein B of oxidized LDL is, in fact, recognized by the macrophage scavenger receptor responsible for foam cell formation. The mechanism of LDL oxidation and the impact on atherogenesis are discussed.

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Introduction

Atherosclerosis is a common disease in western population and plays a central role in the pathophysiology of cardiovascular disease. In the last two decades a series of excellent studies unraveled biochemical mechanisms which provided the background for a theory of atherogenesis. The backbone of this theory is based on lipid laden cells of atherosclerotic lesion (foam cells), on plasma lipoproteins, on a free radical-mediated modification of plasma lipoproteins, and on the scavenger receptor[1]. Foam cells are the main cell type of atherosclerotic lesions and have been identified as differentiated from monocytes migrated from blood and from smooth muscle cells of the arterial wall[2-4]. Among plasma lipoproteins, the low-density lipoprotein (LDL) is the main protein responsible for transport and accumulation of lipids in the arterial wall. Paradoxically, accumulation of LDL in developing foam cells does not occur by the way of classic LDL receptor[5]. Incubation of macrophage with even very high concentrations of LDL does not increase cholesterol content, and patients with familial hypercholesterolemia who totally lack LDL receptors develop atherosclerotic plaques plenty of foam cells[5,6]. LDL needs to be chemically altered/modified to enter the cells of atherosclerotic plaque via an unregulated receptor, the scavenger receptor[5].

Low-density lipoprotein structure and metabolism

The metabolic precursor of LDL is the very low-density lipoprotein (VLDL) constructed in the liver and secreted into the circulation where becomes a mature LDL particle after lipid exchange and remodeling. Lipoprotein lipase, which is present on the surface of the endothelial cells, hydrolyzes VLDL triglycerides and liberates free fatty acids for peripheral tissue needs[7]. With the progressive loss of triglycerides VLDL is transformed into LDL (density, 1.019-1.063 g/ml), which represents the main cholesterol carrying protein in plasma (about 60% of total cholesterol). Each LDL particle has a central lipophilic core composed by cholesteryl esters and triglycerides, and an outer shell of phospholipids and free cholesterol[8]. The apolipoprotein (apo) B, which intercalates the LDL surface and holds about 20% of the total volume[8], acts as a ligand for the receptor that allows cholesterol influx[5]. Cells use cholesterol for building membranes and for synthesizing steroid hormones in adrenal glands.

The B receptor-mediated cholesterol uptake by cells is fine tuned by feedback signals finalized to the maintenance of constant cholesterol levels for cellular requirements[5]. After internalization the LDL particle is fragmented and cholesterol enters the intracellular cholesterol pool. The increase

in intracellular cholesterol concentration suppresses the synthesis of B100 receptors and of the HMG-CoA reductase enzyme. Consequently, cholesterol influx and endogenous cholesterol synthesis are blocked. On the other hand, cholesterol influx activates the acyl-coenzyme A: cholesterol acyltransferase enzyme, which holds down free cholesterol levels[5]. In fact, the excess of free cholesterol is cytotoxic because it forms insoluble crystals. Cholesterol esterification and storage into a pool of inert cytoplasmic cholesteryl esters prevent toxicity.

