Antibodies to Oxidized LDL Predict Coronary Artery Disease in Type 1 Diabetes

A Nested Case-Control Study From the Pittsburgh Epidemiology of Diabetes Complications Study

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The pathogenesis of excess cardiovascular risk in type 1 diabetes is unclear. LDL cholesterol is only weakly predictive, and its concentration is often normal in type 1 diabetes. We therefore examined whether markers of LDL oxidation such as antibodies to oxidized LDL (Ab-OxLDL) and LDL-containing immune complexes, rather than LDL concentration, were predictive of coronary artery disease (CAD) in type 1 diabetes. This nested case-control study from an epidemiologic cohort study included 49 incident cases of myocardial infarction (MI), angina, or CAD death and 49 age-, sex-, and duration-matched control subjects. Ab-OxLDL was measured by enzyme immunoassay and the apolipoprotein B (ApoB) content of immune complexes (ApoB-IC) precipitated by polyethylene glycol by immunoelectrophoresis in baseline stored samples. Ab-OxLDL was inversely, and ApoB-IC directly, related to subsequent CAD. In multivariate analyses, Ab-OxLDL remained a significant independent predictor along with previously recognized predictors, hypertension and Beck depression score. In conclusion, oxidation of LDL and the immune response it elicits may play a role in predicting the development of CAD in type 1 diabetes and explain at least some of the enhanced CAD risk in type 1 diabetes. *Diabetes* 48: 1454–1458, 1999

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Ab-OxLDL, antibodies to oxidized LDL; ApoB, apolipoprotein B; ApoB-IC, apolipoprotein B content of precipitated immune complexes; BBS, borate buffer saline; BDI, Beck Depression Inventory; BSA, bovine serum albumin; CAD, coronary artery disease; EDC, Epidemiology of Diabetes Complications; IC, immune complex; MI, myocardial infarction; OD, optical density; OxLDL, oxidized LDL; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RR, relative risk; WHR, waist-to-hip ratio.

Type 1 diabetes is known to carry a greatly increased risk of coronary artery disease (CAD), often reaching 10-fold or greater (1–3). Until recently, however, little has been known about CAD risk factors in type 1 diabetes (4) apart from a strong association with renal disease (5) and hypertension (6). Recent prospective data from the Pittsburgh Epidemiology of Diabetes Complications (EDC) Study (7) have shown that the renal effect is largely a male phenomenon (a finding confirmed by the cross-sectional results of the Eurodiab Study [8]). Surprisingly, in neither of the latter studies were smoking status or glycemic control significant overall independent predictors (7–9). Nonetheless, high triglycerides, low HDL cholesterol, and hypertension (8) were all independent predictors in Eurodiab (8), while prospectively in men from the Pittsburgh EDC Study, hypertension and low HDL cholesterol were also predictive (7). For women in the Pittsburgh EDC, however, depressive symptomatology, increased waist-to-hip ratio (WHR), and decreased physical activity were independent predictors in addition to longer duration and hypertension.

Levels of these risk factors are not, however, greatly disturbed in type 1 diabetes (10), and are therefore unlikely to explain either the increased CAD risk or its earlier presentation. Focus has therefore turned to qualitative changes in lipoprotein metabolism rather than simply lipoprotein concentrations. It is thought that oxidation of the LDL cholesterol particle enhances its atherogenicity (11) and that, perhaps linked with the excess glycosylation seen in diabetes (12), this process is increased in type 1 diabetes and may explain some of the excess risk (13). To explore this possibility, a nested case-control study was conducted using stored samples from the Pittsburgh EDC Study.

