Protein C activation in NIDDM patients

E. C. Gabazza^{1, 2}, H. Takeya¹, H. Deguchi¹, Y. Sumida², O. Taguchi², K. Murata², K. Nakatani², Y. Yano², M. Mohri³, M. Sata³, T. Shima², J. Nishioka¹, K. Suzuki¹

¹ Department of Molecular Pathobiology, Mie University School of Medicine, Mie-ken, Japan

² Third Department of Internal Medicine, Mie University School of Medicine, Mie-ken, Japan

³ Asahi Chemical Industry Company, Shizuoka, Japan

Summary Enhanced activation of the clotting system has been recently implicated in the pathogenesis of vascular complications in patients with diabetes mellitus. Abnormalities of the anticoagulant system may constitute a potential trigger factor for the haemostatic activation observed in diabetic subjects. The current study aimed to evaluate anticoagulant activity in diabetic patients by assessing the plasma levels of activated protein C-protein C inhibitor complex; and by measuring the anticoagulant response to exogenous thrombomodulin. This study comprised 61 patients (34 men, 27 women) with non-insulin-dependent diabetes mellitus (NIDDM) of whom 22 showed microalbuminuria and 39 normoalbuminuria. Data obtained in 31 non-obese and non-diabetic subjects were available for comparison. The plasma levels of fibrinogen ($p < 0.02$), prothrombin fragment 1 + 2 ($p < 0.05$), fibrin monomer ($p < 0.0001$), protein C antigen ($p < 0.005$), total protein S antigen ($p < 0.02$), soluble thrombomodulin ($p < 0.005$) and soluble E-selectin ($p < 0.005$) were significantly higher in diabetic patients than in healthy subjects. The plasma

level of activated protein C-protein C inhibitor complex (7.4 ± 3.8 vs 3.0 ± 0.4 pmol/l) was significantly higher ($p < 0.0001$) and the anticoagulant response to exogenous thrombomodulin (23.4 ± 2.6 vs 35.3 ± 3.0 ng/ml) was markedly lower ($p = 0.005$) in all diabetic patients than in healthy subjects. Cases with microalbuminuria presented low plasma levels of activated protein C-protein C inhibitor complex (5.5 ± 0.6 vs 8.6 ± 0.7 pmol/l, $p < 0.05$) and significantly decreased values of the anticoagulant response to exogenous thrombomodulin (16.5 ± 2.9 vs 23.4 ± 2.6 %, $p = 0.03$) as compared to those with normoalbuminuria. The present study suggests that the hyper-coagulable state in NIDDM is associated with an increased activation of protein C but with a poor plasma reactivity to the anticoagulant effect of thrombomodulin. [Diabetologia (1996) 39: 1455–1461]

Keywords Protein C, protein S, diabetes mellitus, soluble thrombomodulin, microalbuminuria, activated protein C-protein C inhibitor complex, fibrin monomer, soluble E-selectin.

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Corresponding author: K. Suzuki, Ph.D., Department of Molecular Pathobiology, Mie University School of Medicine, 514 Edobashi 2-174, Tsu-city, Mie-ken, Japan

Abbreviations: NIDDM, Non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; Fbg, fibrinogen; prothrombin F 1 + 2, prothrombin fragment 1 + 2; FM, fibrin monomer; PC, protein C antigen; PCA, protein C activity; total PS, total protein S antigen; free PS, free protein S antigen; sTM, soluble thrombomodulin; sE-S, soluble E-selectin; APC-PCI complex, activated protein C-protein C inhibitor complex; TAR, thrombomodulin-induced anticoagulant activity rate; BSA, bovine serum albumin.

Alteration of the clotting system in diabetic patients is presently the focus of increasing interest due to its potential role in the pathogenesis of large and small-vessel disease in diabetes mellitus. It is believed that enhanced pro-coagulant activity is linked to the excessive cardiovascular morbidity and mortality among diabetic patients which have not been fully explained by major risk factors such as arterial hypertension, cigarette smoking and hypercholesterolaemia. Perturbation of haemostasis has also been implicated in the development of complications such as nephropathy and retinopathy and in the acceleration of atherogenesis observed in diabetic patients [1, 2].

However, the mechanism by which increased activation of the clotting system occurs in diabetes is not clear.

