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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese.

Patients and methods: We sequenced the TM putative promoter, exon, and 3'-untranslated region in DVT patients ($n=118$). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms (–202G>A, 2487A>T, 2729A>C) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5'-UTR, 5'-untranslated region; 3'-UTR, 3'-untranslated region.

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with that in the age, body mass index-adjusted population-based controls.

Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p < 0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p = 0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio=2.76, 95% confidence interval=1.14–6.67; $p = 0.02$). This mutation was in linkage disequilibrium (r -square>0.9) with A455V mutation.

Conclusions: TM mutations, especially those with a haplotype consisting of 2729A>C and A455V missense mutation, affect sTM levels, and may be associated with DVT in Japanese.

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Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of

thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3'-untranslated regions (3'-UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

DVT for genetic variation. By genotyping three polymorphisms (-202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

Patients and methods

DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age: 52.3 ± 16.1 years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3'-UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI

PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

Table 1 Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years \pm S.D.	52.3 \pm 16.1	Nephrotic syndrome, <i>n</i> (%)	0 (0.0)
Women, <i>n</i> (%)	59 (50.0)	Chronic heart failure, <i>n</i> (%)	17 (14.4)
BMI, kg/m ² , mean \pm S.D.	23.7 \pm 3.2	Diabetes Mellitus, <i>n</i> (%)	47 (39.8)
DVT family history, <i>n</i> (%)	8 (6.8)	Hypertlipidemia, <i>n</i> (%)	48 (40.7)
Previous DVT, <i>n</i> (%)	12 (10.2)	Autoimmune disease, <i>n</i> (%)	11 (9.3)
		Inflammatory bowel disease, <i>n</i> (%)	2 (1.7)
Pregnancy, <i>n</i> (%)	5 (4.2)	Estrogen use, <i>n</i> (%)	3 (2.5)
Stroke, <i>n</i> (%)	1 (1.5)	Steroid use, <i>n</i> (%)	9 (7.6)
Prolonged immobility, <i>n</i> (%)	14 (11.9)	Paralysis, <i>n</i> (%)	5 (4.2)
Malignancy, <i>n</i> (%)	16 (13.6)	Myeloproliferative disease, <i>n</i> (%)	1 (0.8)
Major surgery (abd, hip, leg), <i>n</i> (%)	21 (17.8)	Reduced plasminogen activity, <i>n</i> (%)	7 (5.9)
Trauma (pelvis, hip, leg), <i>n</i> (%)	3 (2.5)	Reduced antithrombin activity, <i>n</i> (%)	7 (5.9)
Stasis due to compression, <i>n</i> (%)	6 (5.1)	Reduced protein C activity, <i>n</i> (%)	8 (6.8)
Central venous catheter, <i>n</i> (%)	0 (0.0)	Reduced protein S antigen, <i>n</i> (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin, ACLb2), <i>n</i> (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≥ 126 mg/dl or non-fasting plasma glucose ≥ 200 mg/dl or HbA1c $\geq 6.5\%$ or use of antidiabetic medication; Hypertension, systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive medication; Hypertlipidemia, total cholesterol ≥ 220 mg/dl or use of antihypertlipidemia medication; Myeloproliferative disease, Plt. $>5 \times 10^5$ and Ht. $>55\%$; Reduced plasminogen activity, plasminogen activity $<70\%$; Reduced antithrombin activity, antithrombin activity $<80\%$; Reduced protein C activity, protein C activity $<70\%$; Reduced protein S antigen, protein S antigen $<60\%$.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r -square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means \pm S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t -test or χ^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was estab-

lished at $p < 0.05$. Linkage disequilibrium was calculated using SNPalyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*-832C>A		Promoter		99.6	0.4	gggcagagggcg [c/a] tgggttaggcc	
*-754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggttctctga	
*-265C>A		Exon(5'UTR)		99.6	0.4	aatccgagatg [c/a] ggcatcagccct	
-202G>A	A	Exon(5'UTR)		89.2	10.8	ggagggagggcc [g/a] ggcaattataaa	
*-58G>C		Exon(5'UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcggcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaagggt [g/t] acggtggcgaca	
1754C>T		Exon(3'UTR)		98.7	1.3	aggagcctggct [c/t] cgtccaggagcc	rs13306852
2005G>A	A	Exon(3'UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggaggg	rs3176134
*2230T>C		Exon(3'UTR)		99.6	0.4	tcttggtaatt [t/c] tttttcttagc	
*2487A>T		Exon(3'UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3'UTR)		79.8	20.2	gatgtaaaagg [a/g] ttaattgatgt	rs1042580
2729A>C	B	Exon(3'UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521-3522insT		3'flanking		99.6	0.4	ctcgggtgtgt [-/t] gtctgttcactt	
*3559T>A		3'flanking		99.6	0.4	gccctatttta [t/a] gtcattaaatgg	

LD, mutations in linkage disequilibrium (group A; r -square=0.84, group B r -square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women (n=1215)	Men (n=1032)	p
Age, years \pm S.D.	64.6 \pm 10.7	67.1 \pm 10.9	<0.0001
Systolic blood pressure, mm Hg \pm S.D.	123.5 \pm 19.8	126.1 \pm 17.9	0.0008
Diastolic blood pressure, mm Hg \pm S.D.	74.3 \pm 10.4	77.2 \pm 10.4	<0.0001
Body mass index, kg/m ² \pm S.D.	22.4 \pm 3.2	23.4 \pm 3.0	<0.0001
Total cholesterol, mg/dl \pm S.D.	215.9 \pm 31.6	198.7 \pm 31.5	<0.0001
HDL-cholesterol, mg/dl \pm S.D.	64.4 \pm 15.1	55.2 \pm 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure \geq 140 mm Hg and/or diastolic blood pressure \geq 90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol \geq 220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose \geq 126 mg/dl or non-fasting plasma glucose \geq 200 mg/dl or HbA1c \geq 6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's *t*-test or χ^2 analysis.

mutations were missense mutations (R385K; *n*=2, A455V; *n*=53 heterozygous, *n*=14 homozygous, D468Y; *n*=2). Four mutations within the TM promoter region and the 5'-untranslated region (5'-UTR) (-832C>A, -754G>C, -265C>A, -58G>C) were rare. Twenty-five patients were heterozygous carriers for the -202G>A mutation within the promoter region, which was reported as a -33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (*r*-square>0.8) with 2005G>A in the 3'-UTR. No patients were carriers for previously reported mutations in the lectin-like

domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain (*n*=53 heterozygous, *n*=14 homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (*r*-square>0.9) with the 2729A>C mutation within the 3'-UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women	Men	Total	Women	Men	Total
		<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)
-202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>
-202 G>A	GG	16.9 \pm 1.6		17.0 \pm 1.6		19.2 \pm 1.9		19.6 \pm 1.9	
	GA+AA	17.4 \pm 0.2	0.73	17.4 \pm 0.2	0.77	19.9 \pm 0.2	0.68	19.9 \pm 0.2	0.87
1208 G>A (R385K)	GG	17.4 \pm 0.2		17.4 \pm 0.2		19.9 \pm 0.2		19.9 \pm 0.2	
	GA+AA	16.2 \pm 2.4	0.62	16.0 \pm 2.3	0.54	20.5 \pm 2.2	0.79	20.4 \pm 2.2	0.84
1456 G>T (D468Y)	GG	17.4 \pm 0.2		17.4 \pm 0.2		19.9 \pm 0.2		19.9 \pm 0.2	
	GT+TT	18.1 \pm 1.0	0.51	18.1 \pm 1.0	0.52	22.2 \pm 1.7	0.20	22.6 \pm 1.7	0.11
2487 A>T	AA	17.6 \pm 0.2		17.6 \pm 0.2		20.0 \pm 0.2		20.0 \pm 0.2	
	AT+TT	16.7 \pm 0.4	0.04	16.7 \pm 0.4	0.04	19.6 \pm 0.6	0.54	19.5 \pm 0.6	0.40
2729 A>C	AA	17.9 \pm 0.2		17.9 \pm 0.2		20.4 \pm 0.3		20.3 \pm 0.3	
	AC+CC	16.7 \pm 0.3	<0.01	16.8 \pm 0.3	<0.01	19.4 \pm 0.3	0.03	19.5 \pm 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (-202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects.

