Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening syndrome characterized by thrombocytopenia and microangiopathic hemolytic anemia, and is often associated with neurological dysfunction, renal failure, and fever [1,2]. Unusually large von Willebrand factor (VWF) multimers produced in and then quickly released from vascular endothelial cells, have often been found in patients plasma in familial and nonfamilial TTP [3,4]. VWF is a large glycoprotein which is essential for high-shear stress associated platelet adhesion and aggregation [5]. These large VWF multimers have been thought to interact with circulating platelets, thus resulting in platelet clumping due to an elevated shear stress [3]. Furlan et al. [6] and Tsai [7] independently showed the plasma vWF to be physiologically cleaved by specific metalloprotease. Thereafter, metalloprotease was purified, and cDNA sequencing identified the enzyme as ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) is a metalloprotease, that specifically cleaves the multimeric VWF [8–12].

A severely deficient ADAMTS13 activity (less than 5% of that in normal plasma) is caused by either a mutation of the ADAMTS13 gene [9,13] or by inhibitory antibodies against ADAMTS13 [14–16]. Although measuring the ADAMTS13 activity is important in the TTP diagnosis, the existing methods require time and skill. Kokame et al. [17] developed a synthetic 73-amino-acid peptide, the FRETS-VWF73, and cleavage of this substrate between two modified residues relieves the fluorescence quenching in the intact peptide. Kokame developed a fluorescence resonance energy transfer (FRET) assay for ADAMTS13 activity [17]. This assay is very easy to perform and it is not time consuming, thus suggesting that it is useful for clinical application.

In this study, we measured the ADAMTS13 activity by a FRET assay in the plasma of healthy volunteers and TTP patients and thus examined the usefulness of a diagnosis of TTP.

Materials and methods

The ADAMTS13 activity was measured in 68 healthy volunteers (19 females and 49 males; median age, 33 years; range, 20–54 years). The ADAMTS13 activity was also measured in 38 patients with TTP (21 females and 17 males; median age 46 years; range 1–84 years), in 8 patients from a congenital TTP family (5 females and 3 males; median age 54 years; range 24–78 years), in 24 patients with antiphospholipid antibody syndrome (APS) (19 females and 5 males; median age 52 years; range 25–69 years), and in 29 patients after hematopoietic stem cell transplantation (HSCT) (13 females and 16 males; median age 36 years; range 17–52 years).

The diagnosis of TTP was made on thrombocytopenia due to the consumption, microangiopathic hemolytic anemia, neurological abnormalities, renal function impairment and high fever [16]. APS was diagnosed based on the Sapporo criteria [18]. The study protocol was approved by the Human Ethics Review Committee of Mie University School of Medicine and a signed consent form was obtained from each subject.

Whole blood was collected in tubes containing 1/10 volume of 3.8% sodium citrate as an anticoagulant. Plasma was obtained by centrifugation at 3000×*g* at 4 °C for 15 min.

Fluorescent assay to measure the ADAMTS13 activity

The fluorogenic substrate, FRETS-VWF73, was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) [17]. It was dissolved in 25% dimethlsulfoxide/water to prepare the 100 μmol/l stock solution. The assay was performed according to the method of Kokame et al. [17].

Briefly, pooled human plasma (a range of 0–8 μl as a standard) or 4 μl of each test plasma were diluted in 100 μl of assay buffer (5 mmol/l Bis–Tris, 25 mmol/l $CaCl_2$, 0.005% Tween-20, pH 6.0) in the well of a 96-well white plate (Thermo Elecron Corporation; Waltham, USA). Next, 100 μl of 4 μmol/l FRETS-VWF73 in the assay buffer was added to each well. Fluorescence was measured at 30 °C in a Fluoroskan Ascent FL (Thermo Elecron Corporation; Waltham, USA) equipped with a 340 nm excitation filter and a 450 nm emission filter. Fluorescence was measured every 5 min. The

Table 1 Subjects

	Number	Sex (f:m)	Age (range)
Healthy volunteers	68	19:49	36 (20–54)
TTP	38	21:17	46 (1–84)
Acquired	32	18:14	46 (16–84)
Familial (3 families)	6	3:3	26 (1–55)
TTP family (3 families)	8	5:3	54 (24–78)
Antiphospholipid syndrome	24	19:5	52 (25–69)
Hematopoietic stem cell transplantation	29	13:16	36 (17–52)

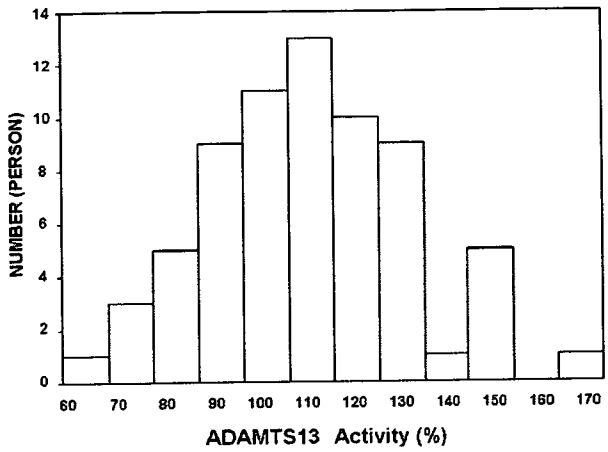

Figure 1 ADAMTS13 activity in healthy volunteers.

reaction rate was calculated by a linear regression analysis of fluorescence over time from 0 to 60 min using a Prism software package (GraphPad Software, San Diego, USA).

The ADAMTS13 activity was determined by the multimetric method which was performed according to the method of Furlan et al. [19,20].

Statistical analysis

The data were expressed as the mean ± standard deviation (S.D.). The differences between the groups were examined for statistical significance using the Mann–Whitney's *U*-test while the correlation between the two variables was tested by Pearson's correlation analysis. A *P* value of less than 0.05 denoted the presence of a statistically significant difference (Table 1).

Results

In healthy volunteers, the plasma levels of the ADAMTS13 activity by FRETS-VWF did not show a

Table 2 TTP patients

No.	Sex	Age	TTP/HUS	FRET	Multimer	Inhibitor	Outcome
1	F	45	TTP	3% >	3% >	(+)	Survive
2	F	17	TTP	3% >	3% >	(+)	Survive
3	F	34	TTP	3% >	3% >	(+)	Survive
4	F	16	TTP	3% >	3% >	(+)	Survive
5	M	38	TTP	3% >	3% >	(+)	Survive
6	M	75	TTP	3% >	3% >	(+)	Survive
7	F	64	TTP	3% >	3% >	(+)	Survive
8	F	17	TTP	3% >	3% >	(+)	Survive
9	F	46	TTP	3% >	3% >	(+)	Survive
10	M	41	TTP	3% >	3% >	(+)	Survive
11	F	45	TTP due to collagen D	3% >	3% >	(+)	Survive
12	F	34	TTP	3% >	3% >	(+)	Survive
13	F	43	TTP	3% >	3% >	(+)	Survive
14	M	55	TTP	3% >	6%	(+)	Survive
15	M	59	TTP	3% >	12%	(+)	Survive
16	M	72	TTP	3% >	3% >	(+)	Death
17	M	79	TTP	3% >	4%	ND	Survive
18	F	50	TTP	14%	28%	ND	Survive
19	M	67	TTP after transplantation	13%	26%	(−)	Death
20	F	71	TTP	28%	60%	(−)	Death
21	F	51	TTP	39%	24%	(−)	Death
22	F	72	TTP	34%	25%	(−)	Death
23	M	17	TTP	49%	70%	(−)	Death
24	F	68	TTP	48%	48%	(−)	Survive
25	F	68	TTP	48	28	(−)	Survive
26	M	48	TTP	58%	28%	(−)	Death
27	M	28	TTP due to collagen D	79	84	(−)	Death
28	F	84	TTP due to drug	80%	52%	(−)	Survive
29	M	69	TTP	97%	70%	(−)	Survive
30	M	44	TTP	100%	26%	ND	Death
31	F	17	TTP with relapse	144%	ND	(−)	Survive
32	F	49	TTP	144%	100%	(−)	Survive
33	M	51	Familial TTP (Family A)	3%>	3%>	(−)	Death
34	F	24	Familial TTP (Family B)	83%	26%	(−)	Survive
35	M	26	Familial TTP (Family B)	113%	42%	(−)	Survive
36	M	1	Familial TTP (Family B)	119%	44%	(−)	Survive
37	F	55	Familial TTP (Family C)	110%	118%	(−)	Survive
38	F	30	Familial TTP (Family C)	120%	150%	(−)	Survive

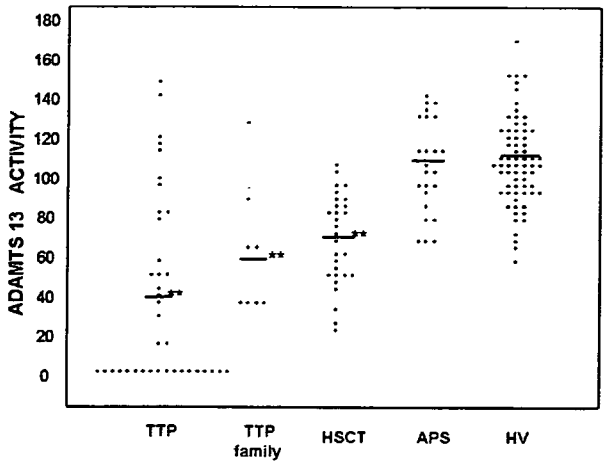

Figure 2 ADAMTS13 activities in TTP, TTP family, HSCT, APS and HV HSCT; hematopoietic stem cell transplantation, APS; antiphospholipid syndrome, HV; healthy volunteer. $^{**}p<0.01$.

