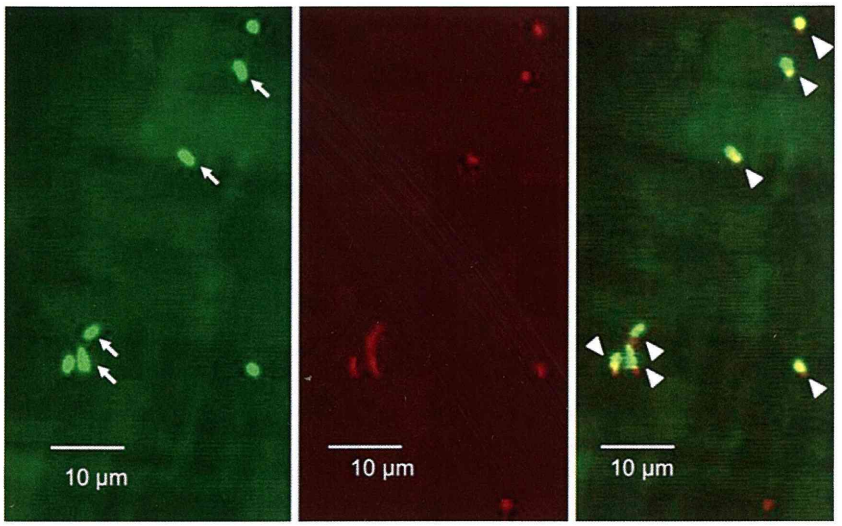

Fig. 1 VWF-dependent platelet adhesion to VECs. A representative figure showing platelets adhered to VECs 25 min after DDAVP administration in GFP-ADAMTS13−/− mice. After intravenous administration of Alexa568-labeled anti-VWF antibody (0.1 ml of 1.0 mg/ml solution) together with DDAVP (0.3 μg/kg body weight), fluorescence of GFP (*green, left*) and Alexa Fluor 568 (*red, middle*) was monitored simultaneously using a W-view system; the right panel is a merged image. Each platelet (*small green dot*) was recognized separately, and some of them were attached (*white arrows*). Most of the spots to which platelets adhered were recognized by Alexa-568-labeled anti-VWF antibody (*white arrow head*). Scale bar shows 10 μm

120 min after DDAVP administration in GFP-ADAMTS13−/− mice or GFP mice (Table 2). The lower dosage of DDAVP employed in the present study, though administered by bolus injection instead of intraperitoneal injection, than previously reported [7] might be a reason for this limited effect.

In vivo analysis of platelets adhesion to $FeCl_3$-injured vascular beds through VWF: role of ADAMTS13 in proteolysis of UL-VWF multimers adhered to an injured vascular wall

$FeCl_3$, at a concentration of 2.5%, induced formation of separated, rather than coalescent, connected platelets aligned on VWF multimer strings, which could be easily evaluated (Fig. 3). The numbers of loci on vascular surfaces injured by $FeCl_3$ to which platelets adhered were similar in GFP-ADAMTS13−/− and GFP mice (46.3±10.2 vs 49.8± 12.3 loci per vascular segment, respectively, mean±SD). The lengths of the adhered strings differed significantly, however, and those in GFP-ADAMTS13−/− mice (mean 25 μm, the 10th–90th percentile 3–50 μm) were approximately 2.5 times longer than those in GFP mice (mean 10 μm, the 10th–90th percentile 2–25 μm, $P<0.01$) (Fig. 4a). The longest string was 105 μm long in GFP-ADAMTS13−/− mice and 50 μm long in GFP mice, respectively. These results reflect the fact that highly active, longer UL-VWF multimers, either circulating in plasma or being secreted from VECs of GFP-ADAMTS13−/− mice, adhered efficiently to the vascular lesion, together with a larger numbers of platelets. Injection of Alexa568-labeled anti-VWF antibody prior to topical use of $FeCl_3$ revealed that multiple strings were bridged by platelets to form longer "platelet strings" (Fig. 5).

The duration time that strings remained attached to the $FeCl_3$-injured vascular surface was measured. The average duration time for VWF strings attached to the vascular surface in the GFP-ADAMTS13−/− mice (mean 1.3 s, the 10th–90th percentile 0.3–6.3 s) was more than four times longer than that for the GFP mice (mean 0.3 s, the 10th–90th percentile 0.2–1.3 s, $P<0.01$) (Fig. 4b). The most stable strings in GFP-ADAMTS13−/− mice survived at least 23.3 s, which was the limit of our observation time in this set of experiments. In GFP mice, the longest string survival time was 6.5 s. These results suggest that the UL-VWF multimers adhered to the vascular lesion were targets for ADAMTS13, and that the lack of this enzyme resulted in sustained adhesion of UL-VWF multimers to the injured vascular wall for longer time periods, together with larger numbers of platelets.

