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Microalbuminuria in IDDM is associated with increased expression of monocyte procoagulant activity

M. Bazzan[1], **G. Gruden**[2], **S. Stella**[1], **A. Vaccarino**[1], **G. Tamponi**[1], **C. Olivetti**[2], **S. Giunti**[2], **P. Cavallo-Perin**[2]

[1] Department of Haematology and Onco-Haematology, University of Turin, Turin, Italy
[2] Department of Internal Medicine, University of Turin, Turin, Italy

Summary Microalbuminuria, the early phase of diabetic nephropathy, is associated with an increased risk of atherothrombosis. Monocytes play an important part in the pathogenesis of atherosclerosis and in the activation of haemostasis. However, procoagulant activity is poorly understood in Type I (insulin-dependent) diabetes mellitus, particularly in the presence of microalbuminuria. This study aimed to evaluate spontaneous and endotoxin-induced monocyte procoagulant activity in insulin-dependent diabetic patients with normoalbuminuria or microalbuminuria. Seventeen patients with microalbuminuria, 28 with normoalbuminuria and 26 healthy control subjects matched for age, sex, body mass index and smoking habit were studied. Mononuclear cells from peripheral venous blood were incubated with or without bacterial lypopolysaccharide. Spontaneous procoagulant activity and procoagulant activity after 3 h and 6 h of incubation were calculated. Spontaneous procoagulant activity values were similar in the three groups. After 3 h and 6 h incubation with bacterial lypopolysaccharide, procoagulant activity values were slightly, but not statistically significantly, higher in the normoalbuminuric diabetic group than in control group, and significantly higher in microalbuminuric diabetic group than in control group ($p < 0.01$). The increased endotoxin-induced monocyte procoagulant activity helps to explain the link between microalbuminuria and the increased risk of atherothrombosis in patients with Type I diabetes. [Diabetologia (1998) 41: 767–771]

Keywords Diabetes, atherosclerosis, procoagulant activity, monocytes

Microalbuminuria, the early phase of diabetic nephropathy, is associated with an increased risk of cardiovascular disease. However, this association cannot be explained fully by the excess of conventional risk factors for atherosclerosis in these patients [1]. Many prothrombotic abnormalities of haemostasis have been found in patients with Type I (insulin-dependent) diabetes mellitus and microalbuminuria: increased plasminogen activator inhibitor 1 and factor VII plasma levels [2], acquired activated protein C resistance [3], increased prothrombin fragment 1 + 2 [4] and increased factors Xa and VIII [5, 6]. Moreover, several haemostatic parameters have been positively correlated with the urinary albumin excretion rate [7].

Peripheral mononuclear cells (PMC), mainly monocytes, can initiate and amplify the coagulation cascade; this activity has been called monocyte procoagulant activity. Monocyte procoagulant activity is mainly linked to tissue factor expression [8]. A lymphocyte subset (T helper, Leu 3a+) produces the monocyte procoagulant inducing factor lymphokine that induces monocyte tissue factor expression [9, 10, 11]. Tissue factor is a membrane glycoprotein that binds and activates factor VII to factor VII a and triggers the coagulation cascade via factors IX a and X a, inducing thrombin generation and fibrin de-

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Corresponding author: Dr. Mario Bazzan, Department of Haematology and Onco-Haematology, via Genova 3, 10126 Torino, Italy

Abbreviations: PMC, Peripheral mononuclear cells; HbA_{1c}, glycated haemoglobin; AGE, advanced glycation end products.

position. Tissue factor expression is almost undetectable in basal conditions, but increases greatly after inflammatory stimuli, such as bacterial lypopolysaccharide, tumor necrosis factor, and interleukin-1 [12, 13, 14], or in conditions characterised by thrombosis and atherosclerosis [8].

Increased spontaneous or endotoxin-induced expression of procoagulant activity has been reported in coronary artery disease [15, 16, 17] and in chronic myeloproliferative diseases [18], which are characterised by frequent thrombotic events. Implication of monocytes in the development of atherothrombotic lesions has also been reported in diabetes. Several mechanisms, such as circulating immunocomplexes [19], poor metabolic control and susceptibility to bacterial infections [20] may be implicated in increased expression of monocyte procoagulant activity in diabetes.

The effect of advanced glycation end products (AGE) could be another mechanism. The AGE receptor (RAGE) has been identified on endothelial cells, monocytes and smooth muscle cells [21, 22, 23]. AGE increase tissue factor expression [24], and high tissue factor plasma concentrations have been reported in patients with diabetes [25]. AGE have a role in the development of atherosclerosis [26] and in the progression of diabetic nephropathy [27].

