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 ± 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 X^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 established 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] tggtgttaggcc	
*−754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggtttcctga	
*−265C>A		Exon(5′UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
−202G>A	A	Exon(5′UTR)		89.2	10.8	ggagggagggcc [g/a] ggcacttataaa	
*−58G>C		Exon(5′UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgcag	
*1197C>T		Exon(EGF4)	H381	99.6	0.4	gcccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
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	tccggcaaggtg [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	tcttggtgaatt [t/c] ttttttcctagc	
*2487A>T		Exon(3′UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3′UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3′UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521−3522insT		3′flanking		99.6	0.4	ctcgggttgtgt [−/t] gtctgttcactt	
*3559T>A		3′flanking		99.6	0.4	gccctcatttta [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 ± S.D.	64.6 ± 10.7	67.1 ± 10.9	<0.0001
Systolic blood pressure, mm Hg ± S.D.	123.5 ± 19.8	126.1 ± 17.9	0.0008
Diastolic blood pressure, mm Hg ± S.D.	74.3 ± 10.4	77.2 ± 10.4	<0.0001
Body mass index, kg/m^2 ± S.D.	22.4 ± 3.2	23.4 ± 3.0	<0.0001
Total cholesterol, mg/dl ± S.D.	215.9 ± 31.6	198.7 ± 31.5	<0.0001
HDL-cholesterol, mg/dl ± S.D.	64.4 ± 15.1	55.2 ± 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 ≧140 mm Hg and/or diastolic blood pressure ≧90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol ≧220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose ≧126 mg/dl or non-fasting plasma glucose ≧200 mg/dl or HbA1c ≧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 X^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
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
−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 ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p	Mean ± SE U/ml	p
−202 G>A	GG	16.9 ± 1.6		17.0 ± 1.6		19.2 ± 1.9		19.6 ± 1.9	
	GA+AA	17.4 ± 0.2	0.73	17.4 ± 0.2	0.77	19.9 ± 0.2	0.68	19.9 ± 0.2	0.87
1208 G>A	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(R385K)	GA+AA	16.2 ± 2.4	0.62	16.0 ± 2.3	0.54	20.5 ± 2.2	0.79	20.4 ± 2.2	0.84
1456 G>T	GG	17.4 ± 0.2		17.4 ± 0.2		19.9 ± 0.2		19.9 ± 0.2	
(D468Y)	GT+TT	18.1 ± 1.0	0.51	18.1 ± 1.0	0.52	22.2 ± 1.7	0.20	22.6 ± 1.7	0.11
2487 A>T	AA	17.6 ± 0.2		17.6 ± 0.2		20.0 ± 0.2		20.0 ± 0.2	
	AT+TT	16.7 ± 0.4	0.04	16.7 ± 0.4	0.04	19.6 ± 0.6	0.54	19.5 ± 0.6	0.40
2729 A>C	AA	17.9 ± 0.2		17.9 ± 0.2		20.4 ± 0.3		20.3 ± 0.3	
	AC+CC	16.7 ± 0.3	<0.01	16.8 ± 0.3	<0.01	19.4 ± 0.3	0.03	19.5 ± 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 ± 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, $p=0.03$), 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)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
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.

Acknowledgements

We would like to thank Ms. Junko Ishikawa of the National Cardiovascular Center for her technical assistance. We also thank Drs. Otosaburo Hishikawa, Yasushi Kotani, Katsuyuki Kawanishi, Toshifumi Mannami, and Mr. Tadashi Fujikawai for their continuous support of our population survey in Suita city. We are grateful to the members of the Satsuki-Junyukai. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; from the Japan Society for the Promotion of Science; and from the Program for Promotion of Fundamental Studies in National Institute of Biomedical Innovation of Japan.

References

[1] Souto JC, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am J Hum Genet* 2000;**67**:1452-9.

[2] Heit JA, Phelps MA, Ward SA, Slusser JP, Petterson TM, De Andrade M. Familial segregation of venous thromboembolism. *J Thromb Haemost* 2004;**2**:731-6.

[3] Rosendaal FR, Bovill EG. Heritability of clotting factors and the revival of the prothrombotic state. *Lancet* 2002;**359**:638-9.

[4] de Lange M, Snieder H, Ariens RA, Spector TD, Grant PJ. The genetics of haemostasis: a twin study. *Lancet* 2001;**357**:101-5.

[5] Ariens RA, de Lange M, Snieder H, Boothby M, Spector TD, Grant PJ. Activation markers of coagulation and fibrinolysis in twins: heritability of the prethrombotic state. *Lancet* 2002;**359**:667-71.

[6] Martinelli I. Risk factors in venous thromboembolism. *Thromb Haemost* 2001;**86**:395-403.

[7] Fujimura H, Kambayashi J, Monden M, Kato H, Miyata T. Coagulation factor V Leiden mutation may have a racial background. *Thromb Haemost* 1995;**74**:1381-2.

[8] Miyata T, Kawasaki T, Fujimura H, Uchida K, Tsushima M, Kato H. The prothrombin gene G20210A mutation is not found among Japanese patients with deep vein thrombosis and healthy individuals. *Blood Coagul Fibrinolysis* 1998;**9**:451-2.

[9] Kinoshita S, Iida H, Inoue S, Watanabe K, Kurihara M, Wada Y, et al. Protein S and protein C gene mutations in Japanese deep vein thrombosis patients. *Clin Biochem* 2005;**38**:908-15.

[10] Kimura R, Honda S, Kawasaki T, Tsuji H, Madoiwa S, Sakata Y, et al. Protein S-K196E mutation as a genetic risk factor for deep vein thrombosis in Japanese patients. *Blood* 2006;**107**:1737-8.

[11] Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc Natl Acad Sci U S A* 1981;**78**:2249-52.

[12] Weiler H, Isermann BH. Thrombomodulin. *J Thromb Haemost* 2003;**1**:1515-24.

[13] Sadler JE, Lentz SR, Sheehan JP, Tsiang M, Wu Q. Structure–function relationships of the thrombin–thrombomodulin interaction. *Haemostasis* 1993;**23(Suppl 1)**:183-93.

[14] Dittman WA, Majerus PW. Structure and function of thrombomodulin: a natural anticoagulant. *Blood* 1990;**75**:329-36.

[15] Weiler-Guettler H, Christie PD, Beeler DL, Healy AM, Hancock WW, Rayburn H, et al. A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. *J Clin Invest* 1998;**101**:1983-91.

[16] Isermann B, Hendrickson SB, Zogg M, Wing M, Cummiskey M, Kisanuki YY, et al. Endothelium-specific loss of murine thrombomodulin disrupts the protein C anticoagulant pathway and causes juvenile-onset thrombosis. *J Clin Invest* 2001;**108**:537-46.

[17] Kamide K, Tanaka C, Takiuchi S, Miwa Y, Yoshii M, Horio T, et al. Six missense mutations of the epithelial sodium channel β and γ subunits in Japanese hypertensives. *Hypertens Res* 2004;**27**:333-8.

[18] Okuda T, Fujioka Y, Kamide K, Kawano Y, Goto Y, Yoshimasa Y, et al. Verification of 525 coding SNPs in 179 hypertension candidate genes in the Japanese population: identification of 159 SNPs in 93 genes. *J Hum Genet* 2002;**47**:387-94.

[19] Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**:1-3.

[20] Mannami T, Konishi M, Baba S, Nishi N, Terao A. Prevalence of asymptomatic carotid atherosclerotic lesions detected by high-resolution ultrasonography and its relation to cardiovascular risk factors in the general population of a Japanese city: the Suita study. *Stroke* 1997;**28**:518-25.

[21] Mannami T, Baba S, Ogata J. Potential of carotid enlargement as a useful indicator affected by high blood pressure in a large general population of a Japanese city: the Suita study. *Stroke* 2000;**31**:2958-65.

[22] Mannami T, Baba S, Ogata J. Strong and significant relationships between aggregation of major coronary risk factors and the acceleration of carotid atherosclerosis in the general population of a Japanese city: the Suita Study. *Arch Intern Med* 2000;**160**:2297-303.

[23] Kokubo Y, Kamide K, Inamoto N, Tanaka C, Banno M, Takiuchi S, et al. Identification of 108 SNPs in TSC, WNK1, and WNK4 and their association with hypertension in a Japanese general population. *J Hum Genet* 2004;**49**:507-15.

[24] Kamide K, Kokubo Y, Yang J, Tanaka C, Hanada H, Takiuchi S, et al. Hypertension susceptibility genes on chromosome 2p24-p25 in a general Japanese population. *J Hypertens* 2005;**23**:955-60.

[25] Tanaka C, Kamide K, Takiuchi S, Miwa Y, Yoshii M, Kawano Y, et al. An alternative fast and convenient genotyping method for the screening of angiotensin converting enzyme gene polymorphisms. *Hypertens Res* 2003;**26**:301-6.

[26] Li YH, Chen CH, Yeh PS, Lin HJ, Chang BI, Lin JC, et al. Functional mutation in the promoter region of thrombomodulin gene in relation to carotid atherosclerosis. *Atherosclerosis* 2001;**154**:713-9.

[27] Ohlin AK, Norlund L, Marlar RA. Thrombomodulin gene variations and thromboembolic disease. *Thromb Haemost* 1997;**78**:396-400.