Thrombogenic tendency results from the disruption of the balance between the prethrombotic and antithrombotic factors that control clotting homeostasis; this imbalance may occur due to an ongoing stimulus to thrombogenesis, a defect of the natural anticoagulant or fibrinolytic process. The most important anticoagulant system is the protein C (PC) pathway. PC is a vitamin K-dependent plasma glycoprotein that, after being proteolytically cleaved by thrombin complexed to thrombomodulin (TM) on the endothelial surface, is converted to activated protein C (APC) which is the enzyme effector of the PC anticoagulant system [3]. APC exerts anticoagulant activity by inactivating factors Va and VIIIa on the membrane of platelets and endothelial cells, and also by stimulating fibrinolysis in concert with protein S (PS) [3]. The biological activity of the anticoagulant PC pathway in diabetes remains controversial; although the plasma antigen levels of PC in diabetes have been previously reported, the degree of PC conversion to its active protease is unclear [4, 5]. An assay for measuring levels of APC in complex with its main inhibitor, PC inhibitor or PCI (APC-PCI complex) has been recently developed [6]. Plasma concentration of APC-PCI complex represents the direct index of *in vivo* ongoing PC activation [7].

TM is an endothelial cell membrane protein that also has antithrombotic effect [8]. Aside from its role in the thrombin-mediated catalytic activation of PC, TM produces additional anticoagulant effects by inhibiting various thrombin-induced thrombogenic activities such as the proteolysis of fibrinogen, activation of factors V and XIII, inactivation of PS and inhibition of platelet aggregation [8]. TM also accelerates the inhibition of thrombin by antithrombin III [9] and PCI [10]. These anticoagulant activities of TM are believed to be impaired in diabetic patients as a result of endothelial injury induced proteolytic cleavage of the membrane-bound TM. However, the reactivity of the foregoing plasma components in diabetic patients to the anticoagulant effects of TM is unclear; to clarify this point, in the current study, we evaluated the response against exogenous TM in plasma of diabetic patients.

The present study aimed to assess: 1) the degree of PC activation as measured by the plasma levels of APC-PCI complex; and 2) the anticoagulant response to exogenous TM in patients with non-insulin-dependent diabetes mellitus (NIDDM).

Subjects and methods

Reagents. The snake venom, reptilase (200 U/ml), was purchased from Pentafarm AG (Basel, Switzerland) and rabbit brain thromboplastin was from Dade Co., (Miami, Fla.,

USA). Bovine serum albumin (BSA) was obtained from Sigma Chemical (St. Louis, Mo., USA) and thrombin chromogenic substrate, S-2238, was from Chromogenix AB, (Mölndal, Sweden). Human prothrombin was purified from plasma as described previously [11], and recombinant human TM prepared as described previously [12]. Anti-PC and anti-PCI antibodies were prepared following previous methods [13, 14]. Streptavidin-peroxidase conjugate was from Amersham International (Amersham, Bucks., UK). Tetramethylbenzidine peroxidase substrate was from Kirkegaard and Perry Laboratories (Bethesda, Md., USA). All other chemicals and reagents used in this study were of the best quality commercially available.

Subjects. This study comprised 61 patients (34 men, 27 women) with NIDDM selected according to the National Diabetes Data Group Criteria from our outpatient clinic [15]. The age (mean $\pm$ SEM) of the patients was 58.0 ± 1.3 years, ranging between 35 and 77 years. The mean ($\pm$ SEM) duration of diabetes was 9.8 ± 1.0 (range 1–31 years). Subjects with clinical or laboratory signs of liver dysfunction, malignancy or a history of coagulation disorder were excluded from the study. Fundi of patients were examined by an ophthalmologist, who found that there were 33 patients with retinopathy and 12 with neuropathy. Thirty-four cases were being treated with diet alone and 27 with oral hypoglycaemic agents. Informed consent was obtained from all subjects. The study was approved by our institution's review board for human experiments and was carried out following the principles of the Helsinki Declaration. Thirty-one healthy non-obese and non-diabetic subjects matched for sex (17 men, 14 women) and age (57.1 ± 3.0 years) served as control subjects. None of the control subjects had a history of arterial hypertension, cardiopathy, thrombotic or liver disease. Diabetic patients were divided into two groups according to the albumin creatinine index, calculated as urinary albumin concentration/urinary creatinine concentration [16]. The albumin creatinine index correlates significantly with the urinary albumin excretion over a period of 24 h [17]. Group I consisted of 39 patients with a normal albumin creatinine index (normoalbuminuria) of less than 30 mg/g creatinine. Group II consisted of 22 patients with an albumin creatinine index in the range of 30–300 mg/g creatinine (microalbuminuria).