In vivo analysis of microthrombus formation at the laser-induced endothelial injury spot

The process of microthrombus formation at the laser-induced endothelial injury spot was monitored by measuring both the fluorescent intensity of GFP platelets and the size of the thrombi. At each time point, volumes of the thrombi in the group of GFP-ADAMTS13−/− mice tended to be larger than those in the GFP mice, although a significant difference was seen only at 300 s (Fig. 6). The largest thrombi were seen in both groups at 30 s after

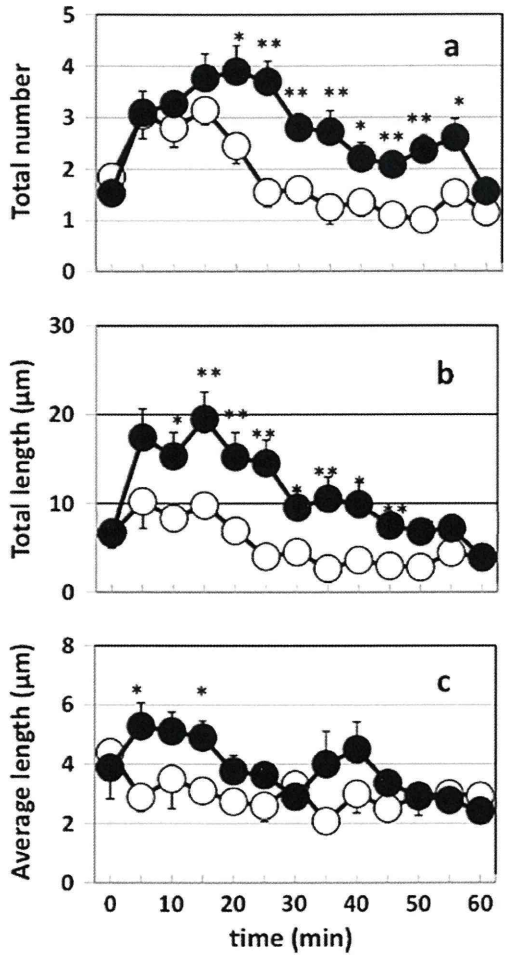

Fig. 2 Platelet adhesion on VECs after DDAVP administration. Numbers of both single platelets and "platelet strings" attached to VECs were counted on each of five venular segments (142×107 μm) of exteriorized mesenteric veins in both GFP-ADAMTS13−/− mice (closed circle, N=6) and GFP mice (open circle, N=4), and results are reported as "total number" (Y-axis, **a**). The diameter of each single platelet as well as the length of each "platelet string" were also measured per venular segment, and results are reported as "total length" (Y-axis, **b**). Their average lengths were calculated in five venular segments (Y-axis, **c**). DDAVP at a dose of 0.3 μg/kg body weight was administered intravenously at point 0. Data are shown as mean±SEM. *P<0.05, **P<0.01

irradiation, and these were 18.8±2.6×10^3 μm^3 in GFP-ADAMTS13−/− mice and 13.1±2.1×10^3 μm^3 in GFP mice, respectively (mean±SEM, n=6) (Fig. 6). The formation of larger thrombi was also shown in an in vitro model

Fig. 3 "Platelet strings" attached to a vascular wall injured by $FeCl_3$. GFP-platelets connected and aligned on a string, i.e., a "platelet string", were observed approximately 1 min after topical application of $FeCl_3$ in GFP-ADAMTS13−/− mice. Application of 2.5% $FeCl_3$ induced separated but not coalescent "platelet string" formation. The continuity of each string was confirmed by evaluating the harmonized motion of connected platelets using digital video images. Scale bar shows 10 μm

after ADAMTS13 depletion using a neutralizing antibody [14]. Differences between the two groups at the laser-induced endothelial injury site, however, appeared smaller when compared with the lengths of "platelet strings" formed on the $FeCl_3$-injured vascular wall. Alexa Fluor 568-labeled anti-VWF antibody was injected to analyze the distribution of VWF in the thrombus in GFP-ADAMTS13−/− mice and in GFP mice. The labeled antibody initially appeared to distribute both in the center and at the periphery of the thrombus, but later it accumulated mainly in the center of the mural-forming thrombus (Fig. 7). A similar distribution was also observed in GFP mice (not shown). The specific binding of this anti-VWF antibody was confirmed by the lack of accumulation of control IgG obtained from non-immunized mice [20]. Bovine serum albumin labeled with Alexa Fluor 568 also did not accumulate in the thrombi (data not shown).

