High Plasma Homocysteine Concentrations Are Associated With Plasma Concentrations of Thrombomodulin in Patients With Type 2 Diabetes and Link Diabetic Nephropathy to Macroangiopathy

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In type 2 diabetic patients with or without nephropathy, we examined relationships between plasma concentrations of total homocysteine (tHcy) and clinical macroangiopathy, as well as endothelial dysfunction indicated by plasma thrombomodulin (TM) concentrations. We studied 103 type 2 diabetic patients including 26 with macroangiopathy (12 patients with coronary artery disease [CAD], 10 with stroke, and 4 with peripheral vascular disease [PVD]). Plasma tHcy was measured by high-performance liquid chromatography. Plasma TM was determined by enzyme immunoassay. As an index of glomerular filtration rate, creatinine clearance (Ccr) also was determined in a 24-hour urine collection. Considering all diabetic patients, plasma tHcy concentrations were significantly higher in those with macroangiopathy than in those without (10.4 ± 3.7 *v* 8.5 ± 2.8 μmol/L, *P* = .0077). By univariate and multivariate analyses, plasma tHcy was correlated inversely with Ccr. Plasma tHcy concentrations were significantly higher in the patients with overt albuminuria than in those with normoalbuminuria or microalbuminuria. After exclusion of patients with renal insufficiency (Ccr < 60 mL/min), differences in plasma tHcy concentrations between patients with and without macroangiopathy were abolished. By multivariate analysis, total cholesterol, urinary albumin, Ccr, C-peptide, and tHcy retained significant influence on the plasma TM. Even in patients with normal renal function (Ccr ≥ 80 mL/min), plasma tHcy was correlated positively with plasma TM. In conclusions, diabetic nephropathy is a main determinant of plasma tHcy elevation in type 2 diabetic patients. Since plasma TM is independently associated with plasma tHcy, in diabetic patients with overt nephropathy, elevation of tHcy reflecting reduced clearance is a likely cause of endothelial dysfunction, resulting in the atherosclerosis underlying development of cardiovascular disease.

HYPERHOMOCYSTEINEMIA is an independent causal factor for coronary artery disease (CAD), stroke, and peripheral vascular disease (PVD).[1-3] A previous study identified moderate hyperhomocysteinemia as a stronger risk factor for cardiovascular disease in patients with type 2 diabetes than in nondiabetic subjects, suggesting that synergistic effects of diabetes and excessive circulating homocysteine (Hcy) accelerate development of atherosclerosis.[4] Elevations of total Hcy (tHcy) in plasma are proportional to the severity of renal dysfunction.[5,6] Additionally, several studies have demonstrated that in patients with type 2 diabetes, the presence of diabetic nephropathy is a major determinant of elevated plasma tHcy concentrations.[7,8]

Mechanisms by which Hcy induces atherosclerosis remain to be determined. However, in vitro and in vivo studies have suggested that Hcy is associated with endothelial damage, smooth muscle proliferation, platelet aggregation, and enhancement of coagulation.[9-11] Furthermore, Woo et al demonstrated impaired endothelial-dependent relaxation in arteries of patients with elevated Hcy concentrations.[12] Considering these findings together, Hcy may be a potent toxic molecule to vascular endothelium.

Thrombomodulin (TM), a thrombin-binding glycoprotein expressed on the endothelial cell surfaces in various tissues,[13] is involved in negative regulation of coagulation through the activation of protein C.[14,15] The soluble form of TM, which arises by proteolytic cleavage from membrane TM on endothelial cells, can be detected in human plasma and urine.[16] Since the plasma TM concentration is elevated in a variety of diseases accompanied by endothelial injury,[17] soluble TM is believed to be a marker for endothelial damage. Several studies have reported that soluble TM is increased in plasma from patients with diabetes mellitus, particularly those with diabetic nephropathy.[18,19]

In the present study of type 2 diabetic patients with or without diabetic nephropathy, we evaluated the association of plasma tHcy with clinical macroangiopathy. We then investigated whether plasma tHcy concentrations are independently associated with endothelial dysfunction as quantitated by plasma concentrations of TM.