Laboratory measurements. For measurement of clotting parameters and soluble E-selectin (sE-S), blood samples were taken from an antecubital vein with plastic syringes and collected in polystyrene tubes containing 3.8% (weight/volume) trisodium citrate (1 ml anti-coagulant/9 ml blood). Blood was then centrifuged at $1200 \times g$ for 20 min at 4°C . Plasma was collected and stored in small aliquots at -80°C until use. Plasma levels of fibrin monomer (FM) and prothrombin fragment 1 + 2 (prothrombin F1 + 2) were measured using the Enzygmun-Test (Boehringer Mannheim, Mannheim, Germany) and Enzygnost F1 + 2 (Behringwerke AG, Marburg, Germany) immunoassay kits, respectively, following the manufacturer's instructions [18]. The intra-assay and inter-assay precisions of the FM assay were 4 and 6%, and of the prothrombin F1 + 2 assay were 4 and 7%, respectively. The plasma concentration of fibrinogen (intra-assay and inter-assay variations $< 10\%$) was measured as previously described [7]. Endothelial cell injury was evaluated by determining the plasma levels of soluble TM (sTM) and soluble E-selectin (sE-S) using commercially available immunoassay kits for sTM (Fuji Chemical Co., Takaoka, Japan) and for sE-S (R&D systems, Minneapolis, Minn., USA) following the manufacturer's instructions [19, 20]. The intra-assay and inter-assay precisions were, respectively, 4.5 and 5.2% for sTM and 4.7 and 5.6%

for sE-S. Plasma levels of PC antigen were measured by a solid-phase immunoassay using a human polyclonal anti-PC and biotin-labelled monoclonal anti-PC antibodies following previously described methods [7]. The intra-assay and the inter-assay coefficients of variation for PC antigen were 5 and 6 %, respectively. PC activity was determined by an amidolytic assay, using Berichrom-Protein C (Berhringwerke AG, Marburg, Germany). Plasma antigen levels of total PS and free PS were determined as described [7, 21]. The intra-assay and the inter-assay variations were less than 10 % for the PC activity, total and free PS assays.

APC-PCI complex, a marker of ongoing PC activation, was measured in plasma by an enzyme-linked immunoassay as described [7]. Briefly, monoclonal anti-PCI antibody (3 µg/ml) was coated on microtitre plates by overnight incubation. Blood samples were prepared as follows: citrated plasma was treated with barium chloride (final 10 mmol/l) and centrifuged at 15 000 rev/min for 5 min. The precipitate dissolved in washing buffer (50 mmol/l Tris-HCl, pH 7.5, 200 mmol/l NaCl, 0.5 mmol/l EDTA, 0.1 % Tween 20 and 0.5 % BSA) was then incubated overnight in anti-PCI-antibody-coated microtitre plate after appropriate washing and blocking of non-specific bindings. After washing, peroxidase-conjugated monoclonal anti-PC antibody (0.5 µg/ml) was added to each microtitre well and incubated for 5 h. After washing, peroxidase substrate was added to each well and absorbance was measured at 450 nm. The inter-assay and the intra-assay coefficients of variability were 5 and 7 %, respectively.

The TM-induced anticoagulant activity rate (TAR) was determined by a modified method as previously described [22]. Briefly, 5 µl of recombinant human TM (final 100 ng/ml) or buffer (25 mmol/l Tris-HCl, pH 7.4) was added to 50 µl of defibrinated plasma and incubated at 37 °C for 1 min. Thrombin formation was triggered by addition of rabbit brain thromboplastin (1:50 dilution) to plasma treated with TM or buffer alone followed by incubation at 37 °C for 10 min. The amount of thrombin generation was detected by adding a specific thrombin substrate, S-2238 (final concentration, 1 mmol/l). Absorbance was then measured at 405 nm. TAR was calculated as follows:

$$\text{TAR (\%)} = \frac{\text{amount of thrombin in TM-added plasma}}{\text{amount of thrombin in buffer-added plasma}} \times 100$$

The intra-assay and the inter-assay precisions were 4.7 and 5 %, respectively.

