Elevation of Plasma Thrombomodulin Level in Diabetic Patients With Early Diabetic Nephropathy

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Thrombomodulin (TM) is a membrane protein in the vascular endothelium, and it plays an important role as a cofactor in the thrombin-catalyzed activation of protein C. It has also been found in human plasma; however, its clinical significance is not known. In this study, fasting plasma TM concentrations in 67 diabetic patients with different degrees of albuminuria (39 men aged 57 ± 8 yr, 28 women aged 57 ± 11 yr; means ± SD) and 34 age- and sex-matched healthy subjects were investigated by use of a one-step sandwich enzyme immunoassay, a new method developed by H.I. and others. As a screening, the patients were divided into three groups according to the first morning urinary concentrations of albumin: group 1, <30 μg/ml (normoalbuminuria); group 2, 30–140 μg/ml (microalbuminuria); group 3, >140 μg/ml (clinical nephropathy). There was no significant difference in plasma TM level between the control group (17.7 ± 3.7 ng/ml, n = 34) and group 1 (16.9 ± 3.4 ng/ml, *n* = 30); however, plasma TM concentrations in group 2 (22.8 ± 3.4 ng/ml, *n* = 22) and group 3 (29.6 ± 6.1 ng/ml, *n* = 15) increased significantly compared with those in the control group and group 1, respectively. As a further investigation, three timed overnight urine collections were made. The patients were allocated to three groups according to their rates of albumin excretion: group I, <20 μg/min (normoalbuminuria); group II, 20–200 μg/min (microalbuminuria); group III >200 μg/min (clinical nephropathy). No significant difference was found in plasma TM level between the control group (17.8 ± 3.8 ng/ml, *n* = 16) and group I (17.8 ± 3.0 ng/ml, *n* = 17); however, plasma concentrations in group II (22.1 ± 2.8 ng/ml, *n* = 18) and group III (29.2 ± 6.0 ng/ml, *n* = 13) increased significantly compared with those in the control group and group I, respectively. No significant differences were found in blood pressure between the control group, group I, and group II. The vascular endothelium could be injured by various metabolic derangements because of diabetes. Accordingly, it is supposed that an injury in the vascular endothelial cell may progress with the advance of diabetic angiopathy, and TM existing on the endothelial membrane surface may be released into the plasma. The vascular permeability that permits glomerular leakage of albumin may also be found in other vessels. Thus, our findings suggest that an increased influx of TM to the plasma may be caused by generalized endothelial damage in patients with early diabetic nephropathy. *Diabetes* 39:983–88, 1990

Thrombomodulin (TM), the membrane glycoprotein existing on the vascular endothelial cell surface, plays an important role as a cofactor in the thrombin-catalyzed activation of protein C (1–3). Activated protein C functions as an anticoagulant by inactivating the coagulation factors V_a and $VIII_a$, which are not inhibited by antithrombin III (4–7). In addition, TM is found to inhibit the procoagulant activities of thrombin, e.g., fibrin formation, factor V activation (8), and platelet activation (9), although human TM is less effective as an inhibitor of the procoagulant actions of thrombin compared with rabbit TM (10). Thrombomodulin also inhibits factor X_a activity in the prothrombinase complex (11). Thus, TM converts thrombin from a procoagulant protease to an anticoagulant and acts as a regulator of intravascular coagulation.

It has been reported that TM is also present in human plasma, and soluble TM is low compared with cellular TM in its intrinsic protein C–activating cofactor activity (12); however, the physiological and pathological significance of circulating TM is not known. There has been no report dealing with diabetic patients who often suffered from thrombotic disease.

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On the other hand, several investigators have proposed that microalbuminuria is a predictor of widespread vascular diseases, not only in diabetic patients (13–17) but also in nondiabetic subjects (18). Therefore, we determined plasma TM concentrations in diabetic patients with different degrees of albuminuria and investigated the relationship with diabetic angiopathy.

