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Modified Forms of LDL in Plasma

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1. Introduction

High blood concentration of low-density lipoprotein (LDL) cholesterol is a major risk factor for early development of atherosclerosis. Massive accumulation of cholesterol in the arterial wall and formation of lipid-laden cells (foam cells) typical of the atheromatous plaque occur after LDL entrapment in the subendothelial space. However, there is a general agreement that native non-modified LDL does not present any of the typical features of an atherogenic lipoprotein; native LDL does not promote foam cell formation and has no inflammatory, proliferative or apoptotic capacity. It is therefore assumed that when LDL is trapped in the arterial wall it is modified by several mechanisms such as lipoperoxidation, non-enzymatic glycosylation, enzymatic lipolysis and/or proteolysis. As a consequence, modified LDL particles acquire inflammatory and apoptotic capacity and are recognizable by scavenger receptors to promote foam cell formation. Although most studies on LDL modification have focused on mechanisms that could occur in the vessel wall, modified LDL particles have been reported in blood. The concentration of these particles is increased in subjects with high cardiovascular risk. The present chapter reviews the possible mechanisms leading to LDL modification. It then focuses on a subfraction of modified LDL particles detected in plasma, named electronegative LDL.

2. Modification of LDL as a key event for atherosclerosis

Pioneering studies by Goldstein and Brown demonstrated the involvement of LDL receptor (LDLr) in the plasma clearance of LDL and its major role in the development of atherosclerosis (Goldstein & Brown, 1985). However, these authors' outstanding observations gave rise to what was called the "cholesterol paradox" (Brown & Goldstein, 1983). Patients with homozygous familial hypercholesterolemia lack functional LDLr. Moreover, LDLr expression is negatively regulated by the intracellular cholesterol content, which results in a tight control of the concentration of esterified cholesterol in the cytoplasm (Brown & Goldstein, 1986). However, atherosclerosis is characterized by an abundance of foam cells loaded with esterified cholesterol droplets. Hence, there must be an alternative pathway leading to the massive accumulation of cholesterol in foam cells of the atherosclerotic lesion. This alternative pathway was discovered through the study of the scavenger receptors (SR) expressed by monocyte-derived macrophages (Krieger, 1992). These receptors have the ability to bind an unusual variety of ligands with the common

characteristic of high electronegative charge. The first SR described was the type A SR (SRA) (Goldstein, 1979). SRA do not recognize native LDL but is able to bind modified forms of LDL; indeed, SRA expression is not regulated by the cytoplasm concentration of cholesterol. Early studies were performed using acetylated LDL (acLDL), a good ligand for SRA. However, acetylation does not occur spontaneously "in vivo". Soon, researchers looked for further modifications of LDL that would increase its negative charge and were able to occur "in vivo". The finding that oxidized LDL (oxLDL) could be internalized through SR and promote the formation of foam cells opened a vast field of research (Steinberg, 1989).

2.1 Oxidative modification of LDL

Oxidative modifications of lipid and proteins are frequent in many pathophysiological processes "in vivo" and it is now well-established that LDL undergoes oxidative modifications that confer to these modified particles a number of atherogenic properties (Witztum & Steinberg, 1991). In the early 80s several researchers independently observed that the incubation of LDL with endothelial cells in culture drastically altered these particles, transforming them into more electronegative LDL, after which they become a ligand for macrophages SR (Henriksen, 1981; Hessler, 1979). These studies concluded that the changes induced by endothelial cells on LDL were due to free radical modification. This was the origin of the oxidative modification hypothesis of atherogenesis (Steinberg, 1989). This hypothesis was gradually strengthened by subsequent findings regarding the properties of oxLDL obtained over the ensuing years. Table 1 summarizes the atherogenic properties observed in oxLDL and their contribution to atherosclerosis.

Characteristic	**Consequence in atherosclerosis**
Impaired binding to the LDL receptor	Decreased plasma clearance
Recognition by SR	Foam cell formation
Increase in the expression of cytokines, chemokines and vascular adhesion molecules	Leukocyte recruitment, inflammation
Increase in the expression of growth factors	Cell proliferation and collagen secretion
Promotion of apoptosis and cytotoxicity	Formation of necrotic core
Immunogenicity	Immunogenic component of atherosclerosis
Inhibition of NO release and function	Vasoconstriction
Increase of tissue factor activity	Thombosis

Table 1. Atherogenic characteristics of oxLDL

Oxidation of LDL primarily attacks the double bonds of unsaturated fatty acids in phospholipids and generates a plethora of lipid-derived compounds, including oxidized cholesterol (oxysterols), oxidized-fragmented phospholipids, lysophosphatidylcholine, hydroperoxids, aldehydes and ketones (Esterbauer, 1990). The protein moiety of LDL can be modified by reacting with lipid-oxidation products. Both aldehydes and ketones, for example, malondialdehyde (MDA) or 4-hydroxynonenal (HNE), have the ability to derivatize lysine and arginine residues in apoB (Fogelman, 1980). This reaction forms an adduct that eliminates the positive charge of these aminoacids and increases the negative charge of the particle, favouring its recognition by SRA (Goldstein, 1979). In addition to SRA, further SRs and other receptors which bind different forms of modified LDLs have

been described in the last two decades (SRBI, CD36, LOX-1, CD68, LRP1, TLR4) (Adachi & Tsujimoto, 2006; Llorente-Cortes & Badimon, 2005; Miller, 2003; Van Berkel, 2000). These receptors play a role in innate immunity, scavenging a number of ligands and extracellular debris. Some of these are also expressed in endothelial cells or in smooth muscle cells (Adachi & Tsujimoto, 2006). The derivatization of lysines has an additional effect on LDL; since a lysine-rich cluster is the LDLr binding site of apoB (residues 3359-3369), the loss of their positive charge abolishes the interaction between oxLDL and LDLr (Boren, 1998a).