normal distribution, and its median value was 106.6% (minimum–maximum; 55–170%) (Fig. 1). In 38 patients with TTP, 6 had familial TTP while 32 had acquired TTP. Ten patients with TTP died within 3 months, while 28 patients had a complete remission. The ADAMTS13 activity of 18 patients (47%) with TTP was less than 5% by FRET assay and the inhibitor for ADAMTS13 was measured in 17 of these patients. 16 patients had an inhibitor for ADAMTS13 and one had familial TTP (Table 2). The ADAMTS13 activity was significantly lower in the patients with TTP (median 13.1%; interquartile range 0–78.5%, $p<0.01$), TTP family (49.4%; 36.2–75.0%, $p<0.01$) and patients with hematopoietic stem cell transplantation (70.0%; 48.4–86.6%, $p<0.01$) than in healthy volunteers (106.7%; 93.7–123.7%). There was no significant difference in the ADAMTS13 activity between patients with APS (70.0%; 48.4–86.6%) and healthy

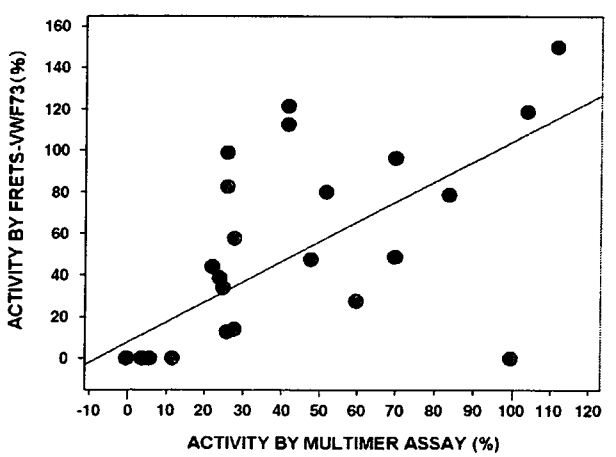

Figure 3 Correlation of ADAMTS13 activity between the FRET assay and multimer assay.

Table 3 Comparison of the ADAMTS13 activity between by the findings of the FRETS-VWF73 and multimer method

		Multimer method		
		0–20%	20–50%	50%<
FRET method	0–20%	18(1)	2	0
	20–50%	0	4	2
	>50%	0	5(3)	6(2)

( ) shows familial TTP.
The ADAMTS13 level by a multimer assay in one case was not measured.

volunteers (Fig. 2). There were 3 families with TTP. The mutation of the ADAMTS13 gene was detected in family A [21] but was not found in the other 2 families. The ADAMTS13 activity by FRETS-VWF73 was more than 50% but according to a multimer assay, it was less than 50% in the family B members.

The ADAMTS13 activity in the members of family C was more than 50%. Marked reduction (less than 3%) of the ADAMTS13 activity was not observed in TTP family, HSCT and APS. The ADAMTS13 activity by FRETS-VWF73 was closely correlated with that determined by the multimer method ($Y=7.913823+0.967015X$, $r=0.816$; $p<0.001$) (Fig. 3). Twenty TTP patients showed less than 20% of ADAMTS13 activity by FRET assay, while 18 showed less than 20% of that by a multimer assay and 2 patients showed 20–50%. In more than 50% of the ADAMTS13 activity by FRET assay, 5 patients showed 20–50% of that by a multimer assay, while 6 showed more than 50% (Table 3).

Discussion

ADAMTS13 was recently identified to be a new hemostatic factor, previously called VWF cleaving protease. Neither the congenital or acquired defects of the enzymatic activity lead to thrombotic thrombocytopenic purpura (TTP). ADAMTS13 specifically cleaves a peptidyl bond between Y1605 and M1606 in the A2 domain of VWF which helps to determine the minimal region which was recognized as a specific substrate by ADAMTS13 [22].

In healthy volunteers, normal range of plasma ADAMTS13 activity by FRETS-VWF ranged from 55% to 170%. The plasma levels of ADAMTS13 did not show a normal distribution, probably because the ADAMTS13 levels were affected by the production in the liver or consumption [3]. The VWF levels decreased in persons with blood type "O" [23].

The ADAMTS13 activity was significantly lower in the patients with TTP and the TTP family, thus indicating that ADAMTS13 plays an important role in the onset of TTP. However, 6 patients had an

ADAMTS13 activity of more than 60%, thus suggesting that the TTP in these patients may have been caused by abnormalities of other factors such as Factor H [24] and CD46 [25]. The activity of ADAMTS13 was low in patients with hematopoietic stem cell transplantation. A decreased activity was reported in patients with hepatic veno-occlusive disease (VOD) after stem cell transplantation [26]. These findings suggest that a reduced amount ADAMTS13 may be a risk factor for the onset of VOD.

The ADAMTS13 activity determined by FRETS-VWF73 was closely correlated with that determined by the multimer method. Especially, in less than 10% of ADAMTS13 activity, these two assays closely correlate. As almost all patients with acquired TTP and showing less than 10% of ADAMTS13 activity had an inhibitor, this FRETS-VWF73 assay may thus be especially useful for TTP patients with an inhibitor. However, there are several discrepancies between the FRET assay and a multimer assay. In 5 cases (3 cases were familial TTP; family B), the ADAMTS13 activity by FRET assay was within the normal range but based on a multimer assay, it was low. This is because a FRET assay can detect the cleaving activity only between Y1605 and M1606 in the A2 domain of VWF, while a multimer assay can detect the cleaving activity of whole VWF. These findings suggest that a FRET assay may miss a few patients with TTP, while a FRET assay may be more sensitive than a multimer assay in some patients.

In TTP patients without an inhibitor, the difference between the two assays may provide important information for a further analysis of ADAMTS13. In addition, an analysis of ADAMTS13 including antigen will thus play an important role in examining various thrombotic diseases.

Acknowledgments

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BRIEF COMMUNICATION

Quantitative Western blot analysis of plasma ADAMTS13 antigen in patients with Upshaw-Schulman syndrome

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Upshaw-Schulman syndrome (USS) was originally reported as a disease complex, characterized by chronic thrombocytopenia and hemolytic anemia, that was dramatically improved by infusions of fresh frozen plasma (FFP) [1–6]. USS is now known to be a hereditary deficiency in the activity of von Willebrand factor-cleaving protease (VWF-CP), also known as ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13), and lacks ADAMTS13 autoantibodies (inhibitors) [7]. In contrast, acquired deficiency of ADAMTS13 activity caused by inhibitors is defined as thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disease characterized by Moschcowitz's pentad [8,9]. Thus, USS is alternatively called congenital TTP, and genetic analysis of *ADAMTS13* has revealed that its mutations are present across the entire gene and not in hot spots [7,10–15]. The *ADAMTS13* gene is located on chromosome 9q34 and USS is a recessive disease, so most USS patients are genetically compound heterozygotes or homozygotes. When expressed in mammalian cells, the *ADAMTS13* gene mutants found in USS patients showed deficient ADAMTS13 activity (ADAMTS13:ACT) that was induced by disturbing the synthesis and/or secretion of the protease. However, these results were left unchecked in the patient plasmas. It was recently shown that the normal plasma level of ADAMTS13 antigen (ADAMTS13:AGN) is approximately 1 μg/ml, according to a sandwich enzyme-linked immunosorbent assay (ELISA) using polyclonal or monoclonal antibodies (mAbs) against ADAMTS13. USS patients exhibit severely reduced levels of ADAMTS13:AGN, resulting in reduced levels of ADAMTS13:ACT [16,17]. However, the investigation of the ADAMTS13 molecules in these patients has not yet been perfomed *in vivo*.

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We therefore analyzed plasma ADAMTS13:AGN in 9 USS patients and their 25 family members, in whom *ADAMTS13* gene mutations were identified by Western blot (WB) using an anti-ADAMTS13 mAb, WH2-11-1. The epitope of this mAb resides on the 4th thrombospondin-1 domain and is reactive by WB under both reducing and non-reducing conditions [18].

Materials and methods

Assays for ADAMTS13:ACT and ADAMTS13 inhibitors

ADAMTS13:ACT and titers of ADAMTS13 inhibitors (ADAMTS13:INH) were assayed by a novel, highly-sensitive ELISA using a murine mAb (N10-146) specifically recognizing Tyr1605 residue of VWF-A2 domain, generated by ADAMTS13 cleavage, and a recombinant GST-VWF73-His polypeptide as a substrate [19,20]. This ELISA had a limit of detection of 0.5% of the normal ADAMTS13:ACT level in normal pooled plasma, and the average plasma level of ADAMTS13:ACT was 99.1 ± 43.0% (mean ± 2SD). Inhibitor titers were expressed as Bethesda units (BU), where one inhibitor unit is defined as the amount necessary to reduce ADAMTS13:ACT levels to 50% of the normal levels. Titers of >0.1 BU/ml, as measured by the novel ELISA, were considered significant [20].

Patients

Nine patients from 9 different families (Families A–I) with histories of USS were enrolled in our study. For each family, diagnoses were confirmed by identifying the *ADAMTS13* gene mutations responsible for the disease, as previously described [10,14,15,21]. Of the 25 USS relatives we tested, 23 were definite carriers and 2 were normal subjects.

