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Extracellular matrix contributes to macrophage foam cell formation and atherosclerosis

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Abstract

During early atherogenesis, low density lipoprotein (LDL) induces the accumulation of cholesterol and oxysterols in arterial macrophages, and in the subendothelial accumulation of atherogenic lipoproteins in extracellular matrix (ECM), takes place.

Retention of LDL and oxidized LDL (Ox-LDL) to ECM is mediated by the ECM proteoglycans (PGs), which in the presence of Lipoprotein Lipase (LPL, acting as a bridging element), bind the lipoproteins. The structures of the ECM proteoglycans, as well as the lipoproteins binding sites for PGs determine the extent of the interaction between ECM PGs and lipoproteins. Following its retention to the ECM, Ox-LDL is taken up by activated macrophages at enhanced rate, leading to cellular accumulation of cholesterol and oxysterols in arterial wall macrophages. Moreover, LDL oxidation in the arterial wall can also take place after lipoprotein retention to ECM PGs. In this case, retained Ox-LDL can be taken up by macrophage after its release from ECM PGs; but also as a complex of Ox-LDL with ECM PGs. The amount and composition of ECM, produced by all major cells of the arterial wall (monocyte-derived macrophages, endothelial cells and smooth muscle cells), determine the extent of lipoproteins cellular uptake. We have demonstrated that under oxidative stress, ECM PGs secretion from macrophages, binding of Ox-LDL to the ECM and uptake of the retained lipoproteins by macrophages are all significantly increased. Altogether, these processes contribute to macrophage foam cell formation and accelerated atherosclerosis.

Key words: extracellular matrix, retention, oxidation, lipoprotein, LDL, oxidized LDL, macrophage, cholesterol, atherosclerosis

Mechanisms involved in early atherosclerosis

The hypotheses implicated for the mechanisms involved in early atherogenesis include endothelial injury, lipoprotein modifications (oxidation, aggregation) and LDL retention [1–4]. However, there is a consensus regarding the fact that whatever process triggers the initiation of atherogenesis, it always leads to macrophage cholesterol accumulation and foam cells formation, the hallmark of early atherogenesis [1, 5].

Foam cells formation results from complex interactions among cells of the arterial wall (endothelial cells, smooth muscle cells — SMCs and monocyte-derived macrophages), arterial lipoproteins (native-LDL, oxidized-LDL and aggregated LDL) and extracellular matrix (ECM) (Fig. 1).

Early atherosclerotic plaque formation begins with the attachment of blood monocytes to the luminal surface of the endothelium, followed by their subsequent migration into the subendothelial space [6, 7]. In the

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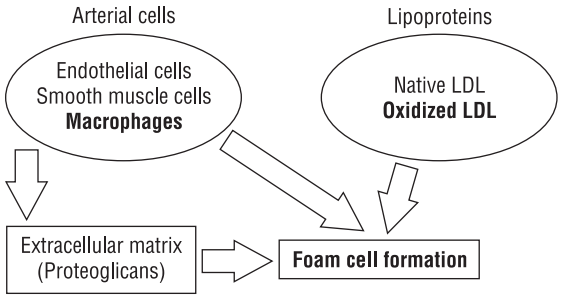

Figure 1. Extracellular matrix and foam cell formation; Foam cells formation results from the interactions among cells of the arterial wall (endothelial cells, SMCs and monocyte--derived macrophages), lipoproteins (LDL, oxidized LDL and aggregated LDL) and the extracellular matrix

arterial wall, monocytes differentiate into macrophages which, under certain conditions such as oxidative stress [8, 9], accumulate massive amounts of lipoprotein-derived cholesterol and oxysterols and are converted into foam cells [2, 10, 11]. Oxidized LDL (Ox-LDL) was shown to be a major contributor to the development of atherosclerosis, as it enhances cellular cholesterol accumulation, as well as inflammatory and thrombotic processes [2, 5, 12, 13]. Extracellular matrix is composed of molecules secreted by cells of the arterial wall and it was shown to participate in foam cell formation, mainly by mediating the retention of atherogenic lipoproteins [4].

Structure and functions of the extracellular matrix

Extracellular matrix of the atherosclerotic lesion consists of collagen (Type I, III, IV, V and VI), elastic fibers (elastin and fibrillin), glycoproteins (fibronectin, fibrin, thrombospondin, tenascin, osteospontin) and proteoglycans (versican, decorin, biglycan, perlecan and hyaluronan) [13, 14].

