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Electronegative low density lipoprotein subform (LDL^-) is increased in type 2 (non-insulin-dependent) microalbuminuric diabetic patients and is closely associated with LDL susceptibility to oxidation

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Abstract There is increasing evidence that diabetes mellitus is characterized by an enhanced lipoprotein oxidation. We have therefore investigated whether a relationship exists between LDL oxidation and microalbuminuria, which is considered an early marker of vascular involvement in type 2 diabetic patients. We selected 12 microalbuminuric and 12 normoalbuminuric type 2 diabetic patients, and 12 control subjects comparable for age, sex and blood pressure values. Oxidatively modified plasma LDL, referred as LDL^-, were measured by ion-exchange HPLC. In vitro susceptibility to oxidation of LDL was evaluated by following the kinetics of conjugated diene formation in the presence of Cu^{++} ions (lag-phase time). Microalbuminuric diabetic patients had a less satisfactory metabolic control and showed a higher plasma triglyceride concentration than both normoalbuminuric diabetic patients (2.21±1.01 vs 1.15±0.39 mmol/l, $P<0.01$) and controls (1.18±0.61 mmol/l, $P<0.01$). The percentage of LDL^- in plasma was significantly increased in microalbuminuric diabetic patients in comparison with both normoalbuminuric diabetic patients (5.24±1.67 vs 3.13±1.22%, $P<0.01$) and controls (2.34±1.03%, $P<0.001$). LDL isolated from microalbuminuric diabetic patients had a significantly shorter lag-phase time in comparison with normoalbuminuric diabetic patients (79±11 vs 97±10 min, $P<0.05$) and controls (120±24 min, $P<0.001$). In diabetic patients a significant linear correlation was observed between the percentage of LDL^- and amount of fructosamine ($r=0.45$, $P<0.05$), HbA_{1c} ($r=0.41$, $P<0.05$), and triglycerides ($r=0.65$, $P<0.001$). An inverse correlation was found between lag-phase time and fructosamine ($r=-0.5$, $P<0.01$) and triglycerides ($r=-0.59$, $P<0.001$). This study shows that microalbuminuric type 2 diabetic patients had evidence of increased LDL oxidation, which seems to be mainly due to a poor metabolic control and a more atherogenic lipid profile.

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Introduction

Epidemiological studies have shown that mortality due to vascular disease is at least doubled in diabetic subjects compared to the general population [1, 2]. Alteration of lipid profile [3], hyperinsulinaemia [4] and hyperglycaemia [5] have been advocated as possible explanations, but the pathogenetic steps of this accelerated atherosclerotic process in diabetic patients are not clear and need to be investigated further.

Oxidative modification of LDL is widely reported to be a contributing factor in the development of atherosclerosis [6, 7]. There is evidence that diabetes is accompanied by enhanced lipoprotein oxidation [8, 9]. Plasma lipid peroxides were found to be particularly elevated in patients with poorly controlled diabetes and macroangiopathy [10] but no increase above normal levels was noted in diabetic patients without vascular complications [11]. Recently, some authors have reported a significant increase of LDL oxidation, evaluated as LDL^- concentration, in diabetic patients in poor metabolic control [12]. This electronegatively charged LDL is an in vivo mildly oxidatively modified lipoprotein, identified in normolipaemic human plasma characterized by an increased content of conjugated dienes and malondialdehyde (MDA) and a decreased vitamin E content [13, 14]. Furthermore LDL^- exhibits cytotoxic effects on endothelial cells, not seen with native LDL alone [15], and might constitute a pro-oxidant state facilitating oxidative reactions in vascular components [16]. Microalbuminuria has been found to be a predictor of cardiovascular mortality in patients with type 1 [17] and type 2 [18] diabetes mellitus and even in non-diabetic subjects [19]. Moreover, microalbuminuria has been found to be associated with an increase of plasma lipid oxidation, evaluated as MDA plasma concentration, in type 2 diabetic patients [20].

