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Enzymatically modified LDL, atherosclerosis and beyond: paving the way to acceptance

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1. ABSTRACT

The eLDL (enzymatically modified LDL) hypothesis proposes that modification of LDL during atherogenesis occurs through the action of ubiquitous hydrolytic enzymes. eLDL is recognized by multiple macrophage receptors. Following cellular uptake, eLDL triggers atherosclerotic lesion initiation with reversion or progression depending on the balance between cholesterol insudation and depletion. The effects of eLDL on cellular constituents of the atherosclerotic lesion comprise both pro- and anti-inflammatory mechanisms. eLDL triggered complement activation is centrally involved in atherosclerosis with the first CRP (C-reactive protein)-dependent activation step to prevail at the early stages of atherogenesis (lesion initiation with reversion), and the second situation to gain dominance as local concentrations of eLDL surpass a critical threshold (lesion initiation with progression). Thus, CRP-mediated lipoprotein removal likely underlies the regression of early lesions, which occurs continuously through life. Perhaps CRP should be considered as an antiatherogenic agent and the question whether it is an innocent bystander or proatherogenic culprit is not really to the point. The observed association between CRP and atherosclerosis might simply be reverse causation: atherosclerotic disease progression induces CRP.

2. LDL (LOW DENSITY LIPOPROTEIN) INSUDATION AND INFLAMMATION – TRIGGERS OF THE ATHEROSCLEROTIC PROCESS

It is widely accepted that the initiation and progression of atherosclerosis from the normal intima to thrombotic occlusion displays all the characteristic features of a chronic inflammatory disease (1). However, a matter of lively debate is the question as to what the causative agent is. Lowering of cholesterol is still the most successful approach to prevent and treat atherosclerosis in humans (2). Is lowering of cholesterol equivalent to removal of the causative agent or, in other words, what link exists between LDL and inflammation?

Atherosclerosis research typically focuses on the evolution of intermediate or advanced atherosclerotic lesions rather than on prelesional stages of atherogenesis. Yet these early events may provide decisive evidence on the triggers of the pathologic process. It is known that plasma proteins seep continuously into the arterial intima even in the absence of overt endothelial injury (3), and spontaneous insudation of LDL was demonstrated some 35 years ago (4, 5). The latter led to the “response to retention” hypothesis, which states that atherosclerosis develops in response to LDL entrapment (6). The fundamental importance of LDL entrapment for initiating lesion formation rather than any