The extracellular matrix participates in several key events during the development of the atherosclerotic lesion which include the following:

1. **Cell adhesion:** Fibronectin, thrombospondin and osteopontin are glycoproteins which are elevated in ECM from atherosclerotic lesions. These glycoproteins possess cell binding domains that serve as attachment sites for vascular cells [14].

2. **Thrombosis and coagulation:** Exposure of the extracellular matrix during plaque rupture or endothelial injury results in platelet adhesion and activation and stimulates thrombosis. Fibronectin and thrombospondin regulate platelet adhesion and aggregation during the generation of a thrombus. Moreover, platelets tend to adhere preferentially to regions of the plaque that are enriched with type I and type III collagen. Whereas the fibrous components of the ECM are prothrombotic, specific glycosaminoglycans of vascular proteoglycans are considered anti-thrombotic [15].

3. **Calcification:** Osteopontin is a calcium binding protein found in atherosclerotic lesions and is mainly secreted by macrophages [16].

4. **Plaque rupture:** Plaque fissuring and subsequent thrombosis often occurs at boundaries between collagen-rich and collagen-poor zones, such as the base of fibrous caps or near lipid-rich areas, especially in areas containing interstitial collagens degrading metalloproteinases [17]. Metalloproteinase are synthesiszed by macrophages, smooth muscle cells (SMC) and mast cells, and are elevated in aneurysmal aortic segments. They are localized to the regions of the plaque that are most prone to rupture such as the plaque shoulder, lipid core or both [18].

5. **Cell migration and proliferation:** Glycoproteins from the ECM which are synthesized by SMC interact with specific cell membrane receptors. Such interactions regulate the proliferative and migratory behavior of SMC during colonization of the thrombus or during the development of the vascular lesion [13]. Moreover, ECM also participates in restenosis and increase the expression of neointimal ECM components (type III collagen, byglican, hyaluronan and versican) [19].

6. **LDL retention:** Arterial ECM contribute to the trapping of LDL in the arterial wall, a phenomenon called "lipoprotein retention" [4, 13]. Extracellular matrix proteoglycans were shown to be responsible for the entrapment of LDL and modified forms of LDL in the arterial wall [20, 21]. Lipoprotein retention mediated by proteoglycans is a major process in arterial ECM-induced foam cell formation. The nature and the structure of the proteoglycans present in ECM, as well as the binding sites for ECM proteoglycans in the lipoprotein molecule determine the level and type of interaction between the ECM proteoglycans and the lipoproteins.

Extracellular matrix proteoglycans synthesis

Proteoglycans (PGs), also known as mucopolysaccharides, represent a class of protein-carbohydrate compounds. They are characterized by glycosaminoglycans (GAGs) that are covalently linked to a peptide chain (the core protein). Proteoglycans from the extracellular matrix include chondroitin sulfate (CS) linked to a core protein versican, dermatan sulfate (DS) with a core protein decorin or byglican, and heparan sulfate (HS) linked to perlecan core protein [13]. Hyaluronan, which is a glycosaminoglycan (not PG) is also found in the ECM.

There are both spatial and temporal changes in each of the ECM components during the evolution of the atherosclerotic plaque. The distribution of proteoglycans in the arterial intima varies with the anatomical location, during intimal thickening and lesion development [13, 14]. The proteoglycan layer of the intima becomes more prominent with physiological intima thickening at branching points and during early atherogenesis. These changes are controlled by a coordinate regulation of the synthesis and turnover of each ECM component by different vascular cells [22]. The development of ECM during atherogenesis is affected by pro-atherogenic stimuli. Predisposing stimuli such as oxidative stress or shear stress can stimulate synthesis of lipoproteins retentive molecules such as PGs. These stimuli can also increase the levels of lipoprotein lipase (LPL), a process which can change the ECM composition [23].

The regional differences in the distribution of proteoglycans during the development of atherosclerotic plaque, suggest different roles for each of the proteoglycans in the atherogenic process. Chondroitin sulfate and hyaluronan are both prominent in the intima of early developing atherosclerotic lesions, whereas dermatan sulfate with byglican and decorin are found in the fibrous cap of atherosclerotic regions [24].

