Review

Pathogenic Role of Modified LDL Antibodies and Immune Complexes in Atherosclerosis

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There is strong evidence supporting a key role of the adaptive immune response in atherosclerosis, given that both activated Th cells producing predominantly interferon-γ and oxidized LDL (oxLDL) and the corresponding antibodies have been isolated from atheromatous plaques. Studies carried out using immune complexes (IC) prepared with human LDL and rabbit antibodies have demonstrated proatherogenic and pro-inflammatory properties, mostly dependent on the engagement of Fcγ receptors I and II in macrophages and macrophage-like cell lines. Following the development of a methodology for isolating modified LDL (mLDL) antibodies from serum and isolated IC, it was confirmed that antibodies reacting with oxLDL and advanced glycation end product-modified LDL are predominantly IgG of subtypes 1 and 3 and that mLDL IC prepared with human reagents possesses pro-inflammatory and proatherogenic properties. In previous studies, LDL separated from isolated IC has been analyzed for its modifications, and the reactivity of antibodies isolated from the same IC with different LDL modifications has been tested. Recently, we obtained strong evidence suggesting that the effects of mLDL IC on phagocytic cells are modulated by the composition of the mLDL. Clinical studies have shown that the level of mLDL in circulating IC is a strong predictor of cardiovascular disease (CVD) and, in diabetic patients, other significant complications, such as nephropathy and retinopathy. In conclusion, there is convincing ex vivo and clinical data supporting the hypothesis that, in humans, the humoral immune response to mLDL is pathogenic rather than protective.


Key words: Modified LDL, Modified LDL antibodies, Immune complexes, Atherosclerosis, Autoimmunity

Introduction

Chronic inflammation is believed to be a major, if not the major, pathogenic factor in human atherosclerosis¹, ². Both innate and adaptive responses have been proposed to play a role in either initiating or perpetuating the inflammatory process associated with atherosclerosis.³⁻⁵. Innate immune mechanisms triggered by the interaction of modified lipoproteins with scavenger receptors and microbial products with toll-like receptors⁵ likely play an important initiating role, supported by a variety of clinical and ex vivo studies in humans, while the adaptive immune response likely plays a major role in perpetuating vascular inflammation.

Oxidized LDL (oxLDL) is taken up by macrophages via receptor-mediated pathways involving several types of scavenger receptors,⁶⁻¹⁰, such as CD36 and others, and contributes to generating reactive oxygen species (ROS)¹¹, which play multiple roles in the inflammatory process, including the persistent generation of oxLDL. Other pro-inflammatory effects of oxLDL, such as chemotactic effects on monocytes¹², ¹³ and enhanced monocyte adhesion to EC in culture¹⁴, ¹⁵, result from the increased expression of VCAM 1 and ICAM 1 in vascular cells¹⁶, ¹⁷, induced directly by undefined pathways or as a consequence of the release of pro-inflammatory cytokines by activated macro-
Advanced glycosylation end product (AGE)-modified LDL can also trigger inflammation. In general, AGE-modified proteins induce increased endothelial cell permeability and procoagulant activity as well as the overexpression of VCAM-1. AGE also contributes to fibroblast proliferation and T-cell activation. In atheromatous plaques, activated T-cells are known to recognize peptides derived from oxLDL, and it seems likely that these cells also recognize peptides from other types of modified LDL (mLDL). Interestingly, 80% of activated T-cells cloned from atheromas produce both interferon-γ and IL-4, 17% produce high levels of IFN-γ and low levels of IL-4 and 2% produce high levels of IL-4 and low levels of IFN-γ. These observations show that the Th1-Th2 dichotomy is not closely followed in humans. Nonetheless, the predominant cytokine released by these cloned T-cells is IFN-γ, which stimulates macrophages in lesions to release higher levels of pro-inflammatory cytokines and chemotactic factors when exposed to modified forms of LDL, thus contributing to the perpetuation of the inflammatory response in the arterial wall.

**Immunogenicity of Modified LDL**

Most modified forms of LDL appear to be immunogenic, both in experimental animals and humans. Steinbrecher, Palinski et al. characterized the immunogenic chemical modifications responsible for antibody formation in experimental animals. In subsequent years, our group conducted a variety of studies focused on the autoimmune response to modified forms of LDL in humans.