RESEARCH DESIGN AND METHODS

The study population is derived from the Pittsburgh EDC Study, a prospective follow-up study of 658 childhood-onset type 1 diabetic subjects diagnosed between 1950 and 1980 and first seen as part of the EDC Study in 1986–1988 when their mean age was 28 years and mean duration of type 1 diabetes 20 years (14,15). In the subsequent 8 years of follow-up (up to 1996), 53 of the 635 subjects without CAD at baseline developed CAD, defined as myocardial infarction (MI) (n = 13, confirmed by Q waves on electrocardiogram or hospital records fulfilling standard criteria [16]), CAD death (n = 4, death certificate), or angina (n = 29, diagnosed by the EDC physician at one or more of the biennial examinations), and there were three cases of angiographically demonstrated CAD ≥50% stenosis. Absence of CAD at baseline was established by the absence of *1*) a history of MI, *2*) Q waves on electrocardiogram, and *3*) clinical diagnosis of angina by the EDC clinic physician. For each case, an age- (± 3 years), sex-, and duration- (± 3 years) matched control subject was identified. Matching was successful for 49 of the cases, giving a study sample of 98 (49 cases, 49 control subjects). Stored plasma samples from

baseline (n = 98), which had been frozen at –70°C since being drawn in 1986–1988, were used for both cases and control subjects. All case samples tested were thus drawn before the first onset of CAD. The interval between blood draw and CAD onset ranged from 6 to 116 months.

Samples were shipped on dry ice overnight from Pittsburgh to the Medical University of South Carolina in Charleston, where the antibody levels against oxidized LDL (Ab-OxLDL) were determined as well as the apolipoprotein B (ApoB) content of precipitated immune complexes (ApoB-IC). Samples were blinded as to case-control status.

Antibodies to modified LDL were determined by the previously reported competitive enzyme immunoassay for Ab-OxLDL (17), which differs from the more commonly used assays (using oxidized/native LDL ratios) by taking account of charge differences between oxidized and native LDL. In brief, flat-bottomed Immulon type 1 enzyme immunoassay plates (Dynatech, Chantilly, VA) were coated with 100 µl OxLDL (7.5 mg/l in 1 mol/l carbonate-bicarbonate buffer, pH 9.6). Serum calibrators, controls, and unknown samples were diluted 1/10 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and afterward separated into two 200-µl aliquots. One of the aliquots was absorbed with an equal volume of OxLDL (400 µg/ml in PBS-BSA), always using the same batch of OxLDL used to coat the plates in the absorption step. The other aliquot (unabsorbed) is mixed with an equal volume of PBS-BSA. The final dilution of these aliquots was 1/20. All samples (absorbed and unabsorbed) are incubated overnight at 4°C then spun at 1,700*g* in an Eppendorf centrifuge (model 5413) for 30 min. The supernatants of the centrifuged samples, unabsorbed and absorbed, were divided into two portions, one to be tested as it was absorbed (1:20 dilution) and the other one to be tested after being diluted 1:2 (final dilution 1:40). Peroxidase-conjugated rabbit anti-human IgG (IgG fraction), reacting with both IgG heavy and light chains (Cappel Organon Teknika, Durham, NC), was used to detect bound immunoglobulins, and 0.5 mmol/l 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Sigma Chemical, St. Louis, MO) was used as substrate. Absorbance was measured at 414 nm in a Vmax enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA). The optical density (OD) values used to calculate antibody concentrations in both standards and unknown samples were obtained by subtracting the OD values of the OxLDL-absorbed from those of the unabsorbed samples. Five serial dilutions of the IgG fraction of a polyclonal rabbit LDL antibody that reacts with both native and oxidized LDL were used to calibrate the assay. The concentration of the antibody in unknown samples represented the average of two determinations (1:20 and 1:40 dilutions) after correction by the corresponding dilution factor. Interassay coefficient of variation for Ab-OxLDL is 14% for low levels and 3.9% for medium to high levels.

Soluble immune complexes were obtained by precipitation with polyethylene glycol (PEG) 6000 (18,19). A freshly prepared solution containing 7% (wt/vol) PEG 6000 in borate buffer saline (BBS) (pH 8.4 and sterilized by filtration through a 0.22-µm filter) was slowly added, under constant mixing, to an equal volume of sera. The samples, containing a final PEG concentration of 3.5%, were incubated overnight at 4°C and then centrifuged at 3000 rpm for 20 min. The precipitates were washed once with 14 ml chilled 3.5% PEG in BBS, centrifuged again, gently resuspended in 2 ml Tyrode's solution (Ca^{2+}, Mg^{2+}), incubated for 15–20 min at 37°C, and resuspended once more. The ApoB content of IC was determined by quantitative immunoelectrophoresis (20). Interassay coefficient of variation for ApoB-IC was 11%. Except where noted, all clinical and other laboratory values were from the same (baseline) cycle of examination that provided the sample for the oxidized LDL determination.