As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 \pm 0.3 U/ml vs. 17.9 \pm 0.2 U/ml, *p*<0.01, men: 19.4 \pm 0.3 U/ml vs. 20.4 \pm 0.3 U/ml, *p*=0.03), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio=2.76, 95% confidence interval=1.14–6.67; *p*=0.02 and odds ratio=2.98, 95% confidence interval=0.21–7.33; *p*=0.02, respectively) (Table 6). This mutation was in linkage disequilibrium (*r*-square>0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3'-UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An *in vitro* study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17β -estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H_2O_2 , or hypochlorous acid generated from H_2O_2 by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3'-UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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Plasma ADAMTS13 Activity May Predict Early Adverse Events in Living Donor Liver Transplantation: Observations in 3 Cases

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A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF). Deficiency of ADAMTS13 increases the unusually large VWF multimers (UL-VWFM), which leads to platelet clumping and/or thrombus formation, resulting in microcirculatory disturbance. We serially determined the activity of plasma ADAMTS13, together with VWF antigen (VWF:Ag) and UL-VWFM, in association with the development of early graft dysfunction in 3 liver transplant recipients and 4 patients with major hepatectomy as controls. In case 1, ADAMTS13 activity decreased markedly from 108% to less than 3% with concomitant thrombocytopenia on posttransplantation day 7, when acute rejection occurred. Simultaneously, UL-VWFM were detected. During the second episode of rejection, VWF:Ag increased to 368% with the appearance of UL-VWFM, while ADAMTS13 activity was as low as 18%, indicating an imbalance between a large amount of UL-VWFM and low activity of ADAMTS13. Administration of fresh frozen plasma (FFP) together with treatment for acute rejection resulted in an improvement of ADAMTS13 activity and disappearance of the UL-VWFM. In case 2, ADAMTS13 activity promptly decreased to 9% with thrombocytopenia on day 1, when ischemia-reperfusion injury occurred. Subsequently, the ADAMTS13 activity increased steadily without appearance of UL-VWFM, and the patient recovered uneventfully. ADAMTS13 activity decreased to 15% immediately after transplantation in case 3 as well. In contrast, ADAMTS13 activity never decreased below 20% in 4 patients with major hepatectomy as controls. In conclusion, these results indicate that the kinetics of ADAMTS13 and UL-VWFM could be good indicators of adverse events after liver transplantation. Our findings not only suggest a novel mechanism for thrombocytopenia, but also provide a useful tool for diagnosis of graft dysfunction in the early stage after transplantation. *Liver Transpl* 12:859-869, 2006.

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Thrombotic microangiopathies (TMAs) are defined as life-threatening generalized disorders, characterized by microangiopathic hemolytic anemia, destructive thrombocytopenia, and organ dysfunction caused by microvascular platelet thrombi.^{1,2} Because of these features, TMAs are usually expressed heterogeneously, and can include thrombotic thrombocytopenic purpura

(TTP) with neurotropic signs prevalent in adults, but not exclusively, and hemolytic-uremic syndrome with predominant nephrotropic signs.^{1,2} TMA is also recognized as a critical complication after solid organ transplantation.³⁻⁹ However, most cases of transplantation-associated TMA are not clearly distinguishable as either TTP or hemolytic-uremic syndrome.

Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; VWF, von Willebrand factor; UL-VWFM, unusually large VWF multimers; FFP, fresh frozen plasma; VWF:Ag, VWF antigen; TTP, thrombotic thrombocytopenic purpura; TMA; thrombotic microangiopathy; ALT, alanine aminotransferase.

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The discovery of a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) has provided a breakthrough in our understanding of TMA pathogenesis. Recent studies indicate that ADAMTS13 is produced mainly in the liver, exclusively the stellate cells (formerly called Ito cells), and then thought to be released into the circulation via the microsinusoidal system,¹⁰ where ADAMTS13 specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 in the A2 domain.¹¹⁻¹⁴ VWF is synthesized in vascular endothelial cells, and released into the plasma as unusually large VWF multimers (UL-VWFM), which have potent biological activities.^{2,15} Under physiological conditions, UL-VWFM are rapidly degraded into smaller VWF multimers by ADAMTS13.^{2,15} Deficiency of the protease increases the level of UL-VWFM in plasma and leads to platelet aggregation and/or thrombus formation, finally resulting in TTP.^{16,17} In fact, the activity of ADAMTS13 is significantly decreased in most patients with TTP, whereas it is relatively preserved in the majority of patients with hemolytic-uremic syndrome.¹⁸

On the other hand, thrombocytopenia is commonly observed during the first week after liver transplantation, with or without apparent TMA.¹⁹⁻²¹ Some clinical studies have demonstrated a significantly poorer prognosis in recipients with severe thrombocytopenia than in those without,^{22,23} suggesting a close relationship of thrombocytopenia to allograft dysfunction including ischemia-reperfusion injury and acute rejection, which are common adverse events in the early period after transplantation. The primary target for these adverse events is vascular endothelial cells, and injury to these cells in the graft liver results in a large amount of VWF production.²⁴⁻²⁷ It is known that circulating VWF levels are markedly high in recipients with poor early graft function.²⁷ Platelet adhesion to the sinusoidal endothelium with a concomitant increase of VWF expression in the reperfused liver is one of the main deleterious effects of cold preservation of liver allografts.²⁶

A few reports have described that ADAMTS13 activity can be used as a marker to diagnose TMA in recipients of liver transplants and renal allografts.^{3,4} However, there is little information about the relationship between ADAMTS13 and allograft dysfunction and thrombocytopenia after liver transplantation. In the present study of 3 living-donor liver transplant recipients, we measured the plasma activity of ADAMTS13 together with VWF and UL-VWFM, and thereby attempted to clarify a potential role of the protease activity in adverse events including ischemia-reperfusion injury and/or acute rejection. As controls, 4 patients with major hepatectomy were also analyzed.