Submitting whole human serum to affinity chromatography in Sepharose columns conjugated with oxLDL or AGE LDL, we isolated the corresponding human antibodies. The predominant antibody isotype for both oxLDL and AGE-LDL antibodies was IgG (subtypes 1 and 3), followed by IgM and IgA, the last of which was detected in very small concentrations.

We have also shown that the average affinity constants for oxLDL and AGE-LDL antibodies isolated from whole sera are lower than the average affinity constant of rabbit oxLDL antibodies, illustrating that human autoantibodies are of lower affinity than antibodies induced by the inoculation of laboratory animals. We also compared the affinity constants of oxLDL antibodies isolated from whole serum or IC in a group of 437 subjects. While the Kd of the antibodies purified from the whole sera was $1.53 \pm 0.13 \times 10^{-8}$ mol/L, the Kd of the antibodies purified from the isolated IC was $0.98 \pm 0.06 \times 10^{-8}$ mol/L, demonstrating that the antibodies involved in IC formation have a higher affinity than those that remain free in the circulation.

To identify the modified forms of LDL that are more prone to trigger autoantibody formation, a study of the reactivity of human autoantibodies was performed by testing the reactivity of IgG fractions isolated from IC precipitated from the sera of 13 subjects with a battery of immobilized modified lipoproteins, including oxLDL, AGE-LDL, malondialdehyde-modified LDL (MDA-LDL), carboxymethyl-lysine-modified LDL (CML-LDL), myeloperoxidase-modified LDL (MPO-LDL), methylglyoxal-modified LDL (MGO-LDL) and hexanoyl-lysine-modified LDL (HEL-LDL). As shown in Fig. 1, MDA-LDL, oxLDL and AGE-LDL were the modifications predominantly recognized by the IgG antibodies isolated from IC. Of the 13 IgG fractions tested, only one reacted weakly with MGO-LDL, which does not appear to be as immunogenic as the other modifications. A more detailed analysis of the mLDLs showed that oxLDL prepared using copper oxidation contained predominantly MDA followed by CML and carboxymethyl-lysine, MDA-LDL prepared by treating LDL with MDA contained exclusively MDA and AGE-LDL prepared using an eight-week incubation of LDL with glucose-6-phosphate had a high content of CML, but also contained CEL and a lower concentration of MDA. The IgG fraction isolated from circulating IC contained antibodies to oxLDL, MDA-LDL, AGE-LDL and CML-LDL, suggesting that MDA-lysine is the major immunogenic epitope of oxLDL and CML-lysine is the major epitope of AGE-LDL. Purified human oxLDL antibodies, on the other hand, reacted almost equally well with oxLDL and MDA-LDL, cross-reacted with MDA-BSA and exhibited borderline reactivity with AGE-LDL. Human AGE-LDL antibodies reacted primarily with AGE-LDL and cross-reacted with oxLDL and CML-LDL. The cross-reactivity of oxLDL antibodies with MDA-BSA suggests that MDA-lysine is highly dominant and only partially dependent on the tertiary structure of the protein to which it is attached. The reactivity of AGE-LDL antibodies with oxLDL apparently resulted from the presence of CML-lysine epitopes in oxLDL. In other words, laboratory-modified lipoproteins have multiple epitopes, some of which are shared between different modifications. It is also important to note that LDL molecules modified in vivo are targets of multiple modifications.
Modified LDL Immune Complexes and Atherosclerosis

In 1988, Klimov's group and our group reported the accumulation of cholesteryl esters in human macrophages incubated with IC prepared with normal human LDL and rabbit antibodies\(^{34, 35}\). We also reported that the primary mechanism of uptake of LDL IC is through Fc receptors and that the ingestion of LDL IC is associated with the paradoxical overexpression of LDL receptors, which leads to the unregulated uptake of LDL\(^{34, 36}\). Meanwhile, both human macrophages and mesangial cells take up LDL IC prepared with human LDL and rabbit or human LDL antibodies predominantly through FcyRII\(^{37,39}\). The uptake of LDL IC by U937 histiocytes involves both FcyRI and IIa\(^{40,41}\). Similar findings were reported by Morganelli \textit{et al.} in a study using bispecific LDL IC prepared with conjugated murine anti-LDL and anti-FcyR antibodies and human macrophages\(^{42}\). In addition, FcyRIII may also be involved in the uptake of oxLDL IC by mesangial cells\(^{38}\).