Fasting plasma glucose concentration was measured by the glucose oxidase method and haemoglobin A_{1c} (HbA_{1c}) was determined by HPLC [23]. Serum and urinary creatinine were measured by the Jaffe reaction [24]. Serum cholesterol and triglycerides were determined by enzymatic techniques.

Statistical analysis

Data are expressed as the mean ± SEM unless otherwise specified. The difference between the mean of two variables was calculated by unpaired Student's *t*-test or Mann-Whitney U-test as appropriate. Kruskal-Wallis analysis of variance with Bonferroni correction was employed for calculating the difference among the means of three or more variables. Correlations were calculated by multivariate analysis [25]. A software package for Macintosh (Statview 4.11, Abacus Concepts Inc., Berkeley, Calif., USA) was employed for the statistical analyses. A *p* less than 0.05 was considered as statistically significant.

Table 1. Clinical profile and laboratory data of diabetic patients

	Diabetic patients	
	(normo-albuminuria)	(micro-albuminuria)
Number of patients (male/female)	39 (22/17)	22 (12/11)
Age (years)	58.2 ± 2.1	59.1 ± 2.1
Diabetes duration (years)	8.2 ± 1.4	12.2 ± 1.4 ^b
Body mass index (kg/m ²)	23.7 ± 0.6	22.4 ± 0.9
Fasting plasma glucose level (mmol/l)	9.8 ± 0.8	11.0 ± 0.9
Haemoglobin A _{1c} (%)	8.3 ± 0.3	9.4 ± 0.5
Urinary albumin concentration (nmol/l)	1.4 ± 0.1	29.4 ± 1.2 ^a
Urinary albumin index (mg/g creatinine)	13.5 ± 1.0	231.7 ± 12.5 ^a
Total cholesterol (mmol/l)	12.0 ± 0.4	12.2 ± 0.6
Triglycerides (mmol/l)	1.8 ± 0.2	1.7 ± 0.3
Blood pressure (mm Hg)		
Systolic	133.2 ± 5.9	139.6 ± 7.7
Diastolic	82.9 ± 4.1	83.1 ± 6.1
Serum creatinine (µmol/l)	76.7 ± 2.1	78.7 ± 6.1

^a *p* < 0.0001, compared to Group I; ^b *p* < 0.01, compared to Group I

Results

The age, sex, BMI, fasting blood glucose levels, serum creatinine, HbA_{1c}, total cholesterol and triglycerides were not significantly different between cases with and without microalbuminuria (Table 1). In addition, no significant differences were observed in the levels of each parameter between patients treated with diet alone, oral hypoglycaemic agent or with a combination of both therapies. As shown in Table 2, the plasma levels of fibrinogen (*p* < 0.02), prothrombin F1 + 2 (*p* < 0.05), FM (*p* < 0.0001), sTM (*p* < 0.005), sE-S (*p* < 0.005), PC antigen (*p* < 0.005), PC activity (*p* < 0.005) and total PS antigen (*p* < 0.02) were markedly elevated in all diabetic patients with diabetes as compared to healthy subjects. The plasma concentration of free PS antigen was, however, significantly lower in diabetic patients than in the healthy control subjects (*p* < 0.005). Comparative evaluation of the foregoing parameters between patients with and without nephropathy disclosed significant increase of the plasma concentrations of fibrinogen (*p* < 0.05), prothrombin F1 + 2 (*p* < 0.05) and sTM (*p* < 0.03) in cases with microalbuminuria as compared to those with normoalbuminuria (Table 2).