METHODS

Plasma levels of ADAMTS13 activity, VWF antigen (VWF:Ag), and UL-VWFM were sequentially evaluated before and after liver transplantation in 3 recipients. Inhibitor activity against ADAMTS13 was assayed on day 7 after transplantation in case 1, and on day 1 after transplan-

tation in case 2. Also, as controls, plasma ADAMTS13 activity and VWF:Ag were measured in 4 patients with normal livers who underwent major hepatectomy in our hospital during July to November 2005. Blood was taken in plastic tubes containing a 1/10th volume of 3.8% sodium citrate, and platelet-poor plasma prepared by centrifugation at 3,000g at 4°C for 15 minutes was stored in aliquots at -80°C until analysis. The activity of plasma ADAMTS13 was assayed by the following 2 methods: 1) VWF-multimer assay using intact VWFM as a substrate according to Furlan et al.,²⁸ with slight modification.¹⁸ The detection limit of the activity with this method was 3%, and the level obtained for 60 normal subjects was $102 \pm 23\%$ (mean \pm standard deviation).¹⁸ 2) Novel enzyme-linked immunosorbent assay using a murine monoclonal antibody specifically recognizing Tyr1605 residue of VWF-A2 domain,²⁹ generated by ADAMTS13 cleavage, and a recombinant GST-VWF73-His polypeptide³⁰ as a substrate. The detection limit of the activity with this enzyme-linked immunosorbent assay was 0.5%, and the normal level obtained for 55 healthy individuals was $99.1 \pm 21.5\%$ (mean \pm standard deviation).²⁹ In the Case Reports and Results section and the Discussion section below, the values determined by VWF-multimer assay are described. Plasma UL-VWFM was evaluated by vertical agarose gel electrophoresis according to the method of Warren et al.,³¹ with modifications. The activity of inhibitor against ADAMTS13 was evaluated using heat-inactivated plasma at 56°C for 30 minutes.^{16,17} Plasma VWF:Ag was measured by a sandwich enzyme immunoassay using a rabbit anti-human VWF polyclonal antibody. The value obtained for healthy subjects in our laboratory (n = 54; 30 males, 24 females, 20-39 yr of age) was $100 \pm 53\%$ (mean \pm standard deviation).

CASE REPORTS AND RESULTS

Case 1

A 27-yr-old man with a diagnosis of Budd-Chiari syndrome was referred to our department for liver transplantation on October 5, 2004. Radiological imaging showed complete occlusion of both the middle and left hepatic veins, together with stenosis of the orifice of the right hepatic vein. The liver had rapidly swollen because of severe liver congestion, and massive ascites was noted before transplantation. Preoperative blood examination revealed a serum total bilirubin level of 4.8 mg/dL, hemoglobin of 10.9 gm/dL, platelet count of 83,000/ μ L, creatinine of 0.7 mg/dL, and alanine aminotransferase (ALT) activity of 23 IU/L (Fig. 1). His prothrombin time was 18 seconds (international normalized ratio: 1.49) and bleeding time was 5 minute. Anticoagulation factors protein C (54%), protein S (56%), and antithrombin III (75%) were not severely decreased. He had no history of hematological disorders, thrombotic events or relevant family diseases. On November 22, the patient underwent living donor liver transplantation, receiving a cross-match-negative and blood group type-identical right liver graft from his brother, who was healthy and had no history of previ-

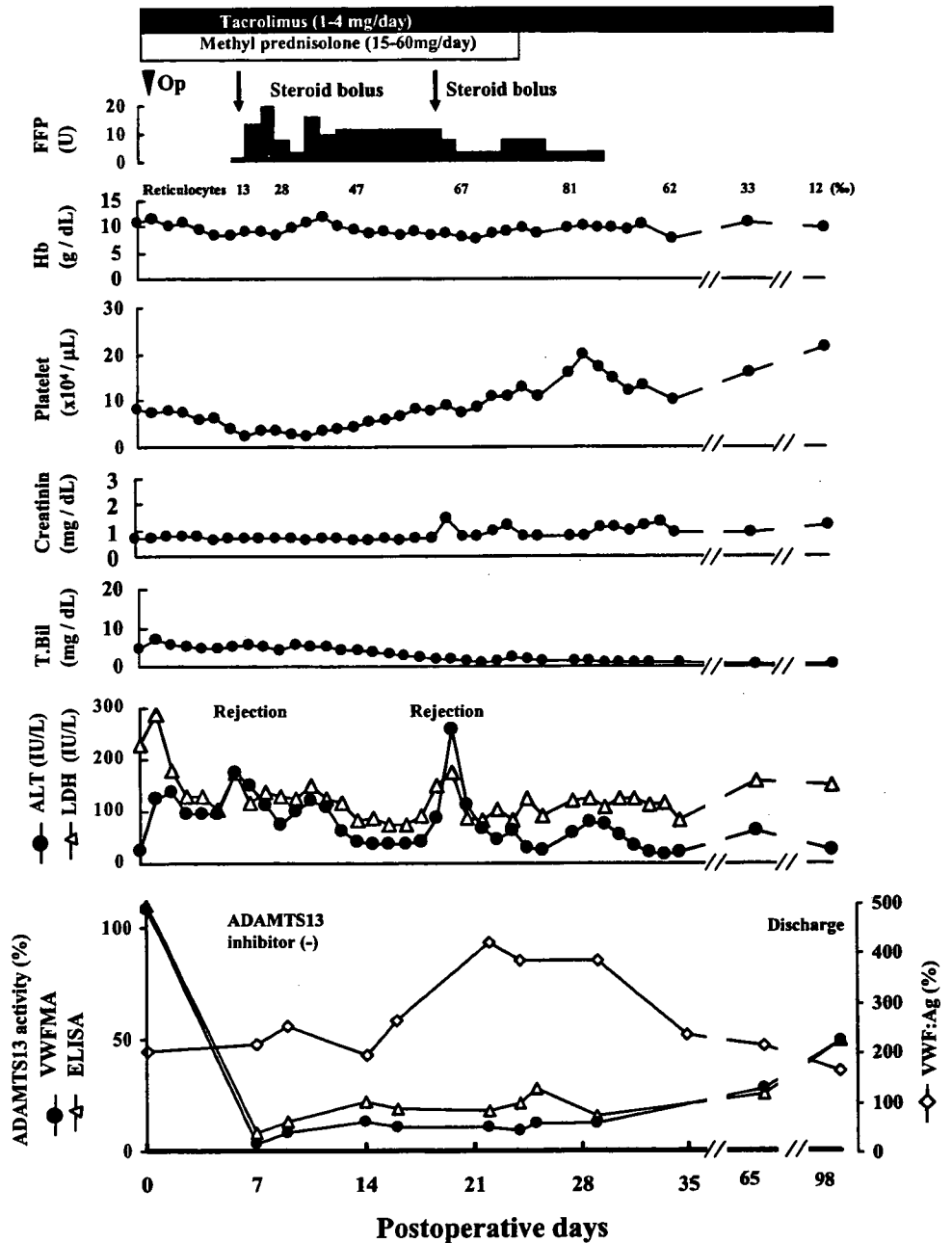


Figure 1. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 1. Serum ALT level was mildly increased on days 1 and 2 because of ischemia-reperfusion injury, decreased thereafter, but rapidly increased again on day 7 due to acute rejection. The platelet count decreased gradually and reached a nadir on day 7, when ADAMTS13 activity decreased markedly to less than 3% from 108% before surgery. No inhibitor against the protease was detected. After the administration of FFP and bolus injection of methylprednisolone to treat acute rejection, ALT level decreased, and the platelet count gradually increased. The activity of ADAMTS13 increased to 22% on day 14. After the first episode of acute rejection, VWF:Ag increased further and reached 368% on day 21, when ALT again increased due to a second episode of acute rejection. Bolus injection of methylprednisolone led to a rapid decrease of ALT and a gradual increase in the platelet count. VWF:Ag decreased gradually, and ADAMTS13 activity finally recovered to 50%, corresponding to the lower limit of the normal range, on day 98.

ous hematological disorders or relevant family diseases. The graft weight and graft-to-recipient weight ratio were 588 gm and 0.93%, respectively. Operative blood loss was 8,410 mL and required 12 units of packed red blood cells and 10 units of fresh frozen plasma (FFP). Platelet concentrate was not required. Posttransplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone. The dose of tacrolimus was adjusted to maintain whole-blood trough levels of 10-15 ng/mL.