Atherogenicity and Pro-Inflammatory Properties of Immune Complexes Containing Modified LDL

The involvement of LDL IC in the pathogenesis of atherosclerosis in humans was first suggested in 1970 by Beaumont and coworkers, who reported massive hyperlipidemia and accelerated atherosclerosis in patients with IgA myeloma in whom monoclonal IgA functioned as an autoantibody against LDL\(^{31}\). In 1978, Fust and coworkers reported the occurrence of LDL antibodies and IC in patients with CVD\(^{32}\). Three years later, Klimov and coworkers reported that complexes formed with human radiolabelled LDL and rabbit anti-ApoB IgG were eliminated from the circulation in normal rabbits two to three times faster than free LDL and that the complexes were taken up by murine peritoneal macrophages more actively than soluble LDL. Furthermore, the cholesterol content of macrophages incubated with LDL IC increased approximately 60-fold over that of the control macrophages\(^{33}\).

Fig. 1. Comparison of the reactivity of the IgG fraction isolated from precipitated LDL IC obtained from 13 patients with type 1 DM against a battery of modified forms of LDL, including oxLDL, AGE-LDL, MDA-LDL, CML-LDL, myeloperoxidase (MPO)-LDL, methylglyoxal (MGO)-LDL and (hexanoyl)lysine (HEL)-LDL. Identical concentrations (0.75 μg/well) of each modified LDL were immobilized in EIA wells and incubated with equal concentrations (10 μg/well) of the antibodies isolated from the patients. Peroxidase-labeled rabbit anti-human IgG was used to detect the bound IgG antibodies. The data are presented as the mean ± 1 SEM for the binding of IgG to each one of the different modifications after subtracting the OD corresponding to the binding to native LDL.
of LDL IC prepared with human LDL and rabbit antibodies by human monocyte-derived macrophages results in the release of IL-1 and TNF and the activation of a respiratory burst, and the effects of LDL IC are more pronounced than those of control IC, such as HDL IC, VLDL IC or KLH IC. Similar results were reported by Kiener et al., who observed that LDL IC prepared with human native or acetylated LDL and rabbit anti-LDL induce the release of TNF by THP-1 cells more effectively than heat-aggregated gammaglobulin and that this effect is not observed if Fab fragments of anti-LDL antibodies are used instead of intact antibodies, further confirming that interaction with FcγR is essential for the macrophage activation induced by LDL IC.

With the development of methodology to isolate human oxLDL antibodies from serum and precipitated IC, we were able to prove that the proatherogenic and pro-inflammatory properties observed when LDL IC are prepared with native LDL and animal apoB antibodies are also observed using IC prepared with human oxLDL and human oxLDL antibodies. Human oxLDL IC, either adsorbed to human red blood cells (RBC) or resuspended following precipitation in the presence of 4% polyethylene glycol (PEG), were found to be more effective than oxLDL in inducing intracellular cholesteryl ester accumulation and TNF release in THP-1 cells. In later studies, human oxLDL IC were shown to activate the complement system and induce the release of pro-inflammatory cytokines at significantly higher concentrations than those induced by oxLDL at the concentration estimated to be present in oxLDL IC from human monocyte-derived macrophages and human MonoMac6 cells. Priming these cells with interferon-γ resulted in the release of significantly higher concentrations of IL-1β, IL-6 and TNF and a significant release of IL-12p70 and IL-10. The concentrations of pro-inflammatory cytokines released by primed MonoMac6 cells were higher after incubation with oxLDL IC than after incubation with identical concentrations of KLH IC.

More recently, Nagarajan reported that incubation of human venous endothelial cells with oxLDL in a first step and rabbit anti-MDA in a second step results in the formation of surface-bound IC that promote the adhesion and activation of U937 cells through FcγRII. The adhesion of U937 cells to IC-coated HUVECs is associated with the secretion of pro-inflammatory chemokines (MCP-1 and IL-8), a factor that plays a significant role in the perpetuation of vascular inflammation.