[28] Franchi F, Biguzzi E, Cetin I, Facchetti F, Radaelli T, Bozzo M, et al. Mutations in the thrombomodulin and endothelial protein C receptor genes in women with late fetal loss. *Br J Haematol* 2001;**114**:641-6.

[29] Faioni EM, Merati G, Peyvandi F, Bettini PM, Mannucci PM. The G1456 to T mutation in the thrombomodulin gene is not frequent in patients with venous thrombosis. *Blood* 1997;**89**:1467.

[30] Ohlin AK, Marlar RA. The first mutation identified in the thrombomodulin gene in a 45-year-old man presenting with thromboembolic disease. *Blood* 1995;**85**:330-6.

[31] Kunz G, Ohlin AK, Adami A, Zoller B, Svensson P, Lane DA. Naturally occurring mutations in the thrombomodulin gene leading to impaired expression and function. *Blood* 2002;**99**:3646-53.

[32] Thude H, Wilkens A, Anders O, Barz D. Analysis of the thrombomodulin gene in patients with venous thrombosis. *Thromb Res* 2002;**107**:109-14.

[33] Aleksic N, Folsom AR, Cushman M, Heckbert SR, Tsai MY, Wu KK. Prospective study of the A455V polymorphism in the thrombomodulin gene, plasma thrombomodulin, and incidence of venous thromboembolism: the LITE Study. *J Thromb Haemost* 2003;**1**:88-94.

[34] Heit JA, Petterson TM, Owen WG, Burke JP, De Andrade M, Melton III LJ. Thrombomodulin gene polymorphisms or haplotypes as potential risk factors for venous thromboembolism: a population-based case-control study. *J Thromb Haemost* 2005;**3**:710-7.

[35] Chao TH, Li YH, Chen JH, Wu HL, Shi GY, Tsai WC, et al. Relation of thrombomodulin gene polymorphisms to acute myocardial infarction in patients < or =50 years of age. *Am J Cardiol* 2004;**93**:204-7.

[36] Takahashi Y, Hosaka Y, Imada K, Adachi T, Niina H, Mochizuki H. Species specificity of the anticoagulant activity of human urinary soluble thrombomodulin. *Thromb Res* 1998;**89**:187-97.

[37] Ohlin AK, Larsson K, Hansson M. Soluble thrombomodulin activity and soluble thrombomodulin antigen in plasma. *J Thromb Haemost* 2005;**3**:976-82.

[38] Yamada N, Wada H, Nakase T, Minamikawa K, Nagaya S, Nakamura M, et al. Hemostatic abnormalities in patients with pulmonary embolism compared with that in deep vein thrombosis. *Blood Coagul Fibrinolysis* 1995;**6**:627-33.

[39] Trifiletti A, Scamardi R, Pizzoleo MA, Soraci S, Nevoso A, Bagnato L, et al. Haemostatic changes in patients with deep vein thrombosis. *Panminerva Med* 1997;**39**: 21-23.

[40] Smith A, Quarmby JW, Collins M, Lockhart SM, Burnand KG. Changes in the levels of soluble adhesion molecules and coagulation factors in patients with deep vein thrombosis. *Thromb Haemost* 1999;**82**:1593-9.

[41] Mohri M, Suzuki M, Sugimoto E, Sata M, Yamamoto S, Maruyama I. Effects of recombinant human soluble thrombomodulin (rhs-TM) on clot-induced coagulation in human plasma. *Thromb Haemost* 1998;**80**:925-9.

[42] Kearon C, Comp P, Douketis J, Royds R, Yamada K, Gent M. Dose–response study of recombinant human soluble thrombomodulin (ART-123) in the prevention of venous thromboembolism after total hip replacement. *J Thromb Haemost* 2005;**3**:962-8.

[43] Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom H, Juneja H, et al. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 1999;**353**:1729-34.

[44] Richardson MA, Berg DT, Calnek DS, Ciaccia AV, Joyce DE, Grinnell BW. 17β-estradiol, but not raloxifene, decreases thrombomodulin in the antithrombotic protein C pathway. *Endocrinology* 2000;**141**:3908-11.

[45] Glaser CB, Morser J, Clarke JH, Blasko E, McLean I, Kuhn I, et al. Oxidation of a specific methionine in thrombomodulin by activated neutrophil products blocks cofactor activity. A potential rapid mechanism for modulation of coagulation. *J Clin Invest* 1992;**90**:2565-73.

[46] Lentz SR, Tsiang M, Sadler JE. Regulation of thrombomodulin by tumor necrosis factor-alpha: comparison of transcriptional and posttranscriptional mechanisms. *Blood* 1991;**77**:542-50.

[47] Lohmueller KE, Pearce CL, Pike M, Lander ES, Hirschhorn JN. Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 2003;**33**:177-82.

Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura

Fumiaki Banno, Koichi Kokame, Tomohiko Okuda, Shigenori Honda, Shigeki Miyata, Hisashi Kato, Yoshiaki Tomiyama, and Toshiyuki Miyata

ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation through cleavage of von Willebrand factor (VWF) multimers. In humans, genetic or acquired deficiency in ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), a condition characterized by thrombocytopenia and hemolytic anemia with microvascular platelet thrombi. In this study, we report characterization of mice bearing a targeted disruption of the *Adamts13* gene. ADAMTS13-deficient mice were born in the expected mendelian distribution; homozygous mice were viable and fertile. Hematologic and histologic analyses failed to detect any evidence of thrombocytopenia, hemolytic anemia, or microvascular thrombosis. However, unusually large VWF multimers were observed in plasma of homozygotes. Thrombus formation on immobilized collagen under flow was significantly elevated in homozygotes in comparison with wild-type mice. Thrombocytopenia was more severely induced in homozygotes than in wild-type mice after intravenous injection of a mixture of collagen and epinephrine. Thus, a complete lack of ADAMTS13 in mice was a prothrombotic state, but it alone was not sufficient to cause TTP-like symptoms. The phenotypic differences of ADAMTS13 deficiencies between humans and mice may reflect differences in hemostatic system functioning in these species. Alternatively, factors in addition to ADAMTS13 deficiency may be necessary for development of TTP. (Blood. 2006;107:3161-3166)

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening systemic disease, characterized by anemia, thrombocytopenia, and microvascular thrombosis.[1-4] Hemolysis, the cause of the anemia, generates pointed red cell fragments, schistocytes. Thrombocytopenia is caused by the consumption of platelets in thrombi, which cause renal and neurologic dysfunction. Without treatment, the mortality rate of affected patients exceeds 90%, but plasma exchange reduces the death rate to approximately 20%.[5]

Our understanding of TTP pathophysiology increased considerably with the identification of ADAMTS13, which specifically cleaves the Tyr^{1605}-Met^{1606} peptidyl bond of von Willebrand factor (VWF).[6-10] VWF is a large glycoprotein that mediates platelet adhesion to vascular lesions. It is mainly synthesized in endothelial cells and secreted into the blood as "unusually large" VWF (UL-VWF) multimers, the highly active forms for platelet adhesion and aggregation.[11,12] ADAMTS13 cleaves UL-VWF multimers into smaller forms under flow, limiting platelet thrombus formation under normal conditions. Severe deficiency in ADAMTS13 activity is observed in most patients with TTP, allowing UL-VWF multimers to persist in the circulation.[1-4] UL-VWF multimers mediate enhanced platelet clumping under shear stress, which is thought to cause the clinical symptoms of TTP. Congenital TTP is associated with mutations in the *ADAMTS13* gene, whereas acquired TTP results from the production of autoantibodies against ADAMTS13. A number of causative mutations for congenital TTP have been identified within the *ADAMTS13* gene.[3,4] In vitro expression studies have confirmed the deleterious effects of mutant ADAMTS13 on proteolytic activity or secretion.[13-15]

Here, we generated a mouse model of ADAMTS13 deficiency by a gene-targeting approach, to further understand the pathophysiologic process of TTP. We found that the complete deficiency in ADAMTS13 is not sufficient to produce in mice the typical TTP phenotype. Other triggers may be needed to provoke the disease.

Materials and methods

Generation of ADAMTS13-deficient mice

The isolation of λ phage genomic clones containing *Adamts13* has been previously described.[16] The targeting vector was constructed from a 12.3-kb fragment including exons 3-12, in which the 3.6-kb *SalI-Eco*RI region containing exons 3-6 was replaced by a neomycin resistance cassette. A

From the Research Institute and Division of Transfusion Medicine, National Cardiovascular Center, Suita, Osaka, Japan; and Graduate School of Medicine, Osaka University, Suita, Osaka, Japan.

Submitted July 12, 2005; accepted December 1, 2005. Prepublished online as *Blood* First Edition Paper, December 20, 2005; DOI 10.1182/blood-2005-07-2765.

Supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare of Japan; the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Japan Society for the Promotion of Science; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biochemical Innovation of Japan.

F.B. designed research, performed research, analyzed data, and wrote the paper; K.K. designed research, performed research, and wrote the paper; T.O. contributed vital analytical tools and interpreted the data; S.H. contributed vital analytical tools and interpreted the data; S.M. contributed vital analytical tools and interpreted the data; H.K. performed research, contributed vital analytical tools, and interpreted the data; Y.T. contributed vital analytical tools and interpreted the data; and T.M. designed research and wrote the paper.