Before transplantation, the activity of ADAMTS13 was 108% of the normal control activity (Figs. 1 and 2), VWF:Ag was 175% (Fig. 1), and UL-VWFM was not detected (Fig. 3). During an uneventful course in the early stage after transplantation, the platelet count de-

creased gradually to 62,000/ μ L on postoperative day 5, and reached a nadir (25,000/ μ L) on day 7, when ADAMTS13 activity decreased markedly to less than 3% (Fig. 2), although no inhibitor against the protease was detected. Simultaneously, the serum ALT level increased to 124 IU/L on day 1 because of ischemia-reperfusion injury, decreased thereafter to 97 IU/L on day 5, and again increased rapidly to 175 IU/L on day 6 due to acute rejection, which was clinically diagnosed (Fig. 1). VWF:Ag was mildly increased to 188% on day 7. UL-VWFM was detectable even on day 1, diminished gradually during days 2 to 4, and again became evident on day 7 (Fig. 3). Based on the activity of ADAMTS13, we considered that TMA was the cause of thrombocytopenia, but the recipient never showed any apparent

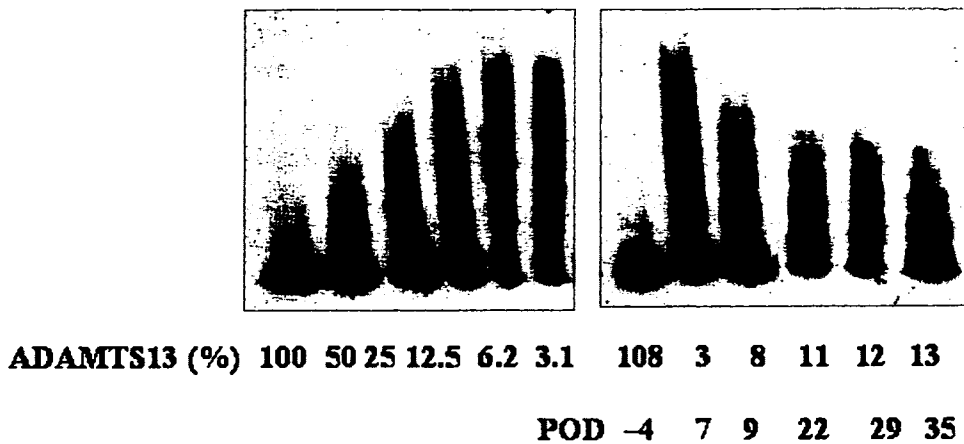


Figure 2. Serial determination of plasma ADAMTS13 activity in case 1. The polymeric size of degraded VWF multimers was analyzed by sodium dodecyl sulfate-1.4% agarose gel electrophoresis, followed by immunoblotting. The standard curve and representative values are shown for plasma samples obtained on preoperative day 4, and on postoperative days 7, 9, 22, 29, and 35. The activity of ADAMTS13 was as extremely low as 3% on day 7, in contrast to the preoperative value of 108%.

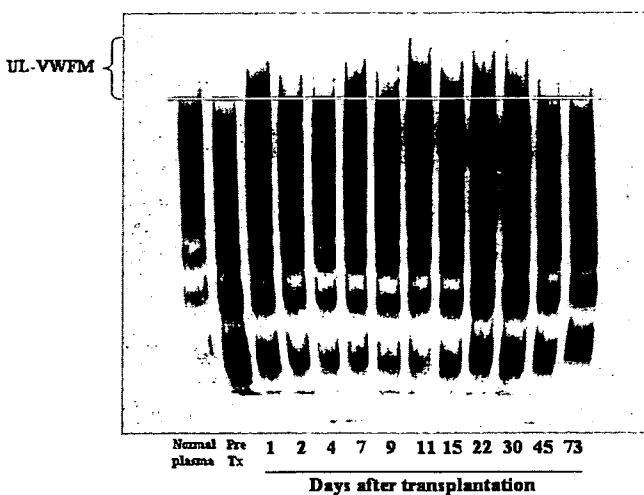


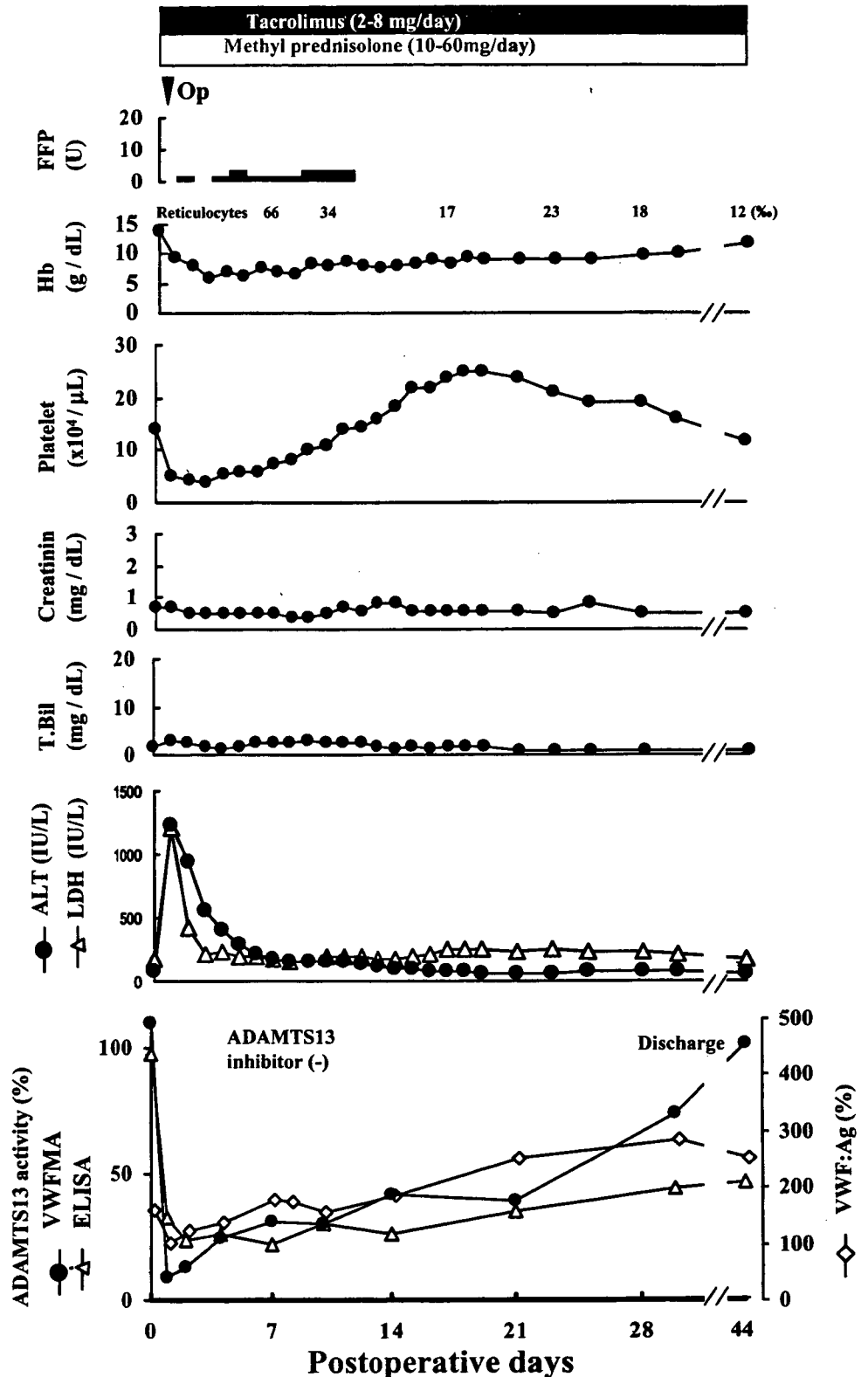
Figure 3. Serial determination of plasma UL-VWFM in case 1 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFM was detectable on day 1 at the time of ischemia-reperfusion injury, thereafter diminishing gradually during days 2 to 4, and again becoming evident on day 7 when acute rejection developed. The UL-VWFM disappeared transiently on day 9, but reappeared on day 11, coinciding with a mild increase in transaminase. UL-VWFM tended to diminish on day 15, but again became prominent on day 22 during the second episode of acute rejection.