Citrated plasma samples taken from USS patients were frozen in aliquots at −80 °C until use. For controls, normal citrated plasma was obtained from 60 healthy individuals (30 females and 30 males, aged 20–40 years) and kept in aliquots at −80 °C. Pooled normal plasma was used as the control standard for this study. These studies were conducted with the approval of the Nara Medical University ethics committee.

Characterization of the murine anti-ADAMTS13 mAb WH2-11-1

A murine anti-ADAMTS13 mAb, termed WH2-11-1 (IgG1-κ), was produced by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) using recombinant (r) full-lengh ADAMTS13 as the immunogen [18]. Monoclonal IgGs were purified on a Protein A column (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions. WH2-11-1 recognizes an epitope on the 4th thrombospondin-1 domain, and this was verified using C-terminal truncated rADAMTS13. This mAb detected plasma ADAMTS13:AGN as a 170-kD band by WB under non-reducing conditions and a single 190-kD band under reducing conditions. However, this mAb showed no significant inhibition of ADAMTS13:ACT. In some WB analyses under non-reducing conditions, another anti-ADAMTS13 mAb with an epitope on the disintegrin domain, A10, was also used [22].

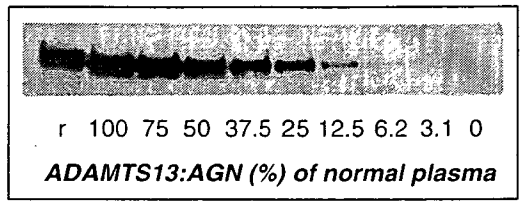
Analysis of plasma ADAMTS13:AGN

We quantified plasma ADAMTS13:AGN by WB. Two microliters of undiluted or diluted plasma samples per lane were analyzed after treatment with sample buffer containing SDS and β-mercaptoethanol, followed by separation by reducing 5% SDS polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were blotted onto polyvinylidene difluoride (PVDF) microporous membranes (Immobilon-P, Millipore, MA, USA) using cyclohexylaminopropanesulfonic acid (CAPS)-NaOH buffer (pH11) [23]. We probed the blots for ADAMTS13:AGN with WH2-11-1 as the primary mAb, followed by secondary staining with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Kirkegaaed and Perry Lab, Gaithersburg, MO). After incubation with Western Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences, Shelton, CT), the blots were exposed to X-ray film. Densitometric analysis of ADAMTS13:AGN was performed for the 190-kD band using NIH imageJ (developed by the National Institutes of Health, http://rsb.info.nih.gov/nih-image/).

Results

When diluted normal plasma was analyzed by WB under reducing conditions, WH2-11-1 detected a single 190-kD band of ADAMTS13:AGN, and the detection limit was determined to be 3% of the normal controls (Fig. 1, top). Densitometric analysis of the 190-kD band showed a nearly straight line on a semi-logarithmic graph (data not shown). Using this assay, the normal range of ADAMTS13:AGN in 60 healthy Japanese subjects (30 females and 30 males, aged 20–40 years) was determined to be 101.6 ± 49.4% (mean ± 2SD).

Next, plasma from 9 USS patients and 25 of their relatives (23 definite carriers and 2 normal subjects), whose *ADAMTS13* gene mutations had been



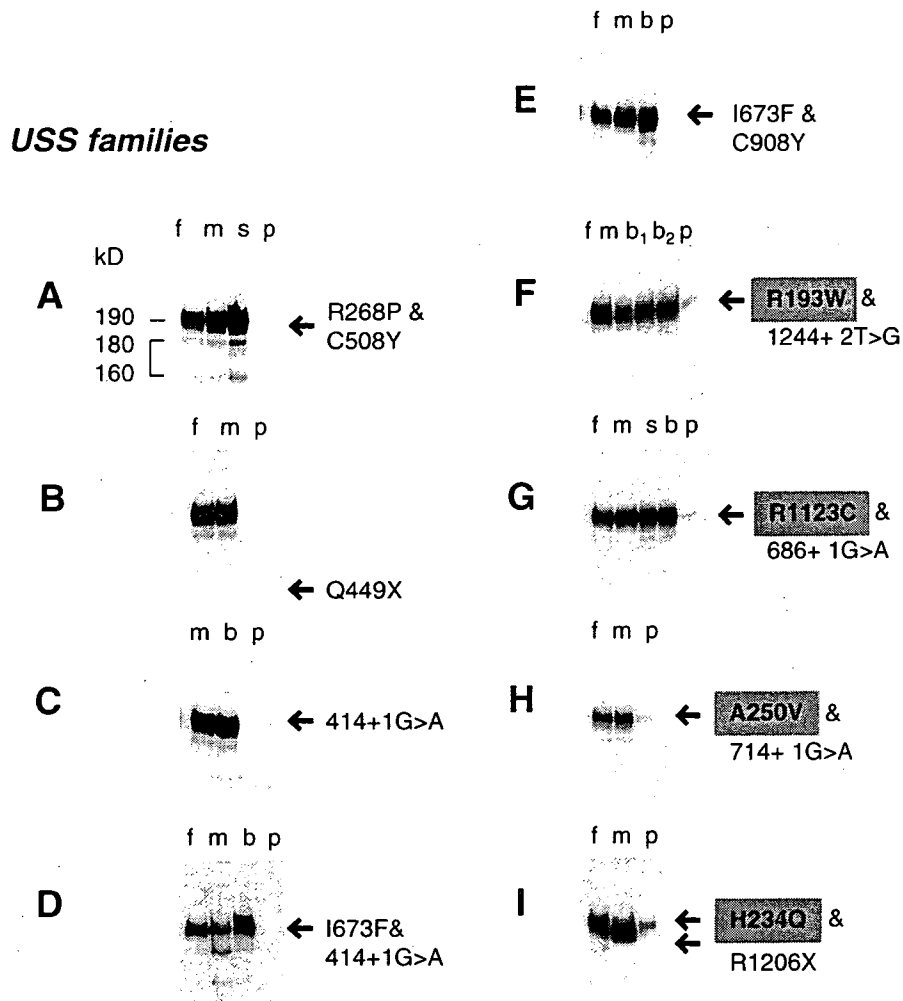


Figure 1 WB analysis under reducing conditions of plasma ADAMTS13:AGN in 9 families with a history of USS. WB analysis of plasma ADAMTS13:AGN in the members of the nine USS families are shown: f (father), m (mother), b(brother), s (sister), and p (patient). Under reducing conditions, recombinant (r) and/or plasma-derived ADAMTS13 from normal individuals is detected as a 190-kD band using an anti-ADAMTS13 mAb, WH2-11-1. Based on serial dilutions of normal plasma, the detection limit was determined to be 3% of the normal controls (top). The 190-kD band is completely absent from the plasma of 5 USS patients (A–E), but faintly detectable in the plasma of 4 patients (F–I) with *ADAMTS13* gene mutations (R193W, R1123C, A250V, and H234Q). Furthermore, several 180–160–kD bands are visible in certain family members under reducing conditions (bottom).

identified (Table 1), were analyzed using this method. It was noteworthy that before analysis, 7 of the 9 USS patients had undetectable levels (<0.5% of the normal control) of plasma ADAMTS13:ACT, while the remaining 2 (patients F and H) showed low but appreciable activities (0.8% and 0.6%) according to a sensitive ADAMTS13:ACT-ELISA. By WB, four missense mutations (R268S, C508Y, I673F, and C908Y) and one intron 4 mutation (414+1G>A) resulted in no appreciable ADAMTS13:AGN in the plasma. Furthermore, one nonsense mutation (Q449X) resulted in the protein being secreted into the culture medium (as a C-terminally truncated 50-kD protein in *in vitro* studies), and this mutant was not detectable in plasma, even with a mAb (A10) directed to the disintegrin domain (data not shown). With regard to another nonsense mutation, R1206X, *in vitro* expression studies have not been done, but the current study has shown that the R1206X mutant protein is absent from patients' plasma.

Table 1 Plasma levels of ADAMTS13:ACT and AGN in patients with USS and its relatives, whose *ADAMTS13* gene mutations were identified

USS families	*ADAMTS13* gene mutations	ADAMTS13: ACT(%) by ELISA	ADAMTS13: AGN(%) by WB	ADAMTS13: ACT/AGN ratio
A				
f	R268P/P475S	4.2	37	0.11
m	C508Y/WT	18	48	0.38
s	P475S/WT	52	80	0.65
p	R268P/C508Y	<0.5	<3	*
B				
f	Q449X/WT	37	37	1.00
m	Q449X/WT	48	48	1.00
p	Q449X/Q449X	<0.5	<3	*
C				
m	414+1G>A/WT	34	46	0.74
b	414+1G>A/WT	41	44	0.93
p	414+1G>A/414+1G>A	<0.5	<3	*
D				
f	I673F/WT	40	40	1.00
m	414+1G>A/WT	40	30	1.33
b	414+1G>A/WT	31	50	0.62
p	414+1G>A/I673F	<0.5	<3	*
E				
f	I673F/WT	20	35	0.57
m	C908Y/WT	33	50	0.66
b	WT/WT	32	67	0.48
p	I673F/C908Y	<0.5	<3	*
F				
f	R193W/WT	17	40	0.43
m	1244+2T>G/WT	10	35	0.29
b1	1244+2T>G/WT	37	48	0.77
b2	WT/WT	50	54	0.93
p	R193W/1244+2T>G	0.8	5	0.16
G				
f	R1123C/WT	32	40	0.80
m	686+1G>A/WT	43	58	0.74
s	686+1G>A/WT	38	62	0.61
b	R1123C/WT	34	64	0.53
p	686+1G>A/R1123C	<0.5	4	*
H				
f	A250V/WT	18	16	1.13
m	714+1G>A/WT	20	23	0.87
p	714+1G>A/A250V	0.6	4	0.15
I				
f	H234Q/WT	24	40	0.60
m	R1206X/WT	18	36	0.50
p	H234Q/R1206X	0.5	6	*
	Normal individuals (mean±2SD)	99.1±43.0	101.6±49.4	

f: father, m: mother, b: brother, s: sister, p: patient.

