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The Level of Autoantibodies against Oxidized LDL is not Associated with the Presence of Coronary Heart Disease or Diabetic Kidney Disease in Patients with Non-insulin-dependent Diabetes Mellitus

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Oxidation of low-density lipoprotein (LDL) may be an important factor in the development of diabetic macrovascular and renal complications. The level of autoantibodies against oxidized LDL (oxLDL-Ab) can be used as an index of LDL oxidation *in vivo*. The purpose of this study was to investigate the association between the level of oxLDL-Ab and the presence of coronary heart disease and renal dysfunction in patients with non-insulin-dependent diabetes mellitus (NIDDM). We determined the plasma levels of oxLDL-Ab in 46 NIDDM patients and 48 well matched non-diabetic control subjects. NIDDM patients had a moderately higher level of oxLDL-Ab than control subjects (0.083 ± 0.051 vs. 0.062 ± 0.045, $p = 0.04$). However, there was no difference in the level of oxLDL-Ab between subjects with and without coronary heart disease, and the level of oxLDL-Ab was not associated with indices of glomerular filtration rate or urinary albumin excretion.

Keywords: Coronary disease, diabetes mellitus, non-insulin-dependent, LDL cholesterol, lipoproteins, oxidative stress

Abbreviations: LDL, low-density lipoprotein; NIDDM, non-insulin-dependent diabetes mellitus; oxLDL-Ab, autoantibodies against oxidized LDL; CHD, coronary heart disease; OD, optical density unit

INTRODUCTION

Atherosclerotic vascular lesions occur earlier and more frequently in patients with non-insulin-dependent diabetes mellitus (NIDDM) than in non-diabetic controls.[1,2] Oxidation of low-density lipoprotein (LDL) is assumed to be a crucial

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step in the formation of atherosclerotic plaque in the vessel wall[3] and also contributes to the development of glomerulosclerosis.[4] Autoantibodies against oxidatively modified LDL have been considered as an index of LDL oxidation *in vivo* and antibodies against malondialdehyde-(MDA-) lysine, or 4-hydroxynonenal-lysine epitopes recognize material in the atherosclerotic lesions both in man and in rabbit.[5,6] The level of autoantibodies against MDA-modified LDL is also associated with the progression of carotid atherosclerosis[7] and has predicted myocardial infarction in non-diabetic population.[8]

The level of autoantibodies against oxidatively modified LDL and its relation to cardiovascular complications has been previously studied in NIDDM patients, but with inconsistent results.[9,10] To further examine the role of LDL oxidation in the development of coronary heart disease and also in diabetic kidney disease, we have studied the level of autoantibodies against copper oxidized LDL (oxLDL-Ab) in 46 NIDDM patients and 48 well matched non-diabetic controls.

TABLE I Demographic and biochemical characteristics of the study groups (mean ± SD)

	NIDDM patients	Control subjects
Gender (male/female)	26/20	24/24
Age (years)	66.4 ± 6.8	65.0 ± 7.6
Smokers % (*n*)	10.9 (5)	16.7 (8)
CHD at the time of study % (*n*)	50.0 (23)*	29.2 (14)
Duration of disease (years)	9.4 ± 0.8	—
BMI (kg/m^2)	30.6 ± 5.6*	27.6 ± 4.3
Fasting blood glucose (mM)	9.7 ± 2.6*	4.7 ± 0.6
HbA_1c (%)	8.2 ± 1.5*	5.6 ± 0.4
Serum fasting insulin (mU/l)	21.5 ± 14.4*	11.3 ± 7.2
Triglycerides (mM)	2.1 ± 1.1*	1.5 ± 0.8
Total cholesterol (mM)	5.2 ± 1.0*	5.9 ± 1.2
HDL cholesterol (mM)	1.0 ± 0.3*	1.2 ± 0.5
LDL cholesterol (mM)	3.2 ± 0.9*	4.0 ± 1.2
Blood pressure, syst/diast (mmHg)	167 ± 21*/87 ± 10	154 ± 22/88 ± 9
Glomerular filtration rate ($ml/min/1.73\,m^2$)	95.2 ± 24.1	87.4 ± 20.1
24-h UAE (mg)	56.9 ± 112.7	31.2 ± 79.2
Serum creatinine (mg/l)	78.9 ± 16.4	82.3 ± 15.6
24-h UAE 30–300 mg (*n*)	14*	1
24-h UAE > 300 mg (*n*)	2	2

*$p < 0.05$ between patients and controls by Student's *t*-test or by χ^2-test.