Discussion

Employing ADAMTS13 gene knockout mice expressing GFP, we analyzed the behavior of intact platelets that were not externally manipulated, e.g., by a fluorescent labeling process, on either normal or injured vascular beds in living animals. We found that VWF multimers attached to VECs after stimulation by DDAVP, a secretagogue of VWF

Table 2 Plasma levels of VWF after DDAVP administration

	Sampling time (minutes)				
	0	5	30	60	120
GFP-ADAMTS13−/− mice	0.372±0.069	0.329±0.025	0.340±0.030	0.377±0.011	0.362±0.010
GFP mice	0.259±0.004	0.219±0.066	0.376±0.003	0.326±0.072	0.368±0.029

Mean±SD, N=12 for GFP-ADAMTS13−/− mice and 10 for GFP mice at time 0, N=3–4 for other time points

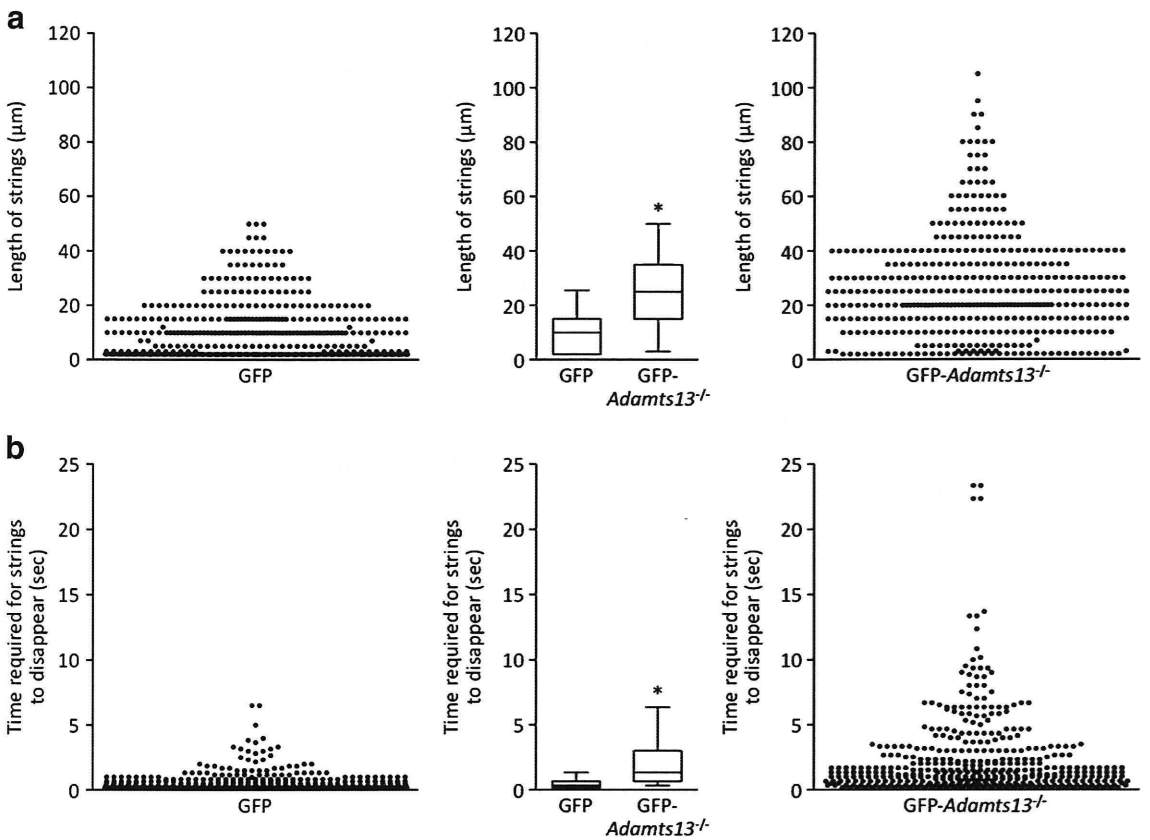

Fig. 4 Quantitative analysis of strings attached to a vascular wall injured by $FeCl_3$. For quantification, 416 strings and 397 strings were observed in GFP-ADAMTS13−/− mice and GFP mice, respectively. The numbers of the strings (*X*-axis) are shown for each length of the strings (*Y*-axis) (**a**). The numbers of strings that disappeared after their initial appearance (*X*-axis) are also shown together with the duration time for VWF strings attached to the vascular surface from their initial appearance (*Y*-axis) (**b**). The length of strings was evaluated when the strings reached their maximum length. The longest persistence times noted were 23.3 s in GFP-ADAMTS13−/− mice and 6.5 s in GFP mice, respectively. Data are also shown as median values together with the values of the 10th and 90th percentiles (*bars*) and of the 25th and 75th percentiles (*boxes*) (**a** and **b**). Statistical significance of differences between the two groups was evaluated by the Mann–Whitney *U* test. **P*<0.01