The degree of PC activation as measured by the plasma levels of APC-PCI complex was significantly (*p* < 0.0001) accelerated in all diabetic patients (7.4 ± 3.8 pmol/l) as compared to healthy subjects (3.0 ± 0.4 pmol/l) (Fig. 1). Comparison of cases with or without nephropathy with the healthy control

Table 2. Clotting variables and markers of endothelial cell injury in diabetic patients

	Healthy subjects	All diabetic patients	Diabetic patients	
			(normoalbuminuria)	(microalbuminuria)
Fibrinogen ($\mu\text{mol/l}$)	10.4 ± 1.1	13.4 ± 0.6^c	12.0 ± 0.7	14.1 ± 0.9^f
Prothrombin F1+2 (nmol/l)	2.5 ± 0.1	3.2 ± 0.3^e	2.9 ± 0.2	3.6 ± 0.6^f
Fibrin monomer (ng/ml)	0.3 ± 0.1	2.1 ± 0.4^a	1.9 ± 0.4	2.2 ± 0.8
Soluble thrombomodulin (pmol/l)	26.4 ± 1.2	34.8 ± 2.9^b	31.2 ± 2.8	41.6 ± 6.1^d
Soluble E-selectin (pmol/l)	384.2 ± 29.4	534.2 ± 31.4^b	556.8 ± 44.6	495.2 ± 37.0
Protein C antigen (%)	92.1 ± 3.9	110.1 ± 4.2^b	109.6 ± 5.5	110.8 ± 6.5
Protein C activity (%)	111.6 ± 4.1	131.6 ± 4.7^b	134.2 ± 6.3	126.9 ± 7.2
Total protein S antigen (%)	71.6 ± 4.2	89.2 ± 4.6^c	85.1 ± 4.5	96.9 ± 10.3
Free protein S antigen (%)	116.4 ± 4.5	102.1 ± 2.2^b	101.2 ± 2.5	103.7 ± 4.1

^a $p < 0.0001$; ^b $p < 0.005$; ^c $p < 0.02$, compared to healthy subjects; ^d $p < 0.03$, compared to normoalbuminuria patients;

^e $p < 0.05$, compared to healthy subjects; ^f $p < 0.05$, compared to normoalbuminuria patients.

Data are mean $\pm$ SEM

Fig. 1. The plasma levels of APC-PCI complex were significantly higher ($p < 0.0001$) in all diabetic patients as compared to healthy subjects. Significant elevation in the plasma levels of APC-PCI complex was observed in either normoalbuminuric (group I; $p < 0.0001$) or microalbuminuric (group II; $p < 0.05$) patients as compared to the healthy control group. However, subjects from group II presented relatively lower ($p < 0.05$) levels of APC-PCI complex than those of group I. Bars indicate the mean values

Fig. 2. The anticoagulant response to exogenous TM (TAR) was significantly lower ($p = 0.005$) in all diabetic patients as compared to healthy subjects. Patients with microalbuminuria (group II) showed significantly lower ($p < 0.0001$) values of TAR as compared to healthy control subjects. Comparative evaluation between the normoalbuminuric (group I) and microalbuminuric (group II) patients, disclosed lower ($p = 0.03$) values of TAR in patients of group II than in those of group I. Bars indicate the mean values

group showed a significant increase of the plasma levels of APC-PCI complex in patients presenting normoalbuminuria (8.6 ± 0.7 pmol/l; $p < 0.0001$) or microalbuminuria (5.5 ± 0.6 pmol/l; $p < 0.05$) compared to healthy subjects, suggesting that in either normoalbuminuric (group I) or microalbuminuric (group II) patients there is a significant enhanced PC activation. However, comparing patients of group I with those of group II, ($p < 0.05$) the plasma levels of APC-PCI complex were relatively lower in subjects with nephropathy than in those without this complication (Fig. 1). The TM anticoagulant activity indicated by TAR was significantly ($p = 0.005$) lower in all cases with diabetic subjects ($23.4 \pm 2.6\%$) than in healthy subjects ($35.3 \pm 3\%$) (Fig. 2). The mean values of TAR decreased significantly in group II patients ($16.5 \pm 2.9\%$; $p < 0.0001$) but weakly in those

of group I ($28.3 \pm 3.8\%$; $p = 0.1$) as compared to healthy subjects. Patients of group II showed significantly lower ($p = 0.03$) values of TAR than those of group I.

