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Oxidized LDL and anti-oxidized LDL antibodies in atherosclerosis – Novel insights and future directions in diagnosis and therapy☆,☆☆

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ABSTRACT

We provide an up-to-date overview of current topics surrounding oxidized low-density lipoprotein (oxLDL) and its related antibodies in the quest to better identify the individuals at risk of cardiovascular disease and atherosclerotic plaques with unfavorable characteristics. We discuss the potential of oxLDL and anti-oxLDL antibodies as serum biomarkers of cardiovascular disease and emerging studies examining the targeting of arterial oxLDL for imaging and therapeutic delivery.

Introduction

With the persistence of cardiovascular disease (CVD) as one of the leading causes of death despite modern therapies [1], it is important to improve on methods of cardiovascular risk stratification as well as to explore opportunities provided by specific targets for diagnostic imaging and therapeutic delivery. With this in mind, the focus of this review will be on recent publications relating to oxidized low-density lipoprotein (LDL) (oxLDL) and antibodies against it. Our aim is to provide a contemporary assessment of the current state and future research avenues in the field.

Background

Atherosclerosis is the key pathophysiological process underpinning CVD. The process is initiated by trapping of LDL in the sub-endothelial space of large- and medium-sized arteries. Ensuing lipid peroxidation and lipoprotein digestion results in the release of reactive aldehydes and bioactive oxidized phospholipids that lead directly or indirectly to endothelial cell dysfunction, increased expression of vascular adhesion molecules and the migration of immune cells into the arterial intima.

OxLDL is a general term that covers heterogeneous oxidative changes to LDL lipid moieties and to Apolipoprotein B (ApoB), the principle protein of the LDL particle. These alterations include reorganization of the phospholipid shell with exposure of phosphorylcholine (PC) and adduction of aldehydes, such as malondialdehyde (MDA), on to ApoB. OxLDL and LDL-derived oxidized phospholipids can stimulate inflammatory activation of macrophages, vascular smooth muscle cells and other cells in the vicinity, acting as 'danger associated molecular patterns' (DAMPs), akin to the 'pathogen associated molecular patterns' (PAMPs) that are found on microbes. Moreover, they provide oxidation-specific epitopes (OSEs) that are recognized by C-reactive protein, complement system proteins and innate "natural" IgM antibodies. Other recognized OSEs include malondialdehyde-acetaldehyde (MAA)-LDL, formed from the condensation of multiple MDA molecules with lysine adducts on LDL, as well as copper-oxidized LDL (Cu-oxLDL), oxidized phosphatidylserine (oxPS) and PC.

The natural IgM antibodies tend to be cross-reactive, recognizing shared epitopes on nucleic acids, cytoskeletal proteins, dead cells and microparticles. Whilst initially homeostatic in facilitating safer clearance of oxLDL and other debris, overdrive of the humoral innate immune system can lead to the development of a mal-adaptive autoimmune response that includes both immunoglobulin class switching (e.g. to IgG), affinity maturation of antibodies and the development of a pathogenic T-lymphocyte response to oxLDL [2,3].

The atherosclerotic process can either be clinically silent, or result in plaque expansion and luminal constriction with ischemic symptoms such as angina. More dangerously, plaques may rupture, leading to a clinically manifest acute coronary syndrome or stroke, depending on its location. There are various factors in play that determine which of these outcomes occur, including the level of inflammation and connective tissue remodelling of the plaque itself, immune and inflammatory factors external to the plaque, as

