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Distribution of IgM and IgG antibodies to oxidized LDL in immune complexes isolated from patients with type 1 diabetes and its relationship with nephropathy

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Abstract

Modified lipoproteins are immunogenic and play a key pathogenic role in vascular disease. Antibodies to oxidized LDL (oxLDL) are mostly of the proinflammatory IgG₁ and IgG₃ isotypes. We measured IgG and IgM oxLDL antibodies in immune complexes (IC) isolated from 36 patients with type 1 diabetes using a nested case-control design. IgG antibodies predominated over IgM antibodies by an 8:1 ratio. IgG antibody concentrations were higher in the nephropathy cases compared to controls ($p=0.09$), but no significant difference was observed because of two patients included in the study who had end-stage renal disease (creatinine > 5mg/dl and glomerular filtration rate (GFR) less than 17 ml/min). After eliminating these patients from the analysis, significant positive associations of IgG antibody concentration with serum creatinine and albumin excretion rate were observed after eliminating two patients with significant renal impairment (serum creatinine > 5 mg/dl). Similarly, a negative correlation with estimated glomerular filtration rate was observed in this subsample of 34 patients. Differences in IgM antibody concentrations by nephropathy classification were not supported by the data. In conclusion, the predominance of pro-inflammatory IgG oxLDL antibodies is associated with existence of diabetic nephropathy, and a protective role of IgM antibodies could not be demonstrated.

Keywords

Autoimmunity; diabetes; nephropathy; oxidized LDL antibodies; immune complexes

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INTRODUCTION

Modified human lipoproteins are immunogenic both in animals and humans.(1;2) From all the modified forms of LDL, oxidized LDL (oxLDL) and malondialdehyde-modified LDL (MDA-LDL) have been most extensively studied. In the case of oxLDL, Steinbrecher as well as Palinski et al. characterized its immunogenic epitopes.(3;4) Human autoantibodies to oxLDL have been purified and characterized.(5–7) The predominant isotype of oxLDL antibodies isolated either from serum (free antibodies) or from precipitated soluble immune complexes (antigen-associated antibodies) is IgG, of subclasses 1 and 3.(7;8) OxLDL-IC have been shown to have proinflammatory properties,(9;10) as expected from the predominance of IgG1 and IgG3 subclasses(11;12) in human oxLDL antibodies. The investigation of the possible pathogenic role of circulating oxLDL antibodies has produced conflicting data. While some groups reported a positive correlation between the levels of free oxLDL antibodies and different endpoints considered as evidence of atherosclerotic vascular disease, progression of carotid atherosclerosis, or risk for the future development of myocardial infarction,(13–19) others failed to show such correlation or showed an inverse correlation.(20–30) On the other hand, there is a significant body of evidence supporting a pathogenic role for immune complexes formed by oxLDL and their corresponding antibodies (oxLDL IC), both in what concerns atherosclerosis and macrovascular disease (5;9;30–34) and nephropathy.(35;36)

Recently, however, several groups have proposed a protective role for the humoral immune response to modified LDL, based on data first obtained in animal models in which the predominant isotype of oxLDL antibody is IgM.(37;38) A protective role for IgM antibodies is logical, given their low affinity, predominant intravascular distribution, and lack of interaction with Fc receptors of phagocytic cells.(11) The same protective role of IgM oxLDL antibodies has been proposed in humans, based on studies reporting a negative correlation between IgM MDA-LDL antibody levels and carotid intima-media thickness (IMT)(39) and with a reduction of coronary ischemia events in patients with unstable angina or non-Q-wave acute myocardial infarction over a 16 week period.(40) However, others have reported that IgM antibodies to oxLDL correlate with a more rapid progression of carotid disease, as judged by IMT measurements.(41)

In an attempt to better define the pathogenic role of oxLDL antibodies we have performed this pilot study on which we measured the relative concentrations of IgG and IgM oxLDL antibodies in IC isolated from the serum of patients with type 1 diabetes mellitus, with and without evidence of diabetic nephropathy, trying to define their proposed pathogenic or protective roles.

MATERIALS AND METHODS

Preparation of human oxLDL

Human LDL was oxidized as previously described.(42) The concentration of human LDL was of 1500mg/L and CuCl₂ was used at a final concentration of 40μmol/L. 200 μmol/L EDTA and 40 μmol/L butylhydroxytoluene (BHT) were added to stop the oxidation reaction. The oxLDL was dialyzed against PBS, pH 7.4 before use.