In 2006, Oksjoki and coworkers reported that oxLDL IC induce the survival of human monocytes cultured in serum-free medium. This effect appears to result from a slowdown of spontaneous apoptosis associated with the release of monocyte colony-stimulating factor (M-CSF) and occurs primarily secondary to the interaction of oxLDL IC with FcγRII. Oksjoki's observations were confirmed by the findings of a study of the effects of the global gene expression following the stimulation of human monocyte-like cells (U937) with oxLDL IC KLH IC and oxLDL alone. That study showed that oxLDL IC have the unique ability to induce the expression of one set of genes involved in the pro-survival functions and, similar to KLH IC, stimulates the expression of genes related to the regulation of transcription, endocytosis, intracellular lipid transport and the inflammatory response, including genes encoding TNF and IL-1β. Additional studies suggest that the pro-inflammatory response in U937 cells induced by oxLDL IC is mediated by the prolonged activation of acid sphingomyelinase.

A study of the trafficking of oxLDL and OxLDL IC in U937 cells reported remarkable differences. While both the lipid and apoB moieties of internalized oxLDL remained colocalized within the endosomal and lysosomal compartments, the lipid moiety of oxLDL IC was localized in the endosomal compartment, while the apolipoprotein moiety was transported to the lysosomal compartment. The sequestration of lipids in the endosomal compartment may explain the slow degradation of the lipid component of LDL IC and appears to be associated with reduced oxidative stress (relative to that observed after oxLDL internalization), likely contributing to the increased survival of U937 cells incubated with oxLDL IC.

The pro-survival effect of oxLDL IC is not common to other types of mLDL IC. While oxLDL IC and MDA-LDL IC induce a similar gene expression and secretion of pro-inflammatory mediators (IL-6, MCP-1), both MDA-LDL and MDA-LDL IC induce a significantly higher release of matrix metalloproteinases than oxLDL or oxLDL IC, the difference being more significant when MDA-LDL IC are compared to oxLDL IC (unpublished data). In contrast, the levels of the secreted inhibitory molecule TIMP-1 remain unaffected by either MDA-LDL IC or oxLDL IC. These data strongly suggest that MDA-LDL IC create favorable conditions for plaque instability and acute cardiovascular events.

Clinical Studies

The involvement of oxLDL IC in the pathogenesis of human atherosclerosis is strongly supported by
the findings of Yla-Herttuala and coworkers, who isolated both oxLDL and the corresponding IgG antibodies from human atheromatous plaques\(^5^{2-55}\). These results and the experimental data supporting the pathogenic role of modified lipoproteins have been applied in clinical studies focusing on the possible correlations between the levels of modified lipoproteins and the manifestations of CVD. However, it is difficult to develop reliable assays for oxLDL and other forms of mLDL, although numerous groups have developed enzyimmunoassays for oxLDL\(^29\), AGE-LDL\(^56\) and MDA-LDL\(^57-60\) antibodies and proceeded by that these antibodies are indirect markers for the clinical manifestations of CVD, generally assuming that these antibodies are indirect markers for the corresponding modified lipoproteins\(^61\). While some groups have found evidence suggesting that the levels of antibodies to oxLDL are correlated with the extent of atherosclerotic CVD and/or the risk of progression of carotid atherosclerosis, other groups, including ourselves, have failed to show such correlations or have even reported inverse correlations\(^5,29\). A lack of correlation between the MDA-LDL antibody titers and atherosclerosis has also been reported\(^60\).

As discussed in detail by us in a previous review\(^29\), one problem with the assays for mLDL antibodies is their lack of standardization; however, even more important is the interference of antigen-antibody complexes formed by mLDL and corresponding antibodies in the assay\(^62-64\). The interference of IC in assays is a well-known phenomenon in infectious disease serology. We previously demonstrated that over 99% of oxLDL and 94% of MDA-LDL in the circulation are precipitated by 4% polyethylene glycol\(^65\), as is characteristic of soluble IC\(^66\). We also previously reported that the antibodies that remain soluble following the precipitation of IC are of lower affinity than those recovered from isolated IC\(^28\) and, for this reason, are less likely to form stable IC and less likely to be pathogenic.