RESEARCH DESIGN AND METHODS

Sixty-seven non-insulin-dependent diabetic subjects were studied. Their ages ranged from 30 to 69 yr (mean ± SD 57 ± 10 yr). The mean ± SD duration of diabetes was 12 ± 6 yr (range 1–28 yr). Thirty-nine of them were men. Fourteen were controlled with diet alone, 22 were controlled with oral hypoglycemic agents, and 31 were treated with insulin injection. None of them had hepatic or autoimmune diseases. Fundi of diabetic subjects were examined by an ophthalmologist. The diabetic subjects with hypercreatininemia (serum creatinine ≧1.4 mg/dl; renal insufficiency) were not contained.

Diabetic neuropathy was diagnosed when a diabetic subject was aware of neuropathic symptoms. Patients with ischemic heart disease had at least one of the following: *1*) a history of myocardial infarction characterized by a typical clinical picture, electrocardiogram (ECG) alterations, and enzymatic changes; or *2*) ischemic alterations in ECG (Minnesota code I, 1–3; IV, 1–4; V, 1–3) with or without chest pain (19). The control group consisted of 34 healthy subjects matched by sex (19 men, 15 women) and age (55 ± 10 yr). The duration of diabetes was taken from the time the patient was diagnosed as diabetic.

Fasting plasma glucose (FPG) was measured by the glucose oxidase method, and HbA_{1c} was determined by high-performance liquid chromatography (20). Serum and urinary creatinine were measured by the routine technique with the Jaffé reaction (21). Blood pressure was measured with a standard clinical sphygmomanometer (cuff 25 × 12 cm). The mean of three measurements within a few months was taken. Body mass index was calculated as weight/height^2 (kg/m^2). Student's *t* test was used to analyze the data.

Determination of plasma TM. TM concentrations in plasma in fasting healthy and diabetic subjects were measured by an enzyme-linked immunoassay with monoclonal antibodies (MoAbs) against human placental TM (22). Each well of a microtiter plate was coated with 2 μg TM-MoAb 20 for 2 h at room temperature and washed 5 times with phosphate-buffered saline (PBS; 10 mM phosphate buffer, pH 7.5, with 0.15 M NaCl) containing 0.05% Tween 20 and then kept with PBS containing 0.2% bovine serum albumin and 0.05% Tween 20 for 1 h at room temperature.

After discarding the buffer, horseradish peroxidase–labeled TM-MoAb 20 and 11 (each final concn 0.3 μg/ml) were placed; this was immediately followed by placement of a test specimen. The plate was incubated 2 h at room temperature and then was washed 5 times with PBS containing 0.05% Tween 20. Subsequently, 200 μl substrate solution with 0.02% H_2O_2 and 0.5 mg/ml *o*-phenylenediamine in 0.1 M citrate buffer (pH 5.0) was placed. The plate was incubated 30 min at room temperature. Color development was terminated with 100 μl of 2 N H_2SO_4. Absorbance at 492 nm was measured with a Titertek Multiskan MK II. The purified placental TM was used as a standard preparation. Two or more determinations were made within a few months, and the average was taken.

Determination of urinary albumin. As a screening, first morning urinary concentrations of albumin were measured. To take account of the high day-to-day variation in urinary albumin excretion, two or more urine collections were made for each subject (23). The level of albuminuria was defined as the median value in these collections determined by an albumin radioimmunoassay kit (Pharmacia, Piscataway, NJ). Urine sterility was checked by urinalysis with a microscope.

The patients were divided into three groups according to the criteria of Mogensen (16). Group 1 consisted of 30 patients with a normal urinary albumin concentration (UAC) of <30 μg/ml. Group 2 consisted of 22 patients with a UAC in the range 30–140 μg/ml (microalbuminuria). Group 3 consisted of 15 patients with a UAC >140 μg/ml (clinical diabetic nephropathy).

For further investigation, three timed overnight urine collections in 48 diabetic subjects examined at screening were made for each subject at home during normal physical activity. Sixteen healthy nondiabetic subjects matched by sex and age served as the control group. The level of albuminuria was defined as the median value in these three collections, determined as described previously. Urine sterility was checked similarly.