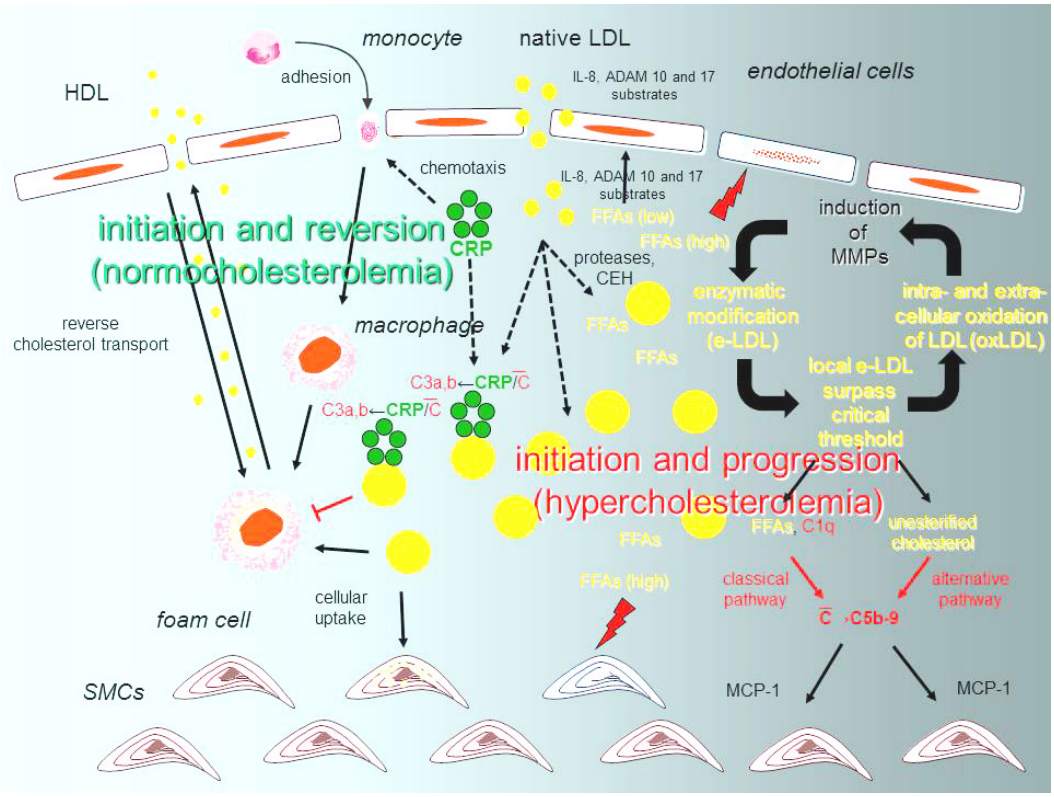

Figure 1. Proposed model of initiation and progression of atherosclerosis with special emphasis on the role of CRP and the complement system. Under normal circumstances (initiation and reversion, normocholesterolemia, left), native LDL entrapped within the arterial intima is enzymatically modified (eLDL), leading to a sequence of events that serve to clear the vessel wall of cholesterol. Binding of CRP to eLDL is the first trigger for complement activation (C), but in this early stage the terminal sequence is spared. The physiological sequence of events is concluded by reverse cholesterol transport. If the capacity of the system is overburdened (initiation and progression, hypercholesterolemia, right), this leads to accumulation of eLDL with subsequent generation of potentially harmful C5b-9 complexes by both the classical and alternative pathway as well as accumulation and oxidation of extracellular LDL particles followed by a wealth of well-documented events like MMP production in surrounding cells and subsequent amplification of enzymatic degradation of LDL. FFAs play multifaceted roles through their dual capacity to exert stimulatory and cytotoxic effects on neighboring cells (modified from 8, 55).

modification of the lipoprotein occurring prior to insudation is highlighted by the demonstration that transgenic mice expressing modified apolipoprotein B that binds poorly to proteoglycans show reduced atheroma development (7).

Nevertheless, native LDL lacks inflammatory properties, and it follows that the lipoprotein must undergo biochemical alterations to become atherogenic. What happens to tissue-stranded LDL? What kind of modification takes place? What drives development of the fatty streak and what tips the balance within early lesions toward development into advanced lesions? The early atherosclerotic lesion is reversible and essentially harmless. What tips the scale towards irreversible, advanced lesions, i. e. the point of no return? In pursuit of an answer, it is important to distinguish between initiation and progression of atherosclerotic lesion development. Central players in advanced stages of atherosclerosis need not to be identical with those responsible for development of early lesions, and *vice versa* (8).

There are numerous reviews on the possible impact of modified lipoproteins on atherosclerosis, indicating that a consensus has not yet been reached and that basic questions still remain unanswered. Among several other concepts, the eLDL (enzymatically modified LDL) hypothesis proposes that modification of LDL occurs through the action of ubiquitous hydrolytic enzymes. In the following, the role of eLDL during both initiation and progression of atherosclerosis is summarized and opposed to the more widespread oxidation hypothesis (Figure 1).

3. MAINSTREAM CONCEPTS ON ATHEROGENESIS

A fundamental question relates to the fate of tissue-stranded native LDL and the nature of the modification that bestows atherogenic properties onto the lipoprotein. The common answer is that oxidative modifications render the lipoprotein atherogenic. Arguments in support of this contention have been summarized by Steinberg (9): 1) oxLDL (oxidized LDL) has

been isolated from atherosclerotic lesions; 2) epitopes reactive with antibodies against oxLDL have been detected in lesions; 3) autoantibodies reactive with oxLDL have been found in patients and experimental animals and the respective antibody titers are reportedly of diagnostic and prognostic value; 4) antioxidants can slow the progression of atherosclerosis in experimental animals.