MATERIALS AND METHODS

We studied 103 type 2 diabetic patients (47 women and 56 men). Patients were referred to the diabetes outpatient clinic for poor glycemic control. The diagnosis of type 2 diabetes was made according to the criteria of the World Health Organization. All patients who fulfilled the following inclusion criteria were considered for the study: (1) no episodes of ketoacidosis; (2) diagnosis of diabetes at an age greater than 30 years; and (3) insulin therapy, if any, not started after until 5 years of known disease. Patient ages and diabetes durations (mean ± SD) were 59.2 ± 12.2 and 11.6 ± 8.2 years, respectively. Patients with thyroid disease were excluded from study, since thyroid hormone affects the metabolism of Hcy. Also excluded were patients who were receiving medications that can affect plasma concentrations of tHcy, such as sex steroid hormone, antiepileptic drugs, and methotrexate.[20]

Macroangiopathy was defined as CAD, stroke, or PVD. CAD was defined as a history of myocardial infarction, coronary artery bypass grafting, or an abnormal coronary angiogram. Stroke was defined as a history of ischemic stroke confirmed by cranial tomography or magnetic resonance imaging. PVD was defined as a history of peripheral artery reconstruction or an ankle- to-brachial systolic blood pressure

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Table 1. Characteristics and Laboratory Data on All Diabetic Patients or Patients With a Ccr of at Least 60 mL/min According to Presence or Absence of Macroangiopathy

	All Patients		Patients With Ccr > 60 mL/min	
	No Macroangiopathy	Macroangiopathy	No macroangiopathy	Macroangiopathy
No. (M/F)	77 (40/37)	26 (16/10)	69 (34/35)	17 (11/6)
Age (yr)	57.5 ± 12.7	64.2 ± 9.0	58.1 ± 12.6	64.1 ± 9.7
BMI (kg/m^2)	23.5 ± 3.7	24.4 ± 4.08	23.1 ± 3.78	24.5 ± 3.80
Diabetes duration (yr)	10.0 (6.0, 15.0)	10.0 (4.5, 14.5)	10.0 (6.0, 15.5)	8.0 (3.0, 10.0)
FPG (mmol/L)	10.2 ± 3.53	11.0 ± 5.45	10.3 ± 3.00	11.8 ± 6.47
HbA_{1c} (%)	9.76 ± 2.03	9.20 ± 1.91	9.90 ± 2.01	9.00 ± 2.15
Ccr (mL/Min)	82.3 ± 30.7	67.3 ± 31.6*	88.6 ± 25.7	84.2 ± 22.7
T.Chol (mmol/L)	5.24 ± 1.11	5.48 ± 2.33	5.10 ± 1.00	5.03 ± 1.44
TG (mmol/L)	1.84 (1.30, 2.60)	2.01 (1.59, 3.05)	1.77 (1.26, 2.44)	1.91 (1.41, 2.32)
HDL-chol (mmol/L)	1.26 ± 0.32	1.18 ± 0.36	1.27 ± 0.33	1.17 ± 0.40
UAE (mg/24h)	36.0 (12.0, 182.0)	54.0 (14.0, 268.0)	24.0 (9.0, 103.5)	22.0 (10.5, 98.0)
TM (FU/mL)	3.46 ± 1.43	4.01 ± 1.25	3.13 ± 0.78	2.87 ± 0.56
Folate (ng/mL)	7.47 ± 3.40	6.07 ± 2.37	7.50 ± 3.24	6.70 ± 2.29
tHcy (μmol/L)	8.5 ± 2.8	10.4 ± 3.7†	8.2 ± 2.5	8.7 ± 2.4.
Hypertension: n (%)	30 (39%)	19 (73%)†	24 (35%)	11 (69%)*
Treatment (D/OHA/Ins)	23/36/10	6/6/4	22/43/12	5/13/8

NOTE. Data are mean ± SD or median and interquartile ranges.