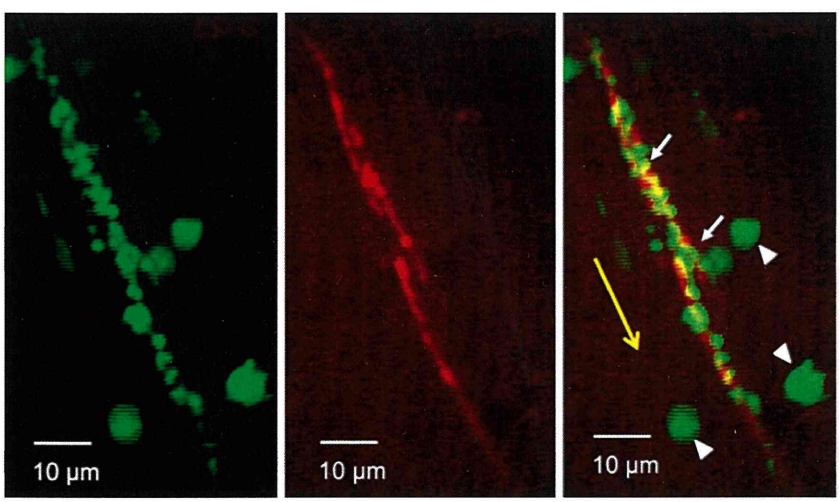

Fig. 5 Elongated UL-VWF multiple strings bridged by platelets. Intravenous administration of Alexa568-labeled anti-VWF antibody followed by topical use of $FeCl_3$ revealed that multiple strings attached to a vascular wall were bridged by platelets to form longer strings in GFP-ADAMTS13−/− mice. Fluorescence of GFP (*green, left*) and Alexa Fluor 568 (*red, middle*) was monitored simultaneously using a W-view system; the right panel is a merged image. Platelets bridging multiple strings are indicated by *white arrows*. White blood cells attached to a vascular wall were also observed (*arrowhead*). The *yellow arrow* shows the direction of blood flow. Scale bar shows 10 μm

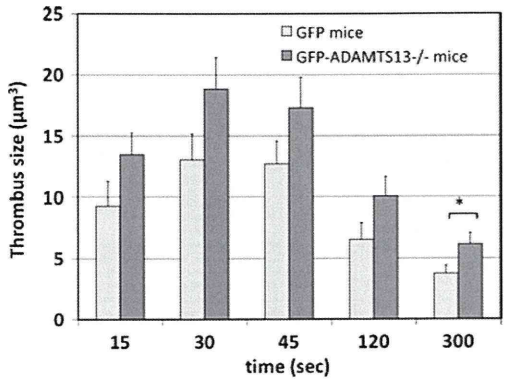

Fig. 6 Size of thrombus formed after laser irradiation. Thrombus formation was triggered by laser irradiation (140 mW, 5 s). Exactly 15, 30, 45, 120, and 300 s after these stimuli, sequences of focal plane images were taken and the area of the thrombus was evaluated by measuring the fluorescent image of GFP platelets. Data are shown as mean±SEM (n=6). *P<0.05

causing its release from storage sites in Weibel-Palade bodies, were longer in GFP-ADAMTS13−/− than in GFP mice, suggesting that ADAMTS13 cleaves UL-VWF multimers during the secretion process, as was previously shown in ADAMTS13−/− mice by stimuli such as histamine and calcium ionophore [10]. Longer platelet strings were also found on the vascular wall after $FeCl_3$ treatment, which is an established technique to expose collagen fibers on the vascular wall by denuding VECs. This suggests that UL-VWF multimers either circulating in plasma or being secreted from VECs of GFP-ADAMTS13−/− mice [6] effectively adhere to the injured vascular wall, together with larger numbers of platelets, compared to GFP mice. Effective and sustained platelet adhesion of long strings composed of several UL-VWF multimers that were bridged by platelets apparently contributed to the accumulation of larger numbers of platelets at the injured vascular wall in ADAMTS13 deficiency.

Several sequential steps are involved in the activation of platelets to form a mural thrombus. The first step is adhesion of platelets to collagen exposed at the injury site, which occurs when VWF binds to GPIbα on the platelet surface [1]. Platelets also adhere to intact or mildly injured VECs, but they detach from these quickly, and repetition of this process is known as rolling [1]. In the present study, the adhesion of non-activated or only minimally activated platelets to VECs was observed at the spots detected using an anti-VWF antibody, following stimulation with DDAVP, a secretagogue known to act on VWF storage sites via V_2 agonist activity [22]. Adhesion of single platelets, as well as several platelets forming "platelet strings", to the spots detected by anti-VWF antibody was observed, consistent with earlier studies [9, 16]. The de novo binding of platelets was also observed upstream of the elongating "platelet strings" whose luminal side tips were already occupied by other platelets (Supplemental Movie and Supplement Fig. 2). Such binding of non-activated or only minimally activated platelets to VWF has been reported previously only under high shear stress conditions in an in vitro study [38], since shear stress-dependent rearrangement of a cryptic binding site in the VWF A1 domain is required before it can bind GPIb [21]. We observed these phenomena in small veins, suggesting that in the secretion of UL-VWF multimers from Weibel-Palade bodies, venous flow is strong enough to alter the conformation of the binding site in the A1 domain. This possibility was suggested in an earlier study [9].