But is this evidence conclusive? In particular, does oxidation explain the development of initial atherosclerotic lesions? Napoli *et al.* analysed fatty streaks in human fetuses (10). Epitopes of oxLDL were found, but these were localized mainly within macrophages. Calara *et al.* reported that a single injection of heterologous native LDL resulted in its accumulation in the arterial wall where it became oxidatively modified within hours (11). However, the lipoprotein was mainly assoa ciated with SMCs (smooth muscle cells). The question of localization, however, is of paramount importance: the primary modification of native LDL should be extracellular, since according to the oxidation hypothesis itself, this modification is required for the lipoprotein to be taken up by macrophages.

Another shortcoming of the oxidation hypothesis is that it does not explain the fact that extracellular cholesterol in early lesions is mainly unesterified and can form crystals (12-15). In marked contrast, the bulk of cholesterol contained in native LDL is esterified with fatty acids. Oxidative modifications do not lead to extensive deesterification of the cholesteryl esters and thus are not in line with a major biochemical finding in early lesions. Finally, the results of human clinical trials with antioxidants were mainly negative, except in selected groups of patients with clearly increased systemic oxidative stress (16).

4. A DIFFERENT CONCEPT ON ATHEROGENESIS – THE ELDL HYPOTHESIS

In the following, I will review the evidence in support of a different concept on atherogenesis (17) distinguishing between atherosclerotic lesion initiation with reversion or lesion initiation with progression. Thereby, emphasis will be placed on new evidence that has emerged in the past 15 years concerning animal models (18-25), *in vivo* evidence (26) as well as proinflammatory effects of eLDL (27-30).

The eLDL hypothesis takes heed of the above mentioned shortcomings of the oxidation hypothesis and proposes that modification of native LDL occurs through the action of ubiquitous hydrolytic enzymes rather than oxidation. As already mentioned above, elegant earlier studies demonstrated that extracellular cholesterol in early lesions is mainly unesterified thus proving hydrolytic enzymatic activity (12-15). The enh zymes, proteases and cholesterol esterase may partly represent lysosomal leakage products of neighbouring cells and incoming macrophages. Indeed, cathepsin D (31) and H (32), plasmin, MMP-9 (matrix metalloprot teinase-9) (33) and cholesterol esterase (34) are pres) ent in atherosclerotic lesions. Moreover, LDL isolated from early lesions has the same biochemical, ultrastructural and immunological properties as eLDL that can be generated *in vitro* by combined treatment of native LDL with an arbitrary protease and cholesterol esterase (33, 35, 36). Finally, in contrast to oxLDL, specific monoclonal antibodies allowed demonstration of extensive extracellular deposits of eLDL in the early lesion. Thereby, the neoepitopes recognized by the two monoclonal antibodies (mAbs) originally used for detection of eLDL appear to become exposed at different stages of enzymatic lipoprotein degradation: mAb AIL-2 recognizes LDL after proteolytic nicking alone, whereas mAb AIL-3 reacts only after combined treatment with a protease and cholesterol esterase. However, mAb AIL-3 stains lesion lipoproteins as strongly as mAb AIL-2 in histological sections indicative of deesterification by enzymatic modification of LDL *in vivo* (37). Such demonstration of extracellular localizal tion is mandatory to any claim that a lipoprotein modification is responsible for inducing foam cell formation.

5. CELLULAR UPTAKE OF ELDL

eLDL is recognized by multiple macrophage receptors and is indeed the most potent naturally occurring foam cell inducer known to date (35, 38, 39). eLDL is taken up much more efficiently than oxLDL by macrophages *in vitro* (35), whereby the receptors involved are multiple (35, 38, 39). Cellular uptake of eLDL by human monocyte-derived macrophages leads to the formation of lipid droplets and preferentially induces cholesterol/sphingomyelin rich membrane microdomains while oxLDL promotes the development of cholesterol/ceramide rich microdomains via activation of the salvage pathway (40). As for smooth muscle cells (SMCs), it was recently demonstrated that eLDL is also highly potent in inducing foam cell formation in both human (41) and murine SMCs. Thereby, eLDL endocytosis is mediated by calcium-dependent macropinocytosis. Interestingly, priming SMCs with eLDL enhances the uptake of oxidized LDL (42). Opsonization has also an impact on cellular uptake of eLDL. For example, it was demonstrated that β-amyloid binding to eLDL enhances cellular cholesterol accumulation as well as β-amyloid deposition in vessel wall macrophages (43).

