Mechanisms of Elevation of Serum and Urinary Concentrations of Soluble Thrombomodulin in Diabetic Patients: Possible Application as a Marker for Vascular Endothelial Injury

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Serum and urinary levels of soluble thrombomodulin (TM) were measured in 71 patients with non–insulin-dependent diabetes mellitus (NIDDM) and 132 age-matched control subjects to elucidate the mechanisms involved in increased TM levels. We compared the TM level with urinary albumin excretion (UAE), creatinine (Cr) clearance, and indices of renal tubular damage such as urinary β_2-microglobulin. Serum TM was significantly higher in diabetic patients versus control subjects ($P < .001$) regardless of whether the patients had diabetic nephropathy. Urinary TM levels were also higher in diabetic patients than in control subjects ($P < .001$). Serum TM in diabetic patients was correlated positively with serum Cr and UAE and inversely with the Cr clearance rate ($P < .001$, respectively). The urinary level of TM in diabetic patients was significantly correlated with 24-hour glucose excretion and the serum level of 1,5-anhydroglucitol (1,5-AG) ($P < .001$). However, no correlations were found between urinary TM levels and renal function in diabetic patients. There was also no correlation between serum and urinary levels of TM in the patients. These results suggest that although the serum TM level is influenced by an impairment of the renal clearance of TM, this parameter may be a useful marker for vascular endothelial injury in diabetic patients. On the other hand, since the elevated urinary level of TM in the patients paralleled their urinary excretion of glucose, urinary TM levels do not correlate with vascular endothelial injury in diabetic patients.

THROMBOMODULIN (TM) is a 105-kd endothelial cell surface glycoprotein involved in the regulation of coagulation at the endothelial surface. This regulation takes place via activation of protein C by formation of the thrombin-TM complex.[1] TM is widely distributed on the endothelium of human arteries, veins, capillaries, and lymphatics in all organs except the brain. The lung, placenta, and kidney exhibit especially high levels of TM.[2] It has been reported that the plasma and urine of humans contain soluble forms of TM that appear to be smaller than cellular TM, indicating the presence of heterogeneous fragments that resemble cellular TM in terms of intrinsic protein C–activating cofactor activity.[3] Several investigators have demonstrated that increased plasma levels of soluble TM are found in patients with disseminated intravascular coagulation, pulmonary thromboembolism, chronic renal failure, acute hepatic failure,[4] systemic lupus erythematosus,[5] and polyarteritis nodosa.[6] These increased plasma levels are thought to be mainly due to an increased amount of degraded fragments from cellular TM resulting from damage to the endothelium by injury or inflammation. This suggests that the plasma level of soluble TM may be a marker for vascular endothelial injury.[4]

It was recently reported that plasma TM is increased in patients with diabetes mellitus, particularly those with diabetic nephropathy.[7] We reported that urinary levels of TM are also increased in patients with diabetes as compared with healthy subjects.[8] In contrast to the close relationship observed between the plasma concentration of TM and the severity of diabetic nephropathy in patients with diabetes mellitus, the mechanism and clinical significance of elevated urinary TM levels in diabetic patients are unclear.

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To clarify the mechanism of the increase in soluble TM in the plasma and urine of patients with diabetes mellitus, we measured serum and urinary levels of this molecule in diabetic patients. We then compared these levels with indices of glomerular function such as creatinine (Cr) clearance, indices of renal tubular function such as urinary β_2-microglobulin and *N*-acetyl-β-D-glucosaminidase (NAG), and parameters of glucose metabolism, including plasma 1,5-anhydroglucitol (1,5-AG), which indicates the level of 24-hour urinary glucose excretion.[9]

SUBJECTS AND METHODS

The study was conducted in 71 Japanese patients with non–insulin-dependent diabetes mellitus (NIDDM) and 132 age-matched healthy controls. None of the subjects had clinical or laboratory evidence of liver disease. Also excluded were smokers and patients with macroangiopathy, such as ischemic heart disease, cerebrovascular disease, and peripheral vascular disease, which may affect serum and urinary TM levels. Serum levels of Cr in diabetic patients were 0.4 to 3.0 mg/dL (mean ± SD, 0.72 ± 0.40 mg/dL).

