Free Radical Activity and Hemostatic Factors in NIDDM Patients With and Without Microalbuminuria

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In non-insulin-dependent diabetes mellitus (NIDDM) patients, microalbuminuria predicts early mortality, predominantly from cardiovascular disease. Increased free radical activity and abnormalities in hemostasis have been implicated in the development of vascular disease. Therefore, we measured markers of free radical activity (nonperoxide-conjugated diene isomer of linoleic acid [PL-9,11-LA'] and lipid peroxides expressed as malondialdehyde [MDA]) along with the hemostatic variables: fibrinogen, von Willebrand factor (vWf), plasminogen activator inhibitor (PAI-1), tissue plasminogen activator (t-PA), and plasmin activity ($B_{\beta 15-42}$) in 24 NIDDM patients (12 patients with microalbuminuria and 12 without microalbuminuria) and in 12 age-matched control subjects. There were no differences in linoleic acid (PL-9,12-LA) concentrations between the three groups. PL-9,11-LA' was elevated in the microalbuminuric patients compared with control subjects ($P < 0.05$), but there was no difference between the two diabetic groups. MDA was elevated in the microalbuminuric diabetic patients compared with those patients without microalbuminuria ($P < 0.05$) and control subjects ($P < 0.001$). MDA was also increased in the patients without microalbuminuria compared with control subjects ($P < 0.01$). Except for $B_{\beta 15-42}$, all the hemostatic variables were increased ($P < 0.05$) in the diabetic patients compared with control subjects. The microalbuminuric diabetic patients had further increases in vWf ($P < 0.03$) and t-PA ($P < 0.03$) compared with patients with microalbuminuria. Our study suggests that there is an increase in free radical activity and abnormalities in hemostatic variables favoring a hypercoagulable state in NIDDM, especially in those with microalbuminuria. These changes may contribute to vascular disease, which is particularly prevalent in NIDDM patients with microalbuminuria.

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Microalbuminuria is an established marker of early renal damage in patients with insulin-dependent diabetes mellitus (1,2). However, in non-insulin-dependent diabetes mellitus (NIDDM), there is a clear association between albuminuria and mortality, with subclinical rises in albumin excretion being predictive of cardiovascular mortality (3–6). The high prevalence of cardiovascular disease in microalbuminuric NIDDM cannot be explained by standard risk factors alone (7), and it is possible that alterations in free radical activity and hemostasis may be important in its pathogenesis (8–13).

The aim of this study was to determine whether markers of free radical activity (nonperoxide isomer–conjugated diene of linoleic acid [PL-9, 11-LA']), its molar ratio to linoleic acid (PL-9, 12-LA), and lipid peroxidation (malondialdehyde [MDA]), along with hemostatic variables (fibrinogen, von Willebrand factor [vWf]), plasminogen activator inhibitor (PAI-1), tissue plasminogen activity (t-PA), and plasmin activity ($B_{\beta 15-42}$) were elevated in NIDDM and also whether any abnormality demonstrated was further altered by microalbuminuria.

RESEARCH DESIGN AND METHODS

Twenty-four NIDDM patients, 12 with normoalbuminuria and 12 with microalbuminuria and 12 comparable control subjects were studied. The study was approved by the local medical ethical committee. Microalbuminuria was determined with an immunoturbidometric assay (14) and was defined as an albumin-creatinine ratio of ≥3.5 with urinary protein concn <200 mg/L. The mean urinary albumin excretion rates from three timed overnight urine collections for the microalbuminuric patients ranged from 42 to 140 μg/min. All patients had easily palpable pe-

TABLE 1
Clinical details of control subjects and diabetic patients

	Age (yr)	*n* (M/F)	Duration of diabetes (yr)	Plasma glucose (mM)	HbA_1 (%)
Control	50 (39–64)	6/6			
Diabetic					
Normoalbuminuric	54 (45–65)	5/7	11 (8–16)	8.2 (6.9–10.7)	9.1 (7.8–10.9)
Microalbuminuric	57 (47–65)	7/5	14 (9–19)	8.0 (6.8–11.1)	9.5 (7.6–11.6)