clinical features including renal dysfunction, neuropsychological symptoms or hemolytic anemia. We therefore administered a large amount of FFP (4 to 20 units daily) from day 7 to day 30 to restore the ADAMTS13 activity. Plasmapheresis was not performed, because no inhibitor against ADAMTS13 was detected. Bolus injection of methylprednisolone (500 mg daily) was added to treat the acute rejection from day 7 to 9, without conversion of tacrolimus to another drug. Thereafter, the platelet count increased gradually to 90,000/ μ L on day 20 without administration of platelet concentrate. The activity of ADAMTS13 also increased to 12% on day 14 (Fig. 2), and this was maintained until day 65. After the first episode of acute rejection around day 7, VWF:Ag increased further and reached 368% on day 21, when ALT level again increased to 259 IU/L due to a second episode of acute rejection. The amount of UL-VWFM

diminished transiently on day 9 during remission of the first acute rejection episode, but increased again on day 11, coinciding with a mild elevation of transaminase. After the amount of UL-VWFM diminished on day 15, it became prominent again on day 22 during the second episode of acute rejection (clinically diagnosed). Bolus injection of methylprednisolone quickly reduced the level of ALT, and the platelet count gradually increased and reached 199,000/ μ L on day 30 (Fig. 1). ADAMTS13 increased gradually to 50%, corresponding to the lower limit of the normal range, and VWF:Ag decreased gradually and returned to the normal range at the time of discharge on day 98 (Fig. 1). UL-VWFM became undetectable until day 45 (Fig. 3). After liver transplantation, the patient lost a large amount of lymphatic fluid via the peritoneal drain, but this gradually decreased and had disappeared completely by about day 65.

Case 2

A 53-yr-old man with a diagnosis of hypercitrullinemia type II was referred to our department for liver transplantation on January 4, 2005. The activity of argininosuccinate synthetase was markedly low (0.36 U/gm liver; normal range 2.59 ± 1.13 U/gm liver), while the quantity of the enzyme was preserved (0.0052 U/mg protein; normal range 0.0033 ± 0.0012 U/mg protein). The serum ammonia level was sometimes higher than 600 μ g/dL, and analysis of plasma amino acid showed a markedly high concentration of citrulline (338 nmol/mol, normal range 17-43 nmol/mol). The patient had several episodes of deep hepatic coma. On February 14, 2005, he underwent auxiliary partial orthotopic liver transplantation using a left liver graft from his wife, because his own liver function was normal except for amino acid metabolism. The day 0 biopsy of the donor liver revealed macrovesicular steatosis. The graft weight and graft-to-recipient weight ratio were 304 gm and 0.56%, respectively. Operative blood loss was 2,900 mL and required 8 units of packed red blood cells and 4 units of FFP. Platelet concentrate was not required. Posttransplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone, as used in case 1.



Preoperative blood examination revealed a serum total bilirubin level of 1.9 mg/dL, hemoglobin 13.8 gm/dL, platelet count 142,000/ μ L, creatinin 0.7 mg/dL, and ALT 85 IU/L (Fig. 4). The coagulation parameters were normal before transplantation, and had no history

of hematological disorders or relevant family diseases. Pre-transplant ADAMTS13 activity was 110% of the normal control activity, VWF:Ag was 142%, and UL-VWFM was undetectable (Fig. 5). On day 1 after transplantation, ALT increased markedly to 1226 IU/L prob-



Figure 5. Serial determination of plasma UL-VWFM in case 2 using 0.9% SDS-agarose gel electrophoresis. UL-VWFM was not detectable during the observation period.

ably because of ischemia-reperfusion injury (Fig. 4). The platelet count decreased to 51,000/ μ L on postoperative day 1 and reached a nadir of 38,000/ μ L on day 3. The activity of ADAMTS13 decreased markedly to 9% on day 1, but no inhibitor against ADAMTS13 was detected. VWF:Ag decreased slightly to 89%. UL-VWFM was not detected (Fig. 5). Although the patient had no signs of TMA including renal dysfunction, neuropsychological symptoms, or hemolytic anemia, a small dose of FFP was administered from days 1 to 11 after transplantation to restore the activity of ADAMTS13. Thereafter, ALT promptly decreased, and the platelet count increased gradually to 182,000/ μ L on day 14. The levels of blood ammonia and citrulline were normalized within 2 weeks after transplantation, suggesting that the auxiliary partial liver graft was functioning very well. The activity of ADAMTS13 increased gradually to 31% on day 7, 42% on day 14, and reached 102% on day 44. VWF:Ag did not increase markedly, and UL-VWFM was never detected during hospitalization (Fig. 5).