Reprints: Toshiyuki Miyata, National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan; e-mail: miyata@ri.ncvc.go.jp.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

diphtheria toxin A fragment expression cassette was inserted into downstream of the 3′-homologous region. The vector was introduced into 129/Sv-derived R-CMTI-1A embryonic stem cells by electroporation. Cells were selected in medium containing G418 (Invitrogen, Carlsbad, CA) and screened by polymerase chain reaction (PCR) and Southern blot analyses. Targeted cells were microinjected into C57BL/6 blastocysts to generate chimeric mice. The resulting male chimeras were bred to wild-type 129/Sv females to produce heterozygous F1 offspring on the 129/Sv genetic background. Heterozygotes were interbred to obtain homozygous mice. Male mice aged 8 to 12 weeks were used for phenotypic analyses. Pregnant female mice aged 8 months were used for renal histology analysis. Female mice aged 15 to 20 weeks (20-30 g) were used for in vivo thrombosis experiments. All animal procedures were performed in accordance with institutional guidelines and were approved by the Animal Care and Use Committee of the National Cardiovascular Center Research Institute.

Genotypic analysis

gDNA, isolated from ear or kidney, was used for genotyping by PCR or Southern blot analyses. For PCR analysis, DNA amplification was performed using a mixture of 3 primers: an intron 2–specific forward primer (5′-ACCCTATCTCTGGCCTGTATTCCT-3′), an intron 3–specific reverse primer (5′-TACTGACTTGTGACCACAAGCCCT-3′), and a neo cassette-specific reverse primer (5′-ATCGAGTCTAGCTTGGCTGGACGT-3′). For Southern blot analysis, a 580-bp fragment upstream of the 5′-homologous region was generated by PCR with primers 5′-TGTCTGCAAGTGCAGTGAGAGGCA-3′ and 5′-AATGAAGATGGCACCAGTGAGGAT-3′ and used for the synthesis of a fluorescein-labeled probe. The probe was hybridized to *Hin*dIII-digested gDNA and detected using a CDP-*Star* detection module (Amersham, Piscataway, NJ).

RT-PCR analysis

Total RNA was prepared from liver using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and subjected to 1-step reverse transcription-PCR (RT-PCR; Qiagen, Hilden, Germany). An exon 21/22-specific sense primer (5′-TTGTGGGAGAGGTCTGAAGGAACT-3′) and an exon 24/25-specific antisense primer (5′-ACAGGAGACAGAGCACTCTGTCCA-3′) were used to amplify ADAMTS13 mRNA.

In situ hybridization

In situ hybridization was performed as described.[17] A 435-bp mouse *Adamts13* cDNA fragment (nucleotides: 679-1113) was used to synthesize digoxigenin-labeled sense and antisense RNA probes by in vitro transcription with a DIG RNA labeling mix (Roche, Basel, Switzerland). The probe was hybridized to liver sections and detected using an anti-DIG AP conjugate (Roche) and NBT/BCIP solution (Roche). Sections were counterstained with Kernechtrot solution.

Measurement of plasma ADAMTS13 activity

With the mice under ether anesthesia, blood was collected from the retro-orbital plexus into tubes containing a 0.1 volume of 3.8% sodium citrate. Plasma was prepared from blood by centrifugation at 800*g* for 15 minutes at room temperature. ADAMTS13 activity was measured using a recombinant substrate, GST-mVWF73-H, as described.[16,18] Activity was also measured using a fluorogenic substrate, FRETS-VWF73 (Peptide Institute, Minoh, Japan).[19]

VWF multimer analysis

Plasma samples, diluted in sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris-HCl, 2% SDS, 2 mM EDTA, 0.02% bromphenol blue, and 43.5% glycerol, pH 6.8) were electrophoresed on a 1% agarose gel (Agarose IEF; Amersham) at a constant current of 15 mA at 4°C. After transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by capillary blotting, the membrane was incubated in peroxidase-conjugated rabbit anti–human VWF (1:500, Dako, Glostrup, Denmark) in 5% skim milk to detect VWF multimers. Bound antibody was detected with Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA) on an image analyzer (Fujifilm, Tokyo, Japan). The chemiluminescent intensities of each lane were scanned using Image Gauge software (Fujifilm); the relative intensity profiles were shown.

Hematologic analysis

Blood cell counts and hematocrit were determined using an automatic cell counter (KX-21NV; Sysmex, Kobe, Japan). Peripheral blood smears were stained with May-Grünwald-Giemsa and examined under light microscopy. Plasma haptoglobin levels were analyzed using a mouse haptoglobin enzyme-linked immunosorbent assay (ELISA) test kit (Life Diagnostics, West Chester, PA).

Plasma VWF antigen was measured by ELISA using antibodies against human VWF. Plasma samples in 1% BSA were applied to rabbit anti–human VWF-coated (Dako) ELISA plates for 2 hours at room temperature. Bound VWF was detected by incubation with peroxidase-conjugated rabbit anti–human VWF (1:4000, Dako) in 1% BSA for 1 hour. Bound antibody was detected using a SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD); the absorbance at 450 nm was measured. A standard curve was constructed from the pooled plasma of 129/Sv mice.

Plasma fibrinogen levels were also measured by ELISA using rabbit anti–human fibrinogen (Dako) and peroxidase-conjugated goat anti–mouse fibrinogen (Nordic Immunological Laboratories, Tilburg, The Netherlands) antibodies. Plasma factor VIII (FVIII) activity was measured using a Testzym FVIII Kit (Daiichi Pure Chemicals, Tokyo, Japan). To assess the ELISA and FVIII activity data, the levels measured in wild-type mice were arbitrarily defined as 100%.

Histologic analysis

The kidneys of pregnant female mice were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin or periodic acid-Schiff reagent. VWF antigen was detected using an ENVISION+ system (Dako) with rabbit anti–human VWF (Dako).

Coagulation tests and bleeding assay

The prothrombin time (PT) and activated partial thromboplastin time (APTT) of plasma samples were determined using Thrombocheck PT (Sysmex) and Thrombocheck APTT (Sysmex) reagents, respectively. Bleeding analysis was performed on mice anesthetized with sodium pentobarbital (50 μg/g). Tails were amputated 3 mm from the tip and immersed in 1 mL PBS at 37°C for 15 minutes. Blood loss was estimated from the comparison of the absorbance of the PBS at 562 nm with that of PBS containing known volumes of mouse blood.

Platelet aggregation analysis

Platelet aggregation was measured using an aggregometer (MC Medical, Tokyo, Japan) as described.[20] Platelet counts in platelet-rich plasma (PRP) were adjusted to $3.0 \times 10^5/\mu L$ by adding platelet-poor plasma (PPP). Aggregation was initiated by addition of acid-insoluble type I collagen (MC Medical) or botrocetin to PRP. PPP was used as a standard indicating 100% aggregation.

Perfusion assay with a parallel plate flow chamber

Platelet thrombus formation in flowing blood on immobilized collagen was analyzed using a parallel plate flow chamber as described.[21,22] Acid-insoluble type I collagen-coated (Sigma, St Louis, MO) glass coverslips were placed in a flow chamber. The chamber was mounted on a fluorescence microscope (Axiovert S100; Carl Zeiss, Oberkochen, Germany) equipped with a 40 ×/0.75 numeric aperture objective lens (Carl Zeiss) and a CCD camera system (DXC-390; Sony, Tokyo, Japan). Blood was collected into tubes containing argatroban (240 μM; Mitsubishi Chemical Corporation, Tokyo, Japan). The fluorescent dye mepacrine (10 μM; Sigma) was added to the blood. Whole blood samples were aspirated through the chamber and across the collagen-coated coverslip by a syringe

pump (Harvard Apparatus, South Natic, MA) at a constant flow rate producing a wall shear rate of 750 s^{-1} The shear rate was calculated from the assumption that the viscosity of mouse blood is equal to that of human blood. To analyze the cumulative thrombus volume, image sets at 1.0-μm z-axis intervals within a defined area (156.4 × 119.6 μm) was captured using MetaMorph software (version 6.1.4; Universal Imaging, West Chester, PA). After blind deconvolution of image sets processed by AutoDeblur software package (version 8.0.2; AutoQuant Imaging, Troy, NY), 3-dimensional volumetric measurements of thrombi were accomplished using VoxBlast software (version 3.0; Vartek, Fairfield, IA).

In vivo thrombosis model

A mixture of 600 ng/g collagen (Nycomed, Roskilde, Denmark) and 60 ng/g epinephrine (Sigma) was injected into tail vein of mice.[23] Blood was collected 15 minutes after the injection and platelet counts were determined.

Statistical analysis

Statistical significance was assessed by the Student t test or the χ^2 test. Differences were considered to be significant at P below .05.

Results

Generation of ADAMTS13-deficient mice

We previously reported 2 strain-specific forms of the mouse *Adamts13* gene.[16] In the 129/Sv strain, the *Adamts13* gene contains 29 exons, as in human *ADAMTS13,* encoding a protein with a similar domain organization as human ADAMTS13. Several strains of mice, including the C57BL/6 strain, harbor a retrotransposon insertion, encoding a variant form of ADAMTS13 that lacks the C-terminal domains. Therefore, we generated and analyzed ADAMTS13-deficient mice on a 129/Sv genetic background.