Following cellular uptake of eLDL, the eLDL hypothesis predicts the following scenario of atherosclerotic lesion initiation with reversion or progression: under normocholesterolemic conditions, the cholesterol removal system comprising cellular uptake and the HDL (high density lipoprotein)-dependent reverse transport pathway is sufficient to deal with

spontaneous insudation of native LDL into the vessel wall with subsequent enzymatic modification. The concept implies that the physiological events leading to macrophage and SMC uptake and reverse transport of eLDL first occur without inflammation (initiation with reversion). In this regard, it departs from all other current concepts of atherogenesis. However, under hypercholesterolemic conditions, when the cholesterol removal system is overburdened due to continuous and excessive tissue-stranding of LDL cholesterol, detrimental effects ensue due to the unhalted activation of innate immune effectors, in particular complement and different subsets of monocyte-derived macrophages (initiation with progression) (17).

6. CELLULAR EFFECTS OF ELDL

The effects of eLDL on cellular constituents of the atherosclerotic lesion, i. e. endothelial cells, monocytes/macrophages, SMCs and T cells, are multifaceted and comprise both pro- and anti-inflammatory mechanisms.

In endothelial cells, eLDL stimulated upregulation of ICAM-1 (intercellular adhesion molecule-1), PECAM-1 (platelet-endothelial cell adhesion molecule-1), P-selectin, and E-selectin with distinct kinetics. Analyses with blocking antibodies indicated that ICAM-1 and P-selectin together mediated approximately 70% of cell adhesion, whereas blocking of PECAM-1 had no effect on adhesion but reduced transmigration to less than 50% of controls. eLDL is thus able to promote the selective adhesion of monocytes and T lymphocytes to the endothelium, stimulate transmigration of these cells, and foster their retention in the vessel wall by increasing their adherence to SMCs (44). Likewise, production of IL-8 (interleukin-8) and simultaneous modulation of NF-kappaB in response to eLDL might also serve to protect the vessel wall and promote silent removal of the insudated lipoprotein (45).

In monocytes/macrophages, eLDL induces upregulation of CLA1/SRB1 (38), the ABCA1 transporter (46), matrix metalloproteinases (47) and cathepsin H (32). Again, an amplifying loop may thus be generated to accelerate local cholesterol removal. Furthermore, eLDL generated foam cells are protected from cell death most likely through the expression of TOSO (named after a Japanese liquor drunk on New Year´s day to celebrate long life and eternal youth) by a FLIP (FLICE-inhibitory protein) independent mechanism (48).

In primary vascular SMCs, eLDL mediated rapid cholesterol loading and foam cell transformation, which was paralleled by a marked dose- and time-dependent expression of PTX3 (pentraxin 3) mRNA and release of the acute-phase protein (49).

Regarding the above mentioned effects of eLDL on cellular constituents of the atherosclerotic lesion, the question is whether inhibition of proinflammatory signaling pathways provides a promising therapeutic tool to prevent inflammatory cascades in atherosclerosis. Using the human leukemia cell line THP-1 and/or primary monocyte-derived macrophages, skepinone-L, the first ATP-competitive p38α MAPK (mitogen-activated protein kinase)/MAPK14 inhibitor with excellent *in vivo* efficacy and selectivity (50), inhibited eLDL induced activation of the p38 MAPK pathway, inhibited eLDL induced expression of both CD36 and ABCA1 without a net effect on foam cell formation, had a cell- and time-dependent effect on eLDL triggered apoptosis and inhibited eLDL stimulated secretion of IL-8 (interleukin-8) and MIP-1β/CCL4 (macrophage inflammatory protein-1β/chemokine, CC motif, ligand 4) (51).