In contrast, the 190-kD band was present for four missense mutations (R193W, R1123C, A250V, and H234Q), but to a much lesser extent than in the normal controls. In addition, in two family members of A-s and D-m, two additional bands at 180 and 160 kD were intensified (Fig. 1).

The results of the densitometric analyses of the plasma levels of the 190-kD ADAMTS13:AGN are summarized in Table 1. Four USS patients had 4–6% antigen, five had less than 3%. The definite carriers of USS (n=23) revealed levels of 43.8±13.7%. We also examined the association of ADAMTS13:ACT (X axis) and ADAMTS13:AGN (Y axis) for both the USS patients and the definite carriers. We found a significant positive correlation between these two values (Y=1.08X+9.1, r^2=0.74, p<0.01) (data not shown).

Discussion

A number of *ADAMTS13* gene mutations have been reported in patients with USS or congenital TTP, but only a limited number of these mutations have been analyzed by gene expression studies using HeLa or HEK293 cells. During our initial studies in HeLa cells,

we observed that *ADAMTS13* with a nonsense mutation, Q449X (found in USS family B), was secreted into the culture medium as a C-terminally truncated 50-kD protein. However, we have shown here that it is not present in plasma. The cause of this discrepancy is not entirely clear, but we presume that the 50-kD protein is more sensitive to proteolytic degradation *in vivo*. The mechanism of proteolytic regulation of ADAMTS13 in normal circulation has not been elucidated, but Crawley et al. showed that three serine proteinases (thrombin, Xa, and plasmin), which are ubiquitously involved in normal hemostasis, cleave ADAMTS13:AGN *in vitro*, leading to a concomitant decrease in ADAMTS13:ACT [24]. Thus, it is reasonable to assume that a proteolytic mechanism might be involved in the rapid clearance of the 50-kD protein from circulation. Furthermore, certain missense mutations (R193W and A250V) led to moderate secretion inhibition [14,15], and other missense mutations of the *ADAMTS13* gene (R268S, C508Y, I673F, and R1123C) showed an almost total lack of secretion despite normal production within cells, suggesting a disturbance of the secretion mechanism in these variants [10,14]. The results presented here largely agree with those obtained from *in vitro* experiments, and in fact USS patients F and H (R193W and A250V) showed a less intense but distinct 190-kD band by WB under reducing conditions. By directly analyzing patient plasma in this study, we have demonstrated that both the missense mutations R1123C and H234Q produce proteins present in circulation but to a much lesser extent than the controls. On the other hand, the protein by nonsense mutation R1206X was not present as a C-terminally truncated protein. These results suggested that R1123C and H234Q mutations might lead to secretion inhibition and the R1206X mutation might show proteolytic clearance. Concerns regarding the potentially increased *in vivo* proteolysis of these ADAMTS13 mutants are important, and should be explored in detail in future studies. In addition, the 23 USS carriers had plasma levels of ADAMTS13:AGN as lower than 50% of normal controls, and these values correlated well with the ADAMTS13:ACT measured in these carriers. In general, the levels of both ADAMTS13:ACT and :AGN in the carriers' plasma therefore appear to reflect the function of a single wild-type allele.

In conclusion, the analysis of plasma ADAMTS13:AGN, as demonstrated here, represents a useful diagnostic tool for USS patients. Further investigation of ADAMTS13:AGN and its mutations in USS would contribute to our understanding of *ADAMTS13 gene* function, and could aid the development of new therapeutic approaches.

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HEMATOLOGY

Plasma Levels of ADAMTS13 Antigen Determined with an Enzyme Immunoassay Using a Neutralizing Monoclonal Antibody Parallel ADAMTS13 Activity Levels

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Abstract

Measurements of plasma ADAMTS13 activity (ADAMTS13:AC) have been used for the diagnosis of patients with thrombotic thrombocytopenic purpura (TTP); however, the clinical usefulness of plasma ADAMTS13 antigen (ADAMTS13:AG) has been controversial, because antigen values vary widely among patients with acquired idiopathic TTP (ai-TTP). We have developed a novel enzyme-linked immunosorbent assay (ELISA) for the determination of plasma ADAMTS13:AG. This highly sensitive ELISA system using a neutralizing monoclonal antibody enables the detection of as little as 0.1% of the level in normal human plasma, corresponding to approximately 1 ng/mL purified plasma ADAMTS13. The mean (± 2 SD) plasma level of ADAMTS13:AG in healthy individuals was 106.4% ± 39.3% (n = 52). Patients with Upshaw-Schulman syndrome (USS) (n = 20) and ai-TTP (n = 30) showed significantly reduced ADAMTS13:AG levels (0.5% ± 1.6% and 1.2% ± 3.4%, respectively). The ADAMTS13:AG level was 48.4% ± 42.6% in USS carriers (n = 40) and <8.3% in ai-TTP patients with <0.5% ADAMTS13:AC. These values were almost parallel to those for ADAMTS13:AC. This ELISA may be useful for the rapid determination of ADAMTS13:AG. Further investigations of this antigen would be helpful in advancing the understanding of the pathogenesis of congenital and acquired TTP.
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Key words: ADAMTS13 antigen; ELISA; Thrombotic thrombocytopenic purpura; Neutralizing monoclonal antibody

1. Introduction

Thrombotic microangiopathies (TMAs) constitute a group of heterogeneous diseases characterized by microangiopathic hemolytic anemia, thrombocytopenia, and microvascular platelet thrombi. TMAs develop in the presence or absence of underlying disease and typically include thrombotic thrombocytopenic purpura (TTP) with predominantly neurotropic clinical signs and hemolytic uremic syndrome with nephrotropic signs [1]. Several investigators have indicated that severely deficient activity of the plasma von Willebrand factor (VWF)-cleaving protease, or ADAMTS13 (a disintegrin and metalloproteinase domain, with thrombospondin type 1 motifs 13) [2-5], was a unique feature of TTP, not found in patients with hemolytic uremic syndrome [6,7]. Patients with congenital TTP, or Upshaw-Schulman syndrome (USS), were subsequently shown to be deficient in ADAMTS13 activity (ADAMTS13:AC) via genetic mutations in the *ADAMTS13* gene, and ADAMTS13:AC deficiency in patients with acquired idiopathic TTP (ai-TTP) was found to be due to neutralizing or nonneutralizing autoantibodies [8,9]. Recently, an enzyme-linked immunosorbent assay (ELISA) that uses rabbit polyclonal antibodies or monoclonal antibodies (MoAbs) against ADAMTS13 was described for the measurement of plasma ADAMTS13 antigen (ADAMTS13:AG). The clinical usefulness of measuring ADAMTS13:AG by ELISA has been controversial, however, because of its limited value in ai-TTP, with the occurrence of autoantibodies against ADAMTS13, and because of the presence of ethnicity-related differences in plasma ADAMTS13:AG levels among healthy donors [10,11]. We measured plasma ADAMTS13:AG concentrations in USS families, ai-TTP patients, and healthy unaffected donors with a newly developed ELISA method that uses neutralizing MoAbs against ADAMTS13.

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2. Materials and Methods

2.1. Patients

2.1.1. USS Patients

Twenty patients with histories of congenital TTP or USS were enrolled in this study. All patients showed completely reduced ADAMTS13:AC levels because of this genetic disorder but did not show appreciable amounts of inhibitor. USS patients are usually compound heterozygotes who receive different *ADAMTS13* gene mutations from unrelated parents; homozygotes are occasionally observed as a product of a consanguineous marriage. Gene analysis identified all parents and asymptomatic siblings of the USS patients as heterozygous for *ADAMTS13* gene mutations and as definite carriers (n = 40).

2.1.2. ai-TTP Patients

The diagnosis of ai-TTP was made in 40 patients on the basis of the following commonly accepted clinical and laboratory findings: (1) thrombocytopenia (platelet count <100 × 10^9/L), (2) microangiopathic hemolytic anemia (hemoglobin level <125 g/L, negative results in the direct Coombs test, and the presence of schistocytes in peripheral blood smears), (3) normal results in a coagulation screening test, (4) the presence of neurotropic signs, and (5) a lack of underlying disease [12]. All ai-TTP patients showed a plasma ADAMTS13:AC of less than 3% of normal by means of a classic VWF multimer assay with its inhibitor. Plasma samples were taken from patients prior to plasma exchange and were sent, together with clinical and laboratory information, to our laboratory from referring hospitals across Japan. Plasma samples were frozen at –80°C in aliquots until use. As controls, we obtained normal citrated-plasma samples from 52 healthy individuals (26 women and 26 men, aged 20-40 years) and kept the samples frozen in aliquots at –80°C. Pooled normal human plasma (NHP) was used as a control standard for this study.