These patients were allocated to three groups according to their albumin excretion rate (AER). Group I consisted of 17 patients with a normal urinary AER of <20 μg/min. Group II consisted of 18 patients with an AER in the range 20–200 μg/min (microalbuminuria). Group III consisted of 13 patients with an AER >200 μg/min (clinical diabetic nephropathy). The albumin creatinine index was calculated as UAC (μg/ml)/urinary creatinine concentration (mg/dl) (24).

RESULTS

Table 1 shows the clinical characteristics and laboratory data of the control and diabetic subjects with different degrees of UAC. No significant differences were found in blood pressure, body mass index, and serum creatinine levels among the groups. The albumin creatinine index (mg/g creatinine) was significantly increased in group 2 (range 26.9–209.0) and group 3 (range 193.5–1010.0) compared with the control group (range 1.7–20.0) and group 1 (range 4.4–24.7) ($P < 0.001$). The UAC in the control group and group 1 ranged from 2.1 to 17.6 μg/ml and from 2.2 to 18.4 μg/ml, respectively. The UACs in group 2 (range 24.4–139.2 μg/ml) and group 3 (range 184.2–594.1 μg/ml) increased significantly compared with the control group and group 1 ($P < 0.001$).

Figure 1 shows the plasma TM levels in the control subjects and diabetic patients grouped according to UAC. There was no significant difference in plasma TM levels between the control group (17.7 ± 3.7 ng/ml) and group 1 (16.9 ± 3.4 ng/ml). Plasma concentrations of TM were significantly increased in group 2 (22.8 ± 3.4 ng/ml) and group 3 (29.6 ± 6.1 ng/ml) compared with group 1 and the control group ($P < 0.001$).

Table 2 shows the clinical characteristics and laboratory data of the control and diabetic subjects with different degrees of AER. All patients allocated to the microalbuminuric

TABLE 1
Clinical characteristics and laboratory data of subjects

	Control	Group 1	Group 2	Group 3
n (M/F)	19/15	18/12	14/8	7/8
Age (yr)	55 ± 10	56 ± 9	58 ± 10	57 ± 10
Diabetes duration (yr)		9 ± 5	12 ± 6	14 ± 7
Fasting plasma glucose (mM)	5.0 ± 0.4	8.2 ± 1.9	8.9 ± 2.2	9.7 ± 3.2
HbA_{1c} (%)	5.9 ± 0.3	8.1 ± 1.6	7.9 ± 1.3	8.7 ± 2.1
Blood pressure (mmHg)				
Systolic	125 ± 13	127 ± 14	132 ± 16	134 ± 13
Diastolic	80 ± 9	82 ± 9	82 ± 7	84 ± 8
Body mass index (kg/m^2)	22.9 ± 2.4	22.0 ± 3.0	22.7 ± 3.2	23.5 ± 3.2
Serum creatinine (mg/dl)	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
Albumin index (mg/g creatinine)	8.1 ± 6.6	10.3 ± 4.8	102.5 ± 43.0*	496.6 ± 297.5*
Urinary albumin concentration (μg/ml)	8.1 ± 4.6	8.6 ± 4.0	71.7 ± 39.4*	282.5 ± 101.4*
Retinopathy (*n*)				
Nil		26	6	0
Background		4	9	10
Proliferative		0	7	5
Neuropathy (*n*)		5	7	11
Ischemic heart disease (*n*)		3	5	4
Mode of therapy (*n*)				
Diet alone		11	2	1
Oral hypoglycemic agent		10	7	5
Insulin		9	13	9

Values are means ± SD.
*$P < 0.001$ vs. control group and group 1.

group according to the UACs also belonged to group II. Sixteen healthy subjects matched by age and sex served as control subjects. There were no significant differences in blood pressure, body mass index, and serum creatinine levels among the control group and groups I–III. The albumin creatinine index was significantly increased in group II (range 26.9–209.0) and group III (range 197.1–1028.4) compared with the control group (range 4.6–23.3) and group I (range 5.2–17.5) ($P < 0.001$).

