Autoantibodies Against Oxidized LDLs and Atherosclerosis in Type 2 Diabetes

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OBJECTIVE — The aim of our study was to examine, in type 2 diabetic patients, the relationship between autoantibodies against oxidatively modified LDL (oxLDL Ab) and two indexes of atherosclerosis, intimal-medial thickness of the common carotid artery (CCA-IMT), which reflects early atherosclerosis, and the ankle-brachial index (ABI), which reflects advanced atherosclerosis.

RESEARCH DESIGN AND METHODS — Thirty newly diagnosed type 2 diabetic patients, 30 type 2 diabetic patients with long duration of disease, and 56 control subjects were studied. To detect oxLDL Ab, the ImmunoLisa Anti-oxLDL Antibody ELISA was used. ABI was estimated at rest by strain-gauge plethysmography. Carotid B-mode imaging was performed on a high-resolution imaging system (ATL HDI 5000).

RESULTS — In patients with long duration of disease, IgG oxLDL Ab were significantly higher and ABI significantly lower compared with the other two groups. We found a correlation between IgG oxLDL Ab and CCA-IMT in all diabetic patients. A significant inverse correlation between IgG oxLDL Ab and ABI only in patients with long duration of disease was seen, demonstrating a close relationship between these autoantibodies and advanced atherosclerosis.

CONCLUSIONS — IgG OxLDL Ab may be markers of the advanced phase of the atherosclerotic process and the response of the immunological system to the oxLDL, which are present within atherosclerotic lesions.

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Macroangiopathy in diabetes consists mainly of an accelerated form of atherosclerosis. Despite the high prevalence of risk factors, no more than 25% of the excess cardiovascular risk in diabetes can be accounted for by known risk factors (1). Other factors such as the formation of advanced glycation end products (2–4), oxidative stress (5), thrombophylic state (6), and the presence of small dense LDL particles (7) may be involved. In vitro studies (8,9) have shown how oxidative LDL modifications are a prerequisite for macrophage uptake and their modification into foam cells. Oxidative LDL modifications render them immunogenic, and autoantibodies against oxidized LDL (oxLDL Ab), particularly against an oxLDL epitope such as malondialdehyde-lysine (10), are found in serum and atheromatous tissue (11–13). In contrast with mouse and rabbit oxLDL-specific Ab, human oxLDL-specific Ab are predominantly IgG (14). OxLDL Ab have been reported to predict the progression of carotid and coronary atherosclerosis (15–18). There are few and controversial cross-sectional or prospective studies (19,20) about the relationship between oxLDL Ab and atherosclerosis in type 2 diabetic patients, but these results do not consider the different degrees of atherosclerosis concerning type 2 diabetic patients. The aim of our study was to evaluate the relationship between oxLDL Ab and both intimal-medial thickness of the common carotid artery (CCA-IMT) and the ankle-brachial index (ABI) (since IMT is a good marker for the early phase, whereas low ABI reflects the advanced phase of atherosclerosis) (21) in newly diagnosed type 2 diabetic patients and those with long duration of disease.

RESEARCH DESIGN AND METHODS

— Two groups of type 2 diabetic patients, as defined by the American Diabetes Association (22), were evaluated: 30 newly diagnosed diabetic patients (group A) and 30 diabetic patients with long duration of disease (a mean disease duration of 10.6 ± 4.4 years; group B). They were recruited among those consecutively seen on an outpatient basis at the diabetes clinic, Department of Medical and Surgical Sciences, University of Padova. A total of 56 sex- and age-matched healthy normotensive individuals (group C) were also enrolled as control subjects (Table 1). A comprehensive medical examination was performed on all subjects, and a medical history check was also made to establish diabetes and the presence of any complications.