Multivariate correlations of pro-coagulant and anticoagulant parameters with clinical and metabolic markers are shown in Table 3. The plasma concentration of APC-PCI complex correlated significantly with the values of albumin creatinine index in all patients with diabetes. The PC activity correlated significantly with the levels of Hb_{1c}, total cholesterol, triglycerides and serum creatinine as shown in Table 3. The plasma levels of APC-PCI complex or the values of TAR did not correlate with any glycaemic control parameter. Plasma APC-PCI complex levels correlated significantly ($r = 0.5$, $p < 0.01$) with the antigen concentration of PC, whereas the TAR values

Table 3. Correlation coefficients of procoagulant and anticoagulant markers with different clinical and laboratory parameters in all diabetic patients

	Fbg	F1+2	FM	PC	PCA	Total PS	Free PS	APC-PCI	TAR
Age	+ 0.3	+ 0.1	+ 0.4 ^a	+ 0.2	− 0.1	+ 0.1	− 0.1	+ 0.1	− 0.2
Diabetes duration	+ 0.5 ^b	+ 0.2	+ 0.2	+ 0.1	− 0.2	+ 0.1	+ 0.1	− 0.1	− 0.1
Fasting plasma glucose	+ 0.2	− 0.1	+ 0.1	+ 0.1	+ 0.1	− 0.2	− 0.1	+ 0.1	+ 0.1
Haemoglobin A _{1c}	+ 0.3 ^c	− 0.2	− 0.1	+ 0.2	+ 0.4 ^b	+ 0.2	+ 0.1	+ 0.1	+ 0.2
Urinary albumin concentration	+ 0.3	− 0.3 ^c	− 0.3 ^c	+ 0.2	− 0.1	+ 0.2	+ 0.1	− 0.3 ^c	− 0.3 ^c
Urinary albumin index	+ 0.4 ^c	− 0.3	+ 0.1	+ 0.1	− 0.1	− 0.1	+ 0.1	− 0.4 ^c	− 0.1
Total cholesterol	− 0.2	− 0.1	− 0.1	+ 0.1	+ 0.5 ^a	+ 0.1	+ 0.2	+ 0.1	− 0.1
Triglycerides	− 0.4 ^b	− 0.1	− 0.1	+ 0.2	+ 0.4 ^b	+ 0.2	− 0.1	+ 0.1	− 0.1
Serum creatinine	+ 0.1	+ 0.2	− 0.2	− 0.2	− 0.3 ^c	+ 0.1	− 0.1	− 0.1	− 0.1

^a $p < 0.003$; ^b $p < 0.02$; ^c $p < 0.05$

Fbg, Fibrinogen; Prothrombin F1+2, prothrombin fragment 1+2; FM, fibrin monomer; PC, protein C antigen; PCA, protein C activity; Total PS, total protein S antigen; Free PS, free

protein S antigen; APC-PCI, activated protein C-protein C inhibitor complex; TAR, thrombomodulin-induced anticoagulant activity rate

showed a significant inverse correlation ($r = -0.3$; $p < 0.05$) with plasma FM levels.

Discussion

The current study showed that the hyper-coagulable state in NIDDM is associated with an increased activation of PC as expressed by the high plasma levels of APC-PCI complex. The anticoagulant effect of exogenous TM (TAR) against the thrombin generation of plasma of diabetic patients was found to be reduced in relation to that of healthy subjects. In addition, a poor anticoagulant response as judged by the decreased formation of APC-PCI complex and low values of TAR were observed in cases presenting microalbuminuria.

Enhanced activation of the clotting system has been previously reported in patients with insulin-dependent diabetes (IDDM) or NIDDM [4]. High plasma concentrations of FM, prothrombin F1 + 2 and fibrinogen found in our NIDDM patients provide confirmatory evidence of these previous reports [26, 27]. This activation of blood coagulation has been recently implicated as an important contributing factor for the occurrence of vascular complications in diabetes [28]. In large epidemiological studies the pro-coagulant factors, fibrinogen and factor VII, have been described to be independent predictors of cardiovascular events in diabetic and non-diabetic subjects, and the detection of microalbuminuria, another well-known independent indicator of much greater risk of premature death from cardiovascular complications, has been demonstrated to correlate significantly with various haemostatic variables [4, 29, 30]. Accordingly, in the present study, the plasma levels of fibrinogen and prothrombin F1 + 2 were also found to be significantly higher in diabetic patients with microalbuminuria than in those with normoalbuminuria.