MATERIALS AND METHODS

Patients

One hundred and fifty recently diagnosed NIDDM outpatients at the municipal health care center and 150 non-diabetic attendants of the same facilities, matched for age and gender, were originally recruited in 1985.[11] The patients fulfilled the WHO diagnostic criteria of NIDDM.[12] Subjects with a serious disease (e.g. cancer) were excluded from both study groups. From these subjects, 46 NIDDM patients and 48 non-diabetic control subjects were chosen at random for this study among the subjects that were re-evaluated after nine years of follow-up. At the time of re-evaluation, 7 patients were on a diet treatment alone, 27 took oral antidiabetic treatment and 12 were on insulin. All subjects gave written informed consent. Demographic and biochemical characteristics of the study subjects are presented in Table I. This study was approved by the ethics committees of the Tampere University Hospital and the Health Care Center of Tampere.

Methods

Overt coronary heart disease (CHD) was defined as a prior myocardial infarction as evaluated by hospital records or by symptoms of angina pectoris or by the presence of one or more of the Minnesota codes[13] 1.1–1.3, 4.1–4.3, 5.1–5.3 or 7.1 in the ECG. The NIDDM patients who had microalbuminuria ($30\,mg/24\,h \leq$ urinary albumin excretion (UAE) $\leq 300\,mg/24\,h$, $n = 14$) or macroalbuminuria ($UAE > 300\,mg/24\,h$, $n = 2$) were recorded.

All biochemical measurements were made from samples collected at the time of the nine-year re-evaluation. Blood samples were drawn from the subjects after they had fasted overnight. Serum cholesterol and triglycerides were determined by the dry slide technique (Ektachem 700 analyzer, Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA). High-density lipoprotein (HDL) cholesterol was measured with the same technique after precipitation of LDL and very low-density lipoprotein (VLDL) with dextran sulphate/magnesium chloride.[14] Serum LDL was calculated with Friedewald's formula[15] in the subjects whose serum triglycerides were under 4.0 mM. Blood glucose, glycosylated hemoglobin (HbA_1c), serum creatinine and 24-h UAE were determined by routine laboratory methods. Serum fasting insulin was determined by radioimmunoassay with a sensitivity of 5 mU/l. The renal glomerular filtration rate was determined by the plasma clearance of ^{51}Cr-EDTA assessed by the single-injection method.[16] The body mass index (BMI) was calculated as body weight (kg)/height^2 (m^2). Cigarette smoking was assessed by questioning by a physician (yes/no for current smoking).

Antibodies against copper oxidized LDL were determined as has been described.[10] The antigens that were used in the assay were native LDL protected against oxidation by 0.27 mM EDTA/20 μM butylated hydroxytoluene (BHT) in phosphate buffered saline, and oxidized LDL produced from the LDL preparation by incubation for 24 h with 20 μM $CuSO_4$. LDL samples were prepared by ultracentrifugation of pooled plasma of ten donors. For enzyme-linked immunosorbent assay, polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μl of native and oxidized LDL antigen at 5 μg/ml for 16 h at 4°C. After removal of unbound antigen and washing of the wells the plates were saturated for 2 h at 4°C with 2% human serum albumin in PBS containing 20 μM BHT. Serum samples of 50 μl, diluted 1 : 50, were added to the wells and incubated overnight at 4°C. After washing the wells six times, a peroxidase conjugated rabbit anti-human monoclonal IgG antibody (Organon, USA, no. 55220 Cappel) was applied (dilution 1 : 4000 in PBS containing 0.27 mM EDTA, 20 μM BHT, 0.05% Tween) for 4 h at 4°C. Freshly made substrate (0.4 mg/ml o-phenylenediamine, Sigma Chemical Co, St. Louis, USA, 0.045% H_2O_2 in 100 mM acetate buffer, pH 5.0) was added and incubated for 5 min at room temperature. The reaction was terminated by adding 50 μl of 2 M H_2SO_4. The optical density (OD) was measured at 492 nm with a microplate reader (Multiskan MCC 340, Labsystems GmbH, Munich, Germany). The results were expressed as the mean OD values from duplicate measurements, and the antibody titer against oxidized LDL was calculated by subtracting the binding to native LDL (natLDL-Ab) from the binding to copper oxidized LDL.