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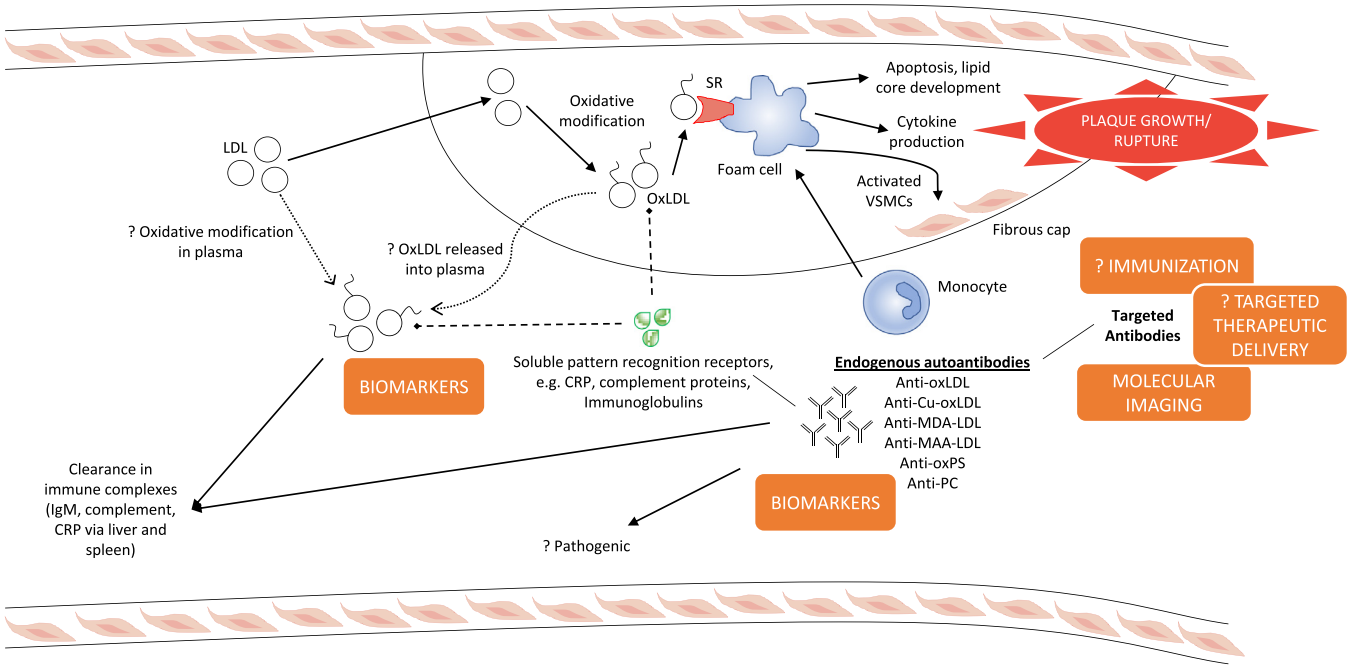

Fig. 1. Schematic summary of the role and potential clinical uses of oxLDL and anti-oxLDL antibodies in atherosclerosis. *LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; SR, scavenger receptor; CRP, C-reactive protein; VSMCs, vascular smooth muscle cells; IgM, immunoglobulin M; anti-Cu-oxLDL, anti-copper-oxLDL; anti-MDA-LDL, anti-malondialdehyde-LDL; anti-MAA-LDL, anti-malondialdehyde-acetaldehyde-LDL; anti-oxPS, anti-oxidised phosphatidylserine; anti-PC, anti-phosphorylcholine.*
LDL is trapped in the sub-endothelial space of arterial walls, where it undergoes oxidative modification to form oxLDL, a heterogenous group comprising various oxidation-specific epitopes (OSEs). These OSEs can be recognized by the humoral immune system, including CRP, complement system proteins and immunoglobulins. Monocytes that have migrated to the sub-endothelial space (driven by pro-inflammatory cytokines), undergo transformation to macrophages, which then uptake oxLDL via scavenger receptors, and become foam cells. Foam cells then perform pro-inflammatory functions, including further monocyte recruitment, cytokine release, activation of vascular smooth muscle cells as well as digestion other cellular debris, leading to apoptosis, development of the necrotic lipid core and resultant plaque growth. Atherosclerotic lesions can continue to grow, causing luminal stenosis and potential clinical angina, or rupture, leading to acute coronary syndromes or strokes.
OxLDL may be released from plaques, or perhaps LDL becomes oxidatively modified in the circulation, with potential pathogenic implications. OxLDL forms immune complexes and is likely to be removed from the circulation via the liver and spleen.
OxLDL, or endogenous antibodies against oxLDL, can be detected in plasma or serum and have been found to relate to CVD, functioning as biomarkers. Targeted antibodies to oxLDL have also been successfully utilized for the imaging of atherosclerotic lesions in pre-clinical studies. Moreover, there is the potential for therapeutic use of anti-oxLDL antibodies, through active or passive immunization as well as drug targeting of the atherosclerotic plaque.

well as alterations in mechanical forces acting on the plaque, such as endothelial shear stress.