Abbreviations: Ccr, creatinine clearance; BMI, body mass index; FPG, fasting plasma glucose; HbA_{Ic}, hemoglobin A_{Ic}; T.Chol, total cholesterol; TG, triglyceride; UAE, urinary albumin excretion; TM, thrombomodulin; tHcy, total homocysteine; D, diet alone; OHA, oral hypoglycemic agents; Ins, insulin.

*$P < .05$, †$P < .01$ *v* No Macroangiopathy.

ratio (ABI) less than 0.50. The ABI was determined as the ratio of ankle systolic blood pressure to the brachial systolic blood pressure, using the form PWV/ABI (AT Co, Komaki, Japan).

Diabetic patients also were placed into 3 groups according to urinary albumin excretion (UAE) in a 24-hour collection, specifically normoalbuminuria (group A), with UAE less than 30 mg/24 h; microalbuminuria (group B), UAE 30 to 299 mg/24 h; macroalbuminuria (group C), more than 300 mg/24 h. As an index of glomerular filtration rate, creatinine clearance (Ccr) was calculated using the same 24-hour urine collection.

Forty-nine of the diabetic patients had hypertension, defined as systolic blood pressure exceeding 140 mm Hg and/or diastolic blood pressure exceeding 90 mm Hg, or alternatively as treatment with antihypertensive agents. These medications included an angiotensin-converting enzyme (ACE) inhibitor (14 patients) and /or a calcium channel blocker (24 patients), and/or an angiotensin receptor blocker (7 patients).

As a control group, 60 nondiabetic subjects were selected to match the overall age and gender distribution of the diabetic group. Their age was 58.1 ± 11.3 years, and their body mass index was 23.8 ± 3.9. All subjects studied gave informed consent for their participation. The study was approved by the Dokkyo University Institutional Review Board.

Venous blood was obtained between 6 and 7 AM after an overnight fast. The length of time between the diagnosis of macroangiopathy and blood sampling was from 2 weeks to 6 months. Concentrations of tHcy were measured in EDTA-anticoagulant plasma using high-performance liquid chromatography. Intra-assay and interassay coefficients of variation (CVs) were 1.44% and 5.09%, respectively. Concentration of soluble TM was measured in citrate-anticoagulant plasma by an enzyme immunoassay (EIA) sandwich method using mouse monoclonal antibodies against human placental TM as previously described.[21] Intra-assay and interassay CVs were 3.60% and 4.53%, respectively. Serum folate concentrations were determined with a commercial kit (ADVIA Centaur Folate, Bayer Medical, Tokyo, Japan). Intra-assay and interassay CVs were 2.27% and 4.75%, respectively.

Statistical Analysis

Data are expressed as the mean ± SD or the median and interquartile ranges. Differences between groups were analyzed by an unpaired *t* test or a 1-way analysis of variance (ANOVA), with the Newman-Keuls multiple comparison test. For nonparametric data, differences between groups were analyzed by the Mann-Whitney *U* test or the Kruskal-Wallis test with Dunn's multiple comparison tests. The significance of differences in prevalence between groups was assessed by chi-square test. A logarithmic transformation of urinary albumin values was used to render the distribution normal for the parametric tests. Multivariate analysis was performed to determine the relationship of plasma TM concentration to age, diabetes duration, glycemic control, renal function, and plasma tHcy. A *P* value less than .05 was accepted as indicating statistical significance.

RESULTS

Plasma concentrations of tHcy were significantly higher in all diabetic patients than in healthy control subjects (8.96 ± 3.04 *v* 6.92 ± 1.36 μmol/L, $P < .0001$). Plasma concentrations of TM were also significantly higher in all diabetic patients than in healthy control subjects (3.60 ± 1.68 *v* 2.98 ± 0.63 FU/mL, $P = .0067$).