Before clinic attendance, participants completed a number of questionnaires including a medical history and the Beck Depression Inventory (BDI) (21), a questionnaire documenting symptoms of depression in which a higher score indicates greater depressive symptomatology.

On arrival at the clinic in the fasted state, subjects underwent sitting blood pressure measurement with a random zero sphygmomanometer according to the Hypertension Detection and Follow-Up Program Protocol (22). The mean of the second and third blood pressures were used in the analyses. Hypertension was defined as a blood pressure of ≥140/90 mmHg or the use of blood pressure medications. Fasting blood samples were taken for a variety of measures as described below.

HbA_1 was originally determined with saline-incubated blood samples and microcolumn cation-exchange chromatography (Isolab, Akron, OH). On 26 October 1987, the HbA_1 technique was changed to high-performance liquid chromatography (Diamat; Bio-Rad, Hercules, CA). Extensive duplicate samples were run with both techniques, and no systematic differences were seen. Readings with the two methods were shown to be almost identical (r = 0.95; Diamat HbA_1 = –0.18 + 1.00 Isolab HbA_1). The absolute difference between the means of the two methods was 0.158 (% HbA_1). Serum cholesterol and triglyceride levels were determined enzymatically (23,24). HDL cholesterol was determined by a heparin and manganese procedure with modification of the Lipid Research Clinics methodology (25,26). LDL cholesterol was calculated with the Friedewald equation if triglycerides were <4.5 mmol/l (27). Two subjects had triglyceride values >4.5 mmol/l. This equation has previously been validated in our laboratory for type 1 diabetes subjects (28). ApoB was determined using a rocket immunoassay (29). Fibrinogen levels were performed via a biuret calorimetric procedure and a clotting method and creatinine with an autoanalyzer (Ectachem 700 xr; Kodak, Rochester, NY).

Three separate timed urine samples (a 24-h, an overnight, and a 4-h clinic) collected in the 2 weeks before examination were assayed for albumin and creatinine. Albumin was determined immunonephelometrically (30,31). Overt nephropathy was defined on the basis of *1*) the presence of renal failure (serum creatinine >440 µmol/l or on dialysis or status after renal transplant), *2*) increased albumin excretion rate (>200 µg/min in two of three timed urine samples), or *3*) in the absence of urine collections, a serum creatinine >180 µmol/l. Microalbuminuria was defined as an albumin excretion rate between 20 and 200 µg/min in two or three of the urine samples. In the 16 subjects with urine samples of questionable completeness, a previously validated albumin-creatinine urinary ratio was used, that is, 0.03–0.3 mg/mg for microalbuminuria and >0.3 for overt nephropathy (32).

Smoking was categorized as being a current cigarette smoker, an ex-smoker (has smoked at least 100 cigarettes but not currently a smoker), an ever-smoker (either of the first two categories), or a never-smoker.

Height was measured using a clinic stadiometer and weight using a balance beam scale. BMI was calculated as weight in kilograms divided by height in centimeters squared. Waist (measured at midpoint between iliac crest and costal margin in the midaxillary line) and hip (maximal gluteal) circumferences were measured in duplicate and their means used to calculate the WHR.

Statistical analysis. The distribution of each variable was examined for normality. Triglycerides were log-transformed to give a more normal distribution. Ab-OxLDL were not normally distributed (48% of samples had no detectable level); nor were the ApoB-IC concentrations. Log transformation (achieved by adding 1 to all values before log transformation, since some subjects had values of "0") improved the normality of both variables and was thus used in statistical testing. Ab-OxLDL were examined as both a categorical variable (yes/no) and a continuous variable. Paired t tests and the McNemar test for matched pairs were used to assess case-control differences univariately. Correlations are presented as Pearson (parametric) after log transformation when appropriate. Multivariate analyses, using Cox proportional hazard models, were examined using each of the significant (P < 0.01) univariate predictors. The model presented was that with the lowest log likelihood ratio and thus provided the best fit.