Besides lipid accumulation, other phenomena occur during the evolution of the disease. Atherosclerosis is a chronic inflammatory process that begins with leukocyte recruitment to the lesion area (Ross, 1999). A number of inflammation mediators, such as cytokines (IL6), chemokines (IL8, MCP1) or vascular adhesion molecules (VCAM, ICAM1, e-selectin) are hyperexpressed in response to oxLDL by all cells involved in atherogenesis, including endothelial cells, monocytes/macrophages, lymphocytes and smooth muscle cells (Berliner, 1997; Tedgui & Mallat, 2006). Related to the inflammatory activity of oxLDL is its ability to promote the expression of growth factors and colony stimulating factors. This proliferative activity is mediated by oxidation-derived lipids, and in smooth muscle cells it induces the change of the normal contractile phenotype to a proliferative phenotype (Auge, 2002). The proliferative phenotype of smooth muscle cells is characterized by the high production of collagen that will contribute decisively to the thickening of the arterial wall (Negishi, 2004).

Another hallmark of the atheromatous plaque in its advanced stages is the presence of a necrotic core formed by debris from dead cells (Stary, 2000). OxLDL also contributes to the formation of this necrotic core, because when the content of esterified cholesterol exceeds the storage capacity of foam cells apoptotic processes are induced (Hessler, 1983). Another mechanism of cytotoxicity is the high content of lysophosphatidylcholine in oxLDL that disrupts the integrity of cytoplasmatic membrane and promotes cell death (Naito, 1994).

OxLDL is immunogenic, and autoantibodies against oxidation-specific epitopes are detected in normal and hyperlipemic subjects (Lopes-Virella & Virella, 2010). The role of these autoantibodies is not well defined and published results are divergent; positive or negative associations between autoantibodies and atherosclerosis have been reported. In general, antibodies of IgG class are presumed to be pro-atherogenic, whereas IgM antibodies would play a protective role (Frostegard, 2010). Further deleterious properties of oxLDL are its capacity to increase the tissue factor activity (Petit, 1999), favouring thrombosis, or the inhibition of the release of nitric oxide (Minuz, 1995), promoting vasoconstriction.

The lipoperoxidative process is sequential and lipids degrade to produce new products which in turn form other molecules (Quehenberger, 1988). As a consequence, oxLDL comprises an extremely heterogeneous group of particles that have different atherogenic characteristics depending on the relative content of each molecule (Esterbauer, 1993). For instance, minimally oxidized LDL (mmLDL) has a high inflammatory activity due to its high content in oxidized phospholipids formed during the early phases of oxidation. However, mmLDL has normal affinity to the LDLr and is not recognized by SR. In contrast, extensively oxidized LDL has relatively low inflammatory activity but it is a major inductor of cytotoxicity due to its high content of oxysterols. Indeed, the more oxidized the LDL the more these particles promote foam cell formation.

2.2 Further modifications of LDL occurring in the arterial wall

Oxidative modification is not the only process affecting the native properties of lipoproteins in the arterial wall. Disappointing results from large-antioxidant trials have led to the concept that alternative mechanisms of modification could be involved (Steinberg, 2009). Current knowledge indicates that although oxidation is still relevant, other processes could contribute substantially to the development of atherosclerosis. A number of modifications have been studied over the past three decades.

Enzymes that are hyperexpressed in the microenvironment of the lesion area, such as lipases (cholesterol esterase (CEase), sphingomyelinase (SMase) or secretary phospholipase A_2 ($sPLA_2$)) or proteases (matrix metalloproteases or cathepsins) can modify LDL (Pentikainen, 2000). In the case of diabetes, non-enzymatic glycosylation could have a major role in LDL modification (Witztum, 1997). The interaction of LDL with the proteoglycans (PG) could modify apoB conformation, destabilizing its conformation and promoting aggregation (Pentikainen, 1997). Other putative physiological processes that could modify LDL are carbamylation (Basnakian, 2010) or desialylation (Tertov, 1990). Table 2 summarizes several possible physiological mechanisms leading to LDL modification.

Mechanisms of modification	Foam cell formation	Inflammation/ proliferation	Cytotoxicity/ apoptosis
Oxidation[1]	+++	+++	+++
Modification by CEase and protease	-/+	++	++
Lipolysis by SMase	+	-	-/+
Lipolysis by PLA_2	-/+	++	+
Non-enzymatic glycosylation	-/+	-/+	-/+
Binding to PG	++	-	-
Carbamylation	++	++	++
Desialylation	++	+	++

Table 2. Atherogenic properties of several modified LDLs. [1] The characteristics of oxLDL, and also those of the other modifications, depend on the extent of modification

2.2.1 Enzymatically-modified LDL (E-LDL)

In an attempt to find a modified LDL alternative to oxLDL, Bhakdi and coworkers performed a series of studies using LDL that was modified by means of one protease and CEase. They found that this "enzymatically-modified LDL" (E-LDL) acquired a number of atherogenic properties (Bhakdi, 1995). As a result of the enzymatic treatment, E-LDL presents mild apoB fragmentation, and it has high free cholesterol and non-esterified fatty acids (NEFA) content (Klouche, 1998). E-LDL induces inflammation, proliferation and apoptosis (Dersch, 2005; Klouche, 1999; Klouche, 2000). The high content of NEFA seems to be major factor responsible for these atherogenic properties (Suriyaphol, 2002). E-LDL adds further atherogenic characteristics because it binds to C-reactive protein and activates the classical complement pathway (Bhakdi, 2004). The activation of complement by E-LDL concurs with the emerging concept of the innate immune response as a potentially important factor in atherosclerosis (Hartvigsen, 2009).