Whilst it is now quite common to refer to “vulnerable patients” and “vulnerable plaques”, these terms remain vague and not well defined. Given their central roles in atherosclerosis pathophysiology, levels of oxLDL and anti-oxLDL antibodies make good candidates for biomarkers of disease burden and predictors of cardiovascular events and thus ‘patient vulnerability’; therefore, modifying the humoral or cellular immune response to oxLDL may well be beneficial. Moreover, regions of vascular wall with greater oxLDL burden may be at higher risk of future plaque rupture [4], potentially making oxLDL an important marker of the ‘vulnerable plaque’, as well as offering opportunities for diagnostic imaging and the targeted delivery of therapeutic agents. Fig. 1 summarizes the role of oxLDL in atherosclerosis biology, as well as potential clinical uses of oxLDL and anti-oxLDL antibodies.

OxLDL and anti-oxLDL as biomarkers of CVD

OxLDL as a biomarker of CVD

Despite the presence of numerous anti-oxidants in blood, it is clear that oxLDL can be identified not just within the atherosclerotic plaque, but also in plasma or serum. LDL has long been identified as a circulating biomarker reflecting general cardiovascular risk, with a reduction in serum levels used clinically as a treatment target. However, there is still scope for more specific biomarkers with pathological relevance to improve the clinical risk prediction of cardiovascular events [5]. Moreover, elevated oxLDL titres may prove to be more causally-associated with cardiovascular events than LDL, due to its central role in atherosclerotic plaque biology, and thus may have potential for improving risk prediction beyond LDL. To this end, elevated levels of circulating oxLDL have been demonstrated in large prospective cohorts, powered for clinical endpoints, to confer an adverse CVD prognosis. For example, in a large population-based survey of 40–79-year-old men and women, the Bruneck Study showed a significant association of OSE biomarkers (including oxidized phospholipids on ApoB-100) and the development of adverse CVD events over 15-year follow up [6]. A recent meta-analysis has supported these findings, reporting that elevated serum oxLDL is associated with an increased risk of CVD events [7]. Moving from the identification of the vulnerable patient to identifying the presence of unfavorable plaque characteristics, circulating MDA-LDL has been associated with the presence of thin-cap fibroatheromas (i.e. plaques viewed as high-risk for rupture), as determined by Optical Coherence Tomography (OCT) [8].

Whilst current investigations detecting oxLDL in the bloodstream have centered around the clinical correlates of elevated levels, establishing whether increased oxLDL levels are epiphenomenal as a result of oxidative stress, or causal in augmenting disease and provoking clinical events (or both), remains a challenge. This question is very relevant when considering potential therapeutic avenues. Another current limitation is that we do not know whether circulating oxLDL is oxidized in the blood, or is being transported from solid tissues. It is interesting that a recent study in patients with stable coronary artery disease (CAD) revealed a

peak rise of oxidized phospholipids immediately following percutaneous coronary intervention (PCI), with immunoglobulins (Ig)s to oxLDL initially falling, then increasing above baseline levels at least 1-week afterwards [9]. This either suggests that oxLDL is liberated into plasma from ruptured atheroma during PCI, or that circulating LDL has become oxidized during the procedure, with potential untoward distant effects. Extending this line of thought, high dose atorvastatin has been found to reduce total plasma oxidized phospholipids complexed with ApoB-100; whereas plasma levels of oxidized phospholipid actually increased when measured per ApoB-100 particle, suggesting enrichment of a smaller subset of ApoB-100 particles. The authors therefore hypothesized that statins may partly exert their beneficial cardiovascular protective effects through mobilization of dangerous pro-inflammatory oxidation species from atherosclerotic lesions [10]. However the effects of statins in this paradigm are still not clear, as high dose rosuvastatin significantly reduced levels of oxLDL (unadjusted for total LDL) versus low dose rosuvastatin in the recently reported Standard versus high-dose therApy with Rosuvastatin for lipiD lowering (SARD) randomized clinical trial [11].