The average length of a "platelet string" adhered to DDAVP-stimulated VECs was approximately 5.3 μm in GFP-ADAMTS13−/− mice and 2.9 μm in GFP mice; this corresponds to 70mers and 38.5mers of VWF, respectively, if we consider the length of one VWF molecule in a multimer to be 75 nm [39]. These correspond to molecular weights of approximately 19,500 and 10,700 kDa, based on

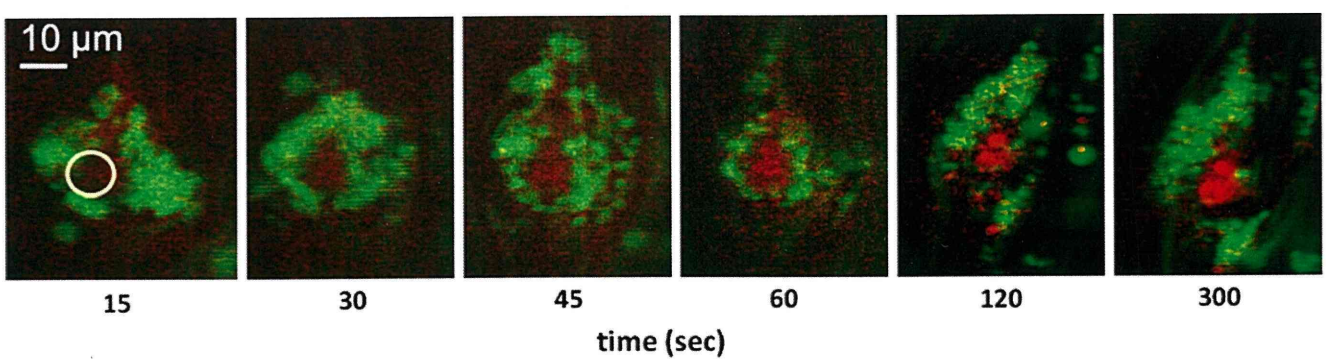

Fig. 7 Localization of VWF in a microthrombus formed by laser irradiation. Accumulation of labeled anti-VWF antibody was evaluated over time as mural thrombi developed in GFP-ADAMTS13−/− mice. The laser-injured area of endothelium is indicated by a *yellow circle* (approximately 10 μm in diameter). Exactly 15, 30, 45, 60, 120, and 300 s after this stimulus, sequences of focal plane images were taken. The focal plane image with the highest intensity of labeled anti-VWF antibody was chosen and evaluated using IPLab software. Scale bar shows 10 μm

the molecular weight of 278 kDa for monomeric VWF [36]. The molecular weight of VWF multimers in normal human subjects ranges from 500 to 20,000 kDa [18]. Thus, the average multimer size in the GFP-ADAMTS13−/− mice was close to the upper limit for UL-VWF in humans, whereas in GFP mice, the average multimer size was within the normal range. The longer length of the platelet strings in GFP-ADAMTS13−/− mice suggests that ADAMTS13 cleaves the UL-VWF multimers even in the vein, which supports a previous in vitro finding that low shear stress is strong enough to cause cleavage of UL-VWF multimers by ADAMTS13 [16]. Thus, lack of ADAMTS13 activity impairs the cleavage of UL-VWF during exocytosis and naturally increases the number of UL-VWF multimers in plasma. The lengths of "platelet strings" adhered to activated VECs by either calcium ionophore or histamine reported previously [10, 31], are similar to those observed after $FeCl_3$ treatment and longer than those observed after DDAVP stimuli in the present study. These results may suggest that DDAVP stimulus is not strong enough to secrete whole VWF strings stored in Weibel-Palade bodies, which can reach 100 μm in length [28]. Another possibility is that the activation of platelet as well as VECs may be required to develop such long strings on VECs in vivo, since both calcium ionophore and histamine are known to enhance platelet aggregation [26].

Next, we examined the behavior of UL-VWF multimers as well as platelets on the injured vascular bed. Exposure of blood vessels to $FeCl_3$, an oxidative chemical agent, is a well-established model of thrombus generation [23]. $FeCl_3$ easily penetrates through the vascular wall and initiates denudation of the endothelium, in which hemolysis and the oxidization of hemoglobin play important roles [45]. The severity of the injury depends on the concentration of $FeCl_3$ as well as the duration of its application to an isolated vein. After trials in which several different concentrations of $FeCl_3$ were applied for different exposure times, $FeCl_3$ application at a concentration of 2.5% for 15 s was chosen, since this procedure produced single and rarely coalescent strings that could be evaluated easily.