Urine was collected over a 24-hour period for analysis. Urinary albumin excretion (UAE) was measured with an immunoturbidimetric assay technique (Hoechst Japan, Tokyo, Japan). The urinary level of β_2-microglobulin was determined by latex-turbidimetric immunoassay (Eiken Chemical, Tokyo, Japan). The urinary enzyme NAG was determined using the MPT-NAG substrate method (Nittobo Medical, Tokyo, Japan). Hemoglobin A_{1c} (HbA_{1c}) levels were measured by high-performance liquid chromatography (Kyoto Daiichikagaku, Kyoto, Japan). Serum concentrations of AG were determined using a chromatographic pyranose oxidase method followed by spectrophotometry (Nippon Kagaku, Tokyo, Japan).

The concentration of soluble TM in serum and urine was measured by enzyme immunoassay (EIA) with monoclonal antibodies against human placental TM (TM monoclonal antibody 2, 11, and 20) as previously described.[10] This EIA sandwich method using three types of monoclonal antibodies is sensitive and reliable for soluble TM assay, measuring almost all subspecies of soluble TM in serum and urine of molecular size 105, 85, 80, 56, 33, and 28 kD, and the coefficient of variation for this method is 4.7% at the low level of TM.[10] Urinary excretion of TM was calculated by dividing the urinary concentration of TM by the urinary concentration of Cr.

Table 1. Clinical Characteristics of the Diabetic Patients and Control Subjects

Characteristic	Diabetic Patients	Control Subjects
No. (male/female)	71 (37/34)	132 (65/67)
Age (yr)	53.0 ± 11.3	55.6 ± 9.2
Body mass index (kg/m²)	22.3 ± 3.7	22.7 ± 1.9
Diabetes duration (yr)	8.1 ± 6.0	—
FPG (mmol/L)	10.5 ± 3.4	—
HbA_{1c} (%)	9.6 ± 2.0	—
1,5-AG (μg/mL)	3.23 ± 3.15	—
Serum TM (U/mL)	24.9 ± 13.2*	16.9 ± 8.0
Urinary TM (U/mg Cr)	41.5 ± 15.2*	34.2 ± 5.7

NOTE. Data are the mean ± SD.
*$P < .001$ *v* control subjects.

Patients with NIDDM were subdivided into three groups according to the rate of UAE in the 24-hour urine collection: normoalbuminuria, UAE less than 30 mg/24 h; microalbuminuria, UAE = 30 to 300 mg/24 h; and macroalbuminuria, UAE greater than 300 mg/24 h.

Data are expressed as the mean ± SD. Differences between data sets were evaluated by a two-tailed unpaired Student's *t* test. Differences in the three groups were compared using Duncan's test. Pearson's correlation coefficient was calculated. A *P* value less than .05 was accepted as statistically significant.

RESULTS

Clinical characteristics and laboratory data for the diabetic patients and control subjects are summarized in Table 1. Serum concentrations of soluble TM were significantly higher in diabetic patients versus control subjects (24.9 ± 13.2 *v* 16.9 ± 8.0 U/mL, $P < .001$). Similarly, urinary concentrations of TM were significantly higher in diabetic patients versus control subjects (41.5 ± 15.2 *v* 34.2 ± 5.7 U/mg Cr, $P < .001$).

In the 71 patients with NIDDM, serum TM levels positively correlated with the duration of diabetes ($P < .05$), the serum Cr concentration ($P < .001$), UAE ($P < .001$), urinary β_2-microglobulin ($P < .001$), and systolic and diastolic blood pressure ($P < .05$, respectively) (Table 2). In this group, the serum levels were inversely correlated with the rate of Cr clearance ($P < .001$; Fig 1). In particular, we observed a close relationship between the serum level of TM and the indices of glomerular function, which suggests that impaired renal clearance may have contributed to the elevated serum level of TM in diabetic patients.