The AER in the control group and group I ranged from 2.0 to 19.8 and from 3.1 to 17.4 μg/min, respectively. The AERs in group II (range 20.1–169.9 μg/min) and group III (range 205.2–748.8 μg/min) increased significantly compared with the control group and group I ($P < 0.001$).

Figure 2 shows the plasma TM levels in the control subjects and diabetic patients allocated according to AER. No significant difference was found in the plasma TM level between the control group (17.8 ± 3.8 ng/min) and group I (17.8 ± 3.0 ng/ml). Plasma concentrations of TM were significantly increased in group II (22.1 ± 2.8 ng/ml) and group III (29.2 ± 6.0 ng/ml) compared with group I and the control group ($P < 0.001$). There was no independent association with the degree of neuropathy or retinopathy. Plasma TM level was not associated with the relatively short-term controlled state of diabetes, because no correlation was found between the plasma TM level and FPG or HbA_{1c}. Furthermore, there was no significant correlation between the plasma TM level and sex, age, or the mode of therapy; however, a significant correlation was found between the plasma TM level and the duration of diabetes ($r = 0.3721$, $P < 0.01$).

DISCUSSION

Although the origin of plasma TM is unclear, we consider two major possibilities for explaining the source of this soluble form of TM. One possibility is that the plasma TM is produced in unidentified cells—specifically those secreted into plasma; however, unlike other molecular markers of the vascular system (e.g., tissue-type plasminogen activator

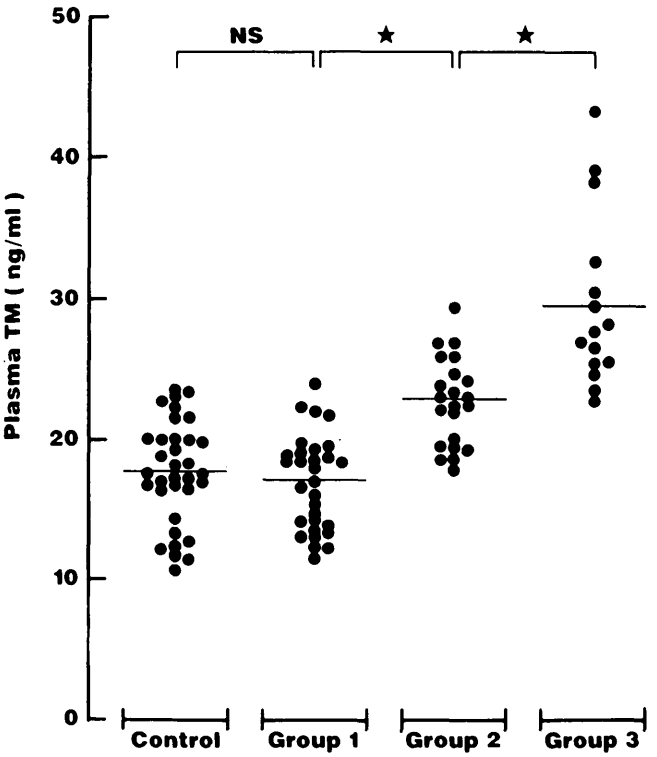

FIG. 1. Plasma thrombomodulin levels in control and diabetic subjects with different levels of urinary albumin concentration (UAC): group 1 (*n* = 30), normal UAC (<30 μg/ml); group 2 (*n* = 22), microalbuminuria (UAC: 30–140 μg/ml); group 3 (*n* = 15), clinical nephropathy (UAC >140 μg/ml). Values in groups 2 and 3 are significantly higher than in control group and group 1. *Horizontal bars* indicate mean values. NS, not significant. *$P < 0.001$.