These studies were conducted following approval by the ethics committee of Nara Medical University.

2.2. Purification of ADAMTS13

NHP was used as the starting material. The method for ADAMTS13 purification has been described in detail elsewhere. In brief, purification entailed the following 3 steps: immunoaffinity chromatography, ion-exchange chromatography, and molecular-sieve chromatography. These steps were carried out at room temperature. Electrophoresis of purified ADAMTS13 revealed a 170-kd band under nonreducing conditions and a 190-kd band under reducing conditions.

2.3. Production and Characterization of 2 Anti-ADAMTS13 Murine MoAbs

The characterization of 2 anti-ADAMTS13 murine MoAbs (A10 and C7) was recently described in detail [13]. In brief, A10 had an epitope on the disintegrin domain and totally inhibited ADAMTS13:AC at a final concentration of 20 μg immunoglobulin G/mL in a static assay system. C7, however, had an epitope on the seventh to eighth thrombospondin-1 domain and did not significantly inhibit ADAMTS13:AC. Furthermore, both MoAbs reacted with ADAMTS13:AG under nonreducing conditions in Western blot analyses but did not react under reducing conditions.

2.4. Analysis of Plasma ADAMTS13:AG

ADAMTS13:AG was measured by sandwich ELISA methods with the 2 anti-ADAMTS13 murine MoAbs (A10 and C7).

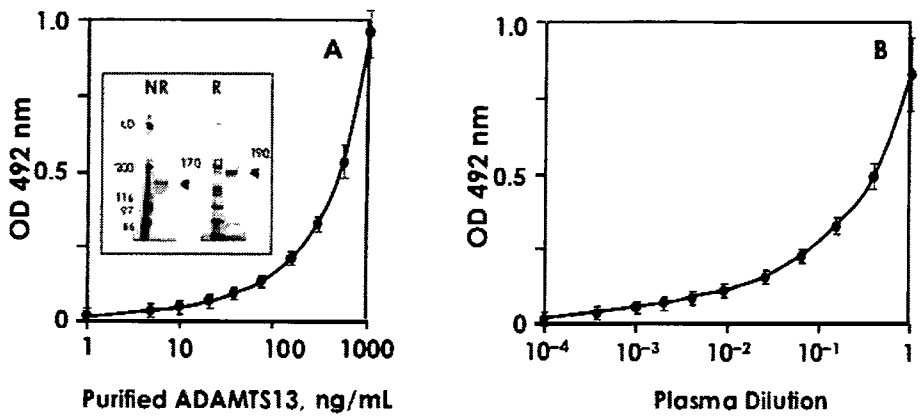

Figure 1. Calibration curves for ADAMTS13 antigen (ADAMTS13:AG) obtained with a novel enzyme-linked immunosorbent assay (ELISA) using a neutralizing monoclonal antibody as a capturing antibody. A, Electrophoresis of ADAMTS13 purified from pooled normal human plasma (NHP) revealed a 170-kd band under nonreducing (NR) conditions and a 190-kd band under reducing (R) conditions. The optical density (OD) at 492 nm for serial dilutions of purified ADAMTS13 measured with the ADAMTS13:AG ELISA increased in a dose-dependent manner; the detection limit was approximately 1 ng/mL. B, Subsequent measurements of serial dilutions of NHP showed the OD at 492 nm to increase in proportion to the NHP concentration, yielding a standard calibration curve for ADAMTS13:AG. The standard curve showed an ADAMTS13 concentration in healthy individuals of 0.95 ± 0.29 μg/mL plasma; the lower limit of detection was identified as 0.1% of the level in NHP. Data are presented as the mean ± SD.

We precoated microtiter plates with A10 MoAb. One hundred microliters of sample was added to the wells of each plate and incubated at 37°C for 3 hours. The wells were washed 3 times with phosphate-buffered saline containing 0.05% polysorbate 20 (Tween 20) (PBS/T), and 100 μL of horseradish peroxidase (HRP)-conjugated C7 MoAb was added to the wells. After incubation at 37°C for 1 hour, the wells were washed 3 times with PBS/T, 100 μL of HRP substrate (*o*-phenylenediamine /hydrogen peroxide) was added, and the wells were incubated for another 30 minutes. The reaction was stopped with 100 μL of 1 M sulfuric acid, and the absorbance was measured at 492 nm. All samples were examined in duplicate, and the results were calculated as the mean of 2 values.

2.5. Assays for ADAMTS13:AC and ADAMTS13 Inhibitors

ADAMTS13:AC and titers of ADAMTS13 inhibitors were assayed with a highly sensitive MoAb-based ELISA [14]. In brief, 100 μL of a solution of a recombinant human VWF fragment (250 ng/mL GST-VWF73-His in PBS with 1% bovine serum albumin) was added to wells of microtiter plates precoated with anti-GST polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) and incubated at 37°C for 1 hour. After 3 washes with PBS/T, 100 μL of plasma sample prediluted 11-fold with reaction buffer (5 mM acetate buffer with 5 mM $MgCl_2$, pH 5.5) was added, and the plates were incubated again at 37°C for 1 hour. The wells were washed 3 times with PBS/T, 100 μL of HRP-conjugated anti-N10 MoAb was added, and the wells were further incubated at 37°C for 1 hour. The wells were then washed 3 times with PBS/T, 100 μL of HRP substrate (*o*-phenylenediamine/hydrogen peroxide) was added, and the plates were incubated for 10 minutes. The reaction was stopped with 100 μL 1 M sulfuric acid, and the absorbance was measured at 492 nm. The inhibitor titer was expressed in Bethesda units, with 1 inhibitor unit defined as the amount necessary to reduce the ADAMTS13:AC to 50% of the control level; titers >0.1 Bethesda U/mL were considered significant. Plasma samples were heat-treated at 56°C for 1 hour and then centrifuged before supernatant levels of ADAMTS13 inhibitor were assessed with these assays.

2.6. Statistical Analysis

All experimental data are presented as the mean ±2 SD. Paired and unpaired comparisons between the 2 groups were performed with the Student *t* test and the Fisher exact test. A 2-tailed *P* value <.05 was considered statistically significant. Analyses were carried out with the StatView statistical software package (version 5.0; SAS Institute, Cary, NC, USA).

3. Results

3.1. ELISA for ADAMTS13:AG

The purified ADAMTS13 sample was analyzed by electrophoresis on a 15% polyacrylamide gel containing sodium dodecyl sulfate; the apparent size of the ADAMTS13:AG band was 170 kd under nonreducing conditions and 190 kd under reducing conditions. With this standard sample, we measured serial dilutions of the purified ADAMTS13 with this novel ELISA. A standard calibration curve for the ADAMTS13:AG concentration revealed the detection limit to be approximately 1 ng/mL (Figure 1A). We subsequently measured ADAMTS13:AG concentrations in serial dilutions of NHP (1:1 to 1:1000) in blocking solution. We plotted corresponding optical density values to obtain a standard calibration curve for determining plasma concentrations of ADAMTS13:AG (Figure 1B). These results revealed the concentration of ADAMTS13:AG in NHP to be 0.95 ± 0.29 μg/mL, and the lower detection limit was 0.1% of the concentration in NHP.

3.2. Measurement of the Plasma Level of ADAMTS13:AG

Using the NHP results as a standard, we identified the plasma level of ADAMTS13:AG in healthy unaffected donors (n = 52) to be 106.4% ± 39.3% of that of NHP. Significantly lower ADAMTS13:AG levels (0.5% ± 1.6%) were found in the patients with USS (n = 20), 8 of whom had undetectable levels (<0.1%), with ADAMTS13:AG concentrations in the remaining 12 patients ranging from 0.1% to 3.8% (median, 0.1%). Definite carriers (n = 40) showed values (48.4% ± 42.6%) approximately half those of healthy donors.

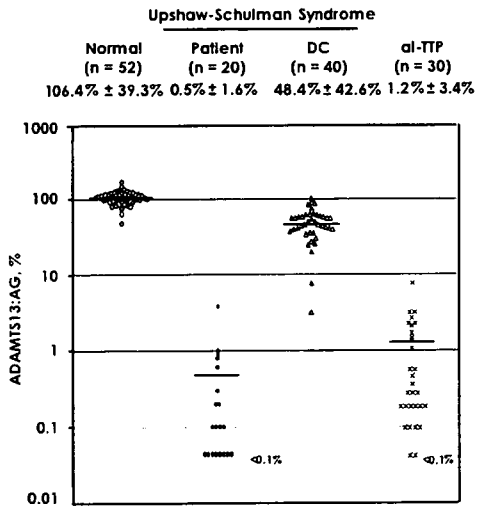

Figure 2. Plasma levels of ADAMTS13 antigen (ADAMTS13:AG) measured by the novel enzyme-linked immunosorbent assay. The standard curve in Figure 1B was used to determine the following plasma ADAMTS13:AG levels (mean ± 2 SD): healthy individuals with wild-type ADAMTS13 (Normal), 106.4% ± 39.3% (n = 52); Upshaw-Schulman syndrome patients (USS), 0.5% ± 1.6% (n = 20); definite USS carriers (DC), 48.4% ± 43.6% (n = 40); patients with acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP), 1.2% ± 3.4% (n = 40). Eight (40%) of 20 USS patients and 2 (5%) of 40 ai-TTP patients showed undetectable ADAMTS13:AG levels (<0.1%). These results indicate that USS and ai-TTP patients had significantly reduced ADAMTS13 levels compared with healthy donors.