Table 1 Clinical characteristics of type 2 diabetic patients and control subjects. Values are mean + SD

	Type 2 diabetic patients without microalbuminuria (n = 12)	Type 2 diabetic patients with microalbuminuria (n = 12)	Control subjects (n = 12)
Male/female	6/6	6/6	6/6
Diabetes duration (years)	15±6	17±9	–
Age (years)	65±9	64±7	65±5
Smokers/non-smokers	4/8	4/8	3/9
Body mass index (kg/m^2)	28.5±6	28.9±4.3	24.7±3.2*
Waist-hip ratio	0.90±0.07	0.93±0.06	0.86±0.05*
Systolic blood pressure (mmHg)	147±19	150±17	145±18
Diastolic blood pressure (mmHg)	90±9	90±10	88±9
Haemoglobin A_{1c} (%)	6.4±1.6	7.8±1.6**	–
Fructosamine (mmol/l)	279±53	341±71***	–
Urine albumin excretion (mg/24 h)	14.6±3.1	84.5±35.9****	–

* $P<0.05$ vs type 2 diabetic patients; ** $P<0.05$ vs type 2 diabetic patients without microalbuminuria; *** $P<0.02$ vs type 2 diabetic patients without microalbuminuria; **** $P<0.01$ vs type 2 diabetic patients without microalbuminuria

The purpose of this study was to evaluate whether there was any association between microalbuminuria, which is considered to be an early marker of macrovascular complications, and both LDL$^-$ concentration and LDL susceptibility to in vitro oxidation in a group of type 2 diabetic patients without clinical evidence of macroangiopathy.

Subjects, materials and methods

Subjects were recruited from patients regularly attending the diabetes department at Venice Regional Hospital. We selected 24 patients (12 male and 12 female) suffering from type 2 diabetes, defined according to National Diabetes Data Group criteria [21], with a mean age of 65±8 (range 40–76 years): 12 of them were microalbuminuric and 12 were normoalbuminuric. Twelve healthy, non-diabetic subjects, attending the health centre of the same hospital (6 men and 6 women, mean age 65±5 years, range 45–72 years) formed the control group. The three groups were comparable for age, sex, blood pressure values; clinical and anthropometric values of all subjects are reported in Table 1. All patients underwent a complete medical history and physical examination specific for diabetes-related sequelae. Diabetes was treated by diet alone in 4 patients, by oral hypoglycaemic agents in 12 and by a combination of hypoglycaemic agents and insulin in 8, with no significant differences between microalbuminuric and normoalbuminuric diabetic patients. Neither patients nor controls had any sign of inflammation or infection. All female diabetic patients were postmenopausal and none were on hormone replacement therapy. There were 4 (33%) current smokers in the microalbuminuric diabetic group, 4 (33%) in the normoalbuminuric diabetic group, and 3 (25%) in the control group (Table 1). Neither patients nor controls were treated with lipid lowering drugs, beta-blockers, thiazide diuretics, or antioxidant vitamin supplements. Patients with clinical evidence of macroangiopathy (coronary artery disease, carotid and lower limb atherosclerotic lesions) were excluded from the study.

We collected three non-consecutive 24-h urine specimens to measure urine albumin excretion (UAE). UAE rate was evaluated by nephelometric method and microalbuminuria was defined as a UAE rate of 30–300 mg per 24 h. After overnight fasting blood samples were taken by venipuncture. Fructosamine was evaluated by spectrophotometry and HbA_{1c} by high performance liquid chromography (HPLC). Total cholesterol (TC) and triglyceride (TG) concentrations were determined enzymatically with specific test kits from Menarini (Milano, Italy). Proteins were evaluated using the method of Lowry et al. [22] with human albumin as the standard. HDL cholesterol (HDL-C) was measured according to Kostner et al. [23]. LDL were separated by sequential preparative ultracentrifugation. Vitamin E was measured in both fresh plasma and freshly separated LDL by HPLC using a fluorescence detector set at an excitation of 292 nm and an emission of 335 nm [24]. MDA was measured as thiobarbituric reacting substance of lipid peroxidation by a fluorimetric method, according to Yagi [25].

Total plasma LDL were separated into unmodified LDL (nLDL) and oxidatively modified LDL (LDL$^-$) by ion exchange HPLC in an MA-7Q column according to Cazzolato et al. [26]. The eluate was monitored by ultraviolet detection at 280 nm and the areas of the peaks were integrated. LDL$^-$ concentration was expressed as a percentage of total LDL. The coefficient of variation of the method was less than 5%. The in vitro oxidability of LDL was assessed by a spectrophotometric technique according to Esterbauer [27]. LDL samples were diluted, in the presence of 2.5 mmol/l of Cu^{++}, to a final cholesterol concentration of 250 mg/ml. Oxidation kinetics were monitored by changes in 234 absorbance on a Beckman DU spectrophotometer equipped with a six-position automatic sample changer. The lag-phase time was defined as the interval between initiation of oxidation (time 0) and the intercept of the tangent of the slope of absorbance during the propagation phase.