Case 3

A 57-year-old man with a diagnosis of hepatitis B virus-related cirrhosis was referred to our department for liver transplantation on June 6, 2005. He had massive uncontrollable ascites and marked jaundice. Preoperative blood examination revealed a serum total bilirubin level of 22.0 mg/dL, hemoglobin of 11.4 g/dL, platelet count of 30,000/ μ L, creatinine of 0.8 mg/dL, and ALT of 32 IU/L (Fig. 1). His prothrombin time was 22.6 seconds (international normalized ratio: 1.97) and his Model for End-Stage Liver Disease score was 25. He had no history of hematological disorders or relevant family diseases. On July 6, the patient underwent living donor liver transplantation, receiving a cross-match-negative and blood group type-compatible (from B donor to AB recipient) right liver graft from his son, who was healthy

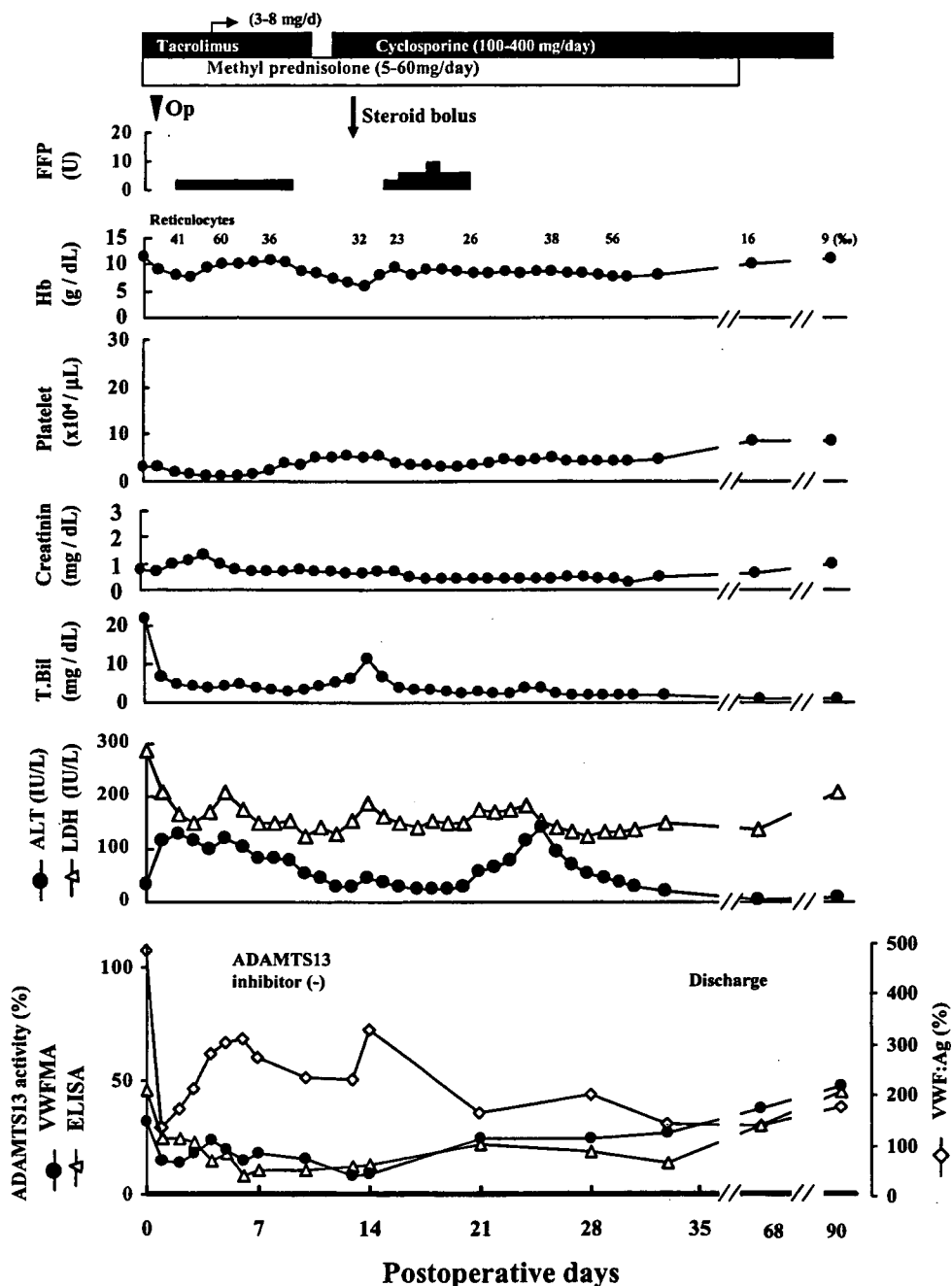
and had no history of previous hematological disorders or relevant family diseases. The graft weight and graft-to-recipient weight ratio were 936 gm and 1.43%, respectively. Operative blood loss was 24,750 mL and required 77 units of packed red blood cells, 70 units of FFP and 5 units of platelet concentrate. The initial post-transplantation immunosuppressive treatment consisted of tacrolimus and methylprednisolone. The dose of tacrolimus was adjusted to maintain whole-blood trough levels of 10-15 ng/mL. Tacrolimus was converted to cyclosporine on day 12 due to tacrolimus-induced leukoencephalopathy, which was diagnosed by magnetic resonance imaging, and recovered completely after conversion to cyclosporine. The dose of cyclosporine was adjusted to maintain whole-blood trough levels of 150-200 ng/mL. The patient was positive for the YMDD-mutant of hepatitis B virus before transplantation and treated with lamivudine and adefovir before and after transplantation. Prophylactic infusion of human hepatitis B immunoglobulin was administered intravenously to prevent hepatitis B recurrence according to the reported protocol.³² Hepatitis B did not recur and the hepatitis B virus-deoxyribonucleic acid levels were below the detection limit after transplantation during observation.

Before transplantation, the activity of ADAMTS13 showed a low level (32%) probably because of severely impaired hepatic functional reserve (Fig. 6). VWF:Ag was markedly increased to more than 500% (Fig. 6), but UL-VWFM was not detected before transplantation (Fig. 7). While initial elevation of ALT due to ischemia-reperfusion was minimal, ADAMTS13 activity decreased markedly to 14% on day 2 after transplantation. His platelet count decreased to 13,000/ μ L during the first week, but platelet concentrate was never administered. VWF:Ag level significantly decreased on day 1 after transplantation, and increased gradually until day 6. ADAMTS13 activity began to increase from day 3, but again markedly decreased to 8% on day 13 when hemolysis due to B cell-mediated graft-vs.-host disease occurred. This hemolysis was considered to be caused by donor-derived antibody against anti-blood type A antigen, because anti-blood type A antigen appeared on day 13 in the peripheral blood. Rapid decrease of hemoglobin associated with increase of total bilirubin and lactate dehydrogenase was observed, but ALT remained normal. Serum creatinine level was not elevated significantly. During this episode, increase of VWF:Ag to 335% was seen, but UL-VWFM was not up-regulated (Fig. 7). This hemolytic reaction was successfully treated with bolus injection of steroid. ADAMTS13 increased to 25% until day 21. Decrease of VWF:Ag was also seen after this treatment. ALT increased mildly around day 25, but we could not determine the specific reason. This increase of ALT recovered without treatment. ADAMTS13 did not increase during this episode, and reached 48% on day 90.