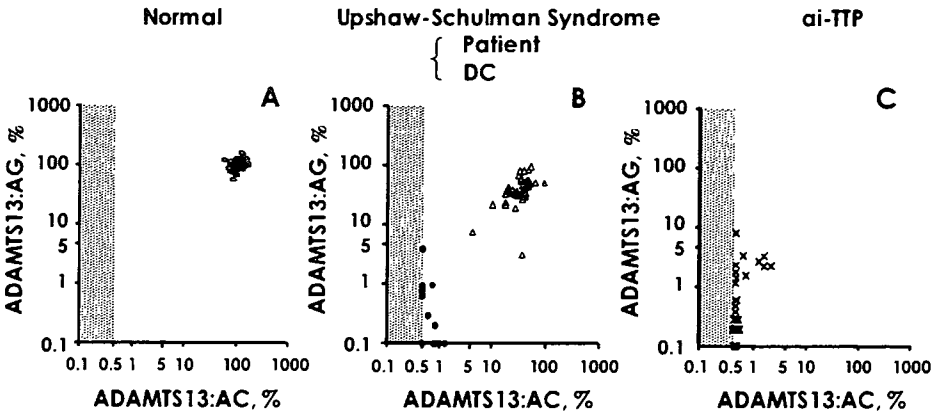

Figure 3. Relationship between plasma levels of ADAMTS13 antigen (ADAMTS13:AG) as measured by the novel enzyme-linked immunosorbent assay and ADAMTS13 activity (ADAMTS13:AC). Plasma ADAMTS13:AC (x-axis) and ADAMTS13:AG (y-axis) showed a significantly positive correlation in (A) healthy donors with wild-type ADAMTS13 (Normal) (n = 52; $y = 0.59x + 47.2$; $r^2 = 0.33$; $P < .05$) and in (B) Upshaw-Schulman syndrome (USS) patients (•, n = 20) plus definite USS carriers (DC) (Δ, n = 40) ($y = 1.12x + 5.8$; $r^2 = 0.66$; $P < .05$). On the other hand, in (C) patients with acquired idiopathic thrombotic thrombocytopenic purpura (ai-TTP) (n = 40), ADAMTS13:AC levels were <2.6% of normal, with the ADAMTS13:AG level ranging from <0.1% to 8.3%. No significant positive correlation was found ($y = 1.1x + 0.5$; $r^2 = 0.10$; $P = .07$).

Patients with ai-TTP (n = 40) also showed significantly reduced ADAMTS13:AG levels (1.2% ± 3.4%). Two patients had undetectable levels, and 38 patients presented ADAMTS13:AG concentrations ranging from 0.1% to 8.3% (median, 4.5%) of those of the NHP standard (Figure 2).

3.3. Relationship between ADAMTS13:AG and ADAMTS13:AC

We also measured the plasma ADAMTS13:AC with a highly sensitive ELISA method, which has previously been described [14]. Healthy individuals, USS patients, USS carriers, and ai-TTP patients had plasma ADAMTS13:AC levels that were 100.1% ± 38.1%, 0.6% ± 0.4%, 35.2% ± 31.2%, and 0.7% ± 1.0%, respectively, of the NHP standard. We found a significant positive correlation between ADAMTS13:AG and ADAMTS13:AC in healthy individuals and USS families ($y = 0.59x + 47.2$ [$r^2 = 0.33$], and $y = 1.12x + 5.8$ [$r^2 = 0.66$], respectively; Figures 3A and 3B); however, a significant positive correlation was not noted in ai-TTP patients ($y = 1.1x + 0.5$; $r^2 = 0.10$; $P = .07$) (Figure 3C).

4. Discussion

ADAMTS13:AC has been measured for the diagnosis and treatment of patients with TMAs via the analysis of multimeric patterns or disulfide-linked cleavage fragments with purified VWF. ELISA-based assays for ADAMTS13:AC that use the VWF73 peptide were recently developed, and these assays are going to become a standard test for the rapid diagnosis of TMAs [14,15]. On the other hand, analyses for assessing ADAMTS13:AG in TMA patients and in healthy individuals have been relatively unchecked. An ELISA-based assay that uses rabbit polyclonal antibodies against ADAMTS13:AG to measure the plasma level of ADAMTS13:AG has been reported, along with its diagnostic usefulness in TMAs [10]. Other investigators have shown that an ELISA-based assay for ADAMTS13:AG that uses murine MoAbs is highly sensitive, with a detection limit of 1.6% of the level in NHP [11]. We have developed a new ADAMTS13:AG sandwich ELISA that uses 2 murine MoAbs: A10 as a capturing antibody and C7 as a detecting antibody. The former is a neutralizing MoAb that recognizes an epitope on the disintegrin-like domain, and the latter is a nonneutralizing MoAb that recognizes an epitope on the seventh to eighth thrombospondin-1 domain. From our analysis of NHP and purified ADAMTS13 derived from NHP, we have found this novel ELISA to be useful for measuring the plasma level of ADAMTS13:AG, with a calculated detection limit of 1 ng/mL of purified ADAMTS13 or 0.1% of the level in NHP. Using this highly sensitive ELISA, we found that USS patients had significantly lower ADAMTS13:AG levels (0.5% ± 1.6%) than those in healthy individuals (106.4% ± 39.3%). Definite carriers of USS showed values approximately half those of noncarriers (48.4% ± 42.6%). These ADAMTS13:AG values closely paralleled ADAMTS13:AC values and showed a positive linear correlation with ADAMTS13:AC ($y = 1.12x + 5.8$; $r^2 = 0.66$). We recently reported that ADAMTS13:AG results for USS patients and their relatives obtained by Western blot analysis largely agreed with those obtained in gene expression studies [15]. These results suggest that this novel sandwich ELISA for ADAMTS13:AG may be convenient and useful as a rapid diagnostic tool for USS or congenital TTP, because both gene expression and Western blot analyses are much more expensive and time consuming.

Although ai-TTP patients also showed significantly reduced ADAMTS13:AG levels (1.2% ± 3.4%), these patients' ADAMTS13:AG values were not significantly correlated with ADAMTS13:AC values. Measurement of ADAMTS13:AG in ai-TTP patients by ELISA has already been reported to be of limited value, because some ai-TTP patients exhibit ADAMTS13:AG values in the normal range even though its inhibitor has markedly reduced the activity level. The discrepancy between ADAMTS13:AC and ADAMTS13:AG values may be due to the presence of the

ADAMTS13-autoantibody complex in the plasma of these patients [10,11]. In this study, however, we did not encounter patients with ADAMTS13:AG values within the normal range (61.2%-165.4%); plasma ADAMTS13:AG levels ranged from <0.1% to 8.3%. These results showed that this novel ELISA method exhibited a better specificity for measuring ADAMTS13:AG. We thought the discrepancy in the present study might be due to a difference in detection limits between these ELISA methods (0.1% versus 0.5%), because 17 patients (57%) showed ADAMTS13:AG values between <0.1% and 0.5%, even though their ADAMTS13:AC values were <0.5%. The 2 MoAbs (A10 and C7) used in this ELISA were able to directly detect immobilized ADAMTS13 in plasma, but only under nonreducing conditions, suggesting that these MoAbs have a high affinity for ADAMTS13 and require the native conformational structure for epitope recognition. Epitope mapping of autoantibodies against ADAMTS13 in patients with ai-TTP revealed that the cysteine-rich spacer domain, the CUB domains, and the first thrombospondin-1 repeat constitute major epitopes for ADAMTS13 autoantibodies [16]. These epitopes for ADAMTS13 autoantibodies in patients with ai-TTP were quite different from those for A10 and C7. Furthermore, the ELISA using C7 as a capturing antibody and A10 as a detecting antibody did not work well, indicating that using a neutralizing MoAb as a capturing antibody was essential for the assay's greater specificity and sensitivity. We speculate that this novel ELISA can distinguish free ADAMTS13 from its immunocomplex because of the conformational change in recognition regions induced by inhibitor binding. Thus, ADAMTS13:AG values determined with this novel ELISA would be reliable for ai-TTP patients.

In this study, the mean ADAMTS13 level in the plasma of healthy Japanese donors was approximately 1 μg/mL, which is equal to that of Caucasians. Healthy Chinese donors, however, have been reported to show significantly lower ADAMTS13:AG levels than Caucasians. Clarification of this issue requires the testing of a much larger population with the standardized ADAMTS13:AG assay.

In conclusion, we have developed a novel ELISA method that uses neutralizing MoAbs against ADAMTS13 to measure plasma levels of ADAMTS13:AG. This ELISA might be available for the determination of ADAMTS13:AG in plasma and should be useful for rapidly diagnosing both congenital and acquired TTP and in devising a treatment strategy for improving the prognosis.

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Two Mechanistic Pathways for Thienopyridine-Associated Thrombotic Thrombocytopenic Purpura

A Report From the SERF-TTP Research Group and the RADAR Project

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Objectives We sought to describe clinical and laboratory findings for a large cohort of patients with thienopyridine-associated thrombotic thrombocytopenic purpura (TTP).

Background The thienopyridine derivatives, ticlopidine and clopidogrel, are the 2 most common drugs associated with TTP in databases maintained by the U.S. Food and Drug Administration (FDA).