The normal intima, and regions prone to lesion development are initially rich in large versican-like PGs. These are the most abundant PGs of the intimal extracellular space and contain chondroitin sulfate as GAGs [25]. The content of chondroitin sulfate proteoglycans (CSPGs) increases with lesion progression and eventually decreases in advanced lesions. The structure of CSPGs changes with lesion development in humans, and CS-rich PGs were also shown to be increased with lesions development in rabbit atheroma. Versican-like PGs, because of their abundance and high affinity apolipoprotein B-100 containing lipoproteins, are most likely the main structure of the intima which interacts with lipoprotein particles that reach this space [26]. In rabbit models of atherosclerosis, the apo B-100 in the intima is colocalized with CS-rich acellular regions [27]. However, other PGs that are less abundant than versican can also form complexes with LDL. Decorin, a small PG, which contains a single dermatan sulfate chain that is interconnected and organizes collagen I and III fibers in the intima, is a prominent component of the fibrous cap. Decorin has been shown to bind LDL as well as Ox-LDL in the presence of LPL. Byglican is also a small PG with two dermatan sulfate GAGs present in lesions, and appears to bind LDL [28]. In the abluminal side the large HSPG type perlecan forms part of the basement membrane that binds the cells to the proteoglycan layer of the intima.

Extracellular matrix can be produced in vitro by arterial cells, including endothelial cells [29] and smooth muscle cells [30]. This extracellular matrix is similar in organization and chemical composition to naturally occurring basal lamina [31]. We have recently demonstrated that macrophages can also deposit an extracellular matrix [32]. Macrophages were previously shown to be able to secrete several components of extracellular matrix, such as collagen, laminin, fibronectin and proteoglycans [33, 34]. Moreover, since macrophages are involved in the formation of the early atherosclerotic lesion [2, 6, 7], the secretion of a macrophage derived ECM could occur already at early stage of atherogenesis, and therefore could be crucial for the lipoprotein retention process.

Retention of native LDL by extracellular matrix

Extracellular matrix proteoglycans are responsible for the entrapment of LDL in the arterial wall [4, 20, 23]. This interaction can be a direct one between glycosaminoglycans and apolipoprotein B-100 or it may involve a bridging molecule such as lipoprotein lipase (LPL) [31, 35, 36]. Once LDL is trapped in the arterial wall, which is a relatively antioxidant-poor environment, it is susceptible to oxidation [37] as well as to aggregation [38, 39]. Oxidation of retained LDL is thought to result in its separation from the ECM, followed by its cellular uptake by arterial macrophages, leading to foam cell formation [37, 40, 41].

Both heparan sulfate and dermatan sulfate (specifically decorin), were shown to participate in the retention of LDL in the subendothelial space [41].

Proteoglycans-lipoproteins complexes that were isolated from fatty streaks or from fibrous plaques of human aorta, induced cholesteryl ester accumulation in macrophages [42]. In addition, complexes of isolated arterial PGs together with lipoproteins are taken up and degraded by macrophages at enhanced rates in comparison to non-bound lipoproteins, and as a consequence, the cells accumulate large amounts of cholesteryl esters [43].

The interaction of LDL with arterial CSPGs was found to increase the susceptibility of LDL to copper-ion-induced oxidation, as well as to cell-mediated LDL oxidation. This was demonstrated by lipid peroxides formation, as well as by the increased anodic electrophoretic mobility [37, 44].

Metabolic studies indicate that cells bind and degrade LDL-PGs complexes by a saturable process with a moderate affinity, and this uptake was shown to be mediated by specific cell surface binding sites [40, 45]. It was suggested that macrophages metabolize LDL-PGs com-

plexes predominantly via a receptor-mediated pathway that was characterized to be a scavenger receptor [46]. However, LDL modified by its association with isolated artery wall PGs was shown to be metabolized via the LDL receptor [40]. The apparent discrepancies between these two studies can be explained by the different nature of the complexes used in these studies. Hurt et al. [40] used complexes with a low ratio of PGs to LDL and the nature of the interaction between the lipoprotein and PGs was reversible, compared to the studies conducted by Viyagopal et al. [46].

Up to eight specific regions in apo B-100 can bind glycosaminoglycans [47]. Weisgraber & Rall identified two fragments, residues 3134–3209 and 3356–3367, that bind to heparin with very high affinity [48]. Camejo et al. confirmed these findings and proposed that residues 3147––3157 (site A) and residues 3359–3367 (site B) may act cooperatively in their association with proteoglycans [49].