As shown in Table 1, we found no difference in age, duration of diabetes, glycemic control, or blood lipid profiles between diabetic patients with and without macroangiopathy. However, Ccr was significantly lower in patients with macroangiopathy than in those without macroangiopathy ($P < .05$; Table 1). Hypertension was more prevalent in patients with macroangiopathy ($P < .01$). Plasma tHcy concentrations were significantly higher in patients with macroangiopathy than in those without macroangiopathy (10.4 ± 3.7 *v* 8.5 ± 2.8 μmol/L, $P = .0077$; Fig 1). We then investigate whether the location of macroangiopathy may contribute to plasma concentrations of

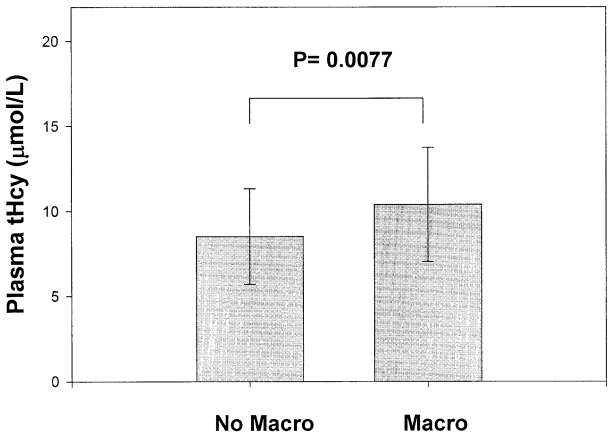

Fig 1. Plasma concentrations of tHcy in all type 2 diabetic patients studied, comparing those with macroangiopathy *v* those without.

tHcy. We could find no significant differences in plasma tHcy concentrations among the patients with CAD, stroke, and PVD (12.0 ± 3.55, 8.51 ± 1.97, and 9.70 ± 3.35 μmol/L, respectively).

By linear regression analyses (Table 2), plasma concentrations of tHcy correlated positively with systolic blood pressure ($P = .0015$), diastolic blood pressure ($P = .0152$), fasting serum C-peptide ($P = .0004$), urinary albumin ($P < .0001$), and plasma TM ($P < .0001$; Fig 2A), considering all diabetic subjects. Additionally, plasma tHcy concentrations correlated negatively with serum high-density lipoprotein (HDL)-cholesterol ($P = .0039$), Ccr ($P < .0001$), and serum folate ($P = .0092$). In diabetic patients with a Ccr of at least 80 mL/min, plasma tHcy also correlated positively with plasma TM ($P = .0016$; Fig 2B).

We next examined whether severity of diabetic nephropathy correlated with plasma concentrations of tHcy in diabetic patients (Table 3). Ccr was lower in groups B (microalbuminuria; $P < .05$) and C (macroalbuminuria; $P < .001$) than in group A (normoalbuminuria), also being lower in group C than in group B ($P < .001$). Plasma concentrations of tHcy were significantly higher in group C than in group A ($P < .001$) or B ($P < .01$). Hypertension was more prevalent in group C ($P < .01$). Macroangiopathy tended to be more prevalent in group C than in group A or B, but the differences fell short of significance.

We then compared plasma concentrations of tHcy between patients with and without macroangiopathy, after exclusion of patients with a Ccr less than 60 mL/min. Among the remaining patients, 17 had macroangiopathy. As shown in Table 1, we found no difference in age, duration of diabetes, glycemic control, blood lipid profiles, or Ccr between diabetic patients with and without macroangiopathy, provided that Ccr was at least 60 mL/min. Hypertension remained more prevalent in patients with macroangiopathy ($P < .05$). No difference was found in plasma tHcy concentrations between the patients with and without macroangiopathy after excluding low-Ccr patients (8.7 ± 2.4 *v* 8.2 ± 2.5 μmol/L, $P = .5067$; Table 1).

To determine independent factors affecting plasma TM concentrations, which reflect endothelial damage, we performed multivariate analysis. In the model that explained 80.7% of variation of plasma TM, total cholesterol, UAE, Ccr, C-peptide, and plasma tHcy were retained as independent factors determining plasma TM in patients with type 2 diabetes (Table 4).