7. COMPLEMENT ACTIVATION

In 1977, it has been demonstrated that free cholesterol activates complement (52) and since then, several reviews summarized the evidence for an important impact of complement activation on atherogenesis (53-56). Thereby, an unexpectedly large number of pathways are operative that may differentially influence the evolution of the atherosclerotic lesion. The terminal sequence with C5b-9 formation is proposed to represent a decisive detrimental factor. The presence of C5b-9 complement complexes was demonstrated in advanced human atherosclerotic lesions by immunohistochemistry in 1985 (57) and quantified by ELISA in 1987 (58). The link between tissue-deposited eLDL and complement activation was elucidated through the isolation of assembled C5b-9 complexes from early adult human atherosclerotic lesions along with a lipoprotein derivative that had complement-activating properties (36). These findings were corroborated and completed by the subsequent *in vitro* generation of this lipoprotein derivative which turned out to be eLDL with potent complement-activating capacity (35) and the immunohistochemical detection of eLDL and its colocalization with the terminal complement complex in the early human atherosclerotic lesion (37). Demonstration of C5b-9 in the early lesion and its colocalization with SMCs (59) and complement-induced release of MCP-1 (monocyte chemotactic protein-1) from human SMCs (60) provided further indications for a possible role of complement activation in atherogenesis. That the terminal complement sequence is indeed centrally involved in atherosclerotic lesion progression was then directly evidenced by the demonstration that complement C6 deficiency protected against diet-induced atherosclerosis in rabbits (61). Likewise, it has been demonstrated that CD59, a key regulator of C5b-9 assembly, offered protection against atherosclerosis

in the context of Apo E deficiency (62, 63). The role of CD55/DAF (decay-accelerating factor), a membrane inhibitor of the C3 convertase, is less clear: while one study reported that CD55 deficiency had no effect on atherogenesis (62) another found that CD55 deficiency protected against atherosclerosis in ApoE-deficient mice apparently via modulation of lipid metabolism (64). Furthermore, in atherosclerotic lesions, apolipoprotein J (clusterin) may subserve protective functions through its capacity to inactivate C5b-9 complement complexes and by reducing the cytotoxic effects of eLDL on cells that gain contact with the lipoprotein (30).

Three independent pathways have been identified via which eLDL triggers complement. The first is the binding of CRP which occurs at low eLDL concentrations and leads to efficient activation of the early complement sequence with cleavage of C3. Conspicuously, progression to the terminal sequence is halted in a manner similar to what has been shown for the CRP-dependent activation of complement on nucleated cells (65). By virtue of its capacity to bind factor H, CRP has been reported to be able to deter the complement sequence at the stage of C3b/C5 (66, 67). Early recruitment of complement by CRP could serve to effect timely removal of eLDL, thus preventing accumulation of the modified lipoprotein with its potentially dangerous cargo of free cholesterol. That the early complement sequence could serve a protective function in atherogenesis is suggested by a recent study in LDL receptor-deficient mice demonstrating that complement C1q deficiency appears to slightly promote lesion development (68). It must be noted, however, that conclusions drawn from mouse models can be no more than tentative because of the oddities of the mouse complement system (21). In fact, the mouse may not be an ideal model to investigate the role of complement in atherogenesis. The same holds true for investigating the role of CRP in atherosclerotic lesion development in the mouse (see below).

With increasing concentration, eLDL then attains an additional dual capacity to activate complement. Firstly, triggering occurs via the alternative pathway, possibly through the presence of large amounts of unesterified cholesterol. There is also immunohistochemical evidence that C5b-9 in atherosclerotic lesions is formed via the alternative pathway (69). Secondly, it has been demonstrated that eLDL is recognized by C1q and activates the classical complement pathway. Thereby, C1q binding to eLDL particles is mediated by the C1q globular domain, which senses unesterified fatty acids generated by cholesterol esterase (70-72). Together, these processes thus guarantee vigorous activation of the complement system as eLDL accumulates. *In vivo*, one might envisage the first CRP-dependent activation step to prevail at the early stages of atherogenesis (lesion initiation with reversion), and the second situation to gain dominance as local concentrations of eLDL surpass a critical threshold (lesion initiation with progression). CRP-dependent activation by eLDL essentially excludes the detrimental terminal C5b-9 sequence and likely subserves the primarily beneficial function of macrophage recruitment, which may occur in conjunction with IL-8, which is coinduced in endothelial cells by eLDL-derived free fatty acids (73). As the rabbit experiments indicate (61) it becomes clear why pathology would be driven particularly when the local eLDL burden exceeds critical limits.