2.2.2 Phospholipase A_2-modified LDL (PLA_2-LDL)

Several studies have focused on the atherogenic effects of LDL modified with different types of secreted phospholipase A_2 ($sPLA_2$) (Divchev & Schieffer, 2008). Some $sPLA_2$ have been detected in atherosclerotic lesions and their products, lysophosphatidylcholine (LPC) and NEFA, induce cytotoxicity at relatively high concentrations by disrupting membrane integrity (Dersch, 2005; Naito, 1994). Moreover, both molecules have inflammatory potential by stimulating the expression of cytokines and chemokines (Sonoki, 2003). In addition, extensively lipolyzed LDL with some (type X), but not all (type IIA or V), $sPLA_2$ are able to induce foam cell formation (Curfs, 2008). An interesting approach was performed by Sparrow and coworkers, which combined $sPLA_2$ and lipoxygenase to modify LDL (Sparrow, 1988). This combination of lipoperoxidation and phospholipolysis generated LDL particles with similar properties to those promoted by endothelial cell-induced oxidative modification. Another atherogenic characteristic of $sPLA_2$-modified LDL is its increased affinity for PG binding due to the exposition of an alternative binding site in apoB that specifically recognizes PG (Boren, 1998b). As occurs with SMase-modified LDL, this would lead to increased subendothelial retention.

PAF-acetylhydrolase (PAF-AH), another PLA_2 with relevance in the metabolism of LDL, merits special mention. This enzyme is transported in plasma bound to lipoproteins (approximately 70% in LDL and 25% in HDL) but, in contrast to $sPLA_2$, its substrate is not native phospholipids but fragmented phospholipids that have been generated by oxidation (Tjoelker & Stafforini, 2000). Thus, LPC content is high in oxLDL due to the action of PAF-AH. However, there is controversy regarding the pro- or anti-atherogenic role of PAF-AH (Tellis & Tselepis, 2009). On one hand, its function should be atheroprotective since it degrades highly-inflammatory fragmented phospholipids. On the other hand, however, the by-products formed are LPC and short-chain NEFA, which also have inflammatory potential, though to a lesser extent than oxidized phospholipids (MacPhee, 1999).

2.2.3 Sphingomyelinase-modified LDL (SMase-LDL)

The hyperexpression of acid and neutral SMases in atherosclerotic lesions has been known for several years (Tabas, 1999). SMase hydrolyzes sphingomyelin (SM) yielding phosphorylcholine and ceramide. Ceramide directly exerts several biological effects, but its main action is to elicit the production of bioactive sphingolipids, such as sphingosine-1-phosphate that plays a major role in apoptosis (van Blitterswijk, 2003). Another action of SMase is to promote extensive LDL aggregation (Oorni, 2000). Aggregation of LDL confers several atherogenic properties. First, aggregated LDL (agLDL) is able to induce foam cell formation, not through SR in this case but through the LDL-receptor related protein 1 (LRP1), a receptor of aggregated lipoproteins highly expressed in smooth muscle cells (Llorente-Cortes & Badimon, 2005). Second, agLDL binds with higher affinity to PG (Oorni, 1998). As discussed below, this binding promotes structural changes in apoB. The association of agLDL to PG precludes the exit of this lipoprotein from the subendothelial space, and consequently favours that agLDL could undergo further modifications.

2.2.4 Glycated LDL (gLDL)

Although non-enzymatic glycosylation of LDL occurs in all subjects, it has stronger consequences in people with diabetes mellitus. Glucose can interact with the free amino groups of lysines and arginines in apoB, forming a Schiff base that rearranges to yield an

Amadori product (Brownlee, 1992). This modification leads to a loss of electropositive charges in glycated LDL (glLDL), decreasing its affinity to the LDLr and consequently prolonging its mean lifetime in plasma (Witztum, 1982). The increase in lifetime can result in further modification of LDL forming advanced glycation end-products (AGE) and producing a form of LDL named AGE-LDL (Menzel, 1997). AGE-LDL can also be internalized by specific receptors (RAGE) (Bucala, 1996). AGE-LDL and glLDL increase chemotactic activity in monocytes, stimulate cell proliferation and enhance platelet aggregation, although the relative contribution that the coexistence of oxidation could have is not well established. The formation of AGE involves oxidative reactions and it has been demonstrated that lipoperoxidation and non-enzymatic glycosylation are mutually potentiated processes (Sobal, 2000). Thus, glycosylation of LDL is not only noxious per se but also because it promotes LDL oxidation.

2.2.5 Proteoglycan-bound LDL (PG-LDL)

PG are the main constituents of the arterial intima. It has been hypothesized that subendothelial retention of lipoproteins due to the binding of LDL to PG is the initial event in atherogenesis, even prior to endothelial dysfunction or inflammation (Williams & Tabas, 2005). The retention itself increases the time of LDL in the subendothelial space and, therefore, the possibility that further modifications occur. Furthermore, the binding LDL-PG also has a direct effect on LDL modification because this binding promotes changes in the structure of apoB that facilitate processes such as oxidation or lipolysis mediated by SMase or $sPLA_2$ (Hevonoja, 2000). On the other hand, LDL-PG complexes are taken up by cells through different types of SR, promoting foam cell formation.