After exposure to $FeCl_3$, "platelet strings" were formed on the injured vascular wall, which were longer in GFP-ADAMTS13−/− mice than in GFP mice, although the numbers of the strings were similar. Thus, both UL-VWF multimers circulating in GFP-ADAMTS13−/− mice and VWF multimers having smaller molecular weights in GFP mice adhered to exposed collagen with similar efficacy. Importantly, VWF strings in GFP-ADAMTS13−/− mice remained intact for a longer time than those in the GFP mice. This suggests that UL-VWF multimers bound to the injured vascular wall are cleaved by ADAMTS13 in vivo.

Interestingly, the platelet strings observed following $FeCl_3$ treatment were longer than those observed on VECs after DDAVP stimulation in both groups of animals. The calculated molecular weights of the strings measured following $FeCl_3$ treatment were approximately 96,000 kDa in GFP-ADAMTS13−/− mice and 37,000 kDa in GFP mice, respectively. Thus, they were larger than circulating VWF multimers in plasma, suggesting that multiple strings were bridged by platelets to form longer strings, as shown in Fig. 5. Platelets are highly activated on the injured vascular surface, so it is possible that they bind more tightly to VWF multimers and can form bridges between several VWF strings. In the absence of ADAMTS13 activity, these long strings were hardly cleaved, and therefore the UL-VWF multimers together with large numbers of platelets accumulated at the injured vascular wall. These phenomena were consistent with the pathogenesis of TTP, which is caused by ADAMTS13 deficiency.

Although there were apparent differences between GFP-ADAMTS13−/− mice and GFP mice in the rates at which platelet-bound strings disappeared from the vascular wall, many of the strings in GFP-ADAMTS13−/− mice remained attached temporarily but then disappeared. This raises the question, therefore, of what factors besides ADAMTS13 influence the survival and disappearance of UL-VWF multimer strings adhered to the vascular wall. Dissociation of the multiple UL-VWF strings bridged by platelets through either GPIbα-dependent or GPIIb/IIIa-dependent process is a possible mechanism. Cleavage of UL-VWF multimers by other proteases besides ADAMTS13 might be another mechanism, as suggested previously [8, 33].

To establish the potential role of ADAMTS13 in the ongoing process of mural thrombus generation in vivo, we compared the size of thrombi generated in GFP-ADAMTS13−/− mice and in GFP mice using a laser-induced thrombus model. Thrombi volumes in the GFP-ADAMTS13−/− mice tended to be larger than those in the GFP mice, but these differences were not large. This is in contrast to the striking differences observed between the two groups in the length of "platelet strings" as well as in their adhesion period following $FeCl_3$ treatment. The size of the laser-induced injury on VECs, which is approximately 10 μm, may not be large enough to accurately model the role of UL-VWF multimers at the injured vascular wall in the initiation of a thrombus. We also analyzed the localization of VWF that accumulated in a growing mural thrombus. An Alexa Fluor 568-labeled anti-VWF antibody initially localized to both the center and the periphery of a mural thrombus and then gradually accumulated mainly in its center over time. Recently, we demonstrated that the magnitude of platelet activation varies depending on their location in the thrombus, and that only the platelets in the center of the thrombus are fully activated to express phosphatidylserine on their outer leaflet

[20]. VWF may have accumulated on these activated platelets by binding to GPIIb/IIIa. A similar uneven distribution of VWF was also reported during thrombus formation in an in vitro study [27]. VWF and fibrinogen may play different roles in thrombus formation after binding to GPIIb/IIIa, as was suggested earlier by experiments showing that several GPIIb/IIIa antagonists having distinct abilities to compete for the binding of fibrinogen and VWF to GPIIb/IIIa [2].

In conclusion, in vivo imaging analysis revealed that ADAMTS13 regulates the disappearance of platelet strings on DDAVP-stimulated VECs and on the $FeCl_3$-injured venous vascular wall through the cleavage of UL-VWF. Lack of ADAMTS13 appeared to facilitate the formation of long-lived and elongated platelet strings adhered at the focally injured vascular wall in vivo.

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Conflicts of interest The authors declare that there are no conflicts of interest associated with study.

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von Willebrand factor-to-ADAMTS13 ratio increases with age in a Japanese population

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ADAMTS13, a plasma metalloprotease, regulates platelet aggregation through shear stress-dependent specific cleavage of von Willebrand factor (VWF) multimers. Severe deficiency of plasma ADAMTS13 activity results in accumulation of unusually high-molecular-weight VWF multimers in plasma, and can cause a systemic disease, thrombotic thrombocytopenic purpura (TTP) [1–3]. Previously, we developed a simple and quantitative assay for measuring ADAMTS13 activity using FRETS-VWF73, a fluorogenic peptide substrate [4]. In this study, we used the assay to measure plasma ADAMTS13 activity in 3616 individuals from the Japanese general population.