DISCUSSION

The present study indicated that in patients with type 2 diabetes, plasma tHcy concentrations were significantly higher in the patients with clinically evident macroangiopathy than in those without macroangiopathy, in agreement with previous studies. Smulders et al reported that fasting tHcy concentrations correlated with prevalence of macrovascular disease in 150 patients with type 2 diabetes.[22] Buysschaert et al also indicated that plasma tHcy elevations in type 2 diabetes were associated with a higher prevalence of macroangiopathy.[23] Although plasma tHcy concentrations are known to be influenced largely by renal function in both nondiabetic and diabetic subjects,[5,6] these previous studies did not evaluate prevalence of macroangiopathy in patients with type 2 diabetes after exclusion of patients with renal insufficiency. The present study also showed that Ccr, an index of glomerular filtration rate, was significantly lower in patients with macroangiopathy than in those without macroangiopathy. Furthermore, multivariate as well as univariate analysis identified Ccr as highly associated with plasma tHcy concentrations in diabetic patients. This suggested that decreased clearance of Hcy by the kidneys resulted in elevated plasma concentrations of tHcy. Previous studies also reported that creatinine clearance is an independent determinant of plasma tHcy in diabetic patients.[8,9] The kidney plays a highly important role in Hcy metabolism of homocysteine; reportedly, 78% of the homocysteine in the blood is removed in the renal tubules.[24] Taken together, these observations indicated that overt diabetic nephropathy is a major determinant of elevated plasma tHcy concentrations.

We therefore compared plasma tHcy concentrations between

Table 2. Univariate Analysis of Relationships Between Plasma Total Homocysteine and Other Variables in Patients With Type 2 Diabetes

Variable	tHcy	
	r	*P* Value
Age (yr)	0.0522	.6058
BMI (kg/m^2)	0.1680	.0964
Diabetes duration (yr)	−0.0612	.5685
SBP (mm Hg)	0.324	.0015
DBP (mm Hg)	0.251	.0152
FPG (mmol/L)	−0.1299	.1975
HbA_{Ic} (%)	−0.1479	.1440
T.Chol (mmol/L)	0.0666	.5103
TG (mmol/L)	0.1659	.1026
HDL-chol (mmol/L)	−0.2892	.0039
Fasting C-peptide (nmol/L)	0.3587	.0004
Ccr (mL/min)	−0.493	<.0001
UAE (log_{10}mg/24 h)	0.404	<.0001
Folate (ng/mL)	−0.263	.0092
TM (FU/mL)	0.561	<.0001