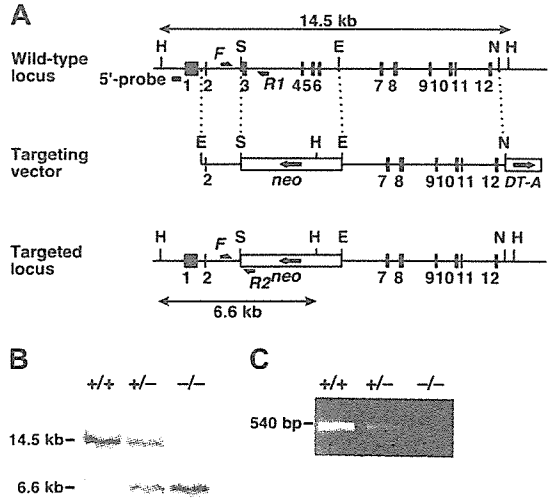

Figure 1. Targeted disruption of the mouse *Adamts13* gene. (A) Structure of the targeted locus in the mouse *Adamts13* gene. Exons are represented by filled boxes. A neomycin-resistance cassette (neo), in the opposite transcriptional orientation, and a forward-oriented diphtheria toxin A fragment expression cassette (DT-A) are indicated. Homologous fragments are indicated by dotted lines; the *Hin*dIII fragments detected by Southern analysis of the wild type and targeted alleles are indicated by double-headed arrows. The sites of primers used for the genotyping PCR (F, R1, and R2) are indicated by arrows. H indicates *Hin*dIII; S, *Sal*I; E, *Eco*RI; N, *Nco*I. (B) Southern blot analysis. gDNA from offspring obtained from heterozygous intercrosses was digested with *Hin*dIII and detected with the 5′-specific probe (wild type: 14.5 kb; targeted allele: 6.6 kb). (C) RT-PCR analysis. Total RNA isolated from mouse liver was reverse-transcribed and amplified using the *Adamts13*-specific primer set to generate a 540-bp fragment.

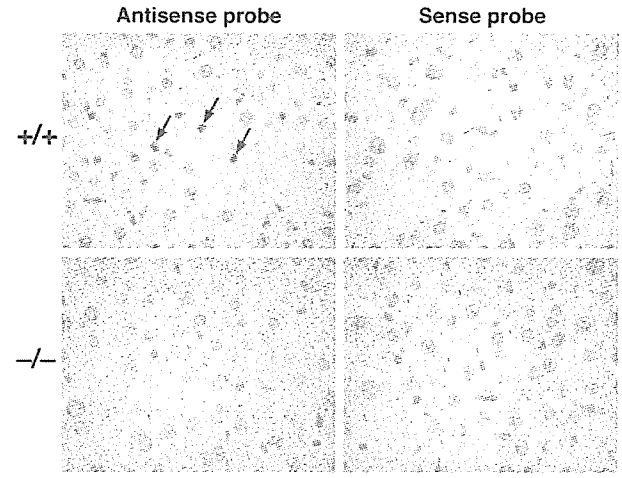

Figure 2. In situ hybridization analysis of ADAMTS13 mRNA. Liver sections from *Adamts13*$^{+/+}$ (top panels) and *Adamts13*$^{-/-}$ (bottom panels) mice were hybridized to the antisense (left panels) or sense (right panels) *Adamts13* RNA probes. The hybridized sections were counterstained with Kernechtrot solution. Typical positive signals are indicated by arrows.

The *Adamts13* gene was disrupted using a targeting vector that eliminated exons 3-6, encoding the catalytic domain (Figure 1A). The expected structure of the targeted locus was confirmed by PCR (data not shown) and Southern blotting (Figure 1B). Elimination of ADAMTS13 mRNA in *Adamts13*$^{-/-}$ mice was verified by RT-PCR of total RNA from liver (Figure 1C), the primary site of synthesis.[16] In situ hybridization analysis also confirmed the loss of ADAMTS13 mRNA in *Adamts13*$^{-/-}$ mice (Figure 2). Because ADAMTS13 is expressed in hepatic stellate cells,[24,25] we detected hybridization with an antisense probe in the nonparenchymal liver cells of *Adamts13*$^{+/+}$ mice. According to their morphology, these cells were hepatic stellate cells. Specific hybridization was not detected in sections from *Adamts13*$^{-/-}$ mice.

No ADAMTS13 enzymatic activity could be detected in plasma samples of *Adamts13*$^{-/-}$ mice by either qualitative (Figure 3A) or quantitative (Figure 3B) methods using GST-mVWF73-H and FRETS-VWF73, respectively, as substrates. Enzymatic activity in *Adamts13*$^{+/-}$ mice was reduced to approximately 35% that seen in *Adamts13*$^{+/+}$ mice (Figure 3B).

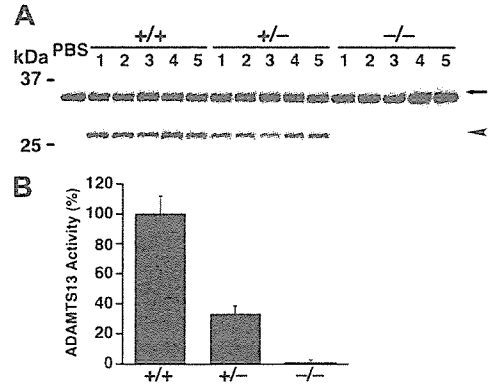

Figure 3. ADAMTS13 activity in plasma. (A) Qualitative assay using a recombinant substrate, GST-mVWF73-H. The substrate and product bands are indicated by arrows and arrowheads, respectively. (B) Quantitative assay using a fluorogenic substrate, FRETS-VWF73. Data are mean ± SD from 4 mice for each genotype. The average activity measured in wild-type mice was defined as 100%.

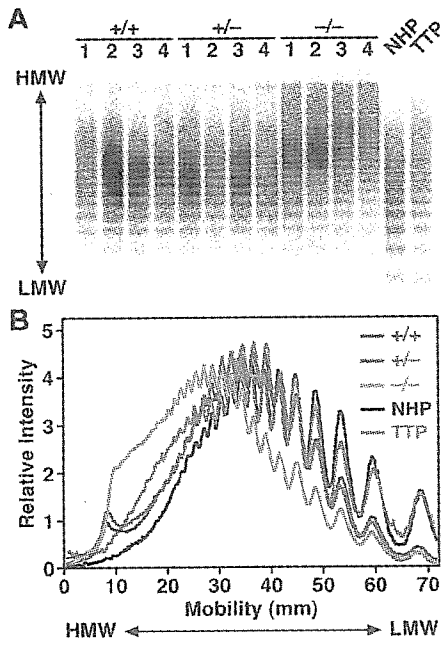

Figure 4. Analysis of plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 μL/lane) from *Adamts13*$^{+/+}$, *Adamts13*$^{+/-}$, and *Adamts13*$^{-/-}$ mice were electrophoresed on SDS-agarose gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. Normal human plasma (NHP) and ADAMTS13-deficient TTP patient plasma (TTP) were analyzed in parallel (0.2 μL/lane). (B) Relative intensities of plasma VWF multimers. The chemiluminescent intensities of the VWF multimer patterns (A) were scanned using image analysis software. HMW indicates high molecular weight; LMW, low molecular weight.

Accumulation of UL-VWF multimers in plasma

In humans, genetic defects in ADAMTS13 lead to the accumulation of UL-VWF multimers in plasma. Analysis of VWF-multimer patterns in plasma detected UL-VWF multimers in *Adamts13*$^{-/-}$ mice (Figure 4), suggesting ADAMTS13 deficiency supports the accumulation of plasma UL-VWF multimers. Because the laddering patterns of VWF multimers in *Adamts13*$^{+/+}$ and *Adamts13*$^{+/-}$ mice were similar, less than half of the normal plasma ADAMTS13 activity (Figure 3B) was sufficient to regulate VWF multimer size. VWF multimers in mice were larger than those in humans (Figure 4B); the multimer sizes seen in *Adamts13*$^{+/+}$ mice were similar to those observed in patients with TTP.

No TTP symptoms in ADAMTS13-deficient mice

Genotyping of 195 offspring of *Adamts13*$^{+/-}$ intercrosses showed the expected 1:2:1 mendelian distribution of *Adamts13*$^{+/+}$ (52 of 195), *Adamts13*$^{+/-}$ (91 of 195), and *Adamts13*$^{-/-}$ (52 of 195). Thus, ADAMTS13 deficiency did not cause embryonic lethality. *Adamts13*$^{-/-}$ mice were viable and fertile. To date, 4 *Adamts13*$^{-/-}$ mice exhibited lateral flexion of upper body; one of them had a cloudy eye. Further study is required to uncover whether this rare phenotype is caused by ADAMTS13 deficiency. Although pregnancy is a triggering event for TTP,[26] deficient females survived pregnancy, delivering viable offspring in normal-sized litters. No significant difference in blood cell counts (Table 1) or plasma haptoglobin levels (Table 2) was observed between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice. Peripheral blood smears from *Adamts13*$^{-/-}$ mice did not show erythrocyte fragmentation (data not shown), demonstrating a lack of spontaneous thrombocytopenia and hemolytic anemia in *Adamts13*$^{-/-}$ mice. The renal histology of *Adamts13*$^{-/-}$ mice during pregnancy did not exhibit microvascular thrombi deposition or excessive accumulation of VWF antigen (data not shown). Thus, *Adamts13* disruption in mice did not cause TTP-like symptoms.