3. Modified LDL in human plasma

For many years it was considered that LDL modification was a phenomenon occurring mainly in the intima layer of the arterial wall. Oxidative modification was the most studied process, and most researchers accepted that the abundance in plasma of soluble molecules with antioxidant capacity (albumin, uric acid, bilirubin, glutathione, ascorbic acid) would inhibit oxidation of lipoproteins. Moreover, the binding of oxLDL by SR expressed in circulating monocytes is known to promote a rapid clearance of extensively oxidized LDL. However, progress in enzyme immunoassay procedures has provided direct evidence of oxLDL in circulating plasma. Besides oxLDL, similar methods have been used to detect other forms of modified LDL in blood. These include glLDL, AGE-LDL, carbamylated LDL (ca-LDL), desialylated LDL and electronegative LDL (LDL(-)).

3.1 Oxidized LDL in plasma

Despite the abundance of antioxidant defences in blood, increased oxidative stress has been described in plasma from patients with atherosclerosis. Early studies reported that increased levels of oxidized lipids in plasma were associated with atherosclerosis development (Avogaro, 1986). In agreement with that, the existence of oxLDL in blood is increased in subjects with high cardiovascular risk (Holvoet, 1999; Holvoet, 1998) or in diseases, such as diabetes, obesity, metabolic syndrome and hyperlipemia (Ishigaki, 2009). Although it cannot be totally ruled out that a part of oxLDL could be formed in blood, it is generally accepted that oxLDL originates primarily in the arterial wall and that the molecules reach the blood from the subendothelial space. For this reason, oxLDL is considered not only a biomarker of

atherosclerosis development but also a reflection of the presence of unstable and ruptured atherosclerotic plaques (Fraley & Tsimikas, 2006). The fact that oxLDL increases temporarily during the acute phase of myocardial infarction or stroke supports this notion (Fraley & Tsimikas, 2006; Nishi, 2002; Uno, 2003) and some studies have raised the possibility that plasma oxLDL could predict future cardiovascular events (Meisinger, 2005). The concentration of oxLDL in plasma is very low, and data reported by several authors are disperse, ranging from 0.01% to 0.5% of total LDL. The heterogeneous nature of oxLDL is one reason to explain variation in the reports of oxLDL concentration, but another could be the use of several antibodies that recognize different epitopes (Ishigaki, 2009). The fact that in vitro oxidation does not reproduce the same epitopes generated during in vivo modification makes it difficult to develop a golden standard.

Several studies have shown that besides its utility as a biomarker, oxLDL in plasma acts as a pathogenic factor. It has been reported that oxLDL contributes to increase the systemic inflammatory status by stimulating the activity of the transcription factor NF-kB in peripheral blood mononuclear cells (Cominacini, 2005). More direct demonstration of the implication of oxLDL in blood has been obtained by increasing the expression of SR, such as SRA1 (Whitman, 2002), LOX-1 (Ishigaki, 2008), or the chimerical fusion protein SRA1-growth hormone (Laukkanen, 2000), which favours oxLDL removal from blood. These studies showed an inhibition of atherosclerosis development. It was recently reported that repeated administration of the chimerical fusion protein Fc-CD68 decreases the extent of atherosclerosis in hyperlipemic mice (Zeibig, 2011). However, these studies have been tested to date in animal models only. Regarding humans, several trials have reported that lipid-lowering therapy in atherosclerotic patients lowers oxLDL, although this decrease is parallel to that of LDL cholesterol (Ky, 2008). However, no trial with a therapy specifically focused on oxLDL is yet available. The development of novel therapies for lowering oxLDL itself is a promising strategy for atherosclerosis treatment.

3.2 Glycated LDL in plasma

The concentration of glLDL in plasma from patients with diabetes is increased, reflecting the hyperglycemia in these patients. This concentration varies depending on the method used for its quantification (ELISA, affinity chromatography), but it is generally higher than that of oxLDL (Cohen, 1993; Reaven, 1995). The proportion of glLDL in diabetics can reach up to 7-8% of total LDL and approximately half in normoglycemic subjects. It is important to note that glLDL is present in all individuals, and even in normoglycemic subjects the concentration in plasma is higher than that of oxLDL. This suggests glLDL plays a role in the development of atherosclerosis even in absence of hyperglycemia. Interestingly, glLDL is more abundant in the subfractions of LDL that are smallest and have the highest density, probably because these small-dense LDL particles are prone to non-enzymatic glycosylation (Younis, 2009). These particles are also more susceptible to oxidation and have a relatively low affinity to the LDL receptor. These properties are related to the strong association between small-dense LDL and high cardiovascular risk (Krauss, 1995).

Alternative pathways can also result in LDL glycosylation without a direct involvement of glucose. Metabolites of glucose such as glyoxal, methylglyoxal or glycaldehyde have a higher reducing capacity as glycating agents (Rabbani & Thornalley, 2011). Minimal modification by methylglyoxal renders LDL particles with atherogenic properties, including binding to PG and susceptibility to aggregation (Rabbani, 2011). LDL particles with this low level of modification are increased in diabetic patients (Rabbani, 2010).

A more advanced form of glLDL is AGE-LDL, in which AGE are formed due to auto-oxidation of Amadori adducts yielding a number of products, such as carboxymethyl lysine or pentosyl lysine (Brownlee, 2000). AGE-LDL has atherogenic characteristics that are similar to oxLDL, probably because their oxidized lipid content is similar. Although it was generally considered that AGE-LDL was generated in the arterial wall this modified form of LDL has also been detected in blood (Lopes-Virella & Virella, 2010). AGE-LDL, like other AGE-containing proteins, is recognized in circulation by RAGE. RAGE activation stimulates cytokyne and growth factors release (Ramasamy, 2009). An excess of stimulation (i.e. an excess of AGE-LDL) plays an essential role in atherogenic alterations.