Samples

Serum samples from patients with Type 1 Diabetes were collected on a subset of the DCCT/EDIC population described in previous reports.(35;43) Our Institutional Review Board and those of all participating DCCT/EDIC centers approved this study. Written informed consent was obtained from all participants. The samples were aliquoted and stored at –70°C. To minimize the number of specimens analyzed for this study, a nested, matched case control

study was designed. Cases were randomly selected from the EDIC cohort that met the following to criteria: (1) estimated glomerular filtration rate (GFR) less than 65 mL/min/1.73 m² at time of collection or at the EDIC visit immediately preceding or following the collection visit; and (2) an albumin excretion rate (AER) $\geq$ 80mg/day at either the previous or following EDIC study visit (because serum samples and timed urine collections are taken in alternating years in EDIC, AER was not measured at the time of the serum collection). Controls were matched on duration of diabetes ($\pm$ 3 years) and gender. Sample size calculations were based on an initial feasibility study of the EDIC cohort in which IgG and IgM oxLDL antibodies in isolated IC were measured. These calculations suggested sample sizes ranging from 22 to 56 would give 80% power to detect the range of correlations (from 0.37 to 0.57) at the $\alpha=0.05$ level of significance. Thus, a 18-matched case and control pairs were selected for this follow up study.

Isolation and fractionation of soluble immune complexes

Soluble IC were precipitated from human serum using 4% (w/v) polyethylene glycol (PEG) 6000, as previously published.(2) This concentration of PEG has been previously found to effectively precipitate all IC containing modified LDL, since the analysis of precipitates and supernatants obtained after centrifugation of the PEG-incubated samples show that modified LDL is only detected in the precipitate, which also contains antibodies to modified LDL.(2) After resuspension, the isolated IC were fractionated by affinity chromatography on immobilized protein G (Protein G-Sepharose 4 Fast Flow, Amersham-Pharmacia biotech, Piscataway, NJ).(8) Two fractions were collected: a washout, containing antigens (e.g., modified lipoproteins) and antibodies of isotypes other than IgG, and an eluate, containing IgG antibodies of multiple specificities. Those fractions were later tested for their contents of specific oxLDL antibodies of the IgG and IgM isotypes. As expected, IgM antibodies were predominantly found in the washout fraction (4.1 ± 3.3 in washout vs. 0.3 ± 0.09 in eluate), and IgG antibodies were almost exclusively found in the eluate (23.8 ± 15.6 in eluate vs. 0.04 ± 0.03 in washout).

Isolation of oxLDL Antibodies from Human Serum to Use as Calibrators

The calibration of the assay required the isolation of IgG and IgM oxLDL antibodies from human serum. The isolation of was performed through a two-step process of affinity chromatography. First, we separated IgG from all other serum proteins (including IgM) by affinity chromatography on immobilized protein G. Both the washout and eluate were dialyzed in 0.01 M NaHCO₃ (pH 8.3) buffer overnight, and were then fractionated on an immobilized oxLDL column.(7) IgM antibodies were obtained from the protein G washout and IgG antibodies were obtained from the protein G eluate. The concentrations of IgG and IgM in the purified antibody preparations eluted from the immobilized oxLDL column were measured by radial immunodiffusion using reagents obtained from The Binding Site (San Diego, CA). The specificity of the purified antibodies was tested by competing their ability to bind to immobilized oxLDL by pre-incubating them with an excess of oxLDL (10 fold over the concentration used to coat the enzymeimmunoassay plates). The reactivity of both antisera was drastically reduced, by 97% in the case of the IgM antibody, and 93% in the case of the IgG antibody.

Assay of IgG and IgM oxLDL antibodies in PEG-precipitated IC

The antibodies to oxLDL of each isotype were determined by enzymeimmunoassay. Immulon Type 1B plates were coated with oxLDL (7.5 μ g/ml). After overnight incubation at 4°C, the plates were blocked with 5% bovine serum albumin in PBS (pH 7.4). Serial dilutions of isolated oxLDL antibodies of the IgG or IgM isotypes, starting at concentrations of 40 μ g/mL and 1.81 μ g/mL, respectively, were used as calibrators. The protein G fractions (washout with IgM and

eluate with IgG) were added to the ox-LDL coated plates as unknowns, each sample assayed in three sequential double dilutions. After incubation at 4°C overnight the plates were washed and affinity purified goat anti human IgG or goat anti-IgM antibodies conjugated with horseradish peroxidase (HRP) (ICN/Cappel, Aurora, OH) were added at a dilution of 1:1000 to the respective plates, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, ABTS] was used as substrate. After color development the plates were read at 414nm and standard curve was drawn from the OD readings of the IgG and IgM anti-oxLDL antibodies and their known concentrations.