By analyzing the proteoglycan-binding activity of recombinant human LDL isolated from transgenic mice, the site B (3359–3369) was shown to be the primary PG-binding site for LDL [50]. Substitution of neutral amino acids for basic amino acids residues in site B (3359–3369) abolished both the receptor-binding and the proteoglycan-binding activities of the recombinant LDL. Chemical modification of the remaining basic residues caused only a marginal further reduction in proteoglycan-binding site of LDL. Although site B was essential for normal receptor binding and proteoglycan binding activities, these two activities could be separated in recombinant LDL with a K3363E mutation. In this latter LDL, a glutamic acid was inserted into the site B basic cluster RKR, and this recombinant LDL had normal receptor-binding but impaired proteoglycans-binding. In contrast, another mutant R3500Q displayed defective receptor binding but interacted normally with PGs. Thus, the PG binding and the receptor binding properties of LDL can be separated by introducing a single point mutation into the apo B-100.

To investigate the atherogenic potential of the direct interaction between apo B-100 and arterial proteoglycans, transgenic mice that express a PG-binding defective LDL were produced. These mice were characterized by a striking reduction in their atherosclerotic lesion, in comparison to mice expressing wild type LDL [51].

Retention of oxidized LDL by extracellular matrix

There has been some controversy regarding the effect of LDL oxidation on its binding to ECM-PGs [52]. Some studies showed that oxidation of LDL particles decrease their ability to bind human aortic PGs [53, 54], whereas in other studies LDL oxidation was shown to enhance its association with HSPGs anchored to endothelial cells matrix [36, 55, 56]. Oxidation of LDL particles was shown to decrease their ability to bind to human aortic proteoglycans [54]. Using several methods to oxidize LDL, the degree of the binding of the particles to proteoglycans decreased as the net negative charge of the lipoprotein particles increased. The increase in the net negative charge of Ox-LDL was accompanied with a loss of free lysine residues [54]. Oxidation also decreased LDL association with the subendothelial extracellular matrix. It was thus suggested that oxidative stress in the vessel wall results in an increased dissociation of the ECM-bound lipoprotein, which in turn makes this oxidized LDL more accessible for binding and uptake by macrophages, leading to cholesterol accumulation and foam cell formation [52].

However, in another study, oxidation of LDL greatly enhances its association with lipoprotein lipase anchored to endothelial cell matrix [55]. Ox-LDL was shown to bind to ECM-anchored LPL with a 4-fold greater affinity than native LDL, and this interaction was dependent on the extent of LDL oxidation [55]. The increased affinity was caused exclusively by modification of the apo B-100 and not the lipid moiety of the lipoprotein, and the apo B-100 arginine residues were shown to play an important role in Ox-LDL retention.

Mild oxidation of lipoproteins was shown to increase their affinity for surfaces covered by heparan sulfate and LPL. Lipoprotein lipase exhibits high affinity for mildly oxidized LDL [56] and it increased the binding and the uptake of mildly oxidized LDL, compared to non-oxidized LDL, by THP-1 monocyte-derived macrophages. Recently, we have shown that macrophage-derived ECM exhibited an increased binding capacity to oxidized LDL compared to native LDL, and this binding was mediated by the ECM heparan sulfate, as well as chondroitin sulfate proteoglycans [36]. It is thus possible that LDL oxidation can also occur under certain conditions prior to the retention of the native lipoprotein. Mild oxidation of LDL increased its binding to chondroitin sulfate, whereas heavy oxidation reduced LDL binding to CS to levels lower than that of native LDL binding to CS. Similar results were previously reported, showing that mild oxidation of LDL or of VLDL increased their ability to bind to heparan sulfate [56]. Chondroitin sulfate proteoglycans (CSPGs) secreted by human monocytes-derived macrophages were shown to bind to mildly oxidized LDL but not to extensively oxidized LDL [57], illustrating that the level of lipoprotein oxidation, as well as the type of LDL oxidation could be of major importance in determining PGs interaction with Ox-LDL.

Role of lipoprotein lipase in lipoproteins retention

Lipoprotein lipase (LPL) certainly plays a central role in the retention of lipoproteins in the arterial wall and the molecule does not need to be catalytically active [4, 23, 35]. We have previously shown [36] that in the absence of LPL, Ox-LDL, as well as native LDL, were able to bind to the ECM layer only to a limited extent. Preincubation of ECM layer with LPL however, led to a substantial increase in the binding of Ox-LDL, as well as that of native LDL to the matrix. Macrophages isolated from human atherosclerotic aorta were shown to express LPL mRNA and to secrete LPL protein [58]. In addition, a recent study showed that LPL was required for Ox-LDL binding to proteoglycans (versican and biglycan) from smooth muscle cells derived ECM [59].