Patients With Major Hepatectomy

Four patients who underwent major hepatectomy were analyzed for ADAMTS13 activity and for VWF:Ag before

Figure 6. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 3. The platelet count was only 30,000/ μ L before operation because of severe liver cirrhosis, and further decreased during the first postoperative week. Serum ALT level was mildly increased on days 1 because of ischemia-reperfusion injury, and decreased slowly. The ADAMTS13 activity decreased markedly to 15% on day 1, and recovered to 24% on day 4. The activity of ADAMTS13 decreased again to 8% on day 13. At that time, severe hemolytic attack developed with significant drop of hemoglobin levels and increase of total bilirubin and lactate dehydrogenase, but ALT did not increase. This was caused by B-cell mediated graft-vs.-host disease with transient increase of antibody against blood type A antigen of the recipient. The ADAMTS13 activity increased after remission of B cell graft-vs.-host disease by bolus injection of steroid. VWF:Ag increased transiently from day 13 to day 14 during B cell graft-vs.-host disease. ADAMTS13 activity finally recovered to 48% on day 90, which was higher than the preoperative level.



and after their operations, for comparison to the liver transplant patients. These 4 patients had normal hepatic parenchyma without cirrhotic change. While VWF:Ag levels were rather higher in these patients than those in cases 1-3 with liver transplantation, ADAMTS13 never decreased below 20% even in the very early phase after operation (Fig. 8).

DISCUSSION

In the present study, we serially determined the plasma values of ADAMTS13 activity, VWF:Ag, and UL-VWFM, and demonstrated their relationship to early adverse events including ischemia-reperfusion injury and/or

acute graft rejection after liver transplantation. In case 1, the activity of plasma ADAMTS13 decreased markedly to less than 3% with concomitant thrombocytopenia on day 7 after transplantation, when acute rejection occurred. UL-VWFM was detected at the time of ischemia-reperfusion injury and also during 2 episodes of acute rejection. In case 2, the activity of the protease decreased markedly to 9% with concomitant thrombocytopenia on day 1, when ischemia-reperfusion injury was observed. The immediate marked decrease of ADAMTS13 was observed also in case 3. On the other hand, changes of ADAMTS13 levels were milder in patients with major hepatectomy than in liver transplant patients. These results indicate that decreased AD-

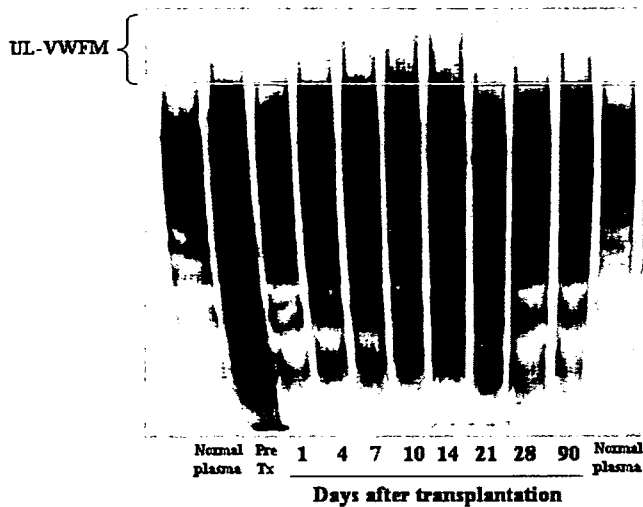


Figure 7. Serial determination of plasma UL-VWFm in case 3 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFm was slightly detectable on day 10, but not significant. The dense band of VWF (not UL-VWFm) might represent very high plasma level of VWF:Ag before transplantation in this patient.

ADAMTS13 activity and the appearance of UL-VWFm are closely related to the development of early posttransplantation allograft dysfunction accompanied by thrombocytopenia.

We analyzed 3 liver transplant recipients, and all of these patients showed a significant decrease of ADAMTS13 with or without upregulation of UL-VWFm during adverse events after transplantation. However, no patient showed the typical clinical signs of TMA including neurological disorder or renal dysfunction during the significant drop in ADAMTS13, while various degrees of thrombocytopenia were associated with the drop in ADAMTS13. These results demonstrate that decrease of ADAMTS13 often occurs after liver transplantation without apparent clinical signs of TMA, and this phenomenon may have a functional relevance to the deterioration of the liver graft presumably due to the mechanism of local TMA within the graft site.

Posttransplantation thrombocytopenia is more often associated with early acute rejection in liver transplant recipients.²² As to the mechanism of the thrombocytopenia, several factors involving sequestration of platelets in the reperfused liver graft, immunologic reactions, increased platelet consumption, reduced platelet production, impaired production of thrombopoietin, medication, or a combination of these have been suspected,^{19-22,33,34} but our findings appear to suggest a novel mechanism of not only early posttransplantation thrombocytopenia, but also early graft dysfunction, which exerts a great influence on the prognosis of recipients.^{22-25,27}

In case 1, we were unable to evaluate the activity of ADAMTS13 during the first few days after surgery, but it could have been low at the time of ischemia-reperfusion injury because of the apparent presence of UL-VWFm on day 1. In case 2, the marked decrease in

ADAMTS13 activity on day 1 may have been due to consumption of the protease because of the weaker VWFm and lower plasma level of VWF compared with the situation before surgery. During the very early stage after transplantation (on day 1), it was noticeable that UL-VWFm was increased in case 1, but decreased in case 2. The decrease of UL-VWFm in case 2 may be explained by the consumption of UL-VWFm in the process of platelet aggregation, probably due to more severe ischemia-reperfusion injury than that in case 1.²⁸ Similarly, the decrease of UL-VWF in plasma was observed in patients with TTP during relapse.³⁵ Another possible explanation may have been the difference in the amount of UL-VWFm released from the damaged sinusoidal endothelium during the surgical procedures, including extirpation of the native diseased liver; case 1 with advanced cirrhosis may have produced a larger amount of UL-VWFm than case 2 with a normal liver, because sinusoidal endothelial damage with capillarization is further augmented with liver fibrosis progresses.^{36,37} Regarding the mechanism responsible for the decrease in ADAMTS13 after liver transplantation, no antibody against ADAMTS13 was detected in either case. Therefore, we speculate that consumption of the protease due to a large amount of UL-VWFm, as seen in case 2, and unknown factors such as proinflammatory cytokines including interleukin-6, which might have inhibited the action of ADAMTS13, were involved.³⁸ In fact, the plasma concentration of interleukin-6 was significantly increased and reached a peak 2 hours after reperfusion of the liver graft.³⁹

In case 3, plasma VWF:Ag was very high before the operation, probably because of sinusoidal endothelial injury due to severe liver cirrhosis.^{36,37} The VWF:Ag rapidly decreased on day 1 after transplantation and UL-VWFm was not detected. We speculate that this significant and immediate decrease of VWF:Ag would be due to the washout effect of a large amount of transfusion during operation. That might be the reason why the VWF:Ag increased gradually during the first postoperative week in spite of the uneventful early recovery of the patient. Although transfusion of a large amount of FFP (70 units) during the operation in case 3 might have increased ADAMTS13 activity, the ADAMTS13 activity on day 1 decreased significantly to 15%. This result suggests that consumption of ADAMTS13 due to ischemia-reperfusion injury during liver transplantation would be so much as to wipe out the effect of 70 units of FFP.