Nonsmoking subjects were defined as those who had never smoked or had stopped smoking at least 3 years before the beginning of the study. The prevalence of smokers was 30% in group A, 50% in group B, and 46% in group C

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Abbreviations: ABI, ankle-brachial index; CCA-IMT, intimal-medial thickness of the common carotid artery; FPG, fasting plasma glucose; oxLDL Ab, oxidatively modified LDL antibody.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Table 1—*Clinical and metabolic parameters of subjects under study*

	Group A	Group B	Group C
n	30	30	56
Age (years)	57 ± 10	63 ± 9	56 ± 8
Sex (men/women)	19/11	21/9	32/24
BMI (kg/m^2)	28.1 ± 5.5	27.4 ± 3.2	27.0 ± 3.7
Duration of diabetes (years)	—	10.6 ± 4.4	—
Smokers (%)	30	50	46
Chronic hypertension (%)	30	42	0
Systolic blood pressure (mmHg)	140 ± 18	140 ± 15	128 ± 18
Diastolic blood pressure (mmHg)	86 ± 9	82 ± 9	76 ± 10
FPG (mg/dl)	130 ± 25*	172 ± 58*	93 ± 10
HbA_{1c} (%)	7.1 ± 1.4†	7.2 ± 3.0†	5.7 ± 0.4
Total cholesterol (mg/dl)	217 ± 35	224 ± 30	213 ± 22
LDL cholesterol (mg/dl)	133 ± 39	133 ± 43	131 ± 19
HDL cholesterol (mg/dl)	42 ± 10	45 ± 10	43 ± 12
Triglycerides (mg/dl)	177 ± 117	153 ± 59	126 ± 83
LDL relative flotation	0.35 ± 0.05	0.35 ± 0.04	0.38 ± 0.03

Data are means ± SD, unless otherwise indicated. *$P < 0.001$ vs. group C; †$P < 0.0001$ vs. group C.

(Table 1). Thirty percent of group A and 42% of group B were taking antihypertensive drugs: ACE inhibitors (four subjects in group A and six in group B), a combination of ACE inhibitors with diuretics (four subjects in group A and six in group B), and a combination of ACE inhibitors with calcium-channel blockers (one subject in group A and one in group B.) Alcohol intake was similar in both diabetic patients and control subjects.

All diabetic patients were on a diet, while 7 patients in group A and 15 in group B were also taking oral hypoglycemic medications. Eleven of the 30 diabetic patients in group B were on multiple-dose insulin regimens (all on regular insulin). Three group A subjects and five group B subjects were taking statins. None of the type 2 diabetic patients were taking vitamins, antioxidants, or drugs directly affecting lipoprotein oxidation. All female subjects included in the study were postmenopausal, and none were on hormone replacement therapy. Informed consent was obtained from all subjects before the study.

Chemical analyses

All analyses were performed on blood samples drawn after an overnight fast of at least 12 h. Samples were immediately assayed. Fasting plasma glucose (FPG) was measured by the glucose-oxidase method (23) and HbA_{1c} by high-performance liquid chromatography (Bio-Rad Laboratories, Milan, Italy) (24). Total, LDL, and HDL cholesterol were measured by enzymatic analytical chemistry (CHOD-PAP method; Roche, Milan, Italy) (25,26), as was plasma triglycerides (GPO-PAP colorimetric enzyme test; Roche) (27).

LDL isolation

LDL particles were isolated from plasma by density gradient ultracentrifugation (28). One aliquot of plasma (1 ml), adjusted to a density of 1.080 g/ml (final volume 4 ml), was layered underneath 9 ml of a 1.006 g/ml NaCl solution, producing a discontinuous salt gradient in a Beckman VTi 65.1 vertical rotor (Beckman Instruments, Palo Alto, CA). Samples were centrifuged at 65,000 rpm for 70 min at 5°C, and the tubes were then fractionated (Fraction Recovery System; Beckman Instruments) from the bottom at a flow rate of 1.7 ml/min, and 37 fractions of 0.35 ml were collected. Total cholesterol was measured in each fraction. LDL relative flotation, a measure of LDL particle buoyancy, was determined by dividing the fraction number containing the highest LDL cholesterol concentration by the total number of collected fractions. Fractions belonging to the LDL lipoprotein peak were pooled and used for further determinations.

Detection of oxLDL Ab

To detect oxLDL Ab, the ImmunoLisa Anti-oxLDL Antibody ELISA (IMMCO Diagnostics, Buffalo, NY) was used. This test is performed as a solid-phase enzyme-linked immunosorbent assay. Results are expressed in enzyme units per milliliter (EU/ml) (29,30). The performance characteristics for this assay were 4.9% CV interassay and 3.5% CV intra-assay. Concentrations of serum IgG antibodies against oxLDL were determined by calibration curves (29). Some findings suggest that malondialdehyde-lysine in LDL is probably the most abundant molecular epitope recognized by autoantibodies of the IgG type directed against oxLDL (31).