RESULTS

Table 1 presents the univariate comparisons for CAD risk factors between cases and control subjects. By design, the cases and control subjects had a similar mean age and duration of diabetes and sex distribution (all matching variables). In addition, although somewhat higher in CAD cases, no significant differences in mean LDL cholesterol, triglyceride, fibrinogen, or ApoB concentrations were seen. Mean HDL cholesterol was lower in CAD cases (P < 0.01). The proportions with hypertension or nephropathy were higher in the CAD cases (P < 0.01), but smoking status did not differ significantly. The level of Ab-OxLDL was, however, significantly lower in the CAD cases (P < 0.01), while ApoB-IC was significantly higher in the CAD cases (P < 0.05). The same pattern was seen for both MI/CAD cases and angina cases when analyzed separately (data not shown). Table 2 shows the correlations between Ab-OxLDL and ApoB-IC and the other CAD risk factors. The only significant correlations seen with Ab-OxLDL were a negative correlation with log triglycerides and a positive correlation with HDL cholesterol. ApoB-IC showed positive correlations with LDL cholesterol and total ApoB and a negative correlation with HDL.

Because of the strong association between CAD and renal disease, Ab-OxLDL and ApoB-IC concentrations in cases and control subjects were examined after stratification by whether the case had overt nephropathy, to see if the differences were independent of nephropathy (Table 3). As can be seen, Ab-OxLDL were lower in cases in both strata (in those both with and without nephropathy), whereas ApoB-IC was only higher in cases with nephropathy.

Multivariate analyses (Table 4) showed hypertension, BDI score, and log Ab-OxLDL to be the only significant independent predictors of CAD status when they and the other significant univariate predictors (HDL, nephropathy) were available. If Ab-OxLDL was not available, only hypertension and BDI score were predictors in the final model, which had a similar log likelihood ratio model, with hypertension having an increased (2.9) relative risk (RR).

TABLE 1
Baseline CAD risk factors by case-control status

	CAD cases	Control subjects
n	49	49
Male	51 (25)	51 (25)
Age (years)	34.0 ± 6.9	33.6 ± 6.9
Diabetes duration (years)	26.3 ± 6.3	26.0 ± 6.4
Hypertension†	42.9 (21)	14.3 (7)
Ever-smoker	62.5 (30)	46.9 (23)
Current smoker	16 (33)	13 (27)
LDL cholesterol (mg/dl)	130.3 ± 36.3	120.5 ± 43.2
HDL cholesterol (mg/dl)*	49.6 ± 12.6	57.2 ± 15.1
Triglyceride (mg/dl)*§	134.3 ± 182.0	109.6 ± 125.6
ApoB (mg/dl)	111.4 ± 28.1	104.0 ± 33.0
Fibrinogen (mg/dl)	325.9 ± 81.1	296.2 ± 105.9
BDI score*	10.0 ± 7.3	7.1 ± 6.2
Log albumin excretion rate (μg/min)†	5.0 ± 2.2	3.5 ± 1.9
Ab-OxLDL		
% >0.00†	43.0 ± 21	76.0 ± 37
ngEq/ml§†	2.5 ± 3.6	5.3 ± 4.7
ApoB-IC (ngEq/ml)†§	165.82 ± 98.9	120.7 ± 86.6
Overt nephropathy (%)‡§	53.1 ± 26	20.4 ± 10
WHR	0.84 ± 0.1	0.83 ± 0.1
HbA_1	10.1 ± 1.7	10.4 ± 2.3

Data are % (*n*) or means ± SD. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. §Statistical testing performed after log transformation.