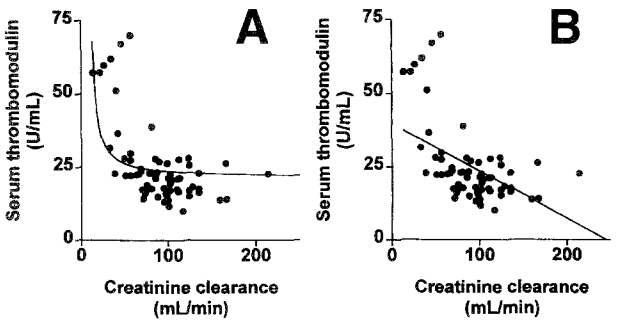

Fig 1. Correlation between serum TM and Cr clearance in patients with NIDDM. (A) One-site binding hyperbola analysis (curvilinear correlation), $R^2 = .281$. (B) Linear regression analysis, $r = -.471$, $P < .001$.

However, no significant correlation was found between serum TM and the markers for glycemic control such as fasting plasma glucose (FPG), HbA_{1c}, AG, and urinary excretion of glucose (Table 2).

No significant correlations were observed between the urinary level of TM and the indices of glomerular function, including the serum Cr concentration, rate of Cr clearance, and UAE, in diabetic patients. However, urinary TM showed a positive correlation with FPG ($P < .001$), HbA_{1c} ($P < .01$), urinary glucose ($P < .001$), and urinary NAG ($P < .05$). Moreover, an inverse correlation was observed between the TM level and the serum level of AG ($P < .001$; Table 2). A strong correlation was observed between the urinary level of TM and the amount of glucose excreted in 24 hours (Fig 2). No significant correlation was observed between serum and urinary levels of TM in patients with NIDDM ($r = -.027$; Fig 3).

We then compared serum TM levels between patients with NIDDM without clinical diabetic nephropathy (ie, normoalbuminuric or microalbuminuric) and control subjects to determine whether serum TM might be a useful and predictive marker for endothelial damage related to diabetic microangiopathy. Normoalbuminuric and microalbuminuric patients both exhibited normal levels of serum Cr and Cr clearance, suggesting that serum TM levels in either subgroup were not influenced by impaired renal clearance of TM. Serum TM was significantly higher in diabetic patients with normoalbuminuria and microalbuminuria versus control subjects ($P < .05$), and urinary TM was also higher in diabetic patients with normoalbuminuria and microalbuminuria than in control subjects ($P < .001$; Table 3).

DISCUSSION

The present study demonstrated that serum levels of soluble TM are influenced to some extent by impaired renal clearance of TM in patients with diabetic nephropathy, especially end-

Table 2. Correlation Between Soluble TM Levels in Serum and Urine and Clinical Parameters in NIDDM Patients

Parameter	Serum TM (U/mL)	Urinary TM (U/mg Cr)
Age (yr)	.087	.121
Diabetes duration (yr)	.274*	−.084
FPG (mmol/L)	.060	.403‡
HbA_{1c} (%)	.010	.320†
AG (μg/mL)	.125	−.493‡
Serum-Cr (mg/dL)	.771‡	−.211
Cr clearance (mL/min)	−.471‡	.082
SBP (mm Hg)	.273*	.092
DBP (mm Hg)	.242*	.110
Urinary glucose (g/24 h)	−.195	.551‡
UAE (mg/24 h)	.687‡	−.146
Urinary β_2MG (μg/mL)	.454‡	−.113
Urinary NAG	.307	.353*

NOTE. Correlation coefficients were determined by linear regression analysis.

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; β_2MG, β_2-microglobulin.

*$P < .05$.
†$P < .01$.
‡$P < .001$.