ABI was estimated at rest by strain-gauge plethysmography, as described before (6). Carotid B-mode imaging was performed on a high-resolution imaging system (ATL HDI 5000). Common internal and external carotid arteries were scanned bilaterally in longitudinal projection. IMT was measured in the CCA on far walls. Characteristic patterns showed two parallel echogenic lines separated by a hypoechogenic space. IMT was defined as the distance from the leading edge of the first echogenic line to the leading edge of the second line. The first line represented luminal-intimal transition, the second medial-adventitial transition. Measurements of maximal IMT were performed in three (anterior, lateral, and posterolateral) longitudinal scans of the far wall on both right and left CCA, 1 cm below the bulb on the side free of plaques (32). CCA-IMT was calculated as the mean of maximal IMT (33). Video-recorded frozen images were analyzed offline by a computerized analyzing system along a 10-mm-long section just proximal to the carotid bulb. The computer program calculated the maximal values of intima-media thickness.

The presence of atherosclerotic lesions (focal plaques with >1.5 mm thickening) was assessed on both right and left internal carotid arteries. The variation coefficient was 6.5 and 5% for inter- and intraobserver variability, respectively.

Statistical analysis

To compare mean values among quantitative variables, the nonparametric Mann-Whitney test was used when two groups were analyzed and the Kruskal-Wallis was used in the case of three groups. To analyze correlations between quantitative variables among different groups of subjects (A = new diagnosis, B = long duration of diabetes, C = control subjects, A+B, A+B+C), Spearman's nonparametric correlation coefficients were ap-

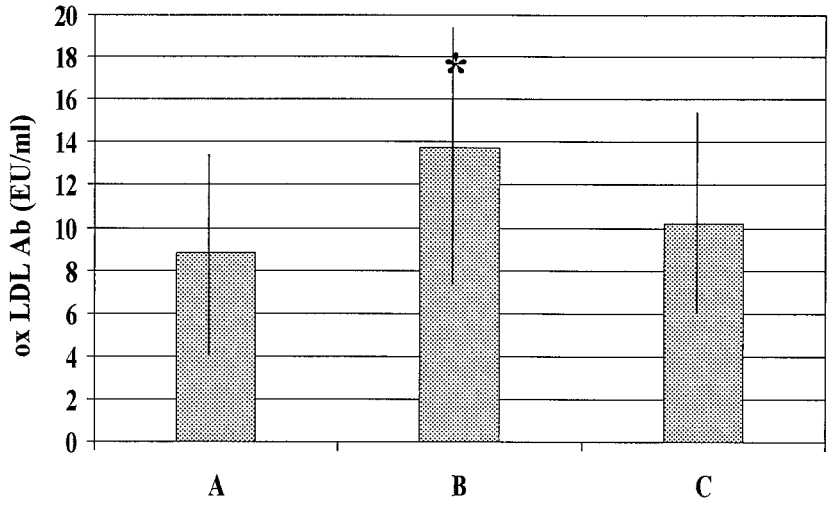

Figure 1—*Mean concentration ± SD of autoantobodies against oxLDL Ab in newly diagnosed type 2 diabetic patients (30 subjects; group A), diabetic patients with long duration of disease (30 subjects; group B), and control subjects (56 subjects; group C) (8.8 ± 5.0 EU/ml, 13.7 ± 6.0 EU/ml, and 10.2 ± 4.6 EU/ml, respectively).* *P = *0.0006 vs. groups A and C.*

plied. To identify the confounding effect of smoking, hypertension, and lipid levels, we calculated Spearman's correlation coefficients of the investigated variables among smokers (ρ_s) and among nonsmokers (ρ_s) and then among hypertensive (ρ_h) and among nonhypertensive (ρ_h) individuals. We then performed a statistical test of hypothesis to compare Spearman's correlation coefficients with the confounding variables ($_sH_0$: $\rho_s = \rho_s$; $_hH_0$: $\rho_h = \rho_h$). When the test indicated that there were no significant differences referable to potential confounding variables, we calculated a single correlation coefficient. Instead, when the test for comparison of correlation coefficients indicated that there were confounding effects of smoking, hypertension, and lipid levels, we constructed correlation coefficients specific for differing subgroups defined by the confounding variable. Statistical analyses were performed using the SAS program (34).