Student's t-test and the χ^2-test were used to compare continuous and discontinuous variables, respectively, between groups. Pearson's correlation matrix was used for correlation analysis. All computations were carried out using Statistica 5.0 software package (Statsoft Inc., Tulsa, OK, USA). A p value < 0.05 was considered as statistically significant.

RESULTS

The level of oxLDL-Ab was somewhat higher in NIDDM patients (0.083 ± 0.051 OD) than in control subjects (0.062 ± 0.045 OD, $p = 0.04$). However, there were no significant correlations between the level of oxLDL-Ab and indices of blood glucose control, i.e., percentage of glycosylated hemoglobin and fasting level of blood glucose, in either of the study groups. The level of oxLDL-Ab did not also differ across the method of antidiabetic therapy of NIDDM patients. The level of oxLDL-Ab was not correlated with any of the serum lipid values.

The prevalence of CHD was higher in NIDDM patients than in controls, as expected (Table I). There were, however, no significant differences in

the level of oxLDL-Ab between subjects with and without CHD in NIDDM patients (0.081 ± 0.049 vs. 0.085 ± 0.054, respectively, $p = 0.79$) or control subjects (0.074 ± 0.041 vs. 0.058 ± 0.046, respectively, $p = 0.26$). The association of oxLDL-Ab and renal function was assessed only in NIDDM patients due to the low number of control subjects with impaired renal function. The level of oxLDL-Ab was not significantly correlated with serum creatinine ($r = 0.09$), 24-h UAE ($r = -0.08$) or glomerular filtration rate ($r = 0.08$). Consequently, there was no significant difference in the level of oxLDL-Ab between patients with UAE > 30 mg/24-h ($n = 16$, 0.097 ± 0.071) and those with normal UAE ($n = 30$, 0.075 ± 0.036, $p = 0.22$).

DISCUSSION

The oxidation of LDL in NIDDM may be enhanced by several mechanisms, including increased generation of free oxygen radicals, by a decrease in the antioxidant activity of plasma and tissues, and by various structural changes in LDL into an atherogenic direction.[17,18] In fact, Bellomo *et al.*[9] first reported an increase in the level of autoantibodies against oxidatively modified and glycated LDL in NIDDM patients. This finding was not, however, supported by the subsequent study of Uusitupa *et al.*[10] that reported an unchanged level of oxLDL-Ab compared to the control group at baseline examination and also after a ten-year follow-up. Thus, further studies exploring the role of oxLDL-Ab in NIDDM were indicated.

In the present study the level of oxLDL-Ab was moderately higher in NIDDM patients after nine years of duration of the disease than in non-diabetic matched controls. The statistical power of the difference between the mean levels of oxLDL-Ab (63% increase in NIDDM patients) was weakened by a high variance of the OD values. The level of oxLDL-Ab was not, however, associated with the presence of CHD or renal dysfunction, as assessed by multiple functional tests. These data are in agreement with the results of previous studies in NIDDM patients: The level of oxLDL-Ab has not differed between patients with and without vascular complications,[9] and has not predicted any cardiovascular event during a ten-year follow-up;[10] and in another study, no association was observed between the level of autoantibodies against MDA-modified LDL and the degree of albuminuria in patients with insulin-dependent diabetes mellitus (IDDM).[19] However, the present study is the first to thoroughly explore the hypothesized association between oxLDL-Ab and diabetic kidney disease in NIDDM patients.

There are several studies where increased lipid peroxidation has been associated with the presence of vascular complications in NIDDM.[20–22] Nevertheless, accumulating evidence suggests that the level of oxLDL-Ab, an index of LDL oxidation *in vivo*, may not be used as an indicator of athero- or glomerulosclerotic processes in diabetic patients. Although LDL oxidation may be increased in NIDDM patients, other factors may cover its role in the development of complications.

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