We used plasma samples from the previously published Suita Study [5–7], an epidemiological study consisting of randomly selected Japanese residents of the city of Suita, which is located in the second largest urban area in Japan. Participants between the ages of 30 and 79 years were randomly selected from the municipality population registry and stratified into groups by sex and age in 10-year increments in 1989. They underwent regular health check-ups between September 1989 and March 1994. Subjects have continued to visit the National Cerebral and Cardiovascular Center every 2 years for regular health check-ups. Our study protocol was approved by the ethical review committee, and only subjects who provided written informed consent for genetic analyses were included.

When the mean of all plasma ADAMTS13 activity values was set at 100%, the standard deviation (SD) was 27% (Fig. 1A). The fifth percentile, 25th percentile, median, 75th percentile and 95th percentile were 61%, 81%, 97%, 116% and 148%, respectively. The mean activity of men (93 ± 24%, mean ± SD, n = 1687) was significantly lower ($P < 0.0001$) than that of women (106 ± 27%, n = 1929), consistent with the previous report [4].

In both men and women, the plasma ADAMTS13 activity tended to decrease with age, especially after age 60 (Fig. 1B). A linear regression model also indicated the decrease with age (regression coefficient of −0.642 and 95% confidence intervals (CI) of −0.740 to −0.544 in men; −0.663 and −0.767 to −0.558 in women). We also measured plasma VWF antigen levels

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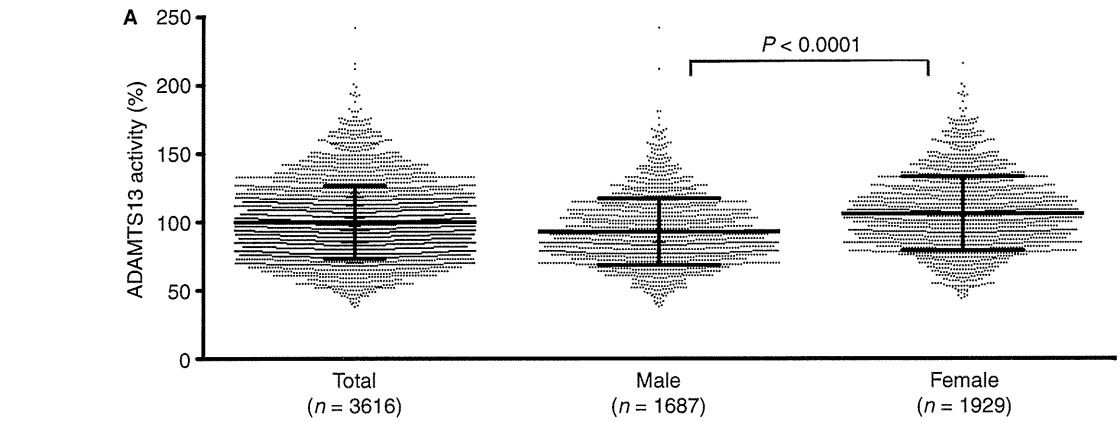
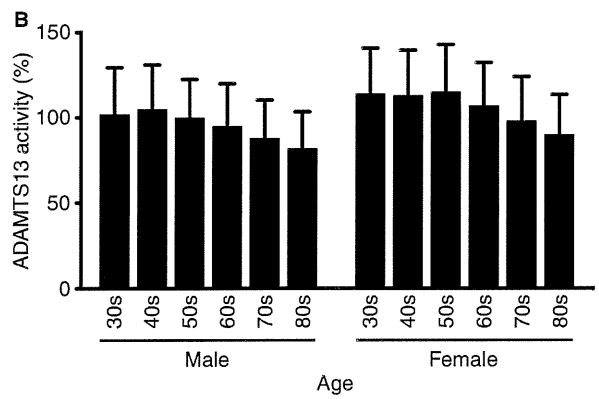
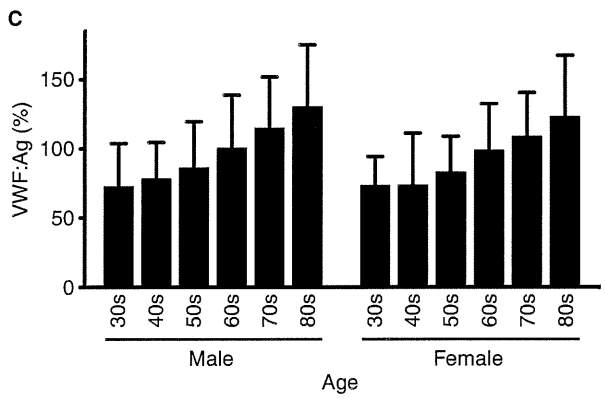
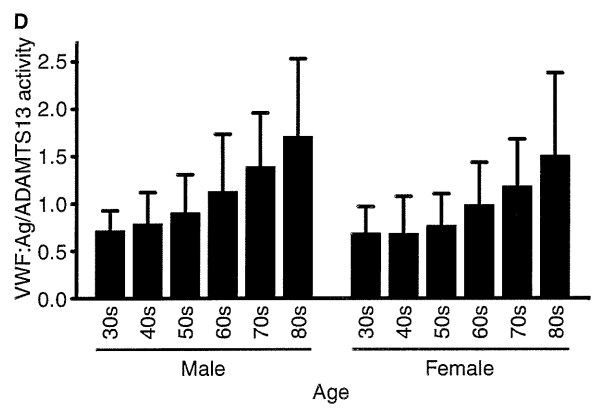