Expression of monocyte procoagulant activity is still poorly understood in diabetes. In one study, it was increased [28], but the diabetic patients investigated were unselected in relation to the type of diabetes (Type I and II), the degree of microangiopathic complications and the presence of infections [28]. Since these factors could have influenced the expression of monocyte procoagulant activity, no conclusions can be drawn [28], and the possible association between monocyte procoagulant activity and microalbuminuria remains to be assessed. The present study aimed to evaluate spontaneous and endotoxin-induced expression of monocyte procoagulant activity in two different, strictly selected groups of patients – Type I patients with normoalbuminuria or microalbuminuria – and healthy subjects.

Materials and methods

Subjects. We studied 17 Type I patients with microalbuminuria and 28 with normal albuminuria. None of the patients had acute or chronic infections (normal C-reactive protein serum concentrations), pre-proliferative or proliferative retinopathy or clinical or echocardiographic evidence of cardiovascular disease. To exclude all non-diabetic causes of an increased albumin excretion rate, and to avoid factors that might affect procoagulant activity, the following inclusion criteria were used for selecting study subjects: negative familial and personal histories of hypertension and non-diabetic renal disease, no echographic evidence of renal abnormalities, normal urine sediments, negative bacterial urine cultures, no drugs other than insulin taken in the past 10 days, normal creatinine clearance and normal values for prothrombin time and partial thromboplastin time.

Patients were considered normoalbuminuric if the albumin excretion rate was < 20 μg/min, and microalbuminuric if the rate was 20–200 μg/min in two of three overnight sterile urine collections. The rate chosen was the median value of the three determinations of albumin excretion. All microalbuminuric patients who met the inclusion criteria were included, whereas patients with normoalbuminuria were selected to obtain a group that was matched for age, sex, diabetes duration, body mass index, smoking habits and glycated haemoglobin (HbA_{1c}) (Table 1). Twenty-six healthy subjects matched with the diabetic patients for age, sex, body mass index and smoking habits comprised the control group.

All procedures followed were in accordance with the Helsinki Declaration of 1975. Informed consent was obtained from all subjects.

Determinations. HbA_{1c} was determined by high pressure liquid chromathography (Diamat, Bio-Rad, Hercu, Calif., USA) and the urinary albumin concentration by RIA (Kabi-Pharmacia, Uppsala, Sweden). Blood samples for PMC determination were drawn (avoiding venous stasis) between 09.00 hours and 10.00 hours – from an antecubital vein and using a 19G needle. After discharging the first 5 ml, the blood samples were placed in pre-cooled vacutainer tubes containing trisodium citrate (0.129 mol) as anticoagulant (1/10 v/v) and immediately put in melting ice.

PMC were separated by Ficoll-Hypaque stratification, and washed twice with phosphate-buffered saline, pH 7.4 [29]. The medium for cell culture was RPMI 1640 (GIBCO), containing 10% fetal bovine serum (GIBCO Life Technologies, Paisley, Scotland) and gentamycin (0.5 mg/l). The working concentration ($1.0 \pm 0.1 \times 10^6$/ml) of cells was obtained by diluting cells in RPMI after Ficoll separation. Cells were counted in an automatic cell counter (Coulter, Microdiff 18), both in peripheral venous blood and after Ficoll separation. The percentage of monocytes in PMC was assayed by a cytofluorometric method (FACS, scan, Becton Dickinson, Milan, Italy) after labelling cells with fluorescinated monoclonal antibody anti-CD 14 (Becton Dickinson). After Ficoll separation, monocyte values (as a percentage of all leucocytes) were 12.9 ± 3.7% in diabetic patients and 13.7 ± 4.6% in control subjects. The mononuclear cell suspension contained fewer than 4 platelets per mononuclear cell and less than 5% polymorphonuclear leucocytes. The remaining cells were lymphocytes. The medium for cell culture was tested by the limulus test assay (QCL-1000, Bio-Whittaker) to exclude endotoxin contamination, and was negative at levels from 0.1 to 0.2 EU/ml.