While the data generated using antibody assays are generally considered to be unreliable by most investigators, the pathological significance of oxLDL antibodies has been further questioned due to the data generated in mouse models, as recently summarized by Amir and Binder, suggesting that antibodies to oxLDL and MDA-LDL have a protective effect against the development of atherosclerosis\(^67\), although some experimental data contradict this conclusion\(^61,68,69\) and other data have been criticized as being overinterpreted\(^70\).

The risk of translating data generated in mice or other animal models directly to human medicine was excellently discussed by Mark Davis in 2008\(^71\). Both the immune system and lipoprotein metabolism of humans and mice have significant differences, compounded by the fact that many studies are conducted with genetically modified animals. Notwithstanding the need to perform experiments in animal models, a great deal of caution is required when extrapolating data generated in very different species, particularly when there are ample data suggesting that the conclusions reached with animal models are not likely to apply to humans, as discussed later in this section.

A more recent view of the protective effects of antibodies against atherosclerosis has focused on antibodies of the IgM isotype. Eighty percent to the total IgM remains intravascular due to its macromolecular nature\(^72\), limiting the ability of IgM antibodies to form pathogenic LDL IC in the subendothelial space, as supported by the fact that antibodies isolated from atheromatous lesions are of the IgG isotype\(^52,54,59\). Another limitation with respect to IgM antibodies is their inability to opsonize through Fc receptors (although they can opsonize through complement receptors if they form IC with attached complement fragments\(^73\)).

Clinical studies regarding the protective role of IgM in humans have yielded conflicting data. While some researchers have reported that the levels of IgM antibodies to oxLDL are inversely related to the degree of carotid atherosclerosis\(^74,75\) and are predictive of the slow development of carotid atherosclerosis\(^75\), others have reported that, in a large series of patients undergoing coronary angiography, the IgG levels were found to be directly associated with angiographically determined coronary heart disease (CAD), while patients with IgM antibodies exhibited an inverse correlation with CAD. However, neither the IgG nor IgM antibody levels were found to be independent predictors of CAD\(^70\). Even more contradictory was the report from Fredrickson \textit{et al.} that the levels of IgM antibodies against MDA-modified peptide 120 are associated with a more rapid progression of carotid intima-media thickness (IMT)\(^77\). In a type 2 diabetes cohort (n = 937) in which we determined the isotype of antibodies separated from isolated IC, only in 1.5% of the patients did the IgM antibody concentrations equal or exceed those of IgG antibodies. The concentrations of antibodies were 85 ± 83 μg/mL (mean ± SD) for IgG and 4.5 ± 7 μg/mL for IgM antibodies\(^78\). We did not find any statistical evidence supporting a protective role of IgM antibodies in relation to CVD, as defined by a variety of end points\(^79\); however, the number of patients with a relatively high concentration of IgM antibodies may have been insufficient to generate statistically significant data.
With the development of monoclonal antibodies to MDA-LDL and oxLDL, approximately one decade ago, there was a surge of interest in developing assays for these two forms of mLDL and investigating their correlation with clinical end points of CVD. An elevated level of oxLDL was proposed to be a biomarker for ischemic heart disease, with better discriminating power to distinguish patients with CAD and healthy subjects than traditional biomarkers. The OxLDL and MDA-LDL levels have also been proposed to be markers for unstable atherosclerotic CVD, however, Niccoli et al., while finding that the oxLDL plasma levels were significantly higher in patients with unstable angina, did not find differences in areas stained with monoclonal antibodies to oxLDL or MDA-LDL in plaque sections obtained via atherectomy from patients with stable or unstable angina.

The results of the Multi-Ethnic Study of Atherosclerosis, MESA, which involved a large cohort of 879 individuals without clinical evidence of CVD, were somewhat disappointing. All patients were non-statin users and examined cross-sectionally. The oxLDL level was measured using a monoclonal antibody 4E6-based ELISA. The presence of subclinical CVD was defined as the occurrence of plaque in carotid arteries with ≥25 stenosis, an ankle-brachial blood pressure index (ABI) of <0.9 and coronary calcification based on an Agatston calcium score of ≥200. While the individuals with subclinical CVD had higher levels of oxLDL, in a multivariate analysis, most of the variation was attributed to other factors, such as dyslipidemia, smoking, ethnicity and gender. Only in 15 individuals with subclinical peripheral vascular disease associated with higher levels of carotid stenosis and coronary artery calcification did the association with the oxLDL level remain significant after correcting for other risk factors.