8. *IN VIVO* EVIDENCE OF THE ELDL HYPOTHESIS

The eLDL hypothesis contends that tissue-stranded native LDL will only become atherogenic after enzymatic transformation to eLDL. The availability of a model to test this basic contention would clearly be highly desirable. Accumulation of native LDL with subsequent modification in the intima begins in childhood and adolescence in the majority of our population and, if unhalted, can lead to development of atherosclerotic lesions. Obviously, investigations on tissues from fetuses and infants might provide valuable clues to first events underlying the initiation of atherosclerosis. To date, a few studies have been undertaken along these lines. Napoli *et al.* described lesion formation in premature fetuses (10) and in children aged 1–13 years (74). These studies did not address eLDL formation, CRP deposition, or complement activation, important issues that were investigated in our own study (26), where the following observations on atherosclerotic lesion initiation and reversion were made: 1) lipoproteins accumulate in the intima before macrophages infiltrate in the early lesion, 2) there is virtually no extracellular lipoprotein modification, either enzymatic or oxidative, within intimal lesions in infancy (<1 year), 3) onset of extracellular enzymatic modification of LDL occurs in the age group between 6 and 15 years and 4) lipoprotein accumulation in the intima does not coincide with activation of the terminal complement cascade but largely coincides with deposition of CRP and C3d in the age group between 6 and 15 years.

In the first year of life, there is obviously a kind of 'inert' lipoprotein insudation into the intima without lipoprotein modification, monocyte/macrophage infiltration and/or inflammation, which explains why genuine atherosclerosis does not occur. This prelesional stage is characterized by the absence of eLDL and C5b-9. All available evidence indicates that native LDL differs from eLDL in lacking the capacity to activate complement (35, 75, 76) and this is tellingly reflected by the above findings.

9. ELDL AND CRP

Bridging the observations on intimal lesions in infancy to the above mentioned pathways of eLDL dependent complement activation, one might envisage the first CRP-dependent activation step to prevail in the lesions from individuals between 6 and 15 years of age (with deposition of eLDL, CRP and C3d but without C5b-9) and other pathways to gain dominance as modified lipoproteins accumulate in adult atherosclerotic lesions. CRP-mediated lipoprotein removal likely underlies the regression of early lesions, which we propose occurs continuously through life.

The interaction of CRP with LDL is considered to be another key property that links CRP with atherosclerosis. However, the data obtained to date are controversial and hence make it difficult to conclude an actual physiological or pathological impact of such interaction. The incompatible findings could be ascribed to the different structural state of CRP and/or LDL. For example, it has been reported that, once CRP is bound to certain ligands, the pentameric structure of CRP is altered so that it can dissociate into monomers (77). Accordingly, the monomeric CRP found in atherosclerotic lesions may be a by-product of a ligand-binding function of CRP. CRP has been shown to prevent the formation of eLDL-loaded macrophage foam cells (78). Thereby, phosphoethanolamine potentiates the binding of CRP to eLDL and, therefore, increases the efficiency of CRP to prevent transformation of macrophages into eLDL-loaded foam cells. Of course, the function of CRP to prevent formation of foam cells may influence the process of atherogenesis (79-81). With regard to oxLDL, it has been reported that CRP binds to oxLDL *in vitro* (82) and some data suggest that CRP may even bind to native LDL (82, 83). However, no data are available to show that any such binding is accompanied by complement activation and indeed, a number of investigations clearly indicate that this is not the case (35, 75, 76, 84).