Aside from coronary disease, serum oxLDL titres have been found to have clinical correlates in cerebrovascular and peripheral circulation. High levels of oxLDL have newly been found to indicate the development of a recurrent incident following an index cerebrovascular event [12], as well as to relate to poor functional outcome and mortality following stroke [13]. Elevated OSE biomarkers have also been found to relate to an augmented risk of peripheral arterial disease in both men and women, independent of other cardiovascular risk factors [14].

Anti-oxLDL antibodies as biomarkers of CVD

Measurement of antibodies to oxLDL may also add to cardiovascular risk stratification and reclassification of patients within risk categories. There is already a large literature on the clinical correlates of anti-oxLDL antibodies [15–19]. However, it is difficult to dissect the associations between coronary heart disease (CHD) and CVD, as many studies have reported the relationship between oxLDL and its related antibodies with CVD. Nonetheless, studies that have reported CHD separately have highlighted the relationship with coronary events, as demonstrated in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) [20].

Contemporary studies reporting levels of anti-oxLDL antibodies with incident CVD mostly agree that IgM antibodies are linked to freedom from events. For example, high levels of IgM anti-oxPS indicated cardiovascular protection amongst Swedish 60-year-olds, most prominently in men [21]. However, the implications of high IgG anti-oxLDL antibody levels are less certain. A recent analysis of the large prospective Dallas Heart Study reported that levels of IgM anti-MDA-LDL reduce with increasing age, whilst IgG anti-MDA-LDL antibody levels tend to increase with age and to independently predict cardiovascular events [22]. Conversely, a recent study from our laboratory demonstrated that high titres of both IgM and IgG-anti-MDA-LDL indicated cardiovascular protection; however, the association was lost after adjustment for total serum IgM and IgG respectively. Indeed, higher levels of both total IgM and total IgG concentrations were associated with protection from coronary events, suggesting a wider homeostatic role for antibodies beyond reaction with oxLDL [20]. A current analysis of the cardiovascular arm of the Malmo Diet and Cancer Study has also shown that high titres of both IgG and IgM anti-ApoB-100 at baseline predict a lower risk of future acute coronary events [19]. However, the question surrounding IgG anti-oxLDL and cardiovascular risk remains unresolved as a meta-analysis of antibodies in atherosclerosis highlighted the positive association of IgG anti-oxLDL with CVD [23]. A further important limitation is that as yet we know little about whether anti-oxLDL antibody levels can be used to predict dangerous morphology of plaques rather than general cardiovascular risk, and we are currently addressing this issue with the Integrated Biomarker and Imaging Study 3 (IBIS-3) [24].

It has been suggested that anti-oxLDL antibodies may also be linked to calcific aortic valve stenosis. However, a recently reported sub-study of the ASTRONOMER (Aortic Stenosis Progression Observation: Measuring Effects of Rosuvastatin) randomised clinical trial established no association between anti-MDA-LDL antibody titres and progression of aortic stenosis or the need for surgical valve intervention [25]. However, this study only considered one of the many antibodies that may play a role in calcific aortic valve disease.

Effects of immunization with oxLDL or anti-oxLDL antibodies

There is considerable interest in boosting protective immunity related to atherosclerosis either by active immunization with oxidized or native LDL or derivatives, or by the passive administration of anti-oxLDL antibodies. Both of these strategies have shown protective potential in atherosclerosis models in mice and rabbits. In the case of active immunization, protection could be the result of augmented cellular immunity as much as, and possibly more than, increasing the antibody response [26–29].