Table 1. Blood cell counts

	Adamts13$^{+/+}$	*Adamts13*$^{-/-}$
Red blood cell count, $\times 10^{12}$/L	8.19 ± 0.41	7.97 ± 0.25
Hemoglobin level, g/L	129 ± 5	126 ± 4
Hematocrit concentration	.426 ± .021	.422 ± .008
Platelet count, $\times 10^{9}$/L	512 ± 42	532 ± 62

Values are mean ± SD of 7 mice in each genotype.

Increased thrombogenesis in ADAMTS13-deficient mice

Plasma VWF antigen levels in *Adamts13*$^{-/-}$ mice were elevated in comparison with those from *Adamts13*$^{+/+}$ mice (Table 2). The activity of plasma FVIII, which correlates with VWF levels, was also significantly increased in *Adamts13*$^{-/-}$ mice (Table 2). The plasma fibrinogen levels, however, were comparable between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (Table 2). PT and APTT suggested the coagulant state in *Adamts13*$^{-/-}$ mice was normal (Table 2). To investigate the effects of ADAMTS13 deficiency on hemostasis in vivo, we measured blood loss after tail transection. There were no significant differences in blood loss between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (Table 2), suggesting UL-VWF multimers did not impair hemostasis.

To uncover a latent prothrombotic state caused by the presence of UL-VWF multimers in *Adamts13*$^{-/-}$ mice, we investigated platelet aggregation under static or flow conditions. We examined agonist-induced platelet aggregation under static conditions. Aggregation responses to botrocetin and collagen in *Adamts13*$^{-/-}$ mice were indistinguishable from those seen in *Adamts13*$^{+/+}$ mice (Figure 5). Thus, an UL-VWF–mediated prothrombotic state could not be detected in *Adamts13*$^{-/-}$ mice under static conditions.

Focusing on thrombus formation under flow, whole blood was perfused over a collagen-coated surface in a parallel plate flow chamber. Even though mice have smaller platelets than humans, thrombus formation was more prominent in mice than in humans, under our flow chamber system. The maximum shear rate to follow up thrombus formation in mouse blood was 750 s^{-1} and we selected this rate for comparing thrombogenesis between the groups. Cumulative thrombus volume was recorded every 0.5 minute after beginning perfusion (Figure 6). Until 3.5 minutes of perfusion, thrombus formation progressed slowly; the thrombus volume did not differ between the *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ groups. After 3.5 minutes, the thrombus grew rapidly in *Adamts13*$^{-/-}$ mice; the thrombus volume at 5.5 minutes was significantly higher in *Adamts13*$^{-/-}$ mice than in *Adamts13*$^{+/+}$ mice. Thus, ADAMTS13 deficiency in mice does not affect the

Table 2. Hematologic and coagulation parameters

	Adamts13$^{+/+}$	*Adamts13*$^{-/-}$
Haptoglobin, %	100 ± 67	103 ± 69
VWF antigen, %	100 ± 23	129 ± 31*
FVIII activity, %	100 ± 10	146 ± 22†
Fibrinogen, %	100 ± 5	98 ± 7
PT, s	16.1 ± 0.8	16.0 ± 1.0
APTT, s	44.2 ± 3.7	43.3 ± 2.5
Blood loss, μL	12.5 ± 8.4	9.5 ± 3.1

Values are mean ± SD of 12 mice in each genotype except for the blood loss, where it is mean ± SD of 18 mice.

*$P < .05$ when compared with *Adamts13*$^{+/+}$ mice

†$P < .001$ when compared with *Adamts 13*$^{+/+}$ mice.

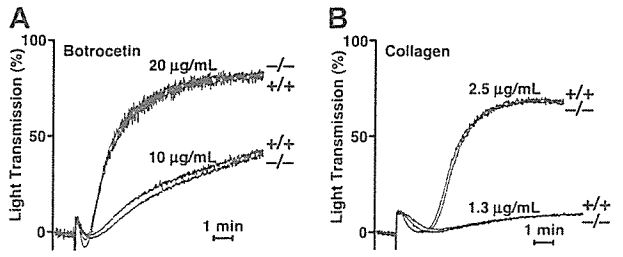

Figure 5. Platelet aggregation under static condition. (A) Botrocetin-induced aggregation. Pooled PRP samples from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice were treated with botrocetin at a final concentration of 10 or 20 μg/mL. Aggregation was measured using an aggregometer at 37°C with constant stirring. (B) Collagen-induced aggregation. Pooled PRP samples were treated with acid-insoluble type I collagen at a final concentration of 1.3 or 2.5 μg/mL. Bars indicate 1 minute. The results of 3 typical experiments are shown.

initial adhesion of platelets to collagen, but enhances thrombus growth under shear stress.

To evaluate in vivo consequence of a lack of ADAMTS13, we examined a model of collagen-induced thrombosis. Under the conditions we examined, the mortality was not different between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (1 of 12 and 1 of 15 died, respectively, $P = .87$ by χ^2 test). However, platelet counts of treated mice were significantly lower in *Adamts13*$^{-/-}$ mice than in *Adamts13*$^{+/+}$ mice (Figure 7), whereas platelet counts of untreated mice were not different between groups. These results indicate that ADAMTS13 deficiency generates prothrombotic state in vivo as well as in vitro.

Discussion

This study suggests 2 perspectives on the etiology of TTP. First, deficiency in ADAMTS13 alone is sufficient to generate UL-VWF multimers in plasma, leading to a prothrombotic state. Second, ADAMTS13 deficiency is insufficient to produce the typical symptoms of TTP in mice. ADAMTS13 deficiency may induce TTP only when combined with other triggering factors.

Under static conditions, platelet aggregation responses to collagen and botrocetin were indistinguishable in ADAMTS13-deficient mice from those seen in wild-type mice, although the plasma VWF multimer size was larger in ADAMTS13-deficient mice. This result is consistent with the previous report that botrocetin is active on rodent platelets, reacting to a broad

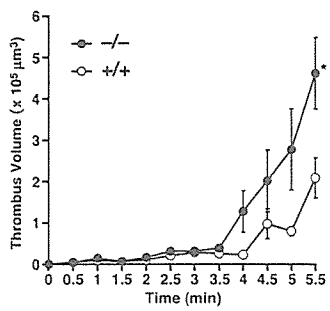

Figure 6. Thrombogenesis on collagen surface under flow. Whole blood from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 750 s^{-1}. The cumulative thrombus volume, analyzed using a multidimensional imaging system, was measured every 0.5 minute until 5.5 minutes. Data are the mean ± SEM of 5 mice for each genotype. *Significant differences at $P < .05$ in comparison with *Adamts13*$^{+/+}$ mice.

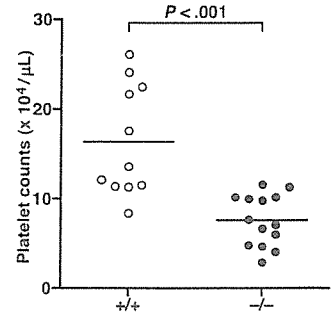

Figure 7. Platelet counts following collagen plus epinephrine challenge. Mice were given injections of 600 ng/g collagen plus 60 ng/g epinephrine via tail vein and platelet counts were measured 15 minutes after injection. Symbols represent platelet counts of a single mouse. Bars represent the mean values of groups. Platelet counts after the challenge were significantly lower in *Adamts13*$^{-/-}$ mice (n = 14) than *Adamts13*$^{+/+}$ mice (n = 11) at 7.7 ± 2.9 × 10^4/μL and 16.4 ± 6.2 × 10^4/μL, respectively (mean ± SD; $P < .001$), whereas platelet counts without challenge were not different between groups (*Adamts13*$^{-/-}$, 86.2 ± 13.2 × 10^4/μL; *Adamts13*$^{+/+}$, 83.7 ± 3.3 × 10^4/μL; mean ± SD of 4 mice).

spectrum of high to low molecular weight VWF multimers.[27] Under flow conditions, however, thrombus formation on a collagen surface was enhanced in ADAMTS13-deficient mice. Although initial platelet adhesion to immobilized collagen was not affected, the growth rate of thrombus was significantly faster in ADAMTS13-deficient mice. In an in vivo thrombosis model, ADAMTS13-deficient mice were more sensitive to collagen-induced thrombocytopenia than wild-type mice, confirming in vitro observation in the flow chamber study. Thus, it was concluded that ADAMTS13 deficiency produces the prothrombotic state. Further study will be necessary to elucidate whether this prothrombotic state is ascribable to hyperreactivity of UL-VWF multimers in ADAMTS13-deficient mice.

Although prolonged coagulation time was not observed, plasma levels of VWF antigen and FVIII activity were elevated in ADAMTS13-deficient mice, potentially reflecting endothelial damage induced by undetectable platelet aggregates. Alternatively, the plasma clearance rate of VWF multimers without cleavage by ADAMTS13 might be slower than cleaved VWF multimers. High levels of VWF antigen are also seen in the plasma of patients with low ADAMTS13 activity.[28]

ADAMTS13 deficiency in mice did not cause a major defect in hemostasis that would lead spontaneously to typical TTP symptoms. ADAMTS13 deficiency may cause a milder prothrombotic state in mice than in humans. The plasma VWF multimer sizes in wild-type mice were larger than those seen in humans, comparable to those in human TTP patients (Figure 4B). Mice lacking VWF exhibit milder tendencies to bleed than patients with type 3 von Willebrand disease.[29] Thus, the dependence of platelet aggregation on VWF might differ in laboratory mice from humans.