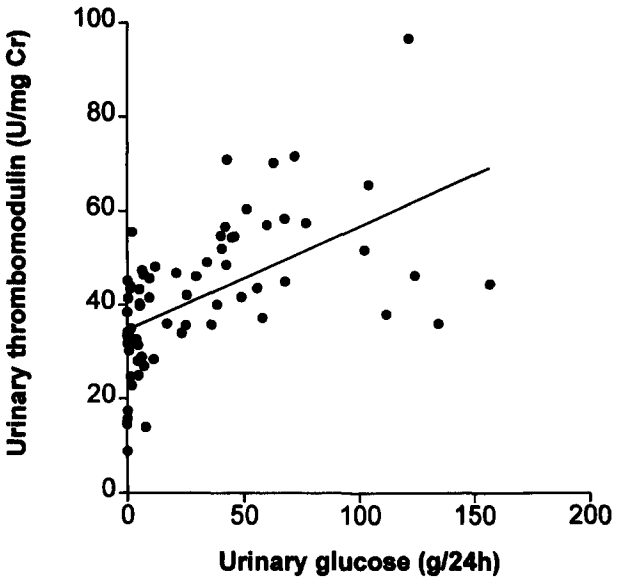

Fig 2. Correlation between urinary TM and the amount of glucose excreted in 24 hours in patients with NIDDM. $r = .551$, $P < .001$.

stage nephropathy, since we observed close correlations between serum TM levels and serum Cr or the rate of Cr clearance in diabetic patients. The elevated serum TM in diabetic overt nephropathy was at least partly related to the retention of TM, ie, delayed clearance of TM from the kidneys. In other words, the marked elevation of serum TM could be attributable to the retention of TM superimposed on the release of TM from vascular endothelial damage in patients with overt diabetic nephropathy. However, the present study also showed that relative to the values in nondiabetic control subjects, serum TM levels were significantly higher in normoalbuminuric and

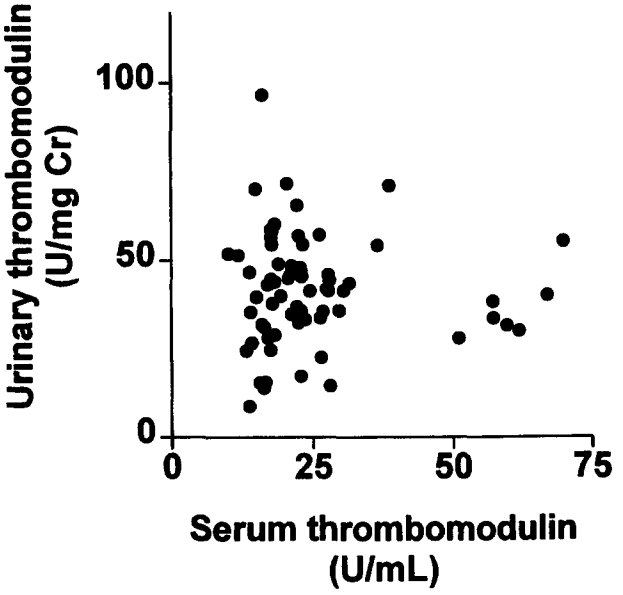

Fig 3. Correlation between serum and urinary TM in patients with NIDDM. $r = -.027$, nonsignificant.

Table 3. Comparison of Soluble TM Levels in Serum and Urine in Control Subjects and Normoalbuminuric or Microalbuminuric NIDDM Patients

Parameter	Control	Normoalbuminuric	Microalbuminuric
No. of subjects	132	34	23
Age (yr)	55.6 ± 9.2	51.5 ± 10.8	56.6 ± 11.5
Serum Cr (mg/dL)	—	0.60 ± 0.15	0.60 ± 0.19
Cr clearance (mL/min)	—	101.0 ± 30.8	97.3 ± 36.3
Serum TM (U/mL)	16.9 ± 8.0	20.5 ± 5.9*	20.3 ± 4.4*
Urinary TM (U/mg Cr)	34.2 ± 5.7	40.5 ± 12.8†	46.2 ± 18.1†