Lipoprotein lipase is secreted by macrophages as a homodimeric form but it rapidly dissociates into inactive monomers, which can be found in the atherosclerotic lesion. It has recently been shown that native LDL binds only to monomeric LPL whereas Ox-LDL, irrespectivly of the type of modification (metal ions, free radical, oxygenases), binds preferably to the dimeric form of LPL [60]. The ratio between dimeric and monomeric LPL in the arterial wall could thus affect the relative retention of Ox-LDL and native LDL to ECM.

Cellular uptake and macrophage foam cell formation by ECM-retained lipoproteins

Studies of macrophage lipoproteins uptake are usually performed by cell incubation with the lipoprotein in an appropriate medium [61–63]. However, in the arterial wall the lipoprotein may not be in a soluble form, but rather attached to the ECM.

A recent study analyzed the events occurring during the interaction of macrophages and matrix-retained aggregated LDL [64]. Using a culture cell model, the matrix-retained lipoprotein was taken up by macrophages, and this model could mimic the *in vivo* interaction of arterial wall macrophages with subendothelial lipoproteins.

We have shown that Ox-LDL that was retained by extracellular matrix can be taken up by activated macrophages (Fig. 2). Oxidized LDL is transferred from the ECM compartment together with its bound PGs to the cellular compartment. Cellular uptake of the ECM-retained Ox--LDL by macrophages can then lead to cholesterol accumulation and foam cell formation, the hallmark of early atherogenesis [65]. We thus proposed an alternative approach to study macrophage uptake of oxidized LDL, different from the classic studies that analyze the uptake of free, non-bound lipoproteins, by the cells.

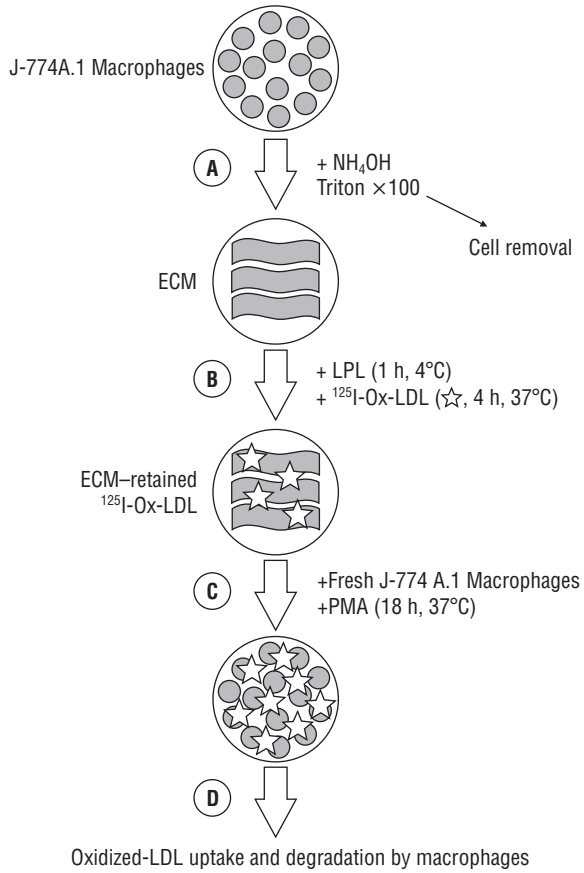

Figure 2. Extracellular matrix-retained Ox-LDL taken up by macrophages is associated with glycosaminoglycans: Methodology approach; **A**) J-774 A.1 macrophages (5×10^5) were plated for 5 d in 35 mm wells in medium supplemented with 5% FCS. Then, by addition of Triton × 100 and ammonium hydroxyde the cell layer was removed leaving the extracellular matrix layer; **B**) After extensive washing with PBS the macrophage derived ECM layer was incubated with 8 μg/ml of lipoprotein lipase in medium with 3% BSA, for 1 h at 4°C. After washes with PBS to remove unbound LPL, ^{125}I-Ox-LDL (or ^{125}I-LDL) were added to the ECM layer for 4 h at 37°C; **C**) Then after washing the ECM layer, fresh macrophages were added to the ECM layer for 18 h at 37°C, in the presence of 100 mM of PMA; **D**) Following the incubation, supernatant medium was removed and analyzed for the determination of Ox-LDL cellular degradation

Activation of macrophages with PMA is required however, for the cellular uptake of ECM-retained Ox--LDL. PMA-induced macrophage activation can possibly increase the synthesis of high affinity receptors for Ox-LDL. Macrophage activation through protein kinase C (PKC) signal transduction pathway can also induce the secretion of hydrolytic enzymes, growth factors and free radicals [66]. These secreted substances can then lead to the release of Ox-LDL-PGs complexes from the matrix, and its subsequent uptake by the cells.