The receptor for oxidized low-density lipoproteins

Circulating LDL are also termed "native LDL" to distinguish them from post-translational modified LDL, which enhance its uptake by macrophage *in vitro*. Modifications of LDL include self-aggregation, complexation with proteoglycans, immunoglobulins, and degradation by hydrolytic enzymes[9-17]. However, none of these modifications has been extensively studied as LDL modification based on oxidation. The papers dealing with oxidized LDL increased exponentially in the last two decades. Oxidized LDL can be produced by several physiological plausible mechanisms that give to oxidation a central role in the theory of atherogenesis[1,5]. Numerous studies have supported the occurrence of oxidized LDL *in vivo*. Oxidized LDL, by-products, and epitopes of LDL oxidation have extensively been demonstrated in atherosclerotic plaques[18-24], and even small amounts of oxidized LDL can be demonstrated in plasma[25-29]. The discovery of oxidized LDL was coincident with studies of cellular uptake of cholesterol[5]. It was evident that not all cell types use the native LDL receptor for cholesterol influx. Macrophages, the main cellular type of atherosclerotic plaque, do not express classic LDL receptors and do not accumulate native LDL at appreciable rate. Initially, it was shown that derivatization of apo B by acetylation of lysine residues rendered LDL suitable for influx into macrophages[6]. Thus, macrophages express a different receptor that is not down-regulated by cholesterol influx, and engulf lipids until becoming foam cells. However, it was not plausible that acetylation of apo B could have occurred *in vivo*. In searching a physiologic mechanism for apo B derivatization, by-products of oxidation of LDL lipids resulted effective derivatizers of apo B. It was shown that LDL incubated in the presence of transition metals induced modification of LDL suitable for macrophage uptake comparable to that of acetylated LDL. The modification of LDL induced by transition metal ions is the result of an oxidation process. Lipid peroxidation is one of the deleterious effects of free radical attack to organic molecules of living organisms. Among lipids, cholesterol and polyunsaturated fatty acids (PUFA) are very sensitive to free radical attack. PUFA are easily attacked at the level of α-methylenic hydrogens with consequent rearrangement, formation of a lipid radical, and triggering of a chain reaction with incorporation of molecular oxygen[8]. By-products of lipid peroxidation, including aldehydes – the most popular being malondialdehyde – and ketones, have enough chemical reactivity to derivatize apo B and to conjugate to other lipids[8]. In addition apo B can be directly modified by oxidative damage[30].

Oxidized LDL exhibit the following chemical properties: increased electrophoretic mobility, increased lisophosphatides, decreased phosphatidylcholine, increased conjugated dienes, hydroperoxides, oxysterols and isoprostanes, and depletion of constitutive antioxidants[8].

Free radical-mediated LDL oxidation has also been produced by physiological plausible mechanisms based on enzymes – lipoxygenase and phospholipase A_2[31] –, redox-cycling of hemoglobin[32], and by cells[33-35].

The original acetyl LDL receptor has been cloned and now designated scavenger receptor A (SRA) and it occurs in two differentially spliced forms, SRAI and SRAII, which have comparable ligand binding capacity[36,37]. The pathogenic role of SRA in atherogenesis has been demonstrated by crossing SRA-targeted mice with apo E-targeted and finding reduction of atherosclerosis lesion severity[38].

The B class of scavenger receptors includes CD36 and SRB1[39,40]. Macrosialin and CD68 have a partial oxidized LDL binding capacity[41,42]. However, taking into account that these receptors are little expressed on plasma membrane, their role in the uptake of oxidized LDL is uncertain.

Pro-atherogenic role of oxidized low-density lipoproteins

Oxidized LDL possess several biological activities, other than the affinity for the scavenger receptor, relevant for atherogenesis. Oxidized LDL induce endothelial dysfunction and the expression of adhesion molecules on endothelial cells[43,44], favor monocyte differentiation[45], are cytotoxic for most cells, counteract biological effects of nitric oxide[46], and are thrombogenic (through platelet activation and increase in tissue factor activity from endothelial cells)[47,48].

Oxidized LDL can stimulate the release of macrophage colony-stimulating factor and monocyte chemoattractant protein-1 by endothelial cells with a potential role in facilitating the development of fatty streaks by recruiting monocytes and facilitating their differentiation into macrophages[49,50].

Isoprostanes, which are generated during LDL oxidation and found in the cellular component of atherosclerotic lesions, exhibit mitogenic activity and induce platelet aggregation and vasoconstriction[51]. In analogy, cholesterol oxidation products have also shown biolog-

ical activities relevant in the atherosclerosis process. 7β-hydroperoxy-5-en-3β-ol, generated during LDL oxidation, is a component of atheroma, is cytotoxic[21,52] and is implicated in the expression of several cytokines, which are of potential relevance in atherosclerosis[52-56].