NOTE. Data are the mean ± SD.
*$P < .05$, †$P < .001$ *v* control.

microalbuminuric NIDDM patients with normal Cr clearance, an index of the glomerular filtration rate (GFR). Furthermore, it is known that diabetic patients with normoalbuminuria and microalbuminuria have a normal or elevated GFR, ie, hyperfiltration,[11] suggesting that the effect of impaired renal clearance of TM may not contribute to the elevation of serum TM in normoalbuminuric and microalbuminuric NIDDM patients. Since serum TM levels in normoalbuminuric and microalbuminuric NIDDM patients were not influenced by an impairment of the renal clearance of TM, the elevation of serum TM in both subgroups of NIDDM patients may be related to the generalized vascular endothelial injury induced by hyperglycemia or/and diabetic premicroangiopathy. It is suggested that this increase in serum TM in diabetic patients is due to the release of TM from injured endothelial cells into the plasma, since levels of soluble TM antigen in conditioned medium are increased by endothelial cell damage[12] and since the increased plasma TM is mainly due to an increase in the smaller molecular form, ie, degraded forms of cellular TM, implying that TM release from endothelial cells is accelerated by proteolytic activity generated on the surface of the endothelium.[3,4] We also speculate that the increase in serum TM in diabetic patients may be derived from the release of systematically damaged endothelial cells, especially from the glomeruli, which are most affected by diabetic microangiopathy and exhibit especially high levels of TM,[2] being also supported by a strong positive correlation between serum TM and UAE. Since TM reactivity is reportedly not found in retinal vessels,[13] one would not expect that diabetic retinopathy contributes to the total serum level of TM. Furthermore, there is no circadian fluctuation of both serum and urinary soluble TM, because soluble TM antigen is not secreted from endothelial cells and is hardly released from endothelial cells under physiological conditions.[10,12] We conclude that serum TM could be a more sensitive and predictive marker for vascular endothelial injury in diabetic patients, especially in diabetic early nephropathy, even when the influence of the delayed clearance of TM is taken into account.

We also found that urinary levels of TM were significantly elevated in diabetic patients compared with control subjects, as we reported previously.[8] Interestingly, unlike serum TM, urinary TM in diabetic patients was little influenced by renal function, since there was no correlation between urinary TM levels and the GFR (ie, Cr clearance) or UAE. Alternatively, no significant correlation was observed between serum and urinary

TM in diabetic patients despite the strong correlation between serum TM and renal glomerular function. However, serum TM increases and urinary TM decreases as the severity of renal insufficiency advances in patients with lupus nephritis.[5] Since the major site of clearance of TM is not only the kidneys but also the liver,[14] we excluded subjects with liver disease from the present study. Therefore, conditions such as hyperglycemia and glucosuria could explain the dissociation between serum and urinary TM observed in diabetic patients.

In contrast to the serum level of TM, the level of TM in the urine of diabetic patients was significantly correlated with markers of short-term glycemic control, especially 24-hour excretion of urinary glucose and serum 1,5-AG levels. This is the first demonstration of a close relationship between the urinary level of TM and the amount of urinary glucose excreted in 24 hours in diabetic patients. Why is urinary TM increased in proportion to the 24-hour urinary excretion of glucose in diabetic patients? It is unlikely that TM in urine is secreted or released from damaged renal tubules, since we did not observe a strong correlation between the urinary level of TM and indices of renal tubular damage such as urinary β_2-microglobulin or NAG. It is possible that the reabsorption of smaller molecular subtypes of TM in renal tubules is inhibited by glucosuria, resulting in the increased urinary excretion of TM in patients with diabetes. That is, osmotic diuresis induced by the acceleration of urinary glucose excretion may transiently impair the renal tubular reabsorptive function,[15] leading to the decreased reabsorption of smaller molecular TM in the renal tubules and the increased excretion of TM into urine. Therefore, it should be interpreted cautiously that urinary TM levels could be used as a simple indicator of vascular endothelial injury in patients with diabetes mellitus.

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