Methods Clinical reports of TTP associated with clopidogrel and ticlopidine were identified from medical records, published case reports, and FDA case reports (n = 128). Duration of thienopyridine exposure, clinical and laboratory findings, and survival were recorded. ADAMTS13 activity (n = 39) and inhibitor (n = 30) were measured for a subset of individuals.

Results Compared with clopidogrel-associated TTP cases (n = 35), ticlopidine-associated TTP cases (n = 93) were more likely to have received more than 2 weeks of drug (90% vs. 26%), to be severely thrombocytopenic (84% vs. 60%), and to have normal renal function (72% vs. 45%) ($p < 0.01$ for each). Compared with TTP patients with ADAMTS13 activity >15% (n = 13), TTP patients with severely deficient ADAMTS13 activity (n = 26) were more likely to have received ticlopidine (92.3% vs. 46.2%, $p < 0.003$). Among patients who developed TTP >2 weeks after thienopyridine, therapeutic plasma exchange (TPE) increased likelihood of survival (84% vs. 38%, $p < 0.05$). Among patients who developed TTP within 2 weeks of starting thienopyridines, survival was 77% with TPE and 78% without.

Conclusions Thrombotic thrombocytopenic purpura is a rare complication of thienopyridine treatment. This drug toxicity appears to occur by 2 different mechanistic pathways, characterized primarily by time of onset before versus after 2 weeks of thienopyridine administration. If TTP occurs after 2 weeks of ticlopidine or clopidogrel therapy, therapeutic plasma exchange must be promptly instituted to enhance likelihood of survival. (J Am Coll Cardiol 2007;50:1138–43) © 2007 by the American College of Cardiology Foundation

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Thrombotic thrombocytopenic purpura (TTP) is a severe, multisystem, thrombotic microangiopathy characterized by thrombocytopenia, microangiopathic hemolytic anemia, renal dysfunction, neurologic abnormalities, and fever (1). About one-fifth of TTP cases are associated with pharmaceuticals (2). The thienopyridine derivatives ticlopidine and clopidogrel are the 2 most commonly reported to the U.S. Food and Drug Administration (3–5) In 1998, we reported 60 cases of ticlopidine-associated TTP, identifying high survival rates after therapeutic plasma exchange (TPE) (4,6). Clopidogrel, a newer thienopyridine derivative, differs in structure from ticlopidine by one methoxycarbonyl group (7). It is now the second most commonly prescribed drug in the U.S. In 2004, we described 39 patients with TTP associated with clopidogrel use, highlighting frequent onset within 2 weeks of drug initiation and high mortality rates despite TPE (5). The manufacturer reported an incidence of one TTP case per 100,000 clopidogrel treated patients (8).

Marked advances in understanding of TTP pathophysiology have occurred recently. One area relates to proteolytic processing of plasma von Willebrand factor (VWF) and characterization of VWF-cleaving protease (VWF) and its inhibitor, an immunoglobulin (Ig)G autoantibody (9,10). In 2001, VWF-cleaving protease was identified as a metalloprotease ADAMTS13, belonging to the ADAMTS (a disintegrin-like and metalloprotease with thrombospondin type 1 motif) family (11). Among idiopathic TTP patients, many have ADAMTS13 deficiency caused by an inhibitory IgG autoantibody. ADAMTS13 activity has been measured for seven patients with ticlopidine-associated TTP, and ADAMTS13 deficiency and autoantibodies to ADAMTS13 were identified in all seven patients (12). Herein, we evaluated clinical, laboratory, and basic science findings for patients with thienopyridine-associated TTP, representing the largest cohort of individuals with this rare syndrome reported to date. Our aim is to identify clinically important differences in presentation and outcome for patients with TTP associated with shorter- versus longer-term administration of ticlopidine and clopidogrel.

Methods

Investigators with the RADAR (Research on Adverse Drug Events and Reports) project identified cases of ticlopidine- and clopidogrel-associated TTP with the use of pharmacovigilance methods that have been described previously (3–5,13,14). Thienopyridine-associated TTP cases were identified from 4 sources: 1) voluntary reports submitted to MedWatch, the Food and Drug Administration's Safety Information and Adverse Event Reporting System (n = 29); 2) published case series or reports from MEDLINE/PubMED, using MeSH terms ticlopidine or clopidogrel, thrombotic microangiopathy, and TTP (n = 40) (4,5,15,16); 3) direct queries of hematologists and apheresis directors in 8 large apheresis centers in geographically dispersed metropolitan areas (Charles Bennett, MD, PhD, Chicago, Illinois; Joseph Kiss, MD, Pittsburgh, Pennsylvania; Thomas Ortel MD, PhD, and Nicholas Bandarenko, MD, Raleigh-Durham, North Carolina; Josh Levy, MD, and Nurit Begani, RN, Los Angeles, California; William Bell, MD, PHD, Baltimore, Maryland; Leo J McCarthy, MD, Indianapolis, Indiana; Jean Connors, MD, Boston, Massachusetts; and Joel Moake, MD, Houston, Texas; n = 42); and 4) a national referral laboratory in Japan (Yoshihiro Fujimura; n = 17). A validated case report form was used to collect data on sociodemographic characteristics, thienopyridine use, clinical data—platelet count (per mm^3), hemoglobin level (g/dl), serum creatinine (mg/dl), neurologic findings (altered mental status, seizure, stroke, or coma)—use of TPE, and survival (4,5). Inclusion criteria were thienopyridine use before the development of thrombocytopenia (platelets <50,000/mm^3) and microangiopathic hemolytic anemia on peripheral blood smear, without the presence of any other identifiable cause, such as disseminated intravascular coagulation, cancer, or preeclampsia. Those cases that did not fulfill or report all of the required inclusion criteria were excluded from analysis.

Abbreviations and Acronyms

TPE = therapeutic plasma exchange
TTP = thrombotic thrombocytopenic purpura
VWF = von Willebrand factor

Assaying of ADAMTS13 activity. Basic laboratory studies were conducted by investigators with the Surveillance Epidemiology and Risk Factors for TTP Study Group (17). Plasma was assayed for ADAMTS13 activity with 3 different methods. Seventeen samples from a Japanese national referral laboratory compared the classic VWF multimer assay measuring the proteolysis of purified VWF into cleaved VWF fragments by sodium dodecyl sulfate agarose gel to a novel enzyme-linked immunoassay technique using monoclonal antibodies directed against the decapeptide of the VWF-A2 domain ending with the C-terminal edge residue Y1605, a cleaved VWF byproduct, and found 100% concordance in determining severe ADAMTS13 deficiency (18). Five samples were measured by collagen binding assay, based on the preferential binding of high-molecular-weight forms of VWF to collagen (15,19). The measurement of ADAMTS13 activity in the remaining 17 plasma samples was performed by measuring proteolysis of purified VWF into VWF fragments by gel electrophoresis (20). Previous studies have reported high levels of concordance in identifying persons with severe ADAMTS13 deficiency using

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these methods for assaying ADAMTS13 levels (21). The inhibitory activity of the IgG autoantibody was determined by mixing TTP plasma samples at various dilutions with normal plasma and measuring the protease activity of the mixture, as previously reported and described (20).

Statistical analysis. Bivariate analysis of factors associated with administration of ticlopidine versus clopidogrel, and shorter- versus longer-term thienopyridine administration, were evaluated with a nonparametric exact methodology called optimal discriminant analysis. Used to analyze binary attributes, optimal discriminant analysis yields results isomorphic with the Fisher exact test and, when used to analyze ordinal attributes, optimal discriminant analysis identifies a threshold value that explicitly maximizes classification accuracy (22). A cut point of 2 weeks or less was determined a priori to define short-term thienopyridine administration based on findings reported previously (3–5). For the subset of patients for whom ADAMTS13 activity levels were measured, optimal discriminant analysis was used to evaluate clinical and laboratory findings associated with severe ADAMTS13 deficiency, characterized as activity levels <15% of normal human plasma as in prior studies (23). However, our findings were qualitatively similar if a cut point of 5% was used, a threshold that was used in some studies (23). A multivariate nonlinear model for predicting survival from TTP was obtained via hierarchically optimal classification tree analysis (21,24). Finally, survival analysis was conducted with Cox proportional hazards survival analysis, with log-rank statistics used to test for differences in the survival outcomes, and Kaplan-Meier analysis for plotting survival curves.

Results

Between 1998 and 2005, 93 ticlopidine- and 35 clopidogrel-associated TTP cases were identified (Table 1). Patients with ticlopidine- and clopidogrel-associated TTP were similar in age (mean 64.2 vs. 58.1 years) and gender (male 53.4% vs. 54.3%) but differed significantly in duration of thienopyridine exposure prior to development of TTP ($p \leq 0.002$) (Fig. 1A). In comparison with patients with clopidogrel-associated TTP, those with ticlopidine-associated TTP were more likely to have received more than 2 weeks of a thienopyridine before TTP (90.3% vs. 25.7%, $p < 0.0001$) and to present with severe thrombocytopenia (platelet count $<20 \times 10^9$/l) (83.9% vs. 60.0%, $p < 0.005$) but less likely to have renal insufficiency (27.8% vs. 55.2%, $p < 0.02$) (Table 1).