Results are medians with ranges in parentheses.

ripheral pulses with no clinical or electrocardiogram evidence of ischemic heart disease. With the exception of two microalbuminuric diabetic patients, who were taking a cardioselective β-blocker for mild hypertension, none of the patients were taking medication other than sulphonylureas. The blood pressure in all patients was <140/90 mmHg. Fundi were examined after mydriasis. Ten patients without microalbuminuria and all patients with microalbuminuria had background retinopathy; no patient had proliferative retinopathy. No patients had significant hyperlipidemia with total cholesterol concn <6.5 mM and triglyceride concn <2.0 mM. The patients and control subjects were venesected between 0900 and 1000 in the fasting state. Blood glucose and HbA_1 were measured with a perspective glucose oxidase analyser (Americal Monitor UK, Burgess Hill, UK) and gel electrophoresis (Corning, Halstead, UK), respectively. The normal range for HbA_1 is 5–8%. Further clinical and laboratory data are shown in Table 1.

Linoleic acid (PL-9, 12-LA) and its diene conjugate (PL-9, 11-LA′) were measured by high-performance liquid chromatography (HPLC) (15,16). Lipid peroxide concentrations were measured in plasma (EDTA) with a modified HPLC method (17). Briefly, modifications were as follows. Plasma (100 μl) was added to 1.5 ml (0.44 M) phosphoric acid and 0.5 ml (42 mM) thiobarbituric acid in a polypropylene tube. The mixture was placed in boiling water for 1 h and then in ice for 5 min. Ethyl acetate/butanol (1.5 ml, 2:2 vol/vol) was added to the tubes, and the contents were mixed for 5 min on a rotary mixer. The tubes were then centrifuged (3000 rpm for 5 min), and 1 ml of the organic layer was removed and evaporated to dryness at 40°C under oxygen-free nitrogen. The residue was redissolved in mobile phase (150 μl), and 50 μl was injected onto the HPLC column. All analyses were conducted in a blinded fashion. All samples were analyzed in a single batch, and plasma specimens were stored at −20°C. The intra-assay coefficients of variation for the determination of the diene conjugate of linoleic acid and the lipid peroxides were 2.1 and 4.3%, respectively.

Blood for hemostatic factors was anticoagulated with 0.11 M trisodium citrate (9:1 vol/vol) and centrifuged at 2000 × *g* at 4°C for 15 min to obtain plasma. Fibrinogen was measured with the method of Clauss (18), with a Coag-A-Mate X2 coagulometer, reagents, and standards (Organon Teknika, Cambridge, UK). vWf was assayed with an enzyme-linked immunosorbent assay (ELISA) technique (Dako, High Wycombe, UK), whereas PAI-1 was measured with a chromogenic substrate assay kit (Kabi Vitrum, Bourne End, UK). Tissue plasminogen activator and $B_{\beta 15-42}$ antigens were measured with an ELISA technique kit (Biopool, Umea, Sweden) and radioimmunoassay technique (19) (IMCO, Stockholm, Sweden).

Statistical analysis. Results are medians and ranges, and the three groups were compared with the Kruskall-Wallis analysis of variance. Multiple regression analysis was used to assess the possible influences of glycemic control (plasma glucose and HbA_1) and albumin excretion rate on the assays of free radical activity and hemostasis.