Procoagulant activity. PCA was assayed on fresh intact cells in RPMI suspension. PMC were more than 94% viable by trypan blue exclusion. PMC, at the adjusted concentration of $1.0 \pm 0.1 \times 10^6$/ml, were incubated at 37 °C in RPMI, either without with or 50 ng/ml bacterial lipopolysaccharide (Calbiochem, Novabiochem, La Jolla, Calif., USA). We assayed procoagulant activity at time 0 (T0) and after 3 h (T3) and 6 h (T6) of incubation at 37 °C, either with or without lipopolysaccharide stimulation. We used a manual clotting assay (two-stage) method. Briefly, after preincubation (2 min at 37 °C) of a 1:1 v/v mixture of PMC with pooled plasma, calcium chloride (25 mmol/l) was added and the clotting time was measured in seconds (recalcification time). Procoagulant activity, expressed in arbitrary milliunits (mU) of thromboplastin, was calculated by comparing the clotting times of a reference curve with times obtained in our data.

Table 1. Characteristics of patients and control subjects (data are mean ± SD)

Characteristics	Healthy control subjects	Insulin-dependent diabetic patients	
		Normoalbuminuric	Microalbuminuric
Number	26	28	17
Sex (M/F)	18/8	18/10	11/6
Age (years)	30 ± 8	29 ± 9	33 ± 10
Smokers (yes/no)	3/24	3/25	2/14
Duration of diabetes (years)	–	16 ± 6	16 ± 4
Body mass index (kg/m^2)	23 ± 3	23 ± 2	24 ± 3
Glycated haemoglobin (%)	–	8.7 ± 2.1	8.9 ± 2.1
Triglycerides (mg/dl)	73 ± 36	70 ± 21	102 ± 50[a]
Total cholesterol (mmol/l)	4.89 ± 0.96	4.81 ± 1.01	5.20 ± 1.38
LDL cholesterol (mmol/l)	2.99 ± 0.75	2.78 ± 0.78	3.48 ± 1.25[b]
HDL cholesterol (mmol/l)	1.48 ± 0.36	1.64 ± 0.34	1.38 ± 0.31[c]
Systolic blood pressure (mmHg)	118 ± 10	115 ± 10	126 ± 20[c]
Diastolic blood pressure (mmHg)	75 ± 7	72 ± 7	81 ± 14[d]
Albumin excretion rate (μg/min)	–	4.1 ± 2.9	91.9 ± 60

[a] $p < 0.01$ *vs* normoalbuminuric patients and control subjects; [b] $p < 0.05$ *vs* normoalbuminuric patients; [c] $p < 0.03$ *vs* normoalbuminuric patients; [d] $p < 0.01$ *vs* normoalbuminuric patients

The reference curve was obtained by recording clotting times of 100 μl of rabbit brain thromboplastin (Ortho, Johnson and Johnson, Milan, Italy) diluted in imydazole buffer (Immuno Vienna, Austria), with 100 μl of pooled plasma, incubated for 2 min at 37 °C, and finally adding 100 μl of calcium chloride 25 mmol/l (two-stage method). Serial dilutions of rabbit brain thromboplastin were used to produce a log-log curve. Pooled plasma was obtained from 20 healthy donors. The recalcification time of undiluted rabbit brain thromboplastin was 32.2 ± 2.0 seconds. This time was arbitrarily assumed to express 1×10^3 mU of procoagulant activity. Cell culture medium recalcification time was 140.0 ± 4.5 seconds, corresponding to 8.0 ± 1.5 mU of procoagulant activity. Control pooled plasma and culture cell medium were tested in duplicate in each experiment. In our experimental system procoagulant activity was factor VII-dependent, as observed in preliminary experiments performed with PMC and single factor-deficient plasma.

Statistical analysis. Procoagulant activity values were expressed as median (range) and analysed by Mann-Whitney U test. Albumin excretion rate data were converted to log values because of their skewed distribution. All other values were analysed by Bonferroni's correction, when significant differences were found, by one way analysis of variance (ANOVA). The threshold of statistical significance was taken as $p < 0.05$.

Results

Clinical and metabolic characteristics of the three groups are reported in Table 1. Plasma lipids (except total cholesterol) and blood pressure values were significantly higher in microalbuminuric than in normoalbuminuric patients. Procoagulant activity values are shown in Table 2. Basal, T3h and T6h procoagulant activity values without lipopolysaccharide stimulation were similar in the three groups. After 3 h and 6 h with lipopolysaccharide incubation, procoagulant activity values were slightly, but not significantly, higher in the normoalbuminuric group than in control group, and significantly higher in microalbuminuric group than in control group ($p < 0.01$). A spontaneous increase in procoagulant activity expression was observed after 6 h in unstimulated cells in the three groups. This phenomenon has already been observed in similar conditions, and could reflect low, but detectable, spontaneous lympho-monocyte activation [18].