Alternatively, ADAMTS13 deficiency may not be sufficient for the development of TTP, even in humans. There is a large variation in the phenotypes of TTP patients with ADAMTS13 deficiency. Most TTP patients with congenital ADAMTS13 deficiency had their first acute episode in the newborn period or early infancy. Only a number of exceptional cases remain asymptomatic until adulthood.[30] Patients with identical *ADAMTS13* genotypes, but different symptoms, have also been described,[31,32] suggesting that the etiology of TTP cannot be explained by a single defect in ADAMTS13. Secondary triggering factors may promote the pathogenic platelet thrombus formation that results in TTP. Indeed,

Motto et al[32] independently reported generation of ADAMTS13-deficient mice and revealed that the injection of shigatoxin, a substance toxic to endothelium, provoked TTP-like symptoms in the ADAMTS13-deficient mice. In the present study, we observed enhanced thrombus formation on collagen surface under flow and promoted thrombocytopenia induced by the injection of a mixture of collagen and epinephrine in ADAMTS13-deficient mice. Genetic defects or environmental factors may stimulate endothelial activation or damage via TTP triggers, such as oxidative stress,[33] infection,[34] antiendothelial cell antibodies,[35] or complement dysfunction.[36,37] ADAMTS13-deficient mice may be useful to identify TTP triggers.

Acknowledgments

We thank Dr Yoshihiro Fujimura (Nara Medical University) for providing the botrocetin, Dr Yuji Arai (National Cardiovascular Center Research Institute) for providing the R-CMTI-1A embryonic stem cells, and Ms Yuko Nobe (National Cardiovascular Center Research Institute) for her technical assistance.

References

1. Moake JL. Thrombotic microangiopathies. N Engl J Med. 2002;347:589-600.
2. Sadler JE, Moake JL, Miyata T, George JN. Recent advances in thrombotic thrombocytopenic purpura. Hematology (Am Soc Hematol Educ Program). 2004:407-423.
3. Kokame K, Miyata T. Genetic defects leading to hereditary thrombotic thrombocytopenic purpura. Semin Hematol. 2004;41:34-40.
4. Soejima K, Nakagaki T. Interplay between ADAMTS13 and von Willebrand factor in inherited and acquired thrombotic microangiopathies. Semin Hematol. 2005;42:56-62.
5. Rock GA, Shumak KH, Buskard NA, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura: Canadian Apheresis Study Group. N Engl J Med. 1991;325:393-397.
6. Furlan M, Robles R, Lämmle B. Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. Blood. 1996;87:4223-4234.
7. Tsai HM. Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. Blood. 1996;87:4235-4244.
8. Soejima K, Mimura N, Hirashima M, et al. A novel human metalloprotease synthesized in the liver and secreted into the blood: possibly, the von Willebrand factor-cleaving protease? J Biochem (Tokyo). 2001;130:475-480.
9. Zheng X, Chung D, Takayama TK, Majerus EM, Sadler JE, Fujikawa K. Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. J Biol Chem. 2001;276:41059-41063.
10. Levy GG, Nichols WC, Lian EC, et al. Mutations in a member of the ADAMTS gene family cause thrombotic thrombocytopenic purpura. Nature. 2001;413:488-494.
11. Kalafatis M, Takahashi Y, Girma JP, Meyer D. Localization of a collagen-interactive domain of human von Willebrand factor between amino acid residues Gly 911 and Glu 1,365. Blood. 1987;70:1577-1583.
12. Federici AB, Bader R, Pagani S, Colibretti ML, De Marco L, Mannucci PM. Binding of von Willebrand factor to glycoproteins Ib and IIb/IIIa complex: affinity is related to multimeric size. Br J Haematol. 1989;73:93-99.
13. Kokame K, Matsumoto M, Soejima K, et al. Mutations and common polymorphisms in ADAMTS13 gene responsible for von Willebrand factor-cleaving protease activity. Proc Natl Acad Sci U S A. 2002;99:11902-11907.
14. Pimanda JE, Maekawa A, Wind T, Paxton J, Chesterman CN, Hogg PJ. Congenital thrombotic thrombocytopenic purpura in association with a mutation in the second CUB domain of ADAMTS13. Blood. 2004;103:627-629.
15. Matsumoto M, Kokame K, Soejima K, et al. Molecular characterization of ADAMTS13 gene mutations in Japanese patients with Upshaw-Schulman syndrome. Blood. 2004;103:1305-1310.
16. Banno F, Kaminaka K, Soejima K, Kokame K, Miyata T. Identification of strain-specific variants of mouse Adamts13 gene encoding von Willebrand factor-cleaving protease. J Biol Chem. 2004;279:30896-30903.
17. Hoshino M, Sone M, Fukata M, et al. Identification of the stef gene that encodes a novel guanine nucleotide exchange factor specific for Rac1. J Biol Chem. 1999;274:17837-17844.
18. Kokame K, Matsumoto M, Fujimura Y, Miyata T. VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13. Blood. 2004;103:607-612.
19. Kokame K, Nobe Y, Kokubo Y, Okayama A, Miyata T. FRETS-VWF73, a first fluorogenic substrate for ADAMTS13 assay. Br J Haematol. 2005;129:93-100.
20. Kato H, Honda S, Yoshida H, et al. SHPS-1 negatively regulates integrin αIIbβ3 function through CD47 without disturbing FAK phosphorylation. J Thromb Haemost. 2005;3:763-774.
21. Tsuji S, Sugimoto M, Miyata S, Kuwahara M, Kinoshita S, Yoshioka A. Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow. Blood. 1999;94:968-975.
22. Sugimoto M, Matsui H, Mizuno T, et al. Mural thrombus generation in type 2A and 2B von Willebrand disease under flow conditions. Blood. 2003;101:915-920.
23 DiMinno G, Silver MJ. Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity by ethyl alcohol. J Pharmacol Exp Ther. 1983;225:57-60.
24. Zhou W, Inada M, Lee TP, et al. ADAMTS13 is expressed in hepatic stellate cells. Lab Invest. 2005;85:780-788.
25. Uemura M, Tatsumi K, Matsumoto M, et al. Localization of ADAMTS13 to the stellate cells of human liver. Blood. 2005;106:922-924.
26. George JN. The association of pregnancy with thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Curr Opin Hematol. 2003;10:339-344.
27. Brinkhous KM, Read MS, Fricke WA, Wagner RH. Botrocetin (venom coagglutinin): reaction with a broad spectrum of multimeric forms of factor VIII macromolecular complex. Proc Natl Acad Sci U S A. 1983;80:1463-1466.
28. Mannucci PM, Canciani MT, Forza I, Lussana F, Lattuada A, Rossi E. Changes in health and disease of the metalloprotease that cleaves von Willebrand factor. Blood. 2001;98:2730-2735.
29. Denis C, Methia N, Frenette PS, et al. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci U S A. 1998;95:9524-9529.
30. Furlan M, Lämmle B. Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease. Best Pract Res Clin Haematol. 2001;14:437-454.
31. Veyradier A, Lavergne JM, Ribba AS, et al. Ten candidate ADAMTS13 mutations in six French families with congenital thrombotic thrombocytopenic purpura (Upshaw-Schulman syndrome). J Thromb Haemost. 2004;2:424-429.
32. Motto DG, Chauhan AK, Zhu G, et al. Shigatoxin triggers thrombotic thrombocytopenic purpura in genetically susceptible ADAMTS13-deficient mice. J Clin Invest. 2005;115:2752-2761.
33. Ruggenenti P, Remuzzi G. The pathophysiology and management of thrombotic thrombocytopenic purpura. Eur J Haematol. 1996;56:191-207.
34. Tarr PI, Gordon CA, Chandler WL. Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. Lancet. 2005;365:1073-1086.
35. Schultz DR, Arnold PI, Jy W, et al. Anti-CD36 autoantibodies in thrombotic thrombocytopenic purpura and other thrombotic disorders: identification of an 85 kD form of CD36 as a target antigen. Br J Haematol. 1998;103:849-857.
36. Manuelian T, Hellwage J, Meri S, et al. Mutations in factor H reduce binding affinity to C3b and heparin and surface attachment to endothelial cells in hemolytic uremic syndrome. J Clin Invest. 2003;111:1181-1190.
37. Noris M, Brioschi S, Caprioli J, et al. Familial haemolytic uraemic syndrome and an MCP mutation. Lancet. 2003;362:1542-1547.

SHORT REPORT

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

Saiho Ko,[1] Eiji Okano,[1] Hiromichi Kanehiro,[1] Masanori Matsumoto,[2] Hiromichi Ishizashi,[2] Masahito Uemura,[3] Yoshihiro Fujimura,[2] Koichi Tanaka,[4] and Yoshiyuki Nakajima[1]

[1]Department of Surgery, [2]Department of Blood Transfusion Medicine, and [3]Third Department of Internal Medicine, Nara Medical University, Kashihara, Nara, Japan, [4] Institute of Biomedical Research and Innovation, Kobe, Japan

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.*

Received October 4, 2005; accepted January 7, 2006.

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.

Supported in part by research grants from the Japanese Ministry of Education, Culture, and Science (to S.K., M.M., H.I, M.U., and Y.F) and the Ministry of Health and Welfare of Japan for Blood Coagulation Abnormalities H17-02 (to Y.F.).

Address reprint requests to: Dr. Saiho Ko, Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. Telephone: 81-744-29-8863; FAX: 81-744-24-6866; E-mail: saihoko@naramed-u.ac.jp

DOI 10.1002/lt.20733
Published online in Wiley InterScience (www.interscience.wiley.com).