The conflicting observations reported by different, and sometimes the same, groups likely reflect the same problems outlined in the discussion of the assays for mLDL antibodies. The fact that the vast majority of circulating oxLDL and MDA-LDL exist as IC is a cause of error that has not been addressed. To our knowledge, no groups have tested whether these assays accurately detect mLDL when testing IC prepared with oxLDL and MDA-LDL and the corresponding human antibodies. On the other hand, the commercial versions of the assays for oxLDL and MDA-LDL, although they generally use the same monoclonal antibodies, have different configurations, and the correlation between the values tested using different kits is poor. In our laboratory, we tested one of the kits for the oxLDL assay and found it to recognize freshly isolated LDL as well as copper-oxidized LDL. Data proving the specificity of the assay must be included in the kits, and the user should verify it as well.

The main focus of our research is to define the pathogenic role of humoral immunity in the pathogenesis of diabetic complications, including CVD, which is also prevalent in the general population. The involvement of IC in the pathogenesis of diabetic complications and CAD was proposed in the early 1980s. However, these early reports were based on data generated using nonspecific IC assays. In 1990, Orekhov and Tertov reported for the first time results based on determining the content of cholesterol in precipitated IC, using cholesterol as a surrogate for LDL. This report was largely ignored, as in that time most groups were interested in developing antibody assays that had the theoretical advantage of specificity. Szondy et al. developed an enzyme immunoassay (EIA) to detect anti-LDL antibodies in PEG-precipitated IC; however, this approach did not resolve the issue of the interference of IC in the detection of antibodies. By the mid-1990s, additional groups reported methods to detect and quantify LDL IC. Boullier and coworkers described an EIA for LDL IC based on the binding of LDL IC to an immobilized anti-apo B monoclonal antibody followed by the detection of IgG and/or IgM associated with the bound LDL. One problem with this assay was that the immobilized monoclonal antibody reacted with high affinity for native LDL and low affinity for oxLDL, and the negative results reported by this group, who attempted to correlate LDL IC titers with coronary stenosis, can be explained by the lack of specificity of the assay. Another potential problem with this type of immunoassay is the interference of anti-IgG autoantibodies, whose occurrence is often unpredictable. The simplicity of the assay has led to its continuing use, with generally inconclusive results. Two EIAs for AGE-LDL IC were reported by Turk et al. One involved precipitation with PEG followed by the assay of AGE or of IgG bound to AGE in solubilized precipitates (a method that does not eliminate the interference of IC with the antigen or antibody assays), while the other involved binding polyclonal anti-AGE to the solid phase and using anti-IgG to measure IgG associated with the captured AGE proteins (including but not exclusively LDL).

In 1997, we published our results of a first attempt to isolate and fractionate circulating LDL IC. We confirmed that our assay could not detect antibodies to oxLDL in resuspended PEG precipitates. However, after fractionating the resuspended precipitates in pro-
tein A/G columns using mildly dissociating conditions, we detected cholesterol and apoB in the washout and oxLDL IgG antibodies in the eluate, as one would expect if IC involving low affinity antibodies were dissociated. We also proved that there was an inverse correlation between the concentration of apoB in isolated and fractionated IC and the serum concentration of oxLDL antibodies. A similar inverse correlation was reported between the serum levels of AGE-proteins and the levels of AGE in isolated IC by Turk et al.

In a later study, the levels of free oxLDL antibodies, total cholesterol and apoB were measured in PEG-precipitated IC in a group of 49 patients with type I diabetes who developed CAD during an 8-year follow-up period and an equal number of patients matched according to age, gender and disease duration who did not develop CAD. The patients who developed CAD had significantly lower levels of free oxLDL antibodies in whole serum and significantly higher levels of cholesterol and Apo B in the PEG precipitates, indicating that they had higher levels of oxLDL IC in the circulation and that a significant fraction of the circulating oxLDL antibodies could not be detected due to IC formation.