Specificity control of purified oxLDL antibodies

Two wells on each enzymeimmunoassay plate were used to test the specificity of the purified oxLDL IgG and IgM antibodies. IgG antibody at 40 µg/mL and IgM antibody at 1.81 µg/mL were absorbed with oxLDL at 75 mg/mL. The reduction in reactivity of both antibodies with immobilized oxLDL after absorption was ≥ 99%.

Statistical analysis

Standard descriptive statistics were used to summarize the demographic data. For all statistical comparisons between the nephropathy cases and controls with respect to the IgG and IgM antibody concentrations, the paired nature of the data was taken into account. In particular, the differences in antibody levels of the IgG and IgM isotype levels were computed within each stratum (i.e., the levels of the control were subtracted from the levels of the matching case), and this difference was tested equal to zero using the Wilcoxon Signed Rank Test. This analysis was supplemented by conditional logistic regression, which allowed for an estimate of the overall effect size as measured by the odds ratio. All statistical analyses were performed using the SAS System version 9.1.3. This study was designed as a pilot study, and accordingly, the type I error conventions for pilot studies were used in the analysis.(44) A type I error rate of 0.15 was selected to effectively allow further evaluation of the IgG and IgM isotype levels in a larger sample. No correction for multiple testing has been applied to the reported p-values.

RESULTS

Table 1 summarizes the clinical and demographic data by case control status for the 18 matched pairs. There were notable differences in several clinical factors such as glycemic control, systolic blood pressure, use of ACE inhibitors, and lipoprotein levels.

Reliability of the measurements of IgG and IgM oxLDL antibodies contained in oxLDL-IC

IgG and IgM antibody measurements in fractionated oxLDL-IC isolated from all 36 participants were performed in triplicate although in a few cases the results reported were based only in duplicated measurements since one of the dilutions tested was outside the calibration curve. The intra-class correlations (ICC), a measure of reliability, for IgG and IgM in oxLDL-IC, respectively, were estimated to be 0.97 and 0.94. These values represent high reliability, and accordingly, the mean of all replicates within subject was computed and used as the outcome for subsequent analyses.

IgG and IgM measurements in isolated oxLDL-IC and their relationship with parameters of kidney function

The descriptive summary of IgM and IgG oxLDL antibody levels in oxLDL-IC is shown in Table 2. In addition to these assessments, the two composite measures (IgG to IgM antibody ratio and the percent of IgM antibodies) are shown in Table 2. IgG was the predominant isotype of oxLDL antibodies in isolated IC, the average ratio between IgG and IgM antibodies in the whole group being of 8:1. Levels of IgG antibodies were higher in the 18 nephropathy

cases ($p=0.09$), but the ratio of IgG and IgM antibodies was not found to differ between nephropathy cases and matched controls ($p=0.30$). Patients without nephropathy tended to have higher percentage of IgM antibodies ($p=0.17$); however, there was no evidence for a difference in the concentration of IgM antibodies in oxLDL-IC between cases and controls ($p=0.70$).

The results of conditional logistic regression analysis supported these findings. For a 1 standard deviation increase in oxLDL IgG antibodies in IC (i.e., IgG increase of 15.26, Table 2), the odds of nephropathy being present increased by a factor of 2.3 (OR=2.34; 95% CI [0.78, 7.03]; $p=0.13$). On the other hand, the odds of nephropathy were increased by 37% for each 12% point reduction in the percentage of IgM antibodies (OR=0.631; 95% CI [0.31, 1.30]; $p=0.21$); however, this finding was a weak trend. The IgG to IgM antibody ratio also showed limited evidence for an association with nephropathy status. For a 1 standard deviation unit increase in the IgG to IgM antibody ratio, the odds of developing nephropathy increased by a factor of 1.69 (OR=1.69; 95% CI [0.75, 3.80]; $p=0.20$). Increases in IgM antibody concentration were not found to show a negative correlation with the development of nephropathy (OR=0.927 for 1 SD change; 95% CI [0.44, 1.96]; $p=0.84$).