RESULTS

The groups were closely age-matched, and the diabetic groups were well matched for duration of disease and glycemic control (Table 1). There was no difference in the concentration of linoleic acid (PL-9, 12-LA) between the control subjects and the two diabetic groups (Table 2). The concentration of the nonperoxide isomer of linoleic acid (PL-9, 11-LA′) was higher in the microalbuminuric diabetic group than in the control subjects ($P < 0.05$). There was no difference in the level of PL-9, 11-LA′ between the normoalbuminuric and the control subjects. The molar ratio (PL-9, 11-LA′, PL-9, 12-LA) was elevated in both the normoalbuminuric ($P < 0.05$) and the microalbuminuric ($P < 0.05$) diabetic groups compared with control subjects; there was no difference between the two diabetic groups. The concentration of MDA was greater in the microalbuminuric diabetic group compared with the normoalbuminuric diabetic group ($P < 0.05$) and the control subjects ($P < 0.001$). The concentration of MDA was also elevated in the normoalbuminuric diabetic group compared with the control subjects ($P < 0.01$). Except for $\beta_{\beta 15-42}$, all hemostatic factors were increased in the diabetic patients ($P < 0.05$) compared with control subjects. The microalbuminuric diabetic patients had further increased levels of t-PA ($P < 0.002$) and vWf ($P < 0.03$) compared with patients without microalbuminuria (Table 3). There was no correlation between the indices of free radical activity or hemostasis and either glycemic control or albumin excretion rate.

TABLE 2
The concentrations of free radical markers in control subjects and diabetic patients

	PL-9,12-LA (μM)	PL-9,11-LA' (μM)	PL-9,11-LA'/ PL-9,12-LA (%)	MDA (μM)
Control	1140	18.6	1.5	1.9
	(660–1580)	(14.1–23.5)	(1.1–2.1)	(1.1–2.5)
Diabetic	1110	19.1	2.1†	2.4‡
Normoalbuminuric	(770–1520)	(9.4–38.2)	(0.7–4.2)	(2.0–2.7)
Microalbuminuric	1290	33.1*	2.3*	3.0§¶
	(960–1510)	(16.9–59.2)	(1.7–5.5)	(2.0–4.0)

Values are medians with ranges in parentheses. PL-9,12-LA, linoleic acid; PL-9,11-LA', nonperoxide isomer–conjugated diene of linoleic acid; MDA, malondialdehyde.
*$P < 0.05$, ¶$P < 0.05$, microalbuminuric vs. normoalbuminuric diabetic patients.
†$P < 0.05$, ‡$P < 0.01$, normoalbuminuric diabetic patients vs. control subjects.
§$P < 0.001$, microalbuminuric diabetic patients vs. control subjects.

DISCUSSION

This cross-sectional study provides further evidence that there is increased free radical activity and an alteration in hemostasis in NIDDM patients (8–12) and that these abnormalities are more pronounced in patients with microalbuminuria. Free radicals are reactive chemical species that have unpaired electrons. Because of their unpaired electrons, the free radical species can react with virtually all cell components. The potential consequences of unimpeded free radical reactions include oxidation and peroxidation of membrane lipids, denaturation of proteins, generation of chemotactic factors, impairment of collagen synthesis, disturbed membrane permeability, and increased inflammatory cell infiltration. As a consequence, free radical activity has been implicated in inflammation, aging, malignant change, and vascular damage (20,21). In addition, it is suggested that the increase in free radical activity demonstrated in diabetes coexists with a reduction in the antioxidant state (9,20) and therefore could potentially increase the deleterious effects of free radicals.

There is no ideal method for the measurement of free radicals in patients. However, the two methods used herein, although indirect, complement one another in that they represent difference indices of free radical–induced damage to lipids. Polyunsaturated lipids are particularly sensitive to free radical attack, which induces a shift of double bonds to the diene-conjugated configuration (23,24). The main diene-conjugated compound in human plasma is the nonperoxide isomer of linoleic acid, PL-9,11-LA' (15,16). Lipid peroxides are predominantly derived from the peroxidation of polyunsaturated fatty acids and their esters. Oxygen is required for peroxidation, and this process appears to be increased in diabetes (25). The measurement of lipid peroxides depends on acid-catalysed thermal decomposition to MDA, which reacts with thiobarbituric acid (TBA) to form a colored adduct. However, other compounds also react with TBA to form products capable of interference in non-HPLC assays (26). In this study, the use of an HPLC assay reduced this interference, making it more specific than previously described assays (21,25,27).