TABLE 2
Clinical characteristics and laboratory data of subjects

	Control	Group I	Group II	Group III
n (M/F)	9/7	10/7	11/7	6/7
Age (yr)	54 ± 8	55 ± 10	58 ± 11	56 ± 10
Diabetes duration (yr)		9 ± 6	12 ± 6	14 ± 7
Fasting plasma glucose (mM)	5.2 ± 0.4	8.6 ± 2.0	8.7 ± 2.4	9.3 ± 3.2
HbA_{1c} (%)	5.9 ± 0.2	8.2 ± 1.5	7.9 ± 1.4	8.3 ± 1.7
Blood pressure (mmHg)				
Systolic	123 ± 13	128 ± 14	132 ± 18	135 ± 13
Diastolic	80 ± 9	84 ± 10	81 ± 7	84 ± 8
Body mass index (kg/m^2)	22.7 ± 2.7	22.6 ± 3.3	22.4 ± 3.1	23.8 ± 3.3
Serum creatinine (mg/dl)	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2
Albumin index (mg/g creatinine)	12.6 ± 7.2	9.3 ± 4.0	99.1 ± 46.4*	454.5 ± 278.8*
Albumin excretion rate (μg/min)	9.2 ± 6.4	8.7 ± 4.4	72.3 ± 46.1*	334.9 ± 188.6*
Retinopathy (*n*)				
Nil		14	6	0
Background		3	6	9
Proliferative		0	6	4
Neuropathy (*n*)		4	6	9
Ischemic heart disease (*n*)		2	5	4
Mode of therapy (*n*)				
Diet alone		4	1	1
Oral hypoglycemic agent		6	6	4
Insulin		7	11	8

Values are means ± SD.
*$P < 0.001$ vs. control group and group I.

[t-PA], its fast-acting inhibitor [PAI-1], and the von Willebrand factor), we suppose that TM is not released from the endothelial cell in a physiological condition, because TM is originally a component of the plasma membrane of the endothelial cell, and the circadian fluctuation of plasma TM is very small (22,25–27).

The second possibility is that the plasma TM reflects proteolysis of endothelial membrane TM as a result of endothelial damage. Because the molecular weight of plasma TM is identical to that of cellular TM treated with elastase, the proteolysis of cellular TM could conceivably account for the circulating form (12). The role of soluble TM in the circulation has been uncertain because the plasma circulation time of soluble TM is unknown.

In this study, we obtained an interesting result when determining plasma TM concentrations in diabetic subjects with different degrees of albuminuria. Plasma TM levels in patients with microalbuminuria increased significantly compared with those of the control subjects and the patients with normoalbuminuria. Generalized endothelial damage in patients with incipient diabetic nephropathy may be the reason for the increase, because the vascular permeability that permits glomerular leakage of albumin may also be found in other vessels (28). It has been reported that the vascular endothelium could be injured by hyperglycemia because of the promotion of glycosylation in functional proteins (29), metabolic derangement of the polyol pathway (30), and changes in the circulating blood properties (31).

It has also been shown that heparan sulfate proteoglycans in plasma membranes of endothelial cells have important antithrombogenic properties; diabetes affects heparan sulfate metabolism and leads to a generalized reduction of normally sulfated heparan sulfate in plasma membranes, resulting in general endothelial dysfunction or damage (32). Furthermore, in diabetic subjects, a decrease in the production of prostacyclin in the vascular endothelial cell and an increase in the production of thromboxane A_2 in platelets have been reported (33–36). In diabetic subjects, therefore,