The correlation of IgG antibody concentrations to markers of renal disease was considered to better describe the observed associations. In the full sample of 36 patients, no linear association of IgG, IgM, IgG:IgM ratio or the percent of IgM was found with serum creatinine, albumin excretion rate, and estimated GFR (all p -values >0.20). However, two observations were highly influential in this analysis as determined by a residual analysis of the simple linear regression models that regressed each of the markers of renal function on IgG antibody concentration. These two patients who had end-stage nephropathy (GFR < 17 mL/min and serum creatinine > 5 mg/dL), were therefore removed from the model as a part of the diagnostic model building process. The exploratory (post hoc) analysis revealed a positive correlation of IgG with serum creatinine (Figure 1a, $r=0.37$, $p=0.032$) and AER (Figure 1a, $r=0.32$, $p=0.069$), and a negative correlation with estimated GFR (Figure 1c, $r=-0.30$, $p=0.088$); however, these findings are limited to patients with serum creatinine levels lower than 5 mg/dL and GFR above 35 mL/min, i.e. patients with nephropathy but not end-stage nephropathy.

DISCUSSION

The present study is unique in two significant areas. First, it is the first study in which human IgG and IgM antibodies involved in soluble oxLDL IC formation have been directly assayed. To achieve this end we isolated circulating IC, separated IgG and IgM immunoglobulins from the isolated IC, and measured specific antibodies to oxLDL in those IgG and IgM fractions. To calibrate the assay we purified IgG and IgM oxLDL antibodies from human sera, which allowed us to express the concentrations of antibodies in unknown samples in weight units. The assay was shown to be specific and to have an acceptable level of reliability. This, in our opinion, is the most accurate approach to these measurements, because it minimizes several pitfalls associated with the measurement of IgM and IgG antibodies in serum samples by enzymeimmunoassay,(26;40) such as the interference of variable concentrations of IgG and IgM antibodies of different affinities in serum samples, competing in their binding to immobilized modified LDL, possible nonspecific absorption of IC and immunoglobulin aggregates unrelated to modified LDL IC, and measurement of low affinity modified LDL antibodies which may have very limited pathogenic potential.

Our results showed that IgG was always the predominant isotype of oxLDL antibody in isolated circulating IC. The predominance of IgG antibodies in oxLDL IC is in total agreement with the predominance of the IgG isotype in circulating oxLDL antibodies isolated from the serum of human subjects.(6;7) The IgG subclass distribution in free oxLDL antibodies purified from diabetic patients and other groups of individuals is consistent in that that the sum of IgG1 and

IgG3 antibodies, with the highest pro-inflammatory potential, clearly exceeds the added concentrations of the less pro-inflammatory subclasses, IgG2 and IgG4.(7;8;11). Therefore, it would be logical to assume that, in humans, the possible protective effect of oxLDL IgM antibodies is unlikely to counterbalance the pathogenic properties of IgG antibodies.

The group of patients with type 1 diabetes included in this study differed by their renal function and notably by their albumin excretion rate, a parameter previously demonstrated to be related to the formation of oxLDL-IC.(35;36) The significant positive association between the levels of oxLDL antibodies of the IgG isotype in LDL-IC and serum creatinine, the also positive correlation between IgG antibody levels and AER, and the negative correlation between IgG antibodies and GFR suggest that high levels of IgG oxLDL in IC have a negative impact in renal function, and are totally in agreement with previous data obtained under less stringent conditions.(35;36). Interestingly, the data also suggest that in patients with end-stage renal disease where progression of nephropathy has reached its endpoint the linearity of the association of IgG antibody in immune complexes is lost. This is not surprising if we consider that marked impairment of renal function, as present in end-stage renal disease, is associated with depression of the immune system.(45–47) Furthermore, any correlation between IgG antibody contents of modified LDL IC and parameters of renal function is likely to be blurred in patients with end-stage renal disease. The group of patients included in this study included equal numbers of matched cases (with confirmed nephropathy) and controls. While systolic blood pressure and use of ACE inhibitors would be expected to differ in diabetic patients at different stages of the development of nephropathy, lipoprotein could be confounding variables, but a conditional logistic regression model with multiple predictors was not considered given the small sample size. The conditional regression analysis did account for the a priori matching of cases with controls. These analyses showed a clear increase in the odds of nephropathy in patients with higher IgG antibody levels, while IgM antibody concentrations and IgG:IgM antibody ratios failed to show a clear correlation with the odds of developing nephropathy. A protective role of IgM antibodies is not supported neither totally ruled out by our data. The protective role for IgM antibodies is logical, given their low affinity, predominant intravascular distribution, and lack of interaction with Fc receptors of phagocytic cells.(11; 48) Data supporting the protective role of IgM antibodies has been obtained in different mouse models, in which oxLDL antibodies are predominantly IgM.(37) Deliberate immunization of hypercholesterolemic mice with *Streptococcus pneumoniae* result in the synthesis of IgM antibodies reactive which phosphorylcholine epitopes shared with oxLDL, which seem to have a protective effect in relation to the development of atherosclerosis.(49) Immunization of both pregnant NZW rabbits and LDLr^(-/-) mice with oxLDL resulted immunization of the progeny, which responded with the synthesis of IgM antibodies and formation of IgM-oxLDL IC, which in turn appeared to have a protective effect against the development of atherosclerosis.(38)