Antioxidants and atherosclerosis

Lipids in LDL are protected from free radical-mediated oxidation by several lipophilic antioxidants. On molar basis, the most represented antioxidant is α-tocopherol (11 nmol/mg protein, equal to 6 molecules α-tocopherol in an LDL particle). Gamma-tocopherol, ubiquinol-10, carotenoids and other antioxidants are present in much smaller amounts[8].

Antioxidants prevent LDL oxidation *in vitro* and the biological effects of oxidized LDL, including macrophage lipid loading and cytotoxicity. Thus, antioxidants have been considered as candidates for inhibiting the evolution of atherosclerotic disease[1]. This hypothesis has also been supported by studies in several experimental animal models in which the administration of antioxidants slows the progression of atherosclerosis[57,58]. Vitamin E and synthetic antioxidants (probucol, butylated hydroxytoluene and diphenylenediamine) have been shown to inhibit the progression of the disease in the LDL receptor-deficient rabbit and mouse, in apo E-deficient mouse, in cholesterol-fed hamster, New Zealand White rabbit and cynomolgus monkey[1,58].

Markers of low-density lipoprotein oxidation and coronary heart disease

In the recent years new technologies have been developed to screen for the existence of enhanced oxidant stress in humans. Isoprostanes are a new family of eicosanoids that derive from oxidation of arachidonic acid[51]. They have been found elevated in atherosclerotic plaque and in several settings associated with coronary atherosclerosis. In particular, isoprostanes have been shown to be elevated in hypercholesterolemic and diabetic patients and in patients undergoing coronary angioplasty[59-61]. An open issue is if isoprostanes are mere markers of oxidant stress or have also biological relevance in the context of atherosclerosis and its complications. Isoprostanes, for instance, are not able to induce platelet aggregation, but in a range of concentration between 10 nM and 10 μM enhance platelet response to subthreshold concentration of different agonists, such as adenosine diphosphate and collagen[62]. These concentrations are higher than those found in conditions characterized by enhanced oxidant stress. However, it has been postulated that they could have a role in case of coincidence of enhanced platelet aggregation and oxidant stress, as in diabetic and hypercholesterolemic patients. A significant correlation was in fact demonstrated between isoprostanes and urinary thromboxane B_2 in this setting[59,60], but a clear-cut cause-effect relationship is still lacking. Another biological effect exerted by isoprostanes is vasoconstriction but also in this case concentrations necessary to lead to this effect are higher than those present in human circulation[63]. Further studies are, therefore, necessary to better understand the role of isoprostanes in the pathophysiology of atherosclerosis.

Cholesterol oxidation products (oxysterols) represent another possible approach to study oxidant stress in humans. Oxysterols derive from enzymatic and non-enzymatic oxidation of cholesterol and are quite abundant in human circulation and in atherosclerotic plaques[52]. Oxysterols may be useful not only as markers of oxidant stress, but also to identify new markers of endothelial damage in the setting of atherosclerosis. 7β-hydroperoxy-5-en-3β-ol, a component of atheroma that is produced during LDL oxidation, is cytotoxic and has been implicated in the expression of several cytokines involved in the atherosclerotic process[21]. Oxysterols may represent an intriguing approach to check for atherosclerosis and its complications also because of a slow elimination from human circulation. In fact, oxysterols are formed in minimally modified LDL, which are not recognized by the scavenger receptor. This could be the reason why the plasma concentration of oxysterols is 3 times higher than that of isoprostanes.

Antibodies against oxidized LDL is another approach that has been largely used to detect atherosclerosis progression, and more recently to screen for chronic and acute coronary heart disease. Antibodies against LDL may be directed against minimally modified LDL or fully oxidized LDL. While minimally modified LDL represent the oxidation of PUFA on phospholipids and cholesterol esters, fully oxidized LDL represent the depletion of antioxidant and apo B-derivatization[64]. It is still uncertain if using one of these two antibodies allows a better interpretation of atherosclerosis progression. Actually, there are conflicting results in the sensitivity of these antibodies in identifying patients with acute coronary syndrome.