The role of circulating LDL IC in the progression of carotid IMT was investigated in patients enrolled in the Epidemiology of Diabetes Interventions and Complications (EDIC) Trial, a follow-up study of the Diabetes Control and Complications Trial (DCCT). Conventional CHD risk factors and the levels of antibodies against modified forms of LDL and LDL-IC (defined by the cholesterol and apoB contents of PEG-precipitated IC) were determined in blood collected from 1,050 patients between 1996 and 1998. B-mode ultrasonography of the internal and common carotid arteries was performed in 1994-1996 and 1998-2000. The levels of cholesterol in IC were significantly higher in the patients who exhibited progression of the internal carotid IMT, and multivariate linear and logistic regression modeling using conventional and non-conventional risk factors showed that the cholesterol content of IC was a significant positive predictor of internal carotid IMT progression.

With the refinement of our methodology for fractionating IC and the development of capture assays for oxLDL, MDA-LDL and AGE-LDL, we were able to determine both specific antibodies to mLDL and the composition of the mLDL involved in IC formation. Therefore, we determined the levels of oxLDL, AGE-LDL and MDA-LDL in IC isolated from the sera of 479 patients in the DCCT/EDIC cohort collected at baseline and analyzed the correlations between these levels and the internal and common carotid IMT measured eight and 14 years later. The multivariate logistic regression models indicated that, comparing individuals in the highest versus lowest quartiles of oxLDL and AGE-LDL in IC, those with higher values of these parameters had a 6.11-fold (CI: 2.51-14.8) and 6.4-fold (CI: 2.53-16.2) increase, respectively, in the odds of having a high carotid IMT after adjusting for conventional risk factors. These odd ratios exceed those of LDL-cholesterol [2.62 (CI:1.24, 5.55)], diastolic blood pressure [1.45 (CI:0.69, 3.03)] and HbA1c [2.33 (CI:1.09, 4.99)]. A parallel study conducted in the same group investigated the correlation between the oxLDL content of isolated IC and coronary artery calcification (CAC) determined on computed tomography. Multivariable regression models indicated that a 1-SD increase in the levels of oxLDL IC was associated with a 37% increase in the risk of having a high CAC score (RR = 1.36; 95% CI: 1.12-1.67) at follow-up after adjusting for DCCT treatment, retinopathy/AER, gender and CT scanning site, as well as baseline age, diabetes duration and HbA1C%. Further adjustment for the smoking status, blood pressure and LDL resulted in a risk ratio of 1.23 (95% CI: 1.01-1.50) that remained statistically significant, indicating that the concentration of oxLDL in IC is independently associated with the development of CAC.

A more recent study of 907 patients recruited as part of the Veterans Administration Administration Diabetes Treatment (VADT) study focused on the main modifications of LDL isolated from PEG-precipitated IC and their value as predictors of cardiovascular events occurring during a follow-up period that averaged 3.7 years. The patients in this population predominantly had type 2 diabetes, and, in contrast to the results obtained in patients with type 1 diabetes, in whom the correlation with atherosclerotic disease was stronger for the levels of oxLDL and AGE-LDL in IC, a stronger correlation was observed in the VADT population between the levels of MDA-LDL and myocardial infarction (MI). The patients in the highest quartile of the MDA concentration in IC were at a higher risk of developing MI than the patients in the lower quartile [HR = 2.4 (1.03, 5.77)], while no differences were observed between the patients in the highest and lowest quartiles for the oxLDL and AGE-LDL concentrations in the IC. The discriminatory power of MDA-LDL in IC exceeded that of LDL-cholesterol, systolic blood pressure and HbA1c. This observation, which is in concordance with Holvoet's data, strongly suggests that MDA-LDL has a unique capacity to
The pathogenic role of immune complexes formed by mLDL and the corresponding antibodies has been well established, by both in vitro and clinical studies, as diagrammatically illustrated in Fig. 2. The levels of mLDL in isolated IC are predictive of the development of CVD. It is also clear that the pathogenic role of IC is not just a reflection of the predominant modification of the LDL involved, because the proatherogenic and pro-inflammatory effects of free mLDL are significantly less pronounced than those of mLDL IC, although there is certainly evidence suggesting that the type of LDL modification modulates the pathogenic effects of mLDL IC. The measurement of mLDL IC carried by IC has a predictive value superior to that of the classical biomarkers currently in use\textsuperscript{103-105}.

Conflicts of Interest

None.
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