Discussion

These results show that procoagulant activity of lipopolysaccharide stimulated monocytes was significantly higher in microalbuminuric patients than in control subjects ($p < 0.01$), and slightly higher in normoalbuminuric Type I diabetes patients than in control subjects. Procoagulant activity values of unstimulated monocytes were similar in the three groups. In a previous study, lipopolysaccharide-induced procoagulant activity was increased in an unselected group of patients with different types of diabetes that included subjects with extremely poor metabolic control or severe chronic complications and infections [28]. Since these factors influence procoagulant activity expression in monocytes [20], our study subjects were strictly selected Type I patients. The value of our results depends on how far the influence of other possible confounding factors can be excluded. Insulin immuno-complexes or transient endotoxin stimulation from bacterial infections, or both, should be considered in diabetic patients who are treated with insulin and could show an increased susceptibility to bacterial infections during poor blood glucose control [19]. It is known that beef and pork insulin immunocomplexes can enhance procoagulant activity [19], but our patients were all treated with human insulin. Furthermore, at the time of the study, none showed poor metabolic control or an intercurrent infection

Table 2. Monocyte procoagulation activity [median (10th–90th centile range)] with or without bacterial lipopolysaccharide stimulation before (T0) and after 3 h (T3) and 6 h (T6) of incubation

Groups	Stimulation status	Procoagulation activity		
		T0	T3	T6
Normoalbuminuric patients (n = 28)	Spontaneous	19.1 (3.3–46.8)	35.4 (12.6–116.6)	53.6 (13.5–205.9)
	After lipopolysaccharide		49.3 (12–190.4)	198.65 (35.2–572.8)
Microalbuminuric patients (n = 17)	Spontaneous	19.3 (2–108)	34.8 (4.4–137.2)	72.6 (12–226.8)
	After lipopolysaccharide		74.5 (32.6–912.8)[a]	278.4 (88–846.3)[a]
Control subjects (n = 26)	Spontaneous	17.85 (6.7–31.5)	32.4 (8.5–73.6)	49.15 (7.2–201.1)
	After lipopolysaccharide		44.4 (11.5–206.6)	117.9 (25.9–445.5)

Mann-Whitney U test, [a] p 0.01 *vs* control subjects

(as ascertained by clinical evaluation, normal C-reactive protein values and negative urine culture). The effect of these factors on results can, therefore, be reasonably excluded by careful selection of patients.

Mononuclear cells from microalbuminuric patients are more responsive to lipopolysaccharide stimulation than cells in control subjects, indicating that microalbuminuria in Type I diabetes is associated with increased expression of monocyte procoagulant activity. An increased lipopolysaccharide-induced monocyte procoagulant activity is still evident in normoalbuminuric patients compared with control subjects, even though the difference between the two groups is not statistically significant. This result suggests that diabetes per se may affect the expression of procoagulant activity. In fact, the in vivo consequences of the observed in vitro abnormalities are unknown. The increase in lipopolysaccharide-induced expression of monocyte procoagulant activity in diabetic patients might be explained by the effects of glycation on structural membrane proteins, as reported in endothelial cells and monocytes in experimental diabetes [21, 22]. The reason for a statistically significant increase in lipopolysaccharide-induced procoagulation activity only in microalbuminuric patients is, however, unclear.

It is possible that the effects of AGE, in inducing tissue factor expression and in promoting kidney damage and atherosclerosis, could explain the link between increased procoagulation activity expression, microalbuminuria and the increased cardiovascular risk in Type I patients. In fact, it has been observed that binding of AGE to the cellular surface receptor (RAGE) induces translocation of the transcription factor NF-kB from the cytoplasm into the nucleus, promoting tissue factor expression [21]. On the other hand, AGE have a role in the development of atherosclerosis in animal models [30] and in microvascular and macrovascular diabetic complications [26], and are closely related to the progression of early morphological kidney damage [27].

Another possible cause of increased cardiovascular risk in microalbuminuric diabetic patients could be the increased prevalence of conventional risk factors, such as hypertension and lipid abnormalities [1]. The increased blood pressure and plasma lipid values in our microalbuminuric patients may support this hypothesis. However, these factors cannot, by themselves, explain fully why microalbuminuria is a potent cardiovascular risk marker – other factors must be considered. In this context the outcome of increased endotoxin-induced monocyte procoagulation activity seems to be an additional critical link between microalbuminuria and atherothrombotic events in diabetic microalbuminuric patients.

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