The protective role of IgM oxLDL antibodies has also been proposed in humans, based on studies reporting a negative correlation between IgM MDA-LDL antibody levels and carotid intima-media thickness (IMT).(39) However, it has been reported that IgM antibodies to MDA-LDL correlate with a more rapid progression of carotid disease, as judged by IMT measurements,(41) and a single report on the effects of pneumococcal vaccination in humans failed to demonstrate the induction of circulating IgM antibodies to oxLDL.(50) In conclusion, our observations suggest that IgG oxLDL antibodies involved in LDL-IC formation play a significant pathogenic role in the development of diabetic nephropathy, in concordance with the results of previous studies focusing on the pathogenic role of LDL-IC in type 1 diabetes.(29;34–36;51–53) The protective role of complexed IgM antibodies could not be proven in this study, also agreeing with previous conflicting data concerning the role of IgM antibodies in humans.(39;41)

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Figure 1.

Graphic representation of the correlation between the levels of IgG oxLDL antibodies in isolated IC and measures of diabetic nephropathy: Panel A) serum creatinine concentrations ($r=0.37$, $p=0.032$, $n=34$); Panel B) albumin excretion rate. ($r=0.32$, $p=0.0691$, $n=34$); and MDRD GFR* ($r=-0.30$, $p=0.09$, $n=34$). The levels of two patients who had extreme nephropathy (GFR < 17 and serum creatinine > 5) and whose levels were highly influential points in the analysis were removed. No linear association was observed with these observations included in the analysis.

*MDRD GFR: Estimated Glomerular Filtration Rate using the formula developed by Levey et al.(54)

Table 1
Descriptive summary of T1DM nephropathy patients and controls.

Variable	T1DM Nephropathy Cases (n=18)		T1DM Nephropathy Controls (n=18)		p-value [§]
	M	SD	M	SD	
Age (yr)	44.8	5.5	42.5	7.3	0.29
Duration of Diabetes (yr)	21.1	4.3	21.0	4.7	0.92
HbA1c (% glycated)	8.97	1.96	7.64	1.07	0.02
Body mass index (kg/m2)	28.0	5.2	27.6	4.6	0.82
LDL (mg/dL)	137.2	32.7	115.5	22.2	0.03
HDL (mg/dL)	53.9	17.5	54.9	13.0	0.84
SBP (mm Hg)	141.6	18.9	118.6	10.8	<0.01
DBP (mm Hg)	79.5	8.1	75.1	8.4	0.12
Internal IMT EDIC Year 6	0.998	0.325	0.864	0.548	0.39
Common IMT EDIC Year 6	0.735	0.183	0.644	0.081	0.08
GFR [¶]	48.5	21.8	93.5	13.7	<0.01
AER [¶]	2503.4	2794.8	16.6	8.0	<0.01
ACE Inhibitor use (N, %)	11	65%	3	18%	0.01
DCCT Intensive Therapy (N, %)	4	22%	10	56%	0.08

[§]P-values determined by two-sample t-test for continuous variables and Fisher's exact test for categorical variables.

[¶]GFR and AER values reported as the minimum (GFR) or maximum (AER) value reported at the previous, current or 1 year post EDIC year.

[†]One observation missing.

[‡]Two observations missing.

Table 2
Descriptive summary of the mean anti-oxLDL IgG and IgM isolated PEG-precipitated immune complexes from T1DM nephropathy patients and controls.

Biomarker	T1DM Overall Sample (n=36)		T1DM Nephropathy Cases (n=18)		T1DM Nephropathy Controls (n=18)		Paired Difference (n=18 pairs)		p-value [†]
	M	SD	M	SD	M	SD	M	SD	
IgG ug/ml	23.82	15.59	26.75	15.89	20.88	15.16	5.87	14.72	0.090
IgM ug/ml	4.01	3.31	3.91	3.24	4.11	3.47	-0.20	4.42	0.702
IgG:IgM ratio	8.08	5.92	9.49	6.63	6.52	4.85	2.97	8.94	0.304
Percent IgM	17.04%	12.07%	14.16%	10.16%	19.92%	13.38%	-5.76%	17.90%	0.167

[†]P-values determined by Wilcoxon Signed Rank Test for paired data.