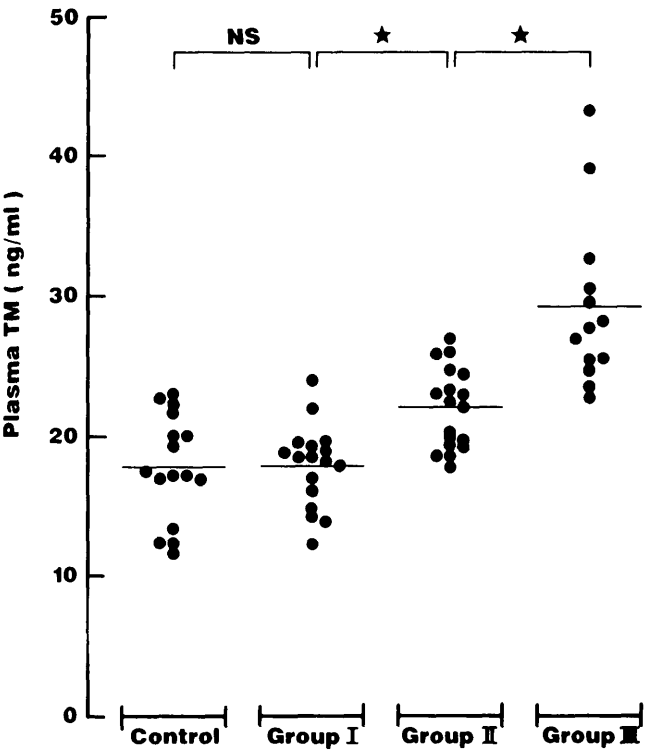

FIG. 2. Plasma thrombomodulin levels in control and diabetic subjects with different degrees of albumin excretion rate (AER): group I (*n* = 17), normal AER (<20 μg/min); group II (*n* = 18), microalbuminuria (AER: 20–200 μg/min); group III (*n* = 13), clinical nephropathy (AER >200 μg/min). Values in groups II and III are significantly higher than in control group and group I. *Horizontal bars* indicate mean values. NS, not significant. *$P < 0.001$.

microthrombi may be easily formed in the microcirculation through the enhancement of platelet aggregation (37,38). Accordingly, it is supposed that an injury in the vascular endothelial cell may progress with the advance of diabetic angiopathy, and TM existing on the endothelial membrane surface may be released into the plasma. With the decreased antithrombin III activity due to nonenzymatic glycosylation of its active site, damaged TM on the endothelial surface may reduce the nonthrombogenic property of endothelium resulting from a balance between procoagulant and anticoagulant activities (39,40).

On the other hand, it has been reported that TM is found on endothelium of arteries, veins, capillaries, and lymphatics in many organs except the brain (41). It has been shown, however, that TM reactivity is found in the choroidal vessel but not in the retinal vessel (42). Therefore, it is unlikely that the elevated plasma TM levels of diabetic patients with retinopathy but not clinical nephropathy may derive from the damaged endothelium of retinal vessels. Conceivably, the raised plasma levels of TM could be explained by a higher prevalence of microalbuminuria among patients with retinopathy. Indeed, an independent association was not found between retinopathy and plasma levels of TM.

It has been emphasized that injury to the endothelium is the initiating event in atherogenesis (43). Therefore, intimal endothelial damage caused by development of advanced lesions of atherosclerosis may at least in part contribute to the increase of the plasma TM level.

Before beginning this study, microalbuminuric patients with resting blood pressure >160/95 mmHg were excluded. Therefore, there was no significant difference in blood pressure between the normoalbuminuric group and the microalbuminuric group. Thus, blood pressure could not account for the elevated plasma TM levels in the microalbuminuric patients. It is thought that the plasma TM concentration is not affected by the degree of plasma glucose level, because the plasma TM level was not correlated with the controlled state of diabetes.

High plasma levels of the von Willebrand factor are widely accepted as an indicator of endothelial damage (44); they have been associated with diabetic retinopathy (45,46) and incipient nephropathy (47,48). As mentioned before, the von Willebrand factor, t-PA, and PAI-1 are synthesized and secreted by the vascular endothelial cell and have a circadian rhythm. Accordingly, their plasma levels may be easily moved by stimuli such as exercise and various pathological states. The von Willebrand factor is also contained in megakaryocytes and platelets, and its plasma levels correlate with the ages of the patients (49–51). Alternatively, TM is originally a component of plasma membrane of the endothelial cell; its plasma levels are stable in a physiological condition, and they do not correlate with the ages of the patients (22). Hence, the elevation of plasma TM levels in diabetic subjects may truly reflect progressing endothelial damage.

Our findings suggest that an increased influx of TM to the plasma may be caused by generalized endothelial damage in patients with early diabetic nephropathy, and we support the hypothesis that albuminuria reflects widespread vascular damage (32). The determination of plasma TM may serve as a new marker for injury caused by diabetes in the general vascular endothelia; however, it is not known whether the clearance of plasma TM is also affected by diabetic microangiopathy.

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