The possible protective role of CRP does not conflict with the fact that CRP represents a powerful predictive factor in cardiovascular risk assessment. A host of epidemiological studies have demonstrated a significant association between elevated serum or plasma CRP concentrations and the prevalence of atherosclerotic vascular disease, the risk of recurrent cardiovascular events among those with established disease or the incidence of first cardiovascular events among those at risk (85). This strong base of epidemiological evidence has led to the hypothesis that CRP is both a marker of and a causal agent in the development of atherosclerosis. However, overburdening of the physiological eLDL removal machinery with atherosclerotic lesion progression is accompanied by interleukin-6 production (86) which could explain the slightly elevated CRP levels. This in turn would serve to augment eLDL removal (even though not sufficient to trigger lesion reversion). Perhaps the pieces of the puzzle concerning the role of CRP in atherosclerosis are trying to be fit into the wrong picture? Perhaps CRP should be considered as an antiatherogenic agent and the question whether it is an innocent bystander or proatherogenic culprit (87, 88) is not really to the point. The observed association between CRP and atherosclerosis might simply be a reverse causation: atherosclerotic disease progression induces CRP.

In past years, many studies attempted to demonstrate an atherogenic effect of CRP in genetically modified mice expressing either hCRP (human CRP) or rbCRP (rabbit CRP) with quite controversial and contradictory results (89): CRP was either proatherogenic (20, 90), had no effect on atherogenesis (18, 21, 23-25) or was even atheroprotective (19). As already mentioned above, the mouse obviously is not an appropriate model for evaluation of CRP and complement because CRP is not an acute phase protein in mice and levels are therefore extremely low compared with humans and rabbits (91). Furthermore, neither hCRP nor rbCRP can activate complement in the mouse (21). Therefore, the hCRP-transgenic rabbit model was selected for this purpose because it lacks the shortcomings of the mouse model concerning CRP and complement pathophysiological functions. Neither high nor low plasma concentrations of hCRP affected aortic or coronary atherosclerotic lesion formation in hCRP-transgenic rabbits (92).

10. INITIATION AND PROGRESSION OF ATHEROSCLEROSIS – ENZYMATIC OR OXIDATIVE MODIFICATION OF LDL?

Given the above-mentioned examples of the different impacts of eLDL and oxLDL on atherosclerotic lesion initiation and progression, we would like to propose the following model that integrates both types of lipoprotein modification (Figure 1) (8). Since LDL continuously becomes entrapped in the arterial intima, a mechanism should exist that removes the stranded lipoprotein. We hypothesize that under normal circumstances, the lipoprotein is indeed enzymatically degraded in the first place and epitopes are exposed to enable the lipoprotein to be recognized and taken up by macrophages. This would lead to a sequence of events that serve to clear the vessel wall of cholesterol and is concluded by the transfer of excess cholesterol from foam cells onto HDL for reverse cholesterol transport. If the amount of insudated LDL exceeds the recycling capacity of the normal intima, i. e. the capacity of the system is overburdened, this would lead to an imbalance between lipoprotein and cholesterol deposition and removal, with subsequent accumulation of extracellular LDL particles (17). If

these are oxidized in the course of their prolonged residence time in the intima, among a wealth of well-documented events, MMP production in surrounding cells would be induced by oxLDL (93) amplifiying enzymatic degradation of LDL and thus initiating a circle of events that accelerates LDL removal. Given the above-mentioned examples of the role of both types of lipoprotein modification in early and advanced atherosclerosis, we propose that eLDL might be more important for the initiation of atherosclerosis, while oxLDL might be more helpful for diagnosis and prognosis of the disease. In this context, oxidative modifications in the vessel wall are considered to occur primarily as a process secondary to inflammation (94). However, without question, further investigations and comparative studies on both eLDL and oxLDL are warranted to corroborate the concept presented here. In any case, different lipoprotein modifications such as enzymatic and oxidative changes do not really compete, but rather complement one another.