TABLE 2
Correlations (*P* value) of antibodies to oxidized LDL and ApoB content of immune complex cholesterol with CAD risk factors

	Log Ab-OxLDL Pearson	Log ApoB-IC Pearson
Age (years)	–0.16 ± 0.23	0.19 ± 0.24
Diabetes duration (years)	–0.19 ± 0.14	0.14 ± 0.15
Blood pressure		
Systolic	–0.14 ± 0.29	–0.02 ± 0.86
Diastolic	–0.10 ± 0.45	0.01 ± 0.89
LDL cholesterol (mg/dl)	–0.07 ± 0.61	0.44 ± 0.00
HDL cholesterol (mg/dl)	0.29 ± 0.03	–0.22 ± 0.03
Log triglyceride (mg/dl)	–0.29 ± 0.03	0.14 ± 0.17
ApoB (mg/dl)	–0.19 ± 0.16	0.22 ± 0.03
Fibrinogen (mg/dl)	–0.07 ± 0.60	0.19 ± 0.06
BDI score	–0.06 ± 0.67	0.02 ± 0.82
HbA_1	–0.02 ± 0.85	0.02 ± 0.84
Number of cigarettes/day	–0.12 ± 0.38	0.03 ± 0.76
Log albumin excretion rate (μg/min)	–0.06 ± 0.63	0.16 ± 0.12
WHR	–0.17 ± 0.09	0.08 ± 0.45

Data are means ± SD.

DISCUSSION

These results provide, to our knowledge, the first prospective evidence that oxidized LDL may play a role in the development of CAD in type 1 diabetes and may thus help to explain the excess CAD seen in type 1 diabetes. It may, at first, seem surprising that decreased, and not increased, antibodies to oxidized LDL were seen in the CAD cases, since several groups have reported increased Ab-OxLDL in atherosclerosis (32–36), including two diabetes studies (37,38). This may reflect an increased consumption of antibody (Ab-OxLDL) in the formation of LDL immune complexes in the CAD cases, since our measure of Ab-OxLDL is of free, and not bound, antibody. It is thus postulated that a greater formation of immune complexes containing oxidized LDL and ApoB is seen in type 1 diabetes, which may be atherogenic by inducing foam cell formation and causing damage to the endothelium (39). Interestingly, the Physicians Health Study also report that antibodies to OxLDL are lower in diabetic subjects than nondiabetic subjects (40), whereas Festa et al. (41) recently showed decreased antibodies to oxidized LDL to be characteristic of type 1 diabetic subjects with microangiopathy. We have also previously reported an inverse correlation between OxLDL antibodies and the extent of atherosclerotic disease in a cross-sectional study involving a relatively small number of patients (17) and lower levels of OxLDL antibodies in a small series of type 1 diabetes (19). The reason for these negative correlations, we speculate, is a higher affinity in type 1 diabetes of antibodies for OxLDL and thus greater immune complex formation, leading to reduced free antibody levels. The

TABLE 3
Ab-OxLDL antibody concentrations and ApoB content of immune complex by case-control status stratified by nephropathy status of case

	CAD cases	Control subjects	*P* value
Case has no nephropathy (*n* = 23)			
Ab-OxLDL (% positive)	43 ± 10	86 ± 20	0.004
Ab-OxLDL (ngEq/ml)*	2.52 ± 3.3	5.4 ± 3.7	0.01
ApoB-IC (ngEq/ml)*	125.3 ± 54.0	123.6 ± 78.8	0.93
Case has nephropathy (*n* = 26)			
Ab-OxLDL (% positive)	42 ± 11	65 ± 17	0.08
Ab-OxLDL (ngEq/ml)*	2.6 ± 3.9	5.1 ± 5.6	0.06
ApoB-IC (ngEq/ml)*	201.7 ± 115.7	118.1 ± 94.5	0.006

Data are means ± SD. *Statistical testing performed after log transformation.

TABLE 4
Multivariate predictors of CAD from Cox model

Variable	Risk ratio (SD interval)	*P* value
Hypertension	2.6 (1.42–4.94)	0.002
BDI score (7)*	1.5 (1.45–1.54)	0.007
Log Ab-OxLDL (0.42)*	0.64 (0.52–0.76)	0.008

*Based on 1 SD interval. Variables available for modeling also included HDL cholesterol, BDI, log albumin excretion rate, and overt nephropathy.

finding that Ab-OxLDL and not the ApoB content of immune complexes content remained in the model may reflect that the assay of ApoB in PEG precipitates is inaccurate: PEG-precipitated IC may not totally resuspend and that may interfere with electrophoretic mobility and diffusion of LDL in the agargel when performing quantitative immunoelectrophoresis. It is interesting to note that in the current study, the ApoB-IC is raised only in cases with nephropathy, suggesting that excess IC formation itself maybe limited to this subgroup of subjects (and may relate to their specific excess risk). As Ab-OxLDL is lowered irrespective of renal status (and the percentage with any Ab-OxLDL), it is likely that oxidative modification per se is predictive irrespective of the absolute degree of IC formation.