3.3 Carbamylated LDL (ca-LDL)

Carbamylation of proteins is a post-translational modification in which amine-containing residues react with cyanate, a compound that derives from urea or from thiocyanate. This modification is relatively frequent in patients with chronic uremia or in heavy smokers. Both situations are closely related to increased cardiovascular risk and carbamylated LDL (ca-LDL) is increased in the plasma in both groups of subjects (Basnakian, 2010). Ca-LDL is recognized by SR, promotes monocyte adhesion to endothelium, stimulates cell proliferation and causes cell injury (Apostolov, 2007; Carracedo, 2011).

3.4 Desialylated LDL (ds-LDL)

Native LDL has high content of sialic acid in the carbohydrate chains attached to apoB. Tertov and colleagues isolated a fraction of desialylated LDL from plasma (Tertov, 1990). This fraction, which was increased in patients with advanced atherosclerosis, induced foam cell formation in cultured smooth muscle cells and presented inflammatory properties (Orekhov, 1991). They suggested that low sialic acid in LDL was a cardiovascular risk factor (Ruelland, 1993) but this idea was not supported by other authors (Cerne, 2002). Later studies revealed that desialylated LDL was oxidized and that the loss of sialic acid was a consequence of oxidative modification (Tertov, 1995).

4. Electronegative LDL, a pool of modified LDL in blood

A common characteristic of most of the previously described modifications is an increase of the negative electric charge. Taking advantage of this property, Avogaro and co-workers fractionated total LDL from human plasma by anion-exchange chromatography, into two subfractions, a major subfraction of native LDL and an electronegatively-charged fraction of LDL (LDL(-)) (Avogaro, 1988). In this first report, LDL(-) accounted for 5-20% of total LDL in normolipemic subjects and presented a number of atherogenic characteristics, including impaired binding to LDLr, high aggregation level, capacity to induce cholesterol accumulation in macrophages and higher conjugated diene (a by-product of lipid peroxidation) content. Since then, a number of studies have tried to elucidate the physico-chemical and biological characteristics of LDL(-) and its relationship with atherosclerosis.

4.1 Origin of LDL(-) – Discrepancies regarding the oxidative origin

Early studies focused on the physico-chemical characteristics LDL(-) concluded, in accordance with the high content of oxidized lipids, that LDL(-) was the in vivo counterpart of in vitro oxidized LDL (Cazzolato, 1991). However, as isolation procedures improved and measures to prevent modification increased, the proportion of LDL(-) and

the content in lipoperoxides decreased (De Castellarnau, 2000; Demuth, 1996; Sevanian, 1997). Discrepancies regarding the increased content in oxidized lipids in LDL(-), continue; some authors do not find differences compared to native LDL (Benitez, 2007b; Demuth, 1996; Sanchez-Quesada, 2003), and others report increased amounts of oxidized lipids in LDL(-) (Asatryan, 2003; Sevanian, 1997; Ziouzenkova, 2002). But in any case, this level of lipoperoxidation is closer to minimally oxidized LDL than to extensively oxidized LDL suggesting that alternative mechanisms should be involved in LDL(-) formation (Fig. 1).

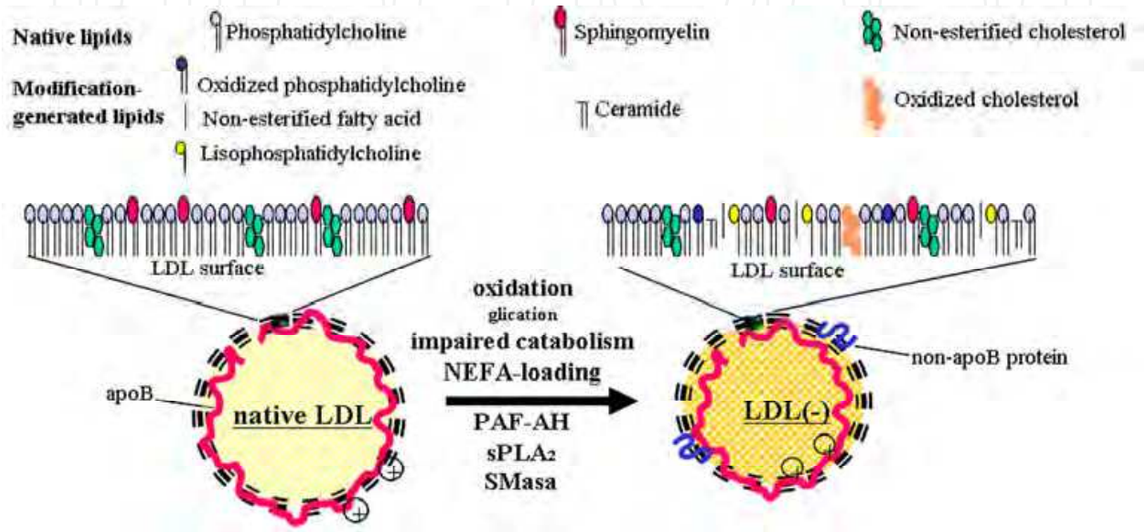

Fig. 1. Formation of LDL(-). The modification of native LDL by one or several mechanisms alters the composition of LDL surface. These alterations include the increase of inflammatory, proliferative and apoptotic lipids and non-apoB proteins in LDL(-), as well as structural abnormalities in apoB.