We evaluated clinical findings, outcomes, and plasma ADAMTS13 activity for 39 thienopyridine-associated TTP patients (Table 1). In comparison with TTP patients with ADAMTS13 activity >15%, those with severely deficient ADAMTS13 activity were more likely to have received ticlopidine (92.3% vs. 46.2%, $p \leq 0.003$) and to be severely thrombocytopenic (96.2% vs. 38.5%, $p < 0.001$) (Table 1) and had a trend toward developing TTP after longer periods of drug exposure (Fig. 1B). Among 30 patients with thienopyridine-associated TTP and plasma available for assays of autoantibody to ADAMTS13, none with normal ADAMTS13 activity had detectable levels of inhibitor, whereas every patient with severe ADAMTS13 deficiency had IgG autoantibodies that inhibited ADAMTS13 activity ($p < 0.0001$). Survival was greater among thienopyridine-associated TTP patients with deficient ADAMTS13 activity levels who underwent TPE compared with those who did not (90.9% vs. 50.0%, $p < 0.05$). Among six ticlopidine-associated and seven clopidogrel-associated TTP patients whose ADAMTS13 levels were >15%, 12 underwent TPE, and only 7 (58.3%) survived.

Overall, the mortality rate for patients with thienopyridine-associated TTP was 25.8%. Univariate associations identified several characteristics significantly associated with an increased mortality risk for the total sample, including abnormal neurologic status ($p < 0.02$), serum creatinine >2.5 mg/dl ($p < 0.04$), and not receiving TPE ($p < 0.0006$). Among patients who developed TTP after >2 weeks of thienopyridine exposure, survival was 2.2-fold greater when treated with TPE (84% vs. 38%, $p < 0.05$). Among patients who developed TTP within 2 weeks of starting thienopyridines, survival was 77%

Table 1 Characteristics of Thienopyridine-Associated TTP Cases

	All Patients (n = 128)	Thienopyridine Rx ≤14 Days (n = 35)	Thienopyridine Rx >14 Days (n = 93)	Ticlopidine (n = 93)	Clopidogrel (n = 35)	ADAMTS13 Deficient (n = 26)	ADAMTS13 Nondeficient (n = 13)
Mean age, yrs (SD)	62.4 (13.9)	59.8 (13.2)	63.4 (14.1)	64.2 (12.9)	58.1 (15.3)	67.1 (12.0)	60.3 (20.2)
Male	53.7%	57.1%	52.3%	53.4%	54.3%	46.2%	61.5%
Thienopyridine Rx ≤14 days	27.3%	—	—	9.7%*	74.3%*	15.4%	38.5%
Thienopyridine Rx >14 days	72.7%	—	—	90.3%	25.7%	84.6%	61.6%
ADAMTS13 deficient	66.7%	44.4%	73.3%	80%*	22.2%*		
Platelet count <20,000/mm^3	77.3%	65.7%†	81.7%†	83.9%*	60.0%*	96.2%*	38.5%*
Creatinine >2.5 mg/dl	35.6%	41.9%	32.9%	27.8%*	55.2%*	26.9%	46.2%
Abnormal neurologic findings	29.1%	33.3%	27.3%	27.8%	32.3%	19.2%	15.4%
Received TPE	76.6%	74.3%	77.4%	74.2%	82.9%	84.6%	92.3%
Survival	74.2%	77.1%	73.1%	75.3%	71.4%	84.6%	61.5%

*$p < 0.05$; †$p < 0.07$.
Rx = treatment; TPE = therapeutic plasma exchange; TTP = thrombotic thrombocytopenic purpura.

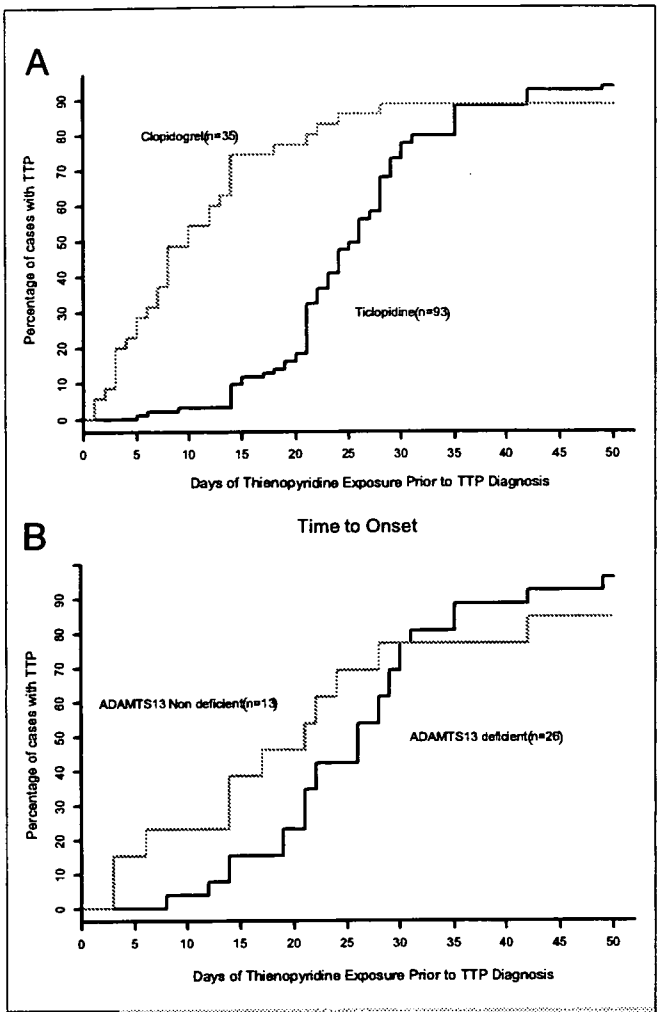

Figure 1 **Duration of Thienopyridine Exposure Prior to TTP Onset**

(A) Thienopyridine-associated thrombotic thrombocytopenic purpura (TTP) onset: ticlopidine versus clopidogrel ($p = 0.0016$). **(B)** Thienopyridine-associated TTP onset: ADAMTS13 deficient versus ADAMTS13 nondeficient ($p > 0.05$).

with TPE and 78% without. A multivariate classification tree analysis model revealed that among thienopyridine-associated TTP patients who received TPE, those patients with ADAMTS13 activity levels >15% at the time of diagnosis of TTP were 4-fold more likely to die (41.9% vs. 9.1%, $p < 0.036$).

Discussion

Our study identifies distinct clinical, laboratory, and outcome differences between ticlopidine- and clopidogrel-associated TTP. More than 90% of the ticlopidine-associated TTP cases develop after more than 2 weeks of thienopyridine use. Among these patients, severe thrombocytopenia and preserved renal function at diagnosis is common, ADAMTS13 activity levels are frequently <15%, and survival is 86% if TPE is administered versus 46% if TPE is not used. These findings are similar to those reported previously for idiopathic TTP cases with severely deficient ADAMTS13 activity levels (16,23,25). In contrast, three-quarters of the clopidogrel-associated TTP cases develop after 2 weeks or less of thienopyridine use. These patients are characterized by mild thrombocytopenia and renal insufficiency at diagnosis, ADAMTS13 activity levels >15%, and survival rates that are similar with versus without TPE (72.4% and 66.7%), findings that are similar to those reported previously for TTP cases with ADAMTS13 activity levels >25%. Our findings suggest 2 mechanistic pathways for thienopyridine-associated TTP, an immunologic pathway associated with more than 2 weeks of thienopyridine use and a nonimmunologic pathway associated with 2 weeks or less of thienopyridine use. In interpreting our study, several factors should be considered.

The results for patients with severe ADAMTS13 deficiency and thienopyridine-associated TTP reinforce previous observations for patients with ticlopidine-associated TTP. Tsai et al. (12) reported 7 ticlopidine-associated TTP patients who had severe ADAMTS13 deficiency and inhibitors to ADAMTS13 at diagnosis, all of whom responded rapidly to TPE. The use of TPE in these patients may result in removal of ADAMTS13 inhibitors and ultra-large VWF multimers, replenishment of ADAMTS13 and VWF, and reduction of cytokines that induce endothelial cell damage and platelet activation (26). Our study also describes cases of thienopyridine-associated TTP cases who do not have severe ADAMTS13 deficiency and whose survival was not influenced by TPE. Preservation of ADAMTS13 activity has been described in patients with post-transplantation thrombotic microangiopathy (27,28) who frequently present with renal insufficiency, moderate thrombocytopenia, and high mortality rates despite TPE. Others have described TTP-like findings among persons with factor V Leiden mutation (29).

Our study has implications for patient safety. First, for the rare individual with a drug-eluting coronary artery stent who develops TTP after the administration of clopidogrel and for whom discontinuation of thienopyridine-therapy could be catastrophic, ticlopidine challenge can be consid-

Table 2 **Outcomes for Ticlopidine- and Clopidogrel-Associated TTP Cases**

	Survival With TPE, %	Survival Without TPE, %
All patients (n = 128)*	81.6	50.0
Ticlopidine (N = 93) *	85.5	45.8
Ticlopidine Rx ≤14 days (n = 9)	100.0	100.0
Ticlopidine Rx >14 days (n = 84)	84.1*	38.1*
Clopidogrel (N = 35)	72.4	66.7
Clopidogrel Rx ≤14 days (n = 26)	70.0	66.7
Clopidogrel Rx >14 days (n = 9)	77.8	—
Thienopyridine Rx ≤14 days (n = 35)	76.9	77.8
Thienopyridine Rx >14 days (n = 93)	83.3*	38.1*

*$p < 0.05$ (for comparison of survival with TPE vs. without TPE).
Abbreviations as in Table 1.