The result of case 2 may be quite important in this study, because the native normal liver was preserved in this patient. The initial drop of ADAMTS13 activity immediately after transplantation was also significant in case 2 as well as case 3, although the production of ADAMTS13 might be preserved by the native right liver with auxiliary partial orthotopic liver transplantation in case 2. This result may suggest that consumption of the ADAMTS13 plays more important role in decrease of ADAMTS13 activity after transplantation during adverse events than decreased ADAMTS13 production due to impaired liver allograft function. Therefore, AD-

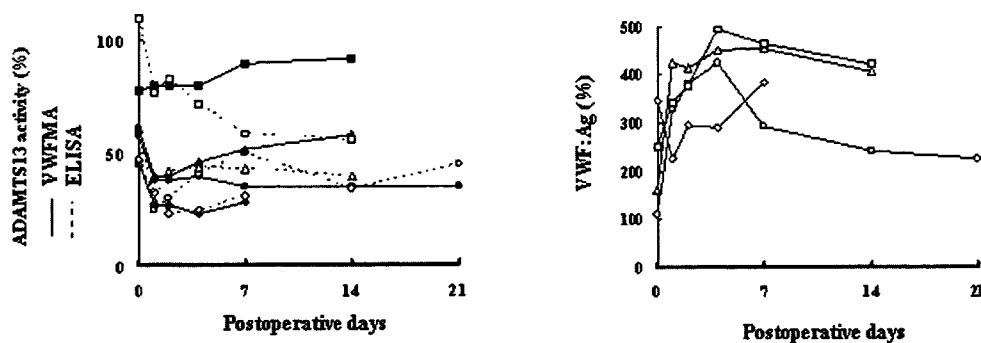


Figure 8. Plasma ADAMTS13 activity (left) and VWF:Ag level (right) after major hepatectomy. Four patients who underwent major hepatectomy were analyzed about ADAMTS13 activity and VWF:Ag for comparison to liver transplantation patients. Circles and triangles show the values of 2 patients who underwent right hepatectomy as living liver donors; Diamonds show a patient with hepatocellular carcinoma who underwent extended right hepatectomy; Squares show a patient with hepatocellular carcinoma who underwent left hepatectomy. Plasma ADAMTS13 activity never decreased below 20%. VWF:Ag levels were rather higher than those in cases 1 to 3 with liver transplantation.

ADAMTS13 may decrease in the initial phase of adverse events before impairment of graft function.

Interestingly, in case 1, VWF:Ag was markedly increased to 368% and UL-VWFM was detected during the second episode of rejection on day 21, while ADAMTS13 activity was as low as 18% and the platelet count was relatively well maintained at $75,000/\mu\text{L}$. These findings indicate that the imbalance of a large amount of UL-VWFM relative to low ADAMTS13 activity could be a good indicator of allograft rejection, even in the absence of severe thrombocytopenia. Thus, our results may be able to explain the fact that liver transplant recipients with increased levels of circulating VWF, a reliable marker of endothelial damage,^{40,41} show poor early graft function.¹⁷

In case 3, ADAMTS13 decreased during hemolysis by B cell-mediated graft-vs.-host disease. However, UL-VWFM was not upregulated during this episode. Differing from ischemia-reperfusion injury or acute rejection, this hemolytic reaction was a systemic reaction due to antibody against blood type A antigen. ADAMTS13, VWF:Ag, and UL-VWFM did not change during nonspecific elevation of ALT around day 25 in case 3. It may be important to analyze ADAMTS13 in combination with UL-VWFM to detect liver transplantation-specific adverse events.

The decrease of ADAMTS13 was milder in patients with major hepatectomy in comparison to liver transplant patients, while VWF:Ag increased higher. This result suggests that significant decrease of ADAMTS13 below 20% would be a liver transplantation-specific event, while the mechanism of difference in changes of ADAMTS13 activity between hepatectomy and liver transplantation is unknown.

The primary target of ischemia-reperfusion injury and allograft rejection is the sinusoidal endothelial cells of the liver graft.^{24-26,42} Deposition of activated platelets on the sinusoidal endothelium with a concomitant increase of VWF expression have been found in the liver immediately after reperfusion or cold preservation.^{24,25} In addition, upregulated VWF expression has been observed in liver allografts during acute rejection.²⁵ Fur-

thermore, recipients with acute rejection show enhanced cytokinemia including tumor necrosis factor- α , which leads to endothelial activation and stimulates the release of UL-VWFM from endothelial cells.^{38,42} VWF, the substrate of ADAMTS13, synthesized in vascular endothelial cells, mediates the initiation and progression of thrombus formation at sites of vascular injury.^{14,40,41,43} VWF is released into plasma as UL-VWFM, which has high platelet aggregation activity. The deficiency of ADAMTS13, together with the excessive release of UL-VWFM from injured graft endothelial cells observed in our study, may cause sinusoidal microcirculatory disturbance and subsequent graft dysfunction.

Various degrees of thrombocytopenia were commonly observed after liver transplantation, especially during the first postoperative week, and many of these patients recover without specific treatment. However, it might be possible that thrombocytopenia is a sign of deterioration of the liver graft in some of the patients with thrombocytopenia, because clinical studies demonstrated that thrombocytopenia was associated with poor prognosis.^{22-25,27} If thrombocytopenia is combined with significant decrease of ADAMTS13, liver graft function may be deteriorated via the TTP like mechanism due to microcirculatory disturbance. Therefore, monitoring of ADAMTS13 would be quite important to judge the necessity of treatment for thrombocytopenia. In case 1, we administered a large amount of FFP from day 7 to prevent further deterioration of thrombocytopenia with TMA mechanism. In cases 2 and 3, a limited dose of FFP was administered as a prophylaxis of graft dysfunction due to TMA-like reaction, because the ADAMTS13 activity significantly decreased. However, it is to be elucidated whether such prophylactic use of FFP based on the ADAMTS13 activity would provide a beneficial effect.

The values of decreased plasma ADAMTS13 activity by VWF-multimer assay in the present study do not appear to be influenced by the elevated plasma UL-VWFM, because the comparable results are drawn by the novel enzyme-linked immunosorbent assay, which is totally insensitive to the presence of intact VWFM.²⁹

This new enzyme-linked immunosorbent assay method would be very useful in clinical application of ADAMTS13 monitoring in liver transplantation, because the results can be obtained within several hours.

At present, FFP is the only available source of ADAMTS13 replacement.¹⁸ Remarkably, in our patients, infusion of FFP, but not platelet concentrate, resulted in gradual improvement of severe thrombocytopenia together with an increase in ADAMTS13 activity. This is an extremely important issue in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation, because administration of platelet concentrate under pathological conditions, including an imbalance between decreased ADAMTS13 activity and enhanced VWF production, would further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFm, leading to multiorgan failure, as seen in TMA.² Platelet concentrate was never administered in case 3 even when the platelet count decreased to 13,000/ μ L on days 4 to 6, because the activity of ADAMTS13 was low. The mechanism of thrombocytopenia associated with early adverse events after liver transplantation is noteworthy. In the post-transplantation period, patients are especially susceptible to TMA because of administration of calcineurin inhibitors including tacrolimus and cyclosporine, which are well-documented to induce TMA.⁴⁴ In case 1, we successfully treated the thrombocytopenia by administering high-dose FFP without conversion of calcineurin inhibitors. Therefore, it would be particularly useful to determine the values of ADAMTS13 and its substrate, VWF:Ag, together with UL-VWFm in the early period after transplantation, not only for the diagnosis of TMA, but also for clarifying the mechanism of thrombocytopenia. Our experience, although based on only 3 liver transplantations and 4 major hepatectomy cases, may provide useful data that are relevant to the diagnosis and treatment of ischemia-reperfusion injury and allograft rejection, as well as for clarifying the pathogenesis of thrombocytopenia after liver transplantation.

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