The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All the subjects gave their informed consent prior to their inclusion in the study.

Results are expressed as mean±standard deviation (SD). Comparisons between type 2 diabetic patients and controls were adjusted for BMI. A non-parametric test was used to evaluate the difference in plasma biochemical parameters in healthy subjects and diabetic patients. P-values less than 0.05 (two-tailed) were considered significant. Correlation coefficients were determined by linear regression analysis. Covariance analysis was performed.

Results

There were no statistical differences in plasma total cholesterol between diabetic patients, with and without microalbuminuria, and controls. Microalbuminuric diabetic patients showed a significantly lower HDL-C plasma value than with normoalbuminuric diabetic patients and controls ($P<0.05$ for both) (Table 2) without a significant difference between diabetic males and females (1.21±0.15 vs 1.30±0.34 mmol/l). Microalbuminuric diabetic patients had triglyceride levels significantly higher than normoalbuminuric diabetic patients and control subjects ($P<0.01$ for both), without a significant difference between diabetic

Table 2 Plasma lipids in type 2 diabetic patients and control subjects. Values are mean + SD

	Type 2 diabetes without microalbuminuria (n = 12)	Type 2 diabetes with microalbuminuria (n = 12)	Control subjects (n = 12)
Total Chol (mmol/l)	5.65 ± 1.48	6.35 ± 1.01	5.97 ± 0.99
HDL-Chol (mmol/l)	1.35 ± 0.30	1.11 ± 0.14 *	1.38 ± 0.29
LDL-Chol (mmol/l)	3.80 ± 0.92	4.20 ± 0.81	4.00 ± 0.90
Triglycerides (mmol/l)	1.15 ± 0.39	2.21 ± 1.01**	1.18 ± 0.61

* $P < 0.05$ vs both normoalbuminuric type 2 diabetic patients and control subjects; ** $P < 0.01$ vs both normoalbuminuric type 2 diabetic patients and control subjects

males and females (1.5 ± 0.9 vs 1.4 ± 0.7 mmol/l). Diabetic patients had a significantly ($P < 0.05$) lower LDL vitamin E, but not plasma vitamin E concentration, than controls, regardless of microalbuminuria, while no differences were detected in MDA levels in both plasma and isolated LDL in the three groups (Table 3). There were no significant differences in plasma and LDL vitamin E and MDA levels between diabetic males and females. Microalbuminuric diabetic patients had a significantly higher percentage of LDL^- in comparison with both normoalbuminuric diabetic patients ($P < 0.01$) and controls ($P < 0.001$), without significant differences between diabetic males and females (3.85 ± 1.06 vs 3.9 ± 1.8%). LDL isolated from microalbuminuric diabetic patients had a significantly shorter lag-phase, when exposed to Cu^{++} ions than that from normoalbuminuric diabetic patients ($P < 0.05$), and controls ($P < 0.001$) (Table 3) without significant differences between males and females (88 ± 14 vs 94 ± 11 min). The above parameters were not evaluated for smokers as the number of patients was too small. No correlation was found between rate of UAE and both the parameters of metabolic control and lipid oxidation. In diabetic patients a significant linear correlation was observed between LDL^- percentage and some parameters of metabolic control such as fructosamine ($r = 0.45$, $P < 0.05$) and HbA_{1c} ($r = 0.41$, $P < 0.05$), while an inverse correlation was found between lag-phase time and fructosamine ($r = -0.5$, $P < 0.01$). A significant positive correlation was observed in diabetic patients between the percentage of LDL^- and triglycerides ($r = 0.65$, $P < 0.001$), while an inverse relationship was found between triglycerides and lag-phase time ($r = -0.59$, $P < 0.001$). Finally a very significant inverse correlation was present between LDL^- percentage and lag-phase time ($r = -0.69$, $P < 0.001$). Covariance analysis did not show a significant effect of microalbuminuria in explaining either the increase of LDL^- or the reduction of lag-phase, even if triglycerides determined a difference in behaviour in the two groups of diabetic patients (LDL^- $F = 4.97$, $P = 0.39$ and lag-phase time $F = 6.31$, $P = 0.023$).