4.1.1 Impaired catabolism of LDL

LDL is a heterogeneous mixture of particles that differ in size (24-28 nm of diameter) and density (1.019-1.063 g/ml). Small-dense particles have fewer lipid molecules while large-buoyant particles have more. It is known that LDL particles at both extremes of the density range have increased electronegative charge compared with mid-density particles (Lund-Katz, 1998). It has been proposed that the origin of LDL(-) could be related with the impairment of the catabolic cascade that transforms VLDL-to-IDL-to-LDL in blood (Sanchez-Quesada, 2004). Such impairment leads to the formation of small-dense or large-buoyant LDL particles. In agreement with this, LDL(-) is most abundant (>80% of LDL(-)) in small-dense particles in normolipemic subjects. Interestingly, in hypercholesterolemic and hypertriglyceridemic patients, LDL(-) are also abundant large-buoyant particles (Sanchez-Quesada, 2002). Another consequence of impaired LDL catabolism is the presence of non-apoB proteins in LDL. Theoretically, the protein moiety of LDL consists of a single copy of a very large protein, apoB. However, it is known that some particles of LDL also contain other proteins. In native LDL, the content of non-apoB proteins is less than 1%. In contrast, LDL(-) contains up to 5% of non-apoB proteins (Bancells, 2010a; Yang, 2003).

4.1.2 Lipolysis

Some characteristics of LDL(-), such as high LPC and NEFA content, suggest that a possible mechanism of formation could be mediated by phospholipases. It has been described that in vitro modification of LDL with $sPLA_2$ renders modified particles that mimics some properties of LDL(-) (Asatryan, 2005; Benitez, 2004a; Benitez, 2004b). On the other hand, PAF-AH, which has a 5-10-fold higher activity in LDL(-) than in native LDL, could also play a role in increasing the content of LPC and NEFA and in the generation of LDL(-) (Gaubatz, 2007; Sanchez-Quesada, 2005). Some type of SMase activity could also be involved in LDL(-) generation since a minor subfraction of LDL(-) is aggregated (Bancells, 2010b). It has been reported that LDL(-) has an intrinsic phospholipase C-like (PLC-like) activity that degrades with high affinity both SM and LPC (Bancells, 2008). The origin of such activity is currently unknown, but it could be due to conformational changes in apoB. One or a combination of these phospholipolityc activities could be involved in the formation of LDL(-).

4.1.3 Content of non-esterified fatty acids (NEFA)

NEFA are transported in blood mainly associated to albumin. However, in some situations that increase NEFA or decrease albumin there is a partition of NEFA towards other proteins, including lipoproteins. In posprandial lipemia or when high energy is required (such as during heavy exercise) the increase of NEFA in blood increases LDL(-) proportion (Benitez, 2002). NEFA content in LDL(-) is three to four-fold higher than in native LDL (Benitez, 2004a; De Castellarnau, 2000; Demuth, 1996). In this context, it has been reported that the main determinant of the electronegativity of LDL(-) is NEFA (Gaubatz, 2007).

4.1.4 Non-enzymatic glycosylation

It would seem reasonable to consider that glLDL contributes in part to the pool of LDL(-), especially in diabetic patients. However, most glycated LDL particles isolable by affinity chromatography do not have a sufficient negative charge to be isolated with LDL(-) and the content of glLDL in LDL(-) is similar to that in native LDL (Benitez, 2007b; Sanchez-Quesada, 2005).

4.1.5 Hemoglobin derivatization and carbamylation

Patients with severe renal failure have a high proportion of LDL(-) (Asatryan, 2003). It was reported that LDL from these patients suffered a cross-linking with hemoglobin, rendering a particle with increased negative charge (Ziouzenkova, 2002). Recent studies on carbamylated LDL, however, suggest that carbamylation could underlie the high proportion of LDL(-) in patients with severe renal disease (Apostolov, 2010).

4.2 Biological properties of LDL(-)

Whatever the mechanism involved in its formation LDL(-), has several potentially atherogenic properties. These include abnormal binding to receptors, inflammatory and cytotoxic properties, high susceptibility to aggregation and increased affinity to PG (Fig. 2).

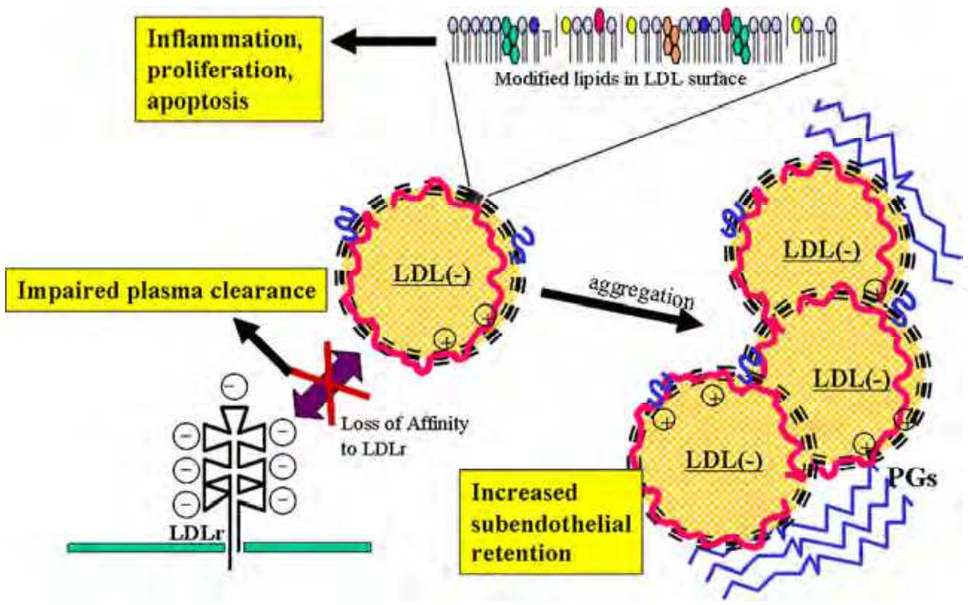

Fig. 2. Atherogenic properties of LDL(-). Modified lipids in LDL(-) surface have inflammatory, proliferative and apoptotic capacity. Aggregation, which is induced by the formation of ceramide, favours the binding to PGs and increases subendothelial retention. The alteration of apoB conformation in LDL(-) induces a partial loss of affinity to the LDLr and impairs its plasma clearance.