11. WIDENING THE IMPACT OF ELDL

Enzymatic modification of LDL drastically increases its cytotoxicity, which could be relevant for the progression of atherosclerotic lesions. This cytotoxicity arises from large amounts of unsaturated FFAs (free fatty acids) that are liberated from cholesterol esters in native LDL during enzymatic modification (27). Low concentrations of FFAs stimulate cytokine production (73) and represent critical regulators of ADAM (a disA integrin and metalloproteinase) function that may assume relevance in many biological settings through their influence on mobility of enzyme and substrate in lipid bilayers (29, 95, 96). High concentrations of FFAs render eLDL cytotoxic to SMCs, endothelial cells and PMNs (polymorphonuclear cells) (27, 28, 30). The potent cytotoxic effects on PMNs may be one reason why these cells are not abundantly present before the development of complicated human atherosclerotic lesions with plaque erosion and rupture (97). Furthermore, eLDL induces rapid foam cell formation in monocytes and upregulates adipophilin mRNA and protein within 2 h of incubation. *Vice versa*, adipophilin facilitates the uptake of FFAs and FFAs increase is related to the early upregulation of adipophilin expression in blood monocytes. FFAs are ligands for PPAR-γ (peroxisome proliferator-activated receptor-γ), and the upregulation of adipophilin mRNA by PPAR-γ agonists like 15d-PGJ2 (15-deoxy-Δ12, 14-prostaglandin J2) and ciglitazone indicates that PPAR-γ may mediate the induction of adipophilin expression in human blood monocytes (98). There is no doubt that FFAs derived from eLDL will be increasingly recognized to assume important roles in atherogenic processes in the future.

A pathogenetic impact of eLDL (and also oxLDL) is not restricted to atherosclerosis. Recently, we demonstrated that subendothelially deposited eLDL is enzymatically transformed into a complement activator at early stages of aortic valve sclerosis development and also taken up by myofibroblasts (99). Very recently, we demonstrated a strong presence of apolipoprotein (a), oxidized phospholipids (OxPL), malondialdehyde-lysine, autotaxin, and macrophages, particularly in advanced lesions rich in cholesterol crystals and calcification. We demonstrated the presence of a constellation of pathologically linked, Lp(a) (lipoprotein(a))-associated molecules in plasma and in aortic valve leaflets of patients with CAVS (calcific aortic valve stenosis) (100).

12. IMPLICATIONS OF THE ELDL HYPOTHESIS

In summary, atherosclerosis is proposed to centrally involve enzymatically degraded lipoproteins and innate immune effectors, so inhibition of these components, in particular with respect to lesion progression might counteract atherogenesis. Of course, it would be highly desirable to have an animal model where it is possible to selectively inhibit enzymatic degradation of eLDL. However, this approach is hindered and probably impossible because of the redundant and unspecific nature of proteolytic nicking required to convert native LDL to eLDL (33). Another approach would be to inhibit either cellular uptake of eLDL or signaling. Recently, we were able to demonstrate that inhibition of a key signaling molecule of the p38 MAPK pathway induced by eLDL cellular uptake, p38α MAPK/MAPK14, by skepinone-L, a novel selective p38α MAPK/MAPK14-inhibitor with multifaceted effects on foam cell formation, apoptosis, and cytokine induction facilitates elucidation of the impact of the complex network of p38 MAPK signaling on atherogenesis and might provide a promising therapeutic tool to prevent inflammatory cascades in atherosclerosis, not least because the *in vivo* potency of skepinone-L has been recently demonstrated (50).

With regard to innate immune effectors, it was demonstrated that complement C6-deficiency protects against diet-induced atherosclerosis (61) and, similar to CD59, apolipoprotein J (ApoJ) may subserve protective functions through its capacity to inactivate C5b-9 complement complexes and also by reducing the cytotoxic effects of eLDL on cells that gain contact with the lipoprotein (30).

Vice versa, any situation leading to overactivation of the immune system probably accelerates atherogenesis in a non-specific fashion. This was clearly demonstrated in experiments where rabbits on a hypercholesterolemic diet were repeatedly challenged with endotoxin (22, 101). These results appear to be reproducible in the mouse (102). We propose that atherosclerosis is a special type of immunopathological disease which evolves as a result of excessive lipoprotein insudation and modification

triggered by a plethora of well known factors including smoking, hypertension, chronic infections, diabetes mellitus, e.t.c.

In this respect I would like to finish with a personal opinion: atherosclerosis is generally considered to be a multifactorial disease, a concept that is not very satisfying, but is rather a platitude. Given the roles of eLDL and oxLDL, I propose that atherosclerosis should be considered as a multi-step rather than a multifactorial disease, with different players becoming important during different stages of the leading cause of mortality in affluent societies around the globe.

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