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 transplantation 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 3,000*g* 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 ± 23% (mean ± 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 polypeptide[30] 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 ± 21.5% (mean ± 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 ± 53% (mean ± 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/μL, 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-

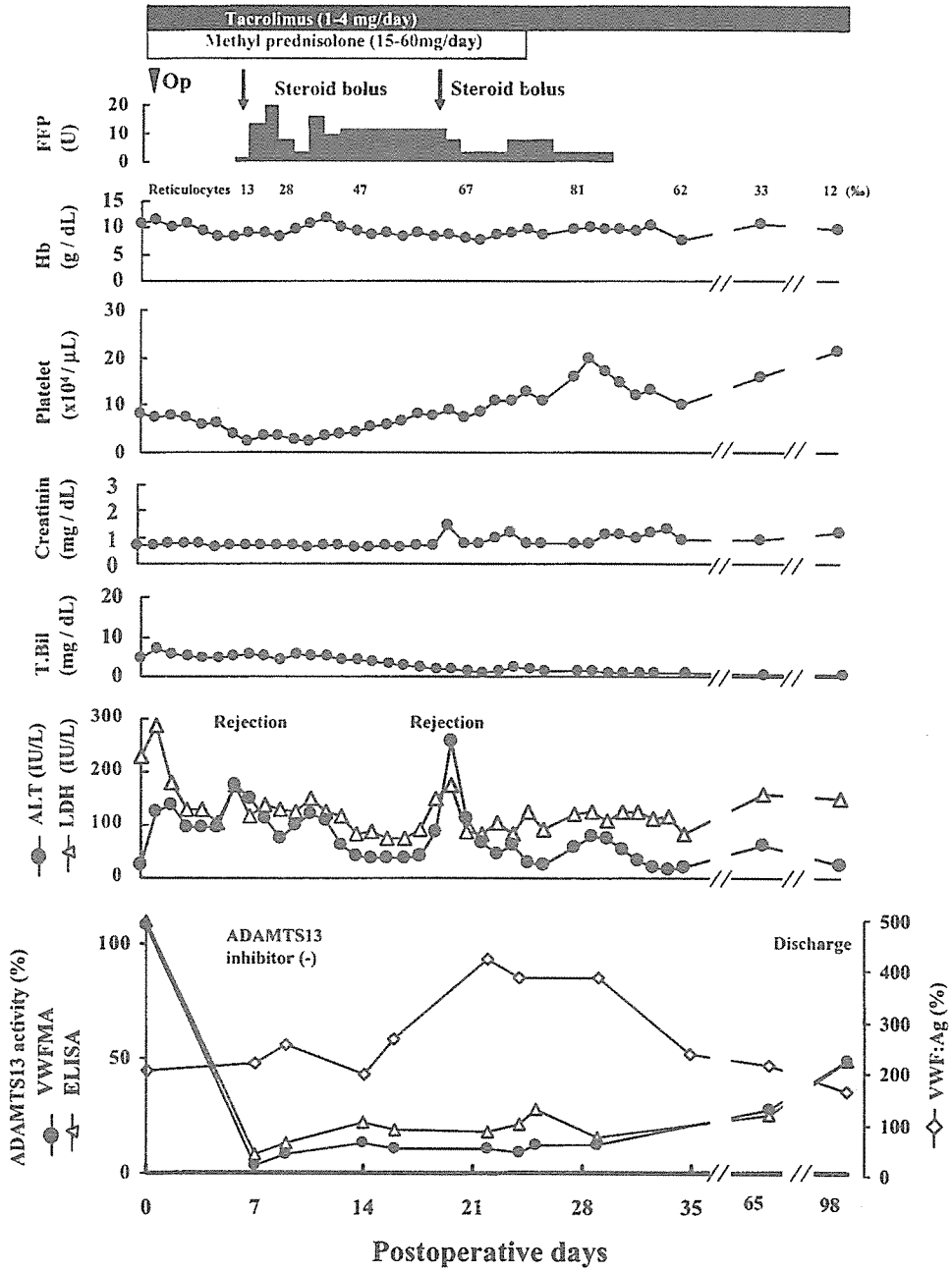

Figure 1. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 1. Serum ALT level was mildly increased on days 1 and 2 because of ischemia-reperfusion injury, decreased thereafter, but rapidly increased again on day 7 due to acute rejection. 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 decreased gradually to 62,000/μL on postoperative day 5, and reached a nadir (25,000/μL) 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

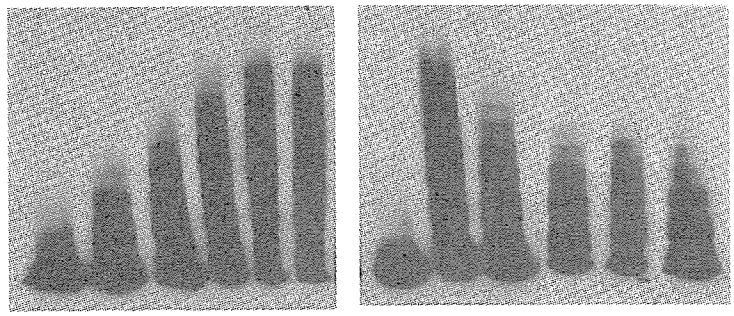

Figure 2. Serial determination of plasma ADAMTS13 activity in case 1. The polymeric size of degraded VWF multimers was analyzed by sodium dodecyl sulfate-1.4% agarose gel electrophoresis, followed by immunoblotting. The standard curve and representative values are shown for plasma samples obtained on 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%.

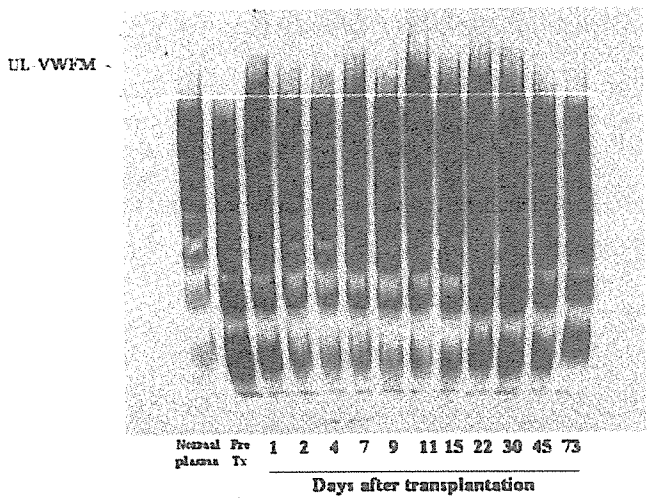

Figure 3. Serial determination of plasma UL-VWFM in case 1 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFM was detectable on day 1 at the time of ischemia-reperfusion injury, thereafter diminishing gradually during days 2 to 4, and again becoming evident on day 7 when acute rejection 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/μL 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/μL 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 μg/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.