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

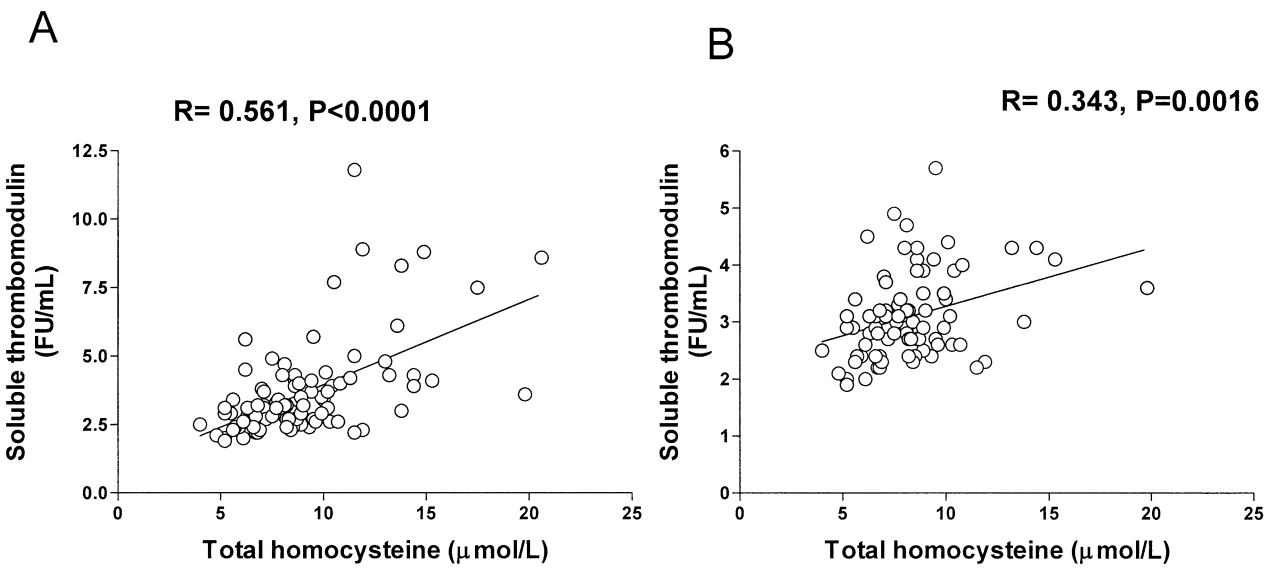

Fig 2. Correlation between plasma concentrations of soluble TM and tHcy in patients with type 2 diabetes. (A) All diabetic patients studied. (B) Diabetic patients with a Ccr ≥ 80mL/min.

patients with and without macroangiopathy after exclusion of patients with a Ccr less than 60 mL/min. No difference was found in plasma tHcy concentrations between the 2 groups. This is the first demonstration that plasma concentrations of tHcy did not differ significantly between diabetic patients with and without macroangiopathy after renal function was taken into account. This result suggests that in diabetic patients without renal insufficiency, factors other than plasma Hcy, such as hypertension, are likely to participate importantly in development of macroangiopathy. The present study showed a higher prevalence of hypertension in patents with macroangiopathy than that in those without macroangiopathy, even after exclusion of patients with renal insufficiency, so hypertension indeed could account for development of macroangiopathy in patients with type 2 diabetes with adequate renal function.

When present, diabetic nephropathy has been suggested to participate directly in development of macroangiopathy. Several studies have reported that proteinuria, especially microalbuminuria, is an independent predictive factor for cardiovascular disease.[25,26] Proteinuria is highly associated with endothelial dysfunction, which results in development of atherosclerosis. The present study also showed higher prevalence of macroangiopathy in diabetic patients with overt albuminuria compared with those with normoalbuminuria or microalbuminuria, but significance was not attained. Furthermore, plasma tHcy concentrations were significantly elevated in patients with overt albuminuria beyond those with normoalbuminuria or microalbuminuria. A previous study also reported that increased plasma tHcy is related to increased severity of diabetic nephropathy.[27] We therefore speculate that in patients with

Table 3. Patient Characteristics and Laboratory Data in Subgroups Defined by Urinary Albumin Excretion

	Group A	Group B	Group C
No. (M/F)	45 (24/21)	40 (21/19)	18 (11/7)
Age (yr)	57.6 ± 14.2	61.4 ± 9.5	58.4 ± 11.7
BMI (kg/m^2)	22.7 ± 3.5	24.3 ± 3.5	24.6 ± 4.9
Diabetes duration (yr)	10.0 (5.0, 15.0)	10.0 (6.0, 15.5)	12.0 (5.0, 15.0)
FPG (mmol/L)	10.5 ± 4.37	10.8 ± 4.23	9.28 ± 2.76
HbA_{1c} (%)	9.76 ± 2.18	9.72 ± 1.81	9.03 ± 1.79
Ccr (mL/min)	93.9 ± 25.1	79.6 ± 26.1*	38.1 ± 19.8‡¶
Folate (ng/mL)	7.52 ± 3.26	7.45 ± 3.24	5.60 ± 2.84
tHcy (μmol/L)	7.93 ± 2.66	8.93 ± 2.64	11.5 ± 3.36‡§
Hypertension: n (%)	15 (33%)	20 (50%)	14 (78%)†
Macroangiopathy: n (%)	9 (20%)	10 (25%)	8 (44%)
Treatment (D/OHA/Ins)	15/23/7	10/22/8	4/9/5

NOTE. Data are means ± SD or median and interquartile ranges.