The most likely sources of the increase in free radical activity are neutrophils and vascular endothelial cells. Neutrophils show increased activation in diabetes as evidenced by an increase in plasma neutrophil elastase concentration (28). Furthermore, free radicals reduce the efficacy of plasma protease inhibitors such as α-1-proteinase inhibitor, resulting in an increase in the potential of the neutrophil lysosomal enzyme elastase to cause vascular damage (29). In addition, vascular endothelium, particularly during periods of ischemia (27,30), can generate significantly increased free radical activity. This may be particularly relevant to diabetes, where endothelial dysfunction is well recognized (31).

Although the changes in lipid peroxidation demonstrated in this study may not be related to the changes in the hemostatic factors, lipid peroxidation per se has been

TABLE 3
Concentrations of hemostatic factors in control subjects and diabetic patients

	Fibrinogen (g/L)	vWf (iμ/ml)	PAI (% normal pool)	tPA (μ/ml)	$B_{\beta 15-42}$ (pmol/ml)
Control	2.6	80	109	6.0	2.1
	(1.7–3.5)	(42–124)	(81–220)	(3.0–8.5)	(1.7–3.0)
Diabetic	2.9*	81*	119*	12.5*	2.6
Normoalbuminuric	(2.1–5.1)	(51–104)	(92–243)	(4.0–21.0)	(1.7–8.5)
Microalbuminuric	3.2*	110*†	160*	14.0*‡	2.6
	(2.3–5.4)	(64–192)	(87–350)	(10.0–27.5)	(1.5–3.8)

Values are medians with ranges in parentheses. vWf, von Willebrand factor; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activity; $B_{\beta 15-42}$, plasmin activity.
*$P < 0.05$, micro- and normoalbuminuric diabetic patients vs. control subjects.
†$P < 0.03$, ‡$P < 0.002$, microalbuminuric vs. normoalbuminuric diabetic patients.

implicated in vascular endothelial damage (21,32). It is intimately involved in prostaglandin biosynthesis, stimulating both cyclooxygenase and thromboxane synthesis and simultaneously inhibiting prostacyclin production, resulting in enhanced platelet aggregation (33–35). Lipid peroxides are also capable of inhibiting antithrombin III activity (36), producing procoagulant activity (37). However, in our study, no significant correlations were noted between lipid peroxidation and the tests of hemostasis, suggesting that the changes demonstrated are unrelated.

We have shown several hemostatic disturbances in diabetic patients that are consistent with previous studies (10,11). These were more marked in patients with microalbuminuria, in accordance with the report of Schmitz and Ingersleu (13). vWf, which was elevated in the microalbuminuric patients, is released from disturbed or damaged endothelial cells. vWf promotes platelet adhesion and aggregation at high shear rates, which in turn tends to promote arterial thrombosis (38). Tissue-type t-PA and its inhibitor (PAI-1) are also released from vascular endothelium. We observed raised levels of both t-PA and PAI-1 in the diabetic patients, especially in those with microalbuminuria. Whether these changes result in changes in plasmin activity is uncertain—there was no significant increases in plasmin activity ($B_{\beta 15-42}$ antigen) in the two diabetic groups. Increased fibrinogen levels in diabetic patients (39,40) may result in part from adhesion of monocytes to damaged endothelial cells, with release of cytokines such as interleukin-6, which increases hepatic synthesis of fibrinogen and other acute-phase proteins. Because both fibrinogen (41) and PAI-1 (42) are risk factors for arterial thrombosis, the high levels in diabetic patients with microalbuminuria may be relevant to their increased risk of cardiovascular disease.

In conclusion, our results support the concept that both increased free radical formation and changes in hemostatic variables related to endothelial damage may be associated with diabetic vascular disease. The prothrombotic processes demonstrated appear to be associated with, and may be implicated in, the development of diabetic vascular disease associated with microalbuminuria.

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