4.2.1 Binding to receptors

LDL(-) presents a partial loss of affinity to the LDLr, a property that could lengthen its half-life in blood (Benitez, 2004b). It was initially believed that this was due to the derivatization of lysines in apoB involved in receptor recognition, in a mechanism similar to oxLDL (MDA-Lys) or glLDL (glucose-Lys). However, recent studies have shown that lysines in LDL(-) are not derivatized but have an altered ionization state due to differences in the conformation of apoB (Blanco, 2010). Regarding SR, LDL(-) binds differently to distinct types of SR. The increment of electronegativity in LDL(-) is not sufficient to allow its binding to SRA in macrophages (Benitez, 2004b). However, it has been reported that LDL(-) binds to another SR, LOX-1, in endothelial cells (Lu, 2009). This binding does not promote foam cell formation, but could mediate the signaling of endothelial apoptosis.

4.2.2 Inflammatory activity

LDL(-) has the ability to activate the transcription factors NF-kB, AP-1 and PPAR, inducing the expression of a number of inflammatory molecules in endothelial cells (Abe, 2007; Benitez, 2006; De Castellarnau, 2000; Ziouzenkova, 2003). These molecules include cytokines (IL6) chemokines (IL8, MCP-1, GRO), vascular adhesion molecules (VCAM) and growth factors (GM-CSF, PDGF). Interestingly, LDL(-) also induces the paradoxical expression of the anti-inflammatory cytokine IL10 in lymphocytes and monocytes (Benitez, 2007a). It has been suggested that IL10 production could be a mechanism to control an excessive inflammatory response limiting the extent of injury. LDL(-) could also play a role in angiogenesis modulation since it stimulates vascular endothelial growth factor (VEGF) expression and inhibits the release of the matrix metalloproteinases MMP2 and MMP9 (Lu,

2008; Tai, 2006). The specific molecules that mediate these actions are not well defined; LPC and NEFA probably play a major role, but the action of oxidized lipids remains under discussion (Abe, 2007; Benitez, 2004a; Chen, 2004). If they are present in LDL(-) they could stimulate inflammatory responses, but it is also possible that high PAF-AH activity in LDL (-) degrades readily oxidized phospholipids (Benitez, 2003). Their degradation products, LPC and short-chain NEFA, would therefore be responsible for triggering inflammation.

4.2.3 Cytotoxicity and apoptosis

LDL(-) induces citotoxicity and apoptosis in endothelial cells and macrophages by different signaling pathways. The mechanisms involved are well defined, especially in endothelial cells, where LOX-1 signaling inhibits fibroblast growth factor 2 (FGF2) transcription and Akt phosphorilation (Chen, 2003; Chen, 2007; Lu, 2008; Tang, 2008; Yang, 2007). In contrast, macrophage apoptosis involves the Fas/FasL signaling pathway and the activation of the transcription factor Nrf2 (Pedrosa, 2010).

4.2.4 Binding to proteoglycans

LDL(-) and PG present a high affinity for binding (Bancells, 2009). This could favor the subendothelial retention of LDL(-) and trigger the inflammatory response. Some characteristics in LDL(-) are involved in this higher affinity. Aggregation of lipoproteins favors PG binding, and LDL(-) has a high tendency to aggregate (Bancells, 2010b). In fact, a subfraction of aggregated LDL(-) is responsible for the binding to PG (Bancells, 2009). This subfraction has an abnormal conformation that exposes an epitope in apoB, known as site Ib, that is an alternative binding site to PGs (Bancells, 2011).

4.3 Physico-chemical characteristics of LDL(-)

4.3.1 Structure

The earliest physical abnormalities reported in LDL(-) were a great heterogeneity in size and density and a high susceptibility to aggregation (Avogaro, 1988). A subpopulation of aggregated LDL(-) has recently been isolated and characterized. This subpopulation, which accounts for only 0.1-0.5 of total LDL in blood, has high affinity to arterial PG (Bancells, 2009). This increased binding seems to be mediated by abnormal conformation of the amino-terminal extreme of apoB (Bancells, 2011). Further evidence of apoB misfolding, in this case affecting LDLr binding, has been obtained by two dimensional nuclear magnetic resonance analyses (Blanco, 2010). LDL(-) is reported to promote aggregation of non-aggregated LDL particles in a process that fits an amyloidogenic model (Parasassi, 2008). It has been reported that the capacity to induce aggregation could be mediated by the PLC-like activity (Bancells, 2010b), although other authors have suggested that plasma $sPLA_2$ could be involved in apoB misfolding (Greco, 2009). Regarding secondary structure of apoB, some authors have reported loss of secondary α-helix structures whereas others did not find differences with native LDL (Asatryan, 2005; Bancells, 2009; Benitez, 2004b; Parasassi, 2001).

4.3.2 Lipids

Although there are contradictory data regarding differences in the lipid content between native LDL and LDL(-) most studies concur in a higher content of triglycerides, NEFA and LPC in LDL(-) (Cazzolato, 1991; De Castellarnau, 2000; Sanchez-Quesada, 2003; Sevanian, 1997; Yang, 2003). The high content of triglycerides reflects impairment of the VLDL-to-IDL-

to-LDL cascade. High NEFA could be a consequence of the increase in NEFA concentration in plasma (posprandial lipemia, intense exercise) (Benitez, 2002; Ursini, 1998), or it could come from lipolysis mediated by phospholipases (Benitez, 2004a). This latter possibility would be the same mechanism of increased LPC content in LDL(-).