*$P < .05$ *v* group A; †$P < .01$ *v* group A; ‡$P < .001$ *v* group A; §$P < .01$ *v* group B; ¶$P < .001$ *v* group B. Group A, UAE < 30 mg/24 h; group B, UAE 30 to 299 mg/24 h; group C, UAE 300 mg/24 h.

Table 4. Multivariate Analysis of Relationships Between Plasma Concentrations of Soluble Thrombomodulin and Selected Clinical Variables in the Diabetic Patients

Variable	Partial Coefficient	*P* Value
Age (yr)	−0.099	.462
Diabetes duration (yr)	0.073	.590
BMI (kg/m^2)	0.091	.503
SBP (mm Hg)	0.003	.982
DBP (mm Hg)	0.110	.414
HbA_{1c} (%)	−0.207	.123
T.Chol (mmol/L)	0.344	.009
TG (mmol/L)	−0.166	.217
HDL-chol (mmol/L)	−0.121	.370
UAE (mg/24 h)	0.517	.000
Ccr (mL/min)	−0.352	.007
C peptide (nmol/L)	−0.358	.006
tHcy (μmol/L)	0.576	.000

NOTE. Adjusted $R^2 = 0.807$.

diabetic overt nephropathy, elevation of plasma tHcy resulting from decreased Hcy clearance may contribute to development of atherosclerosis and its coronary, cerebrovascular, and peripheral vascular consequences.

Serum folate is an important determinant of plasma tHcy concentrations. A lower dietary folate intake is associated with a higher tHcy concentration in adults.[20] By linear regression analysis, the present study also showed an inverse correlation between plasma tHcy and serum folate concentrations in diabetic patients. However, we found no significant differences in serum folate among the patients with diabetic nephropathy groups, although plasma tHcy concentrations were different in the 3 groups. One possible explanation is a limited number of diabetic patients with overt nephropathy. It is also possible that compared with serum folate, renal insufficiency may contribute predominantly to an increased plasma tHcy in diabetic patients with overt nephropathy.

Considering all diabetic patients, the present study showed a strong positive correlation between plasma TM concentrations and tHcy. Several reports have demonstrated that the plasma concentrations of soluble TM are increased in patients with diabetes mellitus, particularly those with diabetic nephropathy.[18-20] Like those of tHcy, plasma TM concentrations are influenced largely by renal function.[21] We therefore evaluated the relationship between plasma tHcy and TM concentrations after exclusion of the patients with a Ccr less than 80 mL/min. We still found a significant positive correlation between concentrations of these 2 molecules when renal function was taken into account. As reported by van den Berg et al, plasma concentrations of endothelial-derived molecules are elevated in hyperhomocysteinemic patients with PVD.[28] Another previous study reported that serum tHcy is weakly associated with plasma concentrations of von Willebrand factor, another marker of endothelial dysfunction, in type 2 diabetic patients.[29] However, no previous reports determined whether plasma concentrations of endothelial markers are independently associated with plasma tHcy in patients with type 2 diabetes. This study is the first to demonstrate by multivariate analysis that plasma tHcy is an independent factor influencing plasma TM. Since plasma TM may be a sensitive marker for vascular endothelial injury,[17] elevated plasma concentrations of tHcy appear likely to promote vascular endothelial damage in patients with type 2 diabetes especially those with overt nephropathy. Hofmann et al have reported that treatment of cultured endothelial cells with the L-Hcy increased release of soluble TM from cell-surface TM into the culture supernatant.[30] In vitro studies showed that Hcy inhibits cell-surface expression of TM on cultured endothelial cells.[31] On the basis of these findings, we speculate that Hcy may directly affect membrane-bound TM on endothelial cells, resulting in increased release of soluble TM into the bloodstream. Accordingly, Hcy may be a potent toxic molecule to vessels. This result suggests Hcy elevation as a possible mechanism linking diabetic nephropathy to development of macroangiopathy.

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