The pathogenic mechanism of the clotting activation in diabetes is not completely clear. Perturbation

of components of the anticoagulant system associated with hyper-glycaemia might play an important role. For instance, hyperglycaemia has been shown to induce depressed biological activity of the anticoagulant protein, antithrombin III, in diabetic and non-diabetic subjects [31]. Structural modification due to non-enzymatic glycation was suggested as the causative factor of this antithrombin III dysfunction [32]. Abnormalities of the anticoagulant PC pathway may also cause hypercoagulability in diabetic patients. The plasma antigen levels of PC and of its cofactor PS were found to be either decreased or increased in various reports depending on the type of diabetes. While reduced plasma levels of PC antigen have been described in IDDM patients, they were found to be elevated in those with NIDDM [4, 33]. Increased plasma levels of PC antigen were also observed in our NIDDM patients confirming these previous observations. Concerning the functional activity of PC, though enhanced anticoagulant response to APC has been described in IDDM, the degree of PC activation in NIDDM patients remains unclear [34]. To clarify the grade of activity of the anticoagulant PC system in NIDDM, we measured the plasma levels of APC-PCI complex, a direct index of ongoing PC conversion to its active enzyme APC. Our data showed for the first time that there is a significant increase in formation of APC-PCI complex in the circulation of NIDDM patients as compared to healthy subjects, suggesting the existence of an accelerated activation of the anticoagulant PC pathway in this group of diabetic patients. A compensatory response to hyper-coagulable state may explain this increased generation of APC-PCI complex in NIDDM. Hyperglycaemia has been considered to be the causative factor of the abnormalities of the PC anticoagulant pathway in IDDM, but in our NIDDM patients, no significant correlation was found between pro-coagulant or anticoagulant parameters with markers of glycaemic control [35].

Although the APC-PCI complex levels increased significantly in microalbuminuric patients as

compared to the healthy control group, they were relatively lower ($p = 0.03$) than those observed in cases with normoalbuminuria. The mechanism of this finding cannot be explained by the results of the current study. It may potentially depend on a lower activation rate of PC due to a severe systemic endothelial cell injury concomitant with the occurrence of microalbuminuria [36]; i.e. aggravation of the systemic microangiopathy may be associated with a progressive diminution of the amount of endothelial surface-bound TM leading to decreased activation of PC. This lesser availability of endothelial membrane-associated TM with subsequent poor PC anticoagulant response may result in further acceleration of the pro-coagulant activity in diabetes. Moreover, decreased activation of PC caused by endothelial cell injury may also diminish the inactivation rate of plasminogen activator inhibitor-1 by APC, leading to enhanced inhibition of fibrinolytic activity and thus to further thrombogenic tendency [37, 38]. Enhanced thrombogenic tendency as shown by the significant elevation of the plasma concentrations of fibrinogen and prothrombin F1 + 2 in cases with microalbuminuria may be explained by this relatively low-grade PC activation [39].

On the other hand, the endothelial membrane-bound TM, besides serving as a co-factor for the thrombin-induced activation of PC, may also exert direct thromboprotective effect by inhibiting the proteolytic and thrombogenic actions of thrombin on other clotting proteins and by accelerating the inhibition of thrombin by plasma serine protease inhibitors such as antithrombin III [9] or PCI [10]. The overall TAR was evaluated by using an *in vitro* assay, and its values were found to be lower in diabetic patients than in normal control subjects. The mechanism of these low TAR values might depend on the inability of TM to inhibit the thrombogenic activity of thrombin or for enhancing the inhibition of thrombin by antithrombin III or PCI, probably due to the occurrence of structural and/or conformational changes of these natural inhibitors in patients with diabetes. This apparent inability of exogenous TM to inhibit thrombin generation by promoting the activity of thrombin inhibitors worsens with the clinical progression of disease, as indicated by the markedly decreased values of TAR in microalbuminuric patients. Further studies are required to clarify the molecular basis of this abnormal TAR in diabetes.

In brief, this investigation showed that the hypercoagulable state in NIDDM is associated with an increased activation of PC but with a low reactivity of plasma of diabetic patients to the anticoagulant effect of TM. In addition, this study suggests that an imbalance between the PC anticoagulant pathway and the coagulation system may be an important causative factor of the enhanced pro-coagulant activity observed in patients presenting microalbuminuria.

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