The significance of these results is enhanced by the fact that previous analyses have shown significant, independent, predictive roles for hypertension, HDL cholesterol, BDI score, and WHR in this population (7). While these predictors are confirmed univariately, the finding that HDL and WHR do not feature in the final model raises the possibility that LDL oxidation may be linked to their final common pathway. However, they did not enter the final model when Ab-OxLDL was unavailable, while hypertension had an increased RR in such a model, suggesting that Ab-OxLDL and hypertension may be associated despite their low correlations (Table 2). It is also likely that other unmeasured factors may contribute to the development of CAD in type 1 diabetes.

Other evidence also suggests that LDL oxidation, as well as the characteristics of the antibodies formed against oxidized LDL (such as their affinity to Ox-LDL and their immunoglobulin isotype), may have significance in terms of atherosclerotic risk in diabetes. In vitro studies demonstrate that different forms of oxidized LDL have the ability to induce intracellular accumulation of cholesterol esters in macrophages and therefore lead to their transformation into foam cells (11,34) as well as the ability to induce the expression of cell adhesion molecules in endothelial cells, thus leading to abnormal endothelial cell–monocyte interactions (35,37).

The relatively few significant correlations of Ab-OxLDL and ApoB-IC are consistent with known CAD risk factors (triglycerides, albumin excretion rate, and ApoB). Interestingly, no relationship with glycemic control has been seen. This contrasts with Festa's study (41), in which a moderate inverse correlation ($r = -0.19$, $P < 0.01$) was seen for antibody levels to oxidized LDL (expressed as a ratio to antibodies against native LDL). The negative correlation of ApoB-IC with HDL and the positive correlation of Ab-OxLDL with HDL may both be consequences of the antioxidant properties attributed to this lipoprotein with a negative effect on the generation of Ox-LDL and on the immune response against it (38).

These analyses also confirm the importance of renal disease and hypertension in predicting CAD in type 1 diabetes. Ab-OxLDL appear to be largely independent of these two risk states (Table 2) which, previous analyses have suggested, are very interchangeable in terms of their predictive power for CAD (7,39). It can thus be speculated that the enhanced risk of CAD in type 1 diabetes has at least two mechanisms, one being by hypertension exacerbated by renal disease and the other related to lipid disturbances including oxidation and immune complex formation, which may play a role as a contributing factor for the development of renal disease (Table 3). Further links with hyperglycemia (beyond the promotion of oxidation), for example the formulation of AGE, may further explain the risk.

There are a number of limitations to the current study. First, the samples have been stored for up to 10 years before analyses. While this may possibly lead to inaccuracies, antibodies tend to be stable over time in frozen samples, particularly at –70°C. Furthermore, it is highly unlikely that storage would differentially affect either cases or control subjects. It should also be noted that storage time was similar for cases and control subjects, and the samples used had not been subjected to repeated thaw-and-freeze cycles.

Another potential limitation is the small sample size (n = 49 pairs). This should not lead to spurious results, as the available subjects show no obvious bias and represent virtually all of the eligible subjects. It will, however, diminish the ability to test for interactions and for detecting significant differences in risk factors with a smaller RR. Nonetheless, we have 80% power to detect for example a difference of HbA_1 of only 0.8% units (with $\alpha = 0.05$), a small but meaningful clinical difference.

In conclusion, we suggest that although these data need to be confirmed and extended, they do represent an exciting development. They suggest a potential qualitative aspect of lipoprotein metabolism that may help explain the remarkably increased atherogenicity of type 1 diabetes despite relatively "normal" lipoprotein concentrations.

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