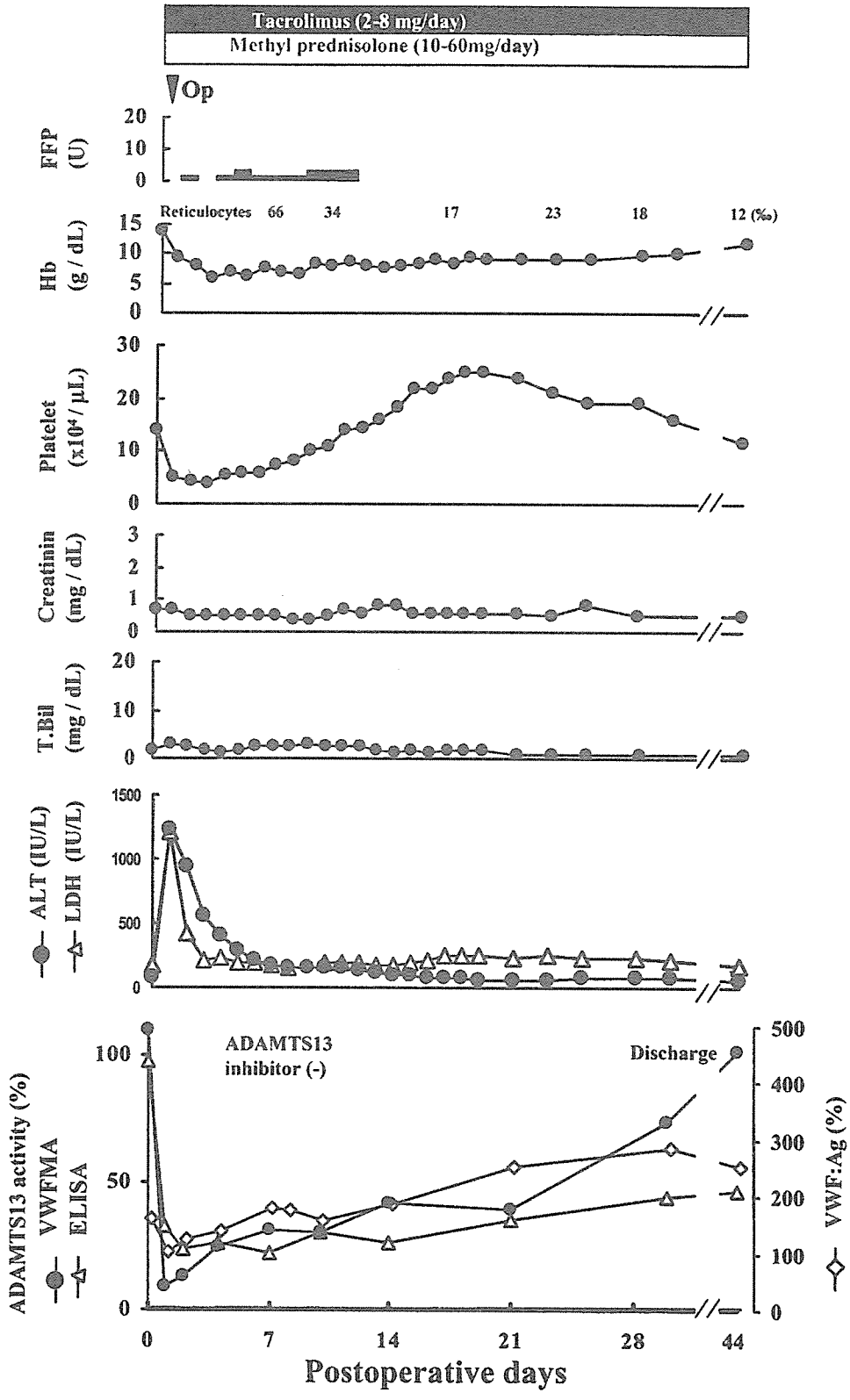

Figure 4. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 2. On postoperative day 1, ALT level was markedly increased because of ischemia-reperfusion injury. The platelet count decreased rapidly on day 1 and reached a nadir on day 3. Simultaneously, ADAMTS13 activity decreased markedly and quickly to 9% on day 1 from the value of 110% before surgery. No inhibitor against the protease was detected. Subsequently the patient's recovery was uneventful. The activity of ADAMTS13 increased to 31% on day 7, 42% on day 14, and reached 102% on day 44. VWF:Ag did not increase significantly.

Preoperative blood examination revealed a serum total bilirubin level of 1.9 mg/dL, hemoglobin 13.8 gm/dL, platelet count 142,000/μL, 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-

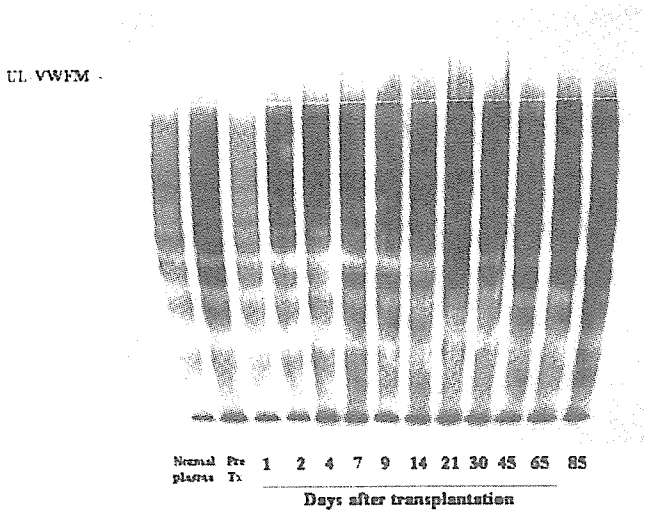

Figure 5. Serial determination of plasma UL-VWFM in case 2 using 0.9% SDS-agarose gel electrophoresis. UL-VWFM was not detectable during the observation period.

ably because of ischemia-reperfusion injury (Fig. 4). The platelet count decreased to 51,000/μL on postoperative day 1 and reached a nadir of 38,000/μL 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/μL 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/μL, 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/ μL 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 begun 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

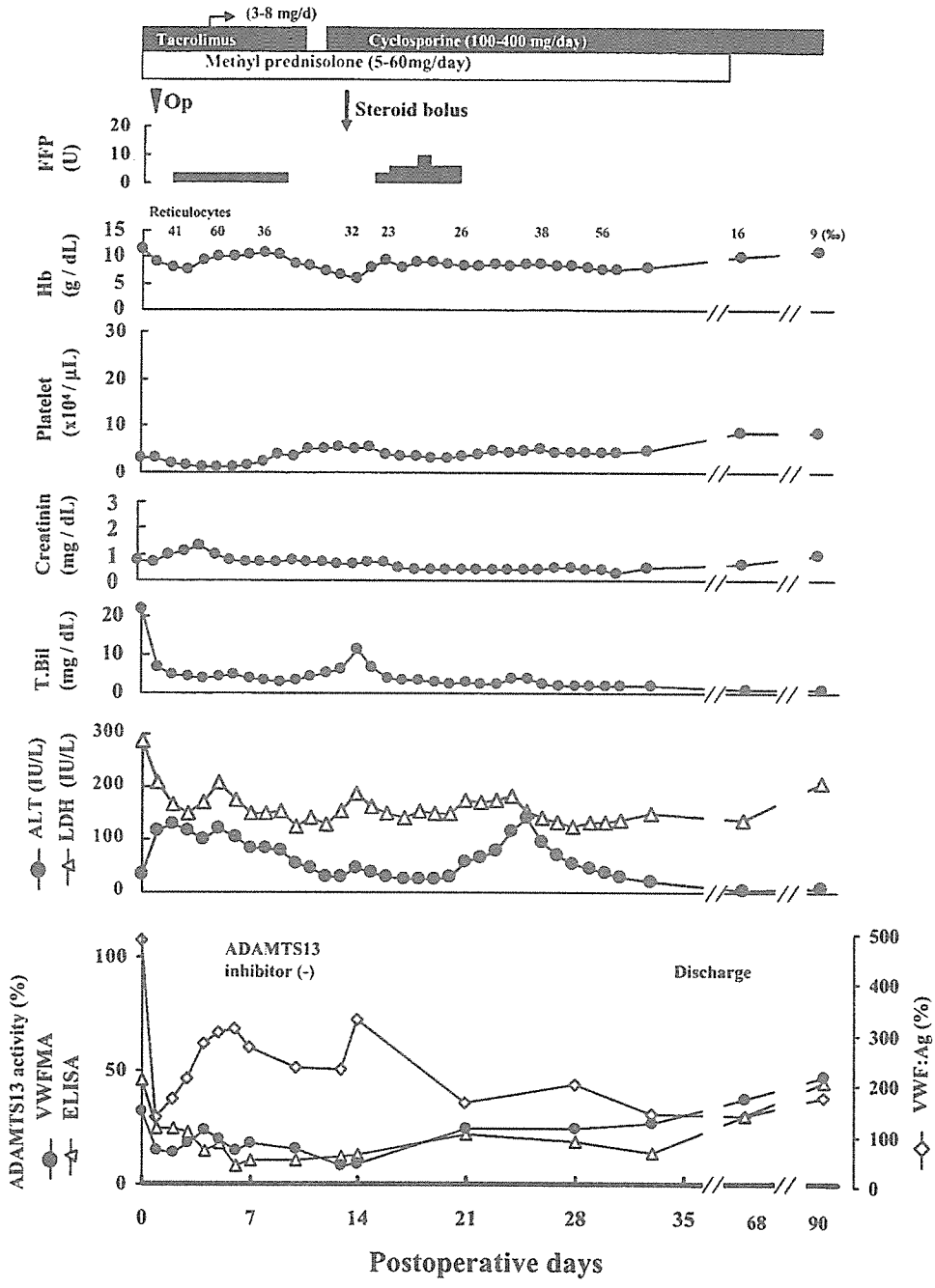

Figure 6. Clinical course and serial changes in plasma ADAMTS13 activity and VWF:Ag level after liver transplantation in case 3. The platelet count was only 30,000/μL before operation because of severe liver cirrhosis, and further decreased during the first postoperative week. Serum ALT level was mildly 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, sever hemolytic attach developed with significant drop of hemoglobin levels and increase of total bilirubin and lactate dehydrogenase, but ALT did not increased. 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-

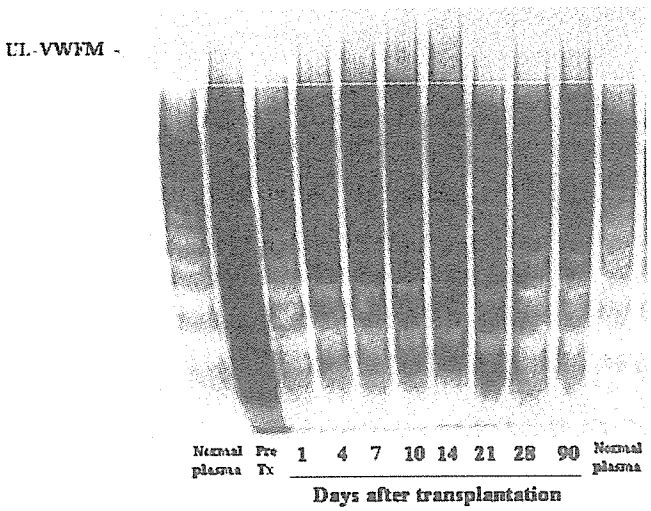

Figure 7. Serial determination of plasma UL-VWFM in case 3 using 0.9% sodium dodecyl sulfate-agarose gel electrophoresis. UL-VWFM was slightly detectable on day 10, but not significant. The dense band of VWF (not UL-VWFM) might represent very high plasma level of VWF:Ag before transplantation in this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

AMTS13 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 as 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 suggest 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-