4.3.3 Proteins

The most abundant proteins in LDL(-) are apoA-I (0.15 molecules/particle of LDL(-)), apoE (0.22), apoC-III (0.37) and apoA-II (0.14), with a content 3-5 fold higher in LDL(-) than in native LDL (Bancells, 2010a). The role of these proteins is unclear but their relevance is probably low. However, other proteins whose absolute content in LDL(-) is lower, such as apoF (0.06 molecules/particle), apoJ (0.01) or PAF-AH (0.004) (Bancells, 2010a; Yang, 2007), but their relative content compared with native LDL is 10, 20 and 100-fold higher, respectively, could have a much more relevant role. ApoF is the physiological inhibitor of cholesteryl ester transfer protein (Morton, 2008), a protein that regulates the catabolism of the VLDL-IDL-LDL cascade. It could therefore be one cause of impaired LDL(-) maturation. ApoJ (also known as clusterin) is an extracellular chaperone that binds to hydrophobic unfolded proteins, favoring their extracellular clearance (Oda, 1995). ApoJ binds mainly to aggregated LDL(-), supporting the presence of misfolded apoB in this subfraction. The role of PAF-AH in LDL(-) would be to deactivate oxidized phospholipids, but its undesirable effect would be the formation of LPC and short-chain NEFA. It has been suggested that the PLC-like activity present in LDL(-) could act in cooperation with PAF-AH degrading LPC (Bancells, 2010b). Therefore, this would be a mechanism to limit the deleterious effects exerted by minimal LDL oxidation on vascular cells.

4.4 Association of LDL(-) with cardiovascular risk

The proportion of LDL(-) is increased in a number of pathologic situations having a cardiovascular risk. Familial hypercholesterolemia and hypertriglyceridemia present a proportion of LDL(-) 3-5 fold higher than normolipemic healthy subjects (Sanchez-Quesada, 2002; Sanchez-Quesada, 1999). These results were obtained using ultracentrifugation plus anion-exchange chromatography, both being laborious and time-consuming techniques. This limits their use for routine analysis of lipoprotein profiles. However, more reliable techniques for rapid analysis have recently been developed, including capillary electrophoresis and ELISA (Santo Faulin Tdo, 2008; Zhang, 2009; Zhang, 2008). These methods have confirmed previous data obtained by anion-exchange chromatography. Statin therapy decreases the proportion of LDL(-) but the process is not parallel to the lipid-lowering effect. This is because total LDL cholesterol decreases very rapidly (in 2 weeks) whereas LDL(-) decreases more slowly (in up to six months) (Sanchez-Quesada, 1999). This suggests that LDL(-) generation not only depends on lipid metabolism but also on other factors such as chronic inflammation.

Both type 1 and type 2 diabetics have a high proportion of LDL(-) (Moro, 1998; Sanchez-Quesada, 1996; Sanchez-Quesada, 2001; Zhang, 2005). This would suggest that non-enzymatic glycosylation could be involved. However, insulin therapy decreases LDL(-) in type 1 diabetes but not in type 2 diabetes. The different response to insulin treatment has been attributed to differences in the systemic inflammation level, which is higher in type 2 patients. This agrees with the finding that pre-diabetic insulin-resistant subjects with high systemic inflammation also have increased LDL(-) proportion (Zhang, 2005). Therefore,

hyperglycemia would not promote the increase of LDL(-) directly but through an increase of systemic inflammation. Another group of subjects with a high proportion of LDL(-) are patients with severe renal disease (Ziouzenkova & Sevanian, 2000). It has been described that α-tocopherol supplementation decreases LDL(-) in hemodialysis patients (Mafra, 2009). Regarding patients with established coronary disease, it has been shown that LDL(-) is increased in patients with angiographically documented coronary artery disease (Tomasik, 2003). Moreover, acute coronary syndromes, such as unstable angina or acute myocardial infarction, have higher levels of LDL(-) than chronic coronary syndromes (Mello, 2011). These observations support the partial subendothelial origin of LDL(-) and open the possibility for LDL(-) to be used as a biomarker of the progression of atherosclerotic lesions.

5. Conclusion

LDL is modified by several mechanisms that confer a number of atherogenic properties to these particles. Although it is believed that such modifications are more frequent in the subendothelial space of the artery wall than in blood, different types of modified LDL have been detected in plasma. LDL(-) is a mixture of modified LDL particles that represent the total pool of modified LDL in plasma. The biological and physico-chemical characteristics of LDL(-) and its association with high cardiovascular risk indicate that this lipoprotein plays a direct role in the development of atherosclerosis. However, although statin and insulin treatment decrease the proportion of LDL(-) , the development of a specific therapy for LDL(-) would be of great interest. Another field of research would be the use of LDL(-) as a biomarker. This could be a promising strategy to evaluate the cardiovascular risk and to monitor the success of distinct therapeutic strategies.

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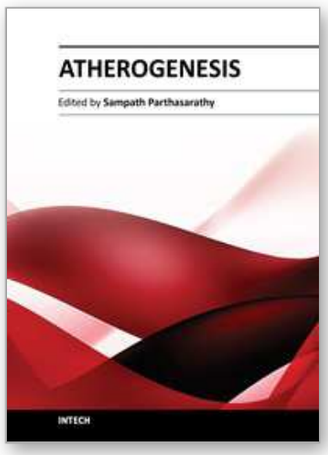

Atherogenesis

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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