RESULTS — The main clinical features and metabolic parameters (means ± SD) in diabetic patients and control subjects are shown in Table 1. Levels of oxLDL Ab were significantly higher in group B compared with group A and control subjects (13.7 ± 6.0 vs. 8.8 ± 5.0 and 10.2 ± 4.6 EU/ml, respectively; $P = 0.0006$; Fig. 1), even after adjustment for hypertension and smoking. Only in group B was there a significant negative correlation between oxLDL Ab and ABI ($\rho = -0.71$; $P < 0.0001$) (Fig. 2). In all diabetic patients, a weak correlation was seen between oxLDL Ab and CCA-IMT ($\rho = 0.31$; $P = 0.04$). These associations persisted after adjustment for cigarette smoking, hypertension, and lipid levels. Statistical analyses of correlations between concentrations of oxLDL Ab and duration of diabetes, FPG, HbA_{1c}, BMI, lipid profiles, cigarette smoking, and hypertension were also performed, but no significant relationships were found.

As indicated by LDL relative flotation, the LDL particles of our diabetic subjects were slightly smaller and denser than those of control subjects, but this difference was not statistically significant. Both groups A and B had significantly greater CCA-IMT ($P < 0.0001$) than control subjects. Only in group B was there a higher prevalence of plaques found at the internal carotid arteries (33%), with respect to the other two groups. ABI was significantly lower in group B than in the other two groups, and this result was not influenced by hypertension or smoking (Table 2). FPG was significantly related to CCA-IMT in diabetic patients and in all subjects participating in the study ($\rho = 0.40$; $P = 0.008$ and $\rho = 0.51$; $P = 0.0001$, respectively). No relationship was observed between FPG and ABI in any group. HbA_{1c} was inversely related to ABI in group B ($\rho = -0.83$; $P = 0.001$), but this relationship was not confirmed when taking hypertension into account. ABI was strongly inversely associated with CCA-IMT only in group B ($\rho = -0.60$; $P = 0.0005$) (Fig. 3), even after adjustment for smoking, hypertension, and lipid levels.

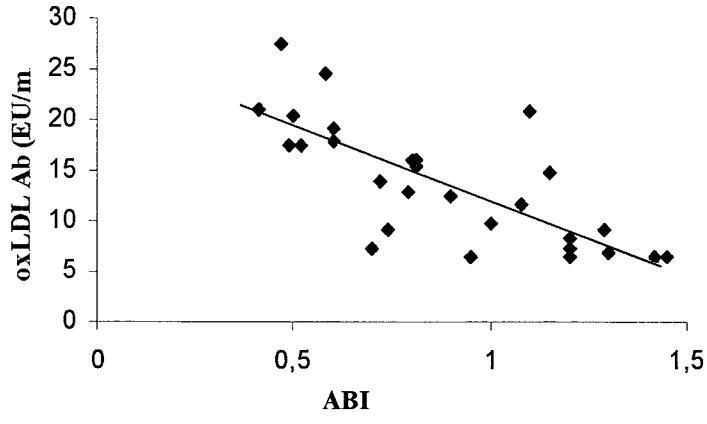

Figure 2—*Relationship between autoantibodies against oxLDL Ab and ABI in diabetic subjects with long duration of disease (ρ = −0.71;* P = *0.0001).*

CONCLUSIONS — The role of the humoral immune response to oxLDL in atherogenesis is unclear, and available studies are contradictory. In patients with diabetes, the presence in the serum of oxLDL Ab has been reported by several investigators. However, there is disagreement among different authors since some studies reported higher levels of oxLDL Ab in both type 1 and type 2 diabetic patients than in control subjects (19,35); other investigators have found comparable levels of these antibodies in type 1 as well as type 2 diabetic patients (20,36, 37). In another study, not significant differences in the levels of free-circulating oxLDL Ab in the diabetic patient group compared with patients with coronary artery disease and control groups have been recorded (38). Our report supports the

Table 2 —*Maximal CCA-IMT, presence of plaques at internal carotid arteries, and ABI in newly diagnosed diabetic patients (group A), in those with long duration of disease (group B), and in normal subjects (group C)*

	Group A	Group B	Group C
n	30	30	56
CCA-IMT (mm)	1.04 ± 0.20*	1.13 ± 0.24*	0.86 ± 0.12
Plaques at internal carotid arteries (%)	7	33	0
ABI	1.11 ± 0.14	0.86 ± 0.29†	1.09 ± 0.12