Discussion

Qualitative modifications of LDL such as oxidation [6, 7] are known to contribute to the development of atherosclerotic vascular damage. Diabetes itself is an important condition of oxidative stress [2] and several studies have shown increased lipoprotein oxidation in diabetic patients [8–10]. However, the interpretation of the previous studies is complicated by the high rate of vascular disease in the evaluated patients. Therefore, we evaluated in a group of type 2 diabetic patients without clinical evidence of macroangiopathy, whether there was any relationship between LDL oxidation and microalbuminuria, which is considered an early marker of vascular complications in diabetic [17, 18] and non-diabetic patients [19]. Diabetic patients with microalbuminuria showed a higher percentage of LDL^-, and LDL isolated from these patients, presented a significant increase in susceptibility to oxidation. However, we did not find a significant relationship between UAE rate and the parameters of in vivo and in vitro LDL oxidation. The most likely explanation for the increased LDL oxidation in microalbuminuric type 2 diabetic patients is that such patients were in poorer metabolic control than those without microalbuminuria. This confirms that both lipoprotein oxidation [9] and microalbuminuria [28] are strongly associated with poor metabolic control. On the other hand, the absence of correlation between LDL oxidation and UAE rate suggests that other pathways may account for the increased LDL oxidation in these patients, particularly the higher triglyceride and the lower HDL-C plasma concentration. It is well-known that this atherogenic lipid profile is associated with

Table 3 Plasma and LDL concentrations of MDA and vitamin E; LDL^- concentration and lag-phase time in type 2 diabetic patients and control subjects. Values are mean + SD

	Type 2 diabetics without microalbuminuria (n = 12)	Type 2 diabetics with microalbuminuria (n = 12)	Control subjects (n = 12)
MDA (μmol/l)	3.45 ± 0.57	3.60 ± 0.61	3.23 ± 1.13
MDA (LDL) (M/M LDL)	0.27 ± 0.07	0.32 ± 0.10	0.29 ± 0.09
Vitamin E (mmol/l)	19.04 ± 4.02	18.9 ± 3.86	20.04 ± 3.45
Vitamin E (LDL) (M/M LDL)	6.25 ± 1.27	5.33 ± 1.22	8.01 ± 3.03*
LDL^- %	3.13 ± 1.22	5.24 ± 1.67**	2.34 ± 1.03
Lag-phase (min)	97 ± 10***	79 ± 11****	120 ± 24

* $P < 0.05$ vs diabetic patients; ** $P < 0.001$ vs control subjects and $P < 0.01$ vs type 2 diabetic patients without microalbuminuria; *** $P < 0.01$ vs control subjects; **** $P < 0.001$ vs control subjects and $P < 0.05$ vs type 2 diabetic patients without microalbuminuria

an increase of the percentage of small and dense LDL [29] which are more readily oxidised and are more atherogenetic [30]. This observation is supported by the fact that we found a strong relationship between hypertriglyceridaemia and both LDL^- concentration and in vitro LDL oxidability in diabetic patients.

Finally, the significant increase of in vitro oxidazability of LDL in microalbuminuric diabetic patients might also be partly explained by the observed reduction of LDL vitamin E concentration and by the increased LDL^- percentage. In fact it is well-known that vitamin E supplementation does protect LDL from oxidation even in type 2 diabetic patients [31], and recently we have observed that the rate of lipid peroxidation and the length of the antioxidant protected phase (lag-phase time) largely depended upon the LDL^- percentage of total LDL [16].

In conclusion, the data of this study show that microalbuminuric type 2 diabetic patients have evidence of increased LDL oxidation, and this seems to be mainly due to a poor metabolic control and a more atherogenetic lipid profile. However, it is likely that the elevated LDL^- levels observed in such patients are an expression of a pro-oxidant state and could facilitate oxidative reactions in vascular components, leading to vascular damage. Further studies with a larger data set are needed to evaluate whether a better metabolic control could reduce oxidative stress and lipoprotein oxidation independently of the presence of microalbuminuria.

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