The reactivity of anti-PC antibodies with both minimally oxidatively modified LDL and pneumococci has attracted considerable interest, and pneumococcal immunization has led to a reduction in atherosclerosis in LDL-receptor deficient ($LDLR^{-/-}$) mice [30]. The Australian Study for the Prevention through Immunization of Cardiovascular Events (AUSPICE) is a large investigation of the effect of pneumococcal polysaccharide vaccine on primary prevention of acute coronary syndromes and ischemic strokes, in 55–60 year olds over 5-year follow up. Recruitment has now finished and is due to report an interim analysis in 2020 [31]. A novel study utilizing immunization of the gingipain A hemagglutinin domain of Porphyromonas gingivalis, a common bacterial agent causing periodontitis, stimulated a strong IgM response to MAA-LDL in $LDLR^{-/-}$ mice. No such IgG response was evoked. Moreover, immunization was found to reduce atherosclerotic plaque size, associated with regulation of anti-inflammatory cytokines [32].

A recent study examined *in vivo* administration of human recombinant IgG anti-MDA-LDL (MLDL1278A) in a porcine model of atherosclerosis. The antibody had no effect on burden of atherosclerosis as assessed on histopathological analysis or fluorodeoxyglucose – positron emission tomography (FDG-PET) imaging [33]. A human phase I-IIa study has also recently been reported – GLACIER (Goal of oxidized LDL and ACtivated macrophage Inhibition by Exposure to a Recombinant antibody). MLDL1278A or placebo was administered to patients with stable carotid artery or aortic disease, with the main outcome being inflammatory activity assessed with FDG-PET at three months after treatment [34]. Whilst this trial was reported as negative, it is possible that treating patients with more active disease may yet be found to be beneficial.

OxLDL as a target for the molecular imaging of atherosclerosis

In the present era, the range of modalities available to image the coronary arteries has grown significantly, with excellent rate of progress. However, each of these modalities has inherent limitations that restrict their clinical utility. The most widely used technique, coronary angiography, involves the selective injection of radio-opaque dye into coronary arteries under fluoroscopic guidance. Unfortunately, this is focused solely on imaging of the vessel lumen and does not examine vascular remodelling or detect plaque components that are crucial for vulnerability. Moreover,

even with the addition of physiological measurements and intravascular imaging techniques, such as intravascular ultrasound, which improve on morphological characterization, we still cannot accurately predict which lesions are likely to rupture [35].

The need to identify plaques at risk is therefore a top priority, and molecular imaging holds considerable promise with clinical implications, both with non-invasive and invasive imaging modalities. As such, various molecular probes have been developed to image the presence of oxLDL in arteries. The first was EO6, a murine IgM monoclonal antibody that reacts with PC [36]. Next, a murine monoclonal IgG MDA2 antibody with specificity for malondialdehyde-lysine, an OSE on oxLDL, was developed. Plaque uptake in LDLR$^{-/-}$ mice was found to correlate with severity of atherosclerosis by autoradiography and immunocytochemistry [37]. Following this, the first human-derived Fv antibody fragment with high specificity for OSEs on oxLDL (MDA-LDL and Cu-oxLDL), was developed, designated IK17. Antibody fragments have advantages over full antibodies in that they induce fewer immunologic reactions, due to lack of Fc regions which interact with cell surface receptors, as well their small size, permitting better plaque penetration for imaging purposes. Moreover, IK17 was identified from human mononuclear mRNA antibody libraries, rather than through murine-derivation, further reducing potential immunologic reactions. This may allow multiple studies on the same patient cohort, permitting lesion surveillance [38].

Both MDA2 and IK17 have been successfully conjugated to gadolinium and manganese in micelles to facilitate magnetic resonance imaging (MRI), producing high-quality imaging with specific uptake in atherosclerotic lesions in experimental animals [39]. Liposomes reacting with an oxLDL scavenger receptor, LOX-1, have also been used in experimental models with successful detection of atherosclerotic lesions via MRI and single photon emission computerized tomography (SPECT) [40]. Molecular imaging with PET has developed more than other modalities for the non-invasive molecular imaging of atherosclerosis, and a Zirconium-89-labelled antigen-binding fragment to MAA epitopes on oxLDL (LA25) has been recently reported. *In vivo* PET/ MR imaging with ^{89}Zr-LA25 in atherosclerotic rabbit aortas demonstrated increased uptake compared to control rabbits, which correlated with near infra-red fluorescence (NIRF), FDG-PET, *ex vivo* gamma counting and autoradiography signals [41].