Oxidized low-density lipoproteins, antioxidants, and cardiovascular disease

The reduction of atherosclerosis lesions in experimental animal models provides a strong support for using antioxidants to prevent and/or reduce the progression of atherosclerosis in man[58]. The oxidized LDL route in plaque resident foam cells and its modulation by vitamin E have been demonstrated *in vivo* in man, in patients undergoing carotid endarterectomy[65]. Radiolabeled LDL intravenously injected have been localized in foam cells of the carotid specimen obtained from en-

darterectomy. The uptake of LDL was almost completely suppressed in foam cells of patients treated for 4 weeks with vitamin E (900 mg daily)[65].

In aggregate, the impact of oxidation on vascular disease provides a strong conceptual basis for using antioxidants at clinical level. It should be reminded that additional support comes from observational studies that showed that dietary intake of vitamin E is inversely related to coronary heart disease[57,58].

Several clinical trials have been conducted with vitamin E to assess if an antioxidant treatment was able to reduce cardiovascular events in patients with previous cardiovascular events or at risk of developing them. Initially, the CHAOS trial showed a significant reduction of cardiovascular events in patients supplemented with vitamin E[66]. However, the lack of effect of vitamin E in subsequent trials raised some concerns on the efficacy of vitamin E in the clinical setting of coronary heart disease[67,68]. The cause of these conflicting results is not clear and several factors have been suggested to play a role. These include: compliance, the trial design, the dosage regimen used – ranging from 50 to about 1600 mg (equivalent to 800 IU) a day – and the source of vitamin E linked to the different bioavailability of natural and synthetic forms of vitamin E[57,58,69]. Compliance is questioned because most studies did not estimate vitamin E plasma levels in placebo and target groups[58]. Plasma vitamin E concentration detected in the ATBC study, which used 50 mg synthetic vitamin E daily, was similar to that observed in the CHAOS study, which used 400 or 800 IU natural vitamin E daily[58].

The dosage regimen is likely of relevant importance in clinical trials as recently supported by the SPACE trial in which 800 IU vitamin E daily significantly reduced vascular events in hemodialysis patients, who are at high risk of cardiovascular complications[70]. Conversely, 300 mg (equivalent to 150 IU) vitamin E daily has been reported to be insufficient to reduce cardiovascular events in patients at risk[71]. Therefore, we should be certain that these trials analyzed subjects in whom the plasma antioxidant capacity was increased by vitamin E treatment. Measurement of plasma vitamin E might be an indirect but useful way to study this issue. In addition, plasma vitamin E should be expressed in relation to cholesterol or blood lipids more than simply indicating its plasma levels[72].

An additional point emerging from clinical trials is that vitamin E bioavailability is never mentioned. Vitamin E is absorbed in the gastrointestinal tract in conjunction with lipids. We have recently demonstrated that in about 30% of subjects vitamin E is not absorbed, if not taken with meal, and that its availability may not be adequate to increase plasma antioxidant activity[73]. Thus, no change in total antioxidant activity was detected despite plasma vitamin E increased by 29%, suggesting the existence of a cut-off above which changes in the antioxidant activity are observed[73].

Conclusions

LDL oxidation and the scavenger receptor are emerged as principal actors in the process of atherosclerosis. Their connections have been deeply investigated *in vitro* and in animal models and the data accumulated constitute the background for understanding atherosclerotic vascular disease. Antioxidants are conceptually the best candidates to interfere with the process and among them vitamin E, as a natural non-toxic compound, has received much attention. However, the intense research in the topic of LDL oxidation has unanswered the question of the relevance of LDL oxidation at clinical level. Clinical trials did not include biochemical markers of oxidant stress for selecting patients who potentially would benefit from antioxidant treatment, nor compliance of antioxidant treatment was available. Until recently, commonly employed indices of lipid peroxidation were based on *ex vivo* systems or unspecific analytes. Accordingly, much caution should be used in the interpretation of data based on unspecific assays for LDL oxidation. The availability of specific and sensitive markers of lipid peroxidation, including isoprostanes and oxysterols, measurable by mass spectrometry should be widely used at clinical level to identify patients with increased oxidant stress. In addition, antioxidant treatment should be monitored by measuring plasma concentrations of the supplemented vitamin, and eventually the changes in plasma antioxidant activity.

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