The cellular uptake of ECM-retained Ox-LDL is thus accompanied by the uptake of ECM-GAGs [65]. Since

lipoprotein retention was previously shown to be mediated by binding to ECM proteoglycans [20, 21, 23], it seems that the proteoglycan GAGs are detached from the core protein and are then taken up by the cells bound to Ox-LDL. In arterial lesions of atherosclerotic animals, the presence of PGs-lipoproteins complexes was shown [27, 67]. These complexes were previously shown to be taken up at enhanced rate by macrophages and to cause cellular cholesterol accumulation and foam cell formation [37, 40, 41].

It can therefore be postulated that oxidation of LDL may take place in the arterial wall even with no preceding retention of native LDL to ECM. Since LDL oxidation was shown to result in increased association of the lipoprotein with extracellular matrix [36, 55, 56], LDL oxidation can possibly trigger the trapping of Ox-LDL in the arterial wall. Thus, oxidation of LDL can occur also prior to its retention, unlike the dogma of LDL oxidation only after its retention to ECM (Fig. 3).

Conclusions

The extracellular matrix plays a central role in the formation of macrophage foam cells during early atherogenesis. Lipoproteins retention, which is mediated by ECM proteoglycans (PGs) is probably a major process in foam cell formation. The nature and the structure of ECM proteoglycans, as well as the lipoproteins binding sites for PGs determine the extent of the interaction between ECM PGs and lipoproteins. Retention of lipoproteins to ECM proteoglycans was shown for both native LDL an oxidized LDL (Ox-LDL). This latter ECM-retained Ox--LDL is taken up by activated macrophages at enhanced rate, leading to cellular accumulation of cholesterol and oxysterols in arterial wall macrophages, a key process in early atherosclerosis. The content and composition of ECM produced by all major cells of the arterial wall (monocyte-derived macrophages, endothelial cells and smooth muscle cells) determine the extent of lipoproteins cellular uptake. Under oxidative stress, ECM secretion, binding of oxidized LDL to ECM and arterial macrophage uptake of the retained lipoproteins are all significantly increased, contributing to macrophage foam cell formation and to accelerated atherosclerosis.

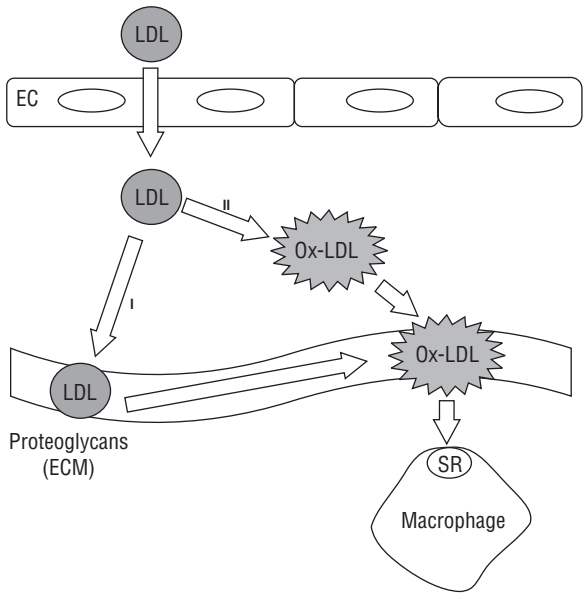

Figure 3. Oxidation of retained and non-retained LDL and macrophage foam cell formation; I) Following the entry of LDL into the arterial wall, proteoglycans ECM can bind the lipoprotein leading to LDL retention followed by its oxidation and cellular uptake by macrophages; II) Oxidation of LDL (Ox-LDL) however, can also occur prior to its retention, and the ECM-retained Ox-LDL can then be taken up by macrophages via their scavenger receptors (SR)

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