Intravascular imaging using NIRF, an intravascular catheter-based optical imaging modality utilizing the near infra-red spectrum, combined with molecular probes, is a pre-clinical modality with great potential for clinical translation. Hybrid imaging with NIRF and OCT has newly been used successfully in human coronary arteries [42]. We have recently explored this technology to develop a well characterized, in-house generated monoclonal IgG autoantibody to MDA-LDL, LO1, as a NIRF molecular targeting agent in atherosclerosis. Using LDLR$^{-/-}$ mice and high-fat fed diet rabbits with balloon-injured aortas as experimental models, we have demonstrated that LO1 tagged with a NIRF-reporting fluorophore can target atherosclerotic lesions in these models [43].

Targeted therapy to atherosclerotic plaques

Aside from the diagnostic utility, targeting oxLDL may be used for delivery of nanoparticles with therapeutic cargo to the plaque, allowing the directed treatment of atherosclerotic lesions at risk. This could allow smaller doses for therapeutic efficacy and reduce systemic side effects, with great potential for clinical application. This will be of utmost importance when using novel anti-inflammatory agents, where cost and systemic susceptibility to infection concerns will be paramount. As yet, few published attempts have been made to achieve targeted drug delivery to the atherosclerotic plaque, and none have directly targeted oxLDL. However, a high-density lipoprotein nanoparticle containing lipophilic simvastatin has been used to target atherosclerotic lesions in Apolipoprotein E-knock-out mice. A therapeutic cargo of statins was utilized given their anti-inflammatory properties and low oral systemic bioavailability. The nanoparticles were demonstrated to accumulate in atherosclerotic plaques, which reduced progression of lesion size on MRI, as well as lesion inflammation on *in vivo* fluorescence molecular tomography with computed tomography (FMT-CT) and *ex vivo* immunohistochemical analysis [44].

Future directions

There has been significant development in the understanding and pre-clinical utility of oxLDL, and its related antibodies, as both a biomarker and target for imaging. However, the translation of this to the clinical setting has not yet been achieved, nor the best imaging modality identified with which to do so. It may well be that a combination of non-invasive techniques enables risk stratification of sub-selected patient populations, whilst those at greatest risk from plaque rupture may undergo invasive imaging to pinpoint which lesions require treatment. At the present time that treatment would be PCI, but in the future, targeted drug delivery using oxLDL-tagged nanoparticles may be feasible. To accomplish this, either humanisation of the currently available murine antibodies or fragments, or discovery of novel human antibodies, needs to be performed, with associated pharmacodynamic and pharmacokinetic studies. Furthermore, the future may lie in hybrid molecular targeting platforms, benefitting from the examination of different aspects of the atherosclerotic cascade to provide more complete lesion assessment. This for example may include a combination of inflammation, oxLDL accumulation and matrix metalloproteinase activity markers, that in concert would provide a more detailed representation of plaque biological activity.

The interplay between the immune system and atherogenesis also requires further clarification. If oxLDL is indeed causal in accelerating plaque rupture, and IgM anti-oxLDL antibodies convey protection, then therapeutic implications are readily explorable. Identifying how IgM mechanistically performs its reported advantageous homeostatic functions will be important. Moreover, the roles of IgG, amongst other antibodies, in atherosclerosis needs considerable elucidation. Greater understanding of how currently available cardioprotective drugs, such as statins, perform their beneficial effects at the level of the atherosclerotic plaque, and how they interact with the immune system, still is in need of further study.

Conclusions

OxLDL plays important roles in the development of atherosclerosis. There is growing support for circulating oxLDL and anti-oxLDL antibodies as clinical biomarkers for CVD risk prediction, although their true significance and mechanistic involvement remain to be ascertained. *In vivo* pre-clinical efficacy and practicality of imaging with anti-oxLDL antibody molecular probes has been demonstrated with a variety of imaging modalities. The clinical translation of these, with the most appropriate hybrid imaging techniques, as well as potential utility in drug-targeting, could be revolutionary.

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