Data are means ± SD, unless otherwise indicated. *$P < 0.0001$ vs. group C; †$P = 0.003$ vs. groups A and C.

hypothesis that the humoral immune response could play a different role in different stages of the development of atherosclerosis, in the presence of various other risk factors. Two indicators of macrovascular disease, to evaluate different stages of atherosclerosis, were used: IMT (early atherosclerosis) and ABI (lower ABI represents advanced atherosclerosis) in two groups of newly diagnosed type 2 diabetic patients and with long duration of disease, with particular attention to the relationship between the values of these parameters and oxLDL Ab. IgG oxLDL Ab concentrations were similar in both newly diagnosed type 2 diabetic patients and in control subjects, indicating that type 2 diabetes in itself is not responsible for the increased production of this type of autoantibodies. Instead, high levels of IgG oxLDL Ab were found in diabetic patients with long duration of disease and advanced atherosclerosis, and a significant inverse correlation was seen between oxLDL Ab and ABI only in this group of patients (group B).

In accordance with reports in the literature (39), we found a greater CCA-IMT in all diabetic patients compared with control subjects. Conversely, signs of advanced atherosclerosis, such as lower ABI and higher prevalence of plaques in internal carotid arteries, prevailed in diabetic patients with long duration of disease, and only in this group did we find a significant inverse correlation between CCA-IMT and ABI in accordance with the ARIC Study (21), independently of other vascular risk factors examined. This evidence indicates that, in diabetic patients, after 10 years of disease, thickness of CCA and ABI are associated. The correlation between CCA-IMT and oxLDL Ab in diabetic patients pooled together, but not in diabetic groups evaluated separately, showed that CCA-IMT is not closely related to the different concentrations of oxLDL Ab found in the two groups of diabetic patients. This result was probably influenced by the fact that group B has higher CCA-IMT than group A, although this difference is not significant, and the same group has higher oxLDL Ab titers. The correlation between CCA-IMT and FPG in diabetic patients and in all subjects suggests the role of glyco-oxidative stress only in the early steps of atherosclerosis in type 2 diabetic patients, while IgG oxLDL Ab are produced as a result of the oxidative biological processes involving LDL particles within advanced (but not early) atherosclerotic lesions, later becoming potential markers of the advanced phase of the atherosclerotic process. According to Maggi et al. (31), our findings might indicate that autoantibody levels represent an epiphenomenon of the antigenic induction of LDL peroxidation, which is caused by inflammatory cells present within atherosclerotic lesions. Some authors (7) have demonstrated that small dense LDL particles are more susceptible to oxidative modifications. In our subjects, LDL relative flotation (which is an index of LDL size and density) was not related to oxLDL Ab, thus indicating that this type of autoantibody is not associated with the physical properties of LDL. Moreover, oxLDL Ab were not correlated with plasma lipid concentration, diabetes duration, or other metabolic parameters, indicating that production of oxLDL Ab is not affected by these factors. However, other causes of heightened oxidation or reduction in defenses, such as a low paraoxonase activity, in diabetes should also be considered (40,41).

In conclusion, our results contribute to the hypothesis that the oxLDL Ab concentrations in diabetic patients are not associated with the early stages of atherosclerosis (as identified by CCA-IMT), but they are associated with the presence of advanced lesions (as identified by a low ABI).

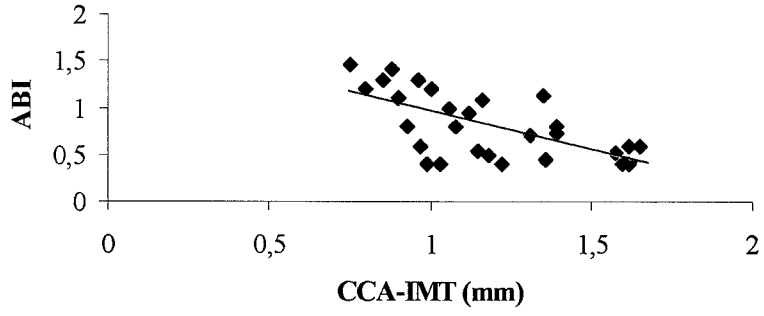

Figure 3—*Relationship between ABI and CCA-IMT in diabetic patients with long duration of disease ($\rho = -0.60$; P = 0.0005).*

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