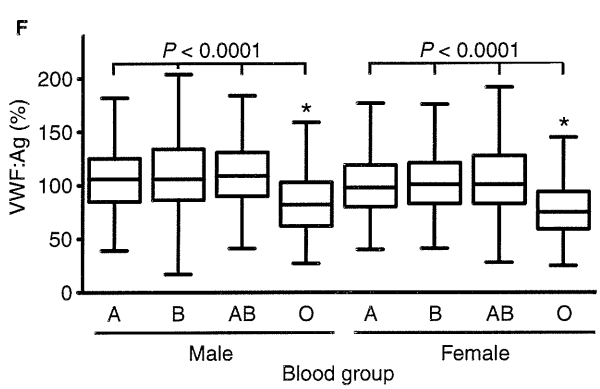






E

		Q1	Q2	Q3	Q4
Male $n = 1687$	ADAMTS13 activity (%)	38–76	76–90	90–109	109–242
	VWF:Ag (%) ± SE	107 ± 11	106 ± 11	108 ± 11	111 ± 11
		$P = 0.153$			
Female $n = 1929$	ADAMTS13 activity (%)	44–86	86–104	104–123	123–216
	VWF:Ag (%) ± SE	103 ± 9	99 ± 10	99 ± 10	101 ± 9
		$P = 0.670$			



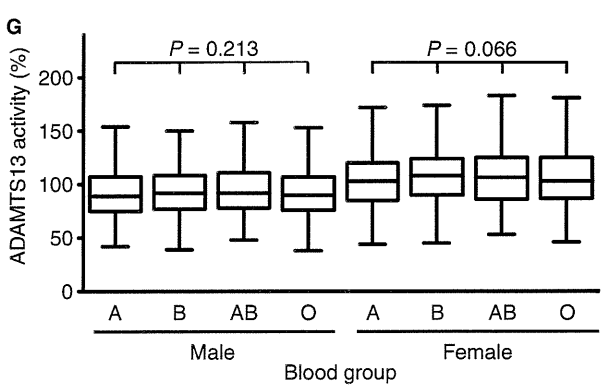


Fig. 1. Plasma ADAMTS13 activity in a Japanese general population. (A) Scatter dot plot of total, male and female plasma ADAMTS13 activity. The mean of all values was set at 100%. Lines indicate the means with SD. *P*, *t*-test. (B, C, D) Age-specific ADAMTS13 activity (B), VWF:Ag (C) and VWF:Ag-to-ADAMTS13 activity ratio (D). The mean of all VWF:Ag values was set at 100%. Error bars indicate SD. (E) Analysis of VWF:Ag by quartiles of plasma ADAMTS13 activity. *P*, ANCOVA. (F, G) Box-and-whisker plot of blood group-specific VWF:Ag (F) and ADAMTS13 activity (G). *P*, Kruskal–Wallis test. *Only blood group O had significantly different VWF:Ag from other blood groups.

(VWF:Ag) of all subjects using an immuno-turbidimetric assay, STA LIATEST VWF (Diagnostica Stago, Parsippany, NJ, USA). In both men and women, VWF:Ag increased with age (Fig. 1C), as reported previously [8]. The linear regression coefficient was 1.37 (95% CI, 1.21–1.52) in men and 1.30 (1.17–1.42) in women. Because of combined effects of the increase in VWF:Ag and the decrease in ADAMTS13 activity, the VWF:Ag-to-ADAMTS13 activity ratio was dramatically increased with age (Fig. 1D). This may partly explain the prothrombotic state of elderly men and women, because the imbalance between VWF and ADAMTS13 may be involved in thrombotic diseases such as acute myocardial infarction [9], advanced liver cirrhosis [10] and coronary artery disease [11]. As the FRETS-VWF73 assay itself was not affected by VWF concentration in plasma samples (0–160 $\mu g\ mL^{-1}$, data not shown), the reduced ADAMTS13 activity in the plasma of elderly subjects was not considered to be due to the assay-dependent artifactual phenomenon. In fact, when age-adjusted VWF:Ag was compared among quartiles of ADAMTS13 activity in the population using SPSS Statistics (IBM, Tokyo, Japan), no significant association between VWF:Ag and ADAMTS13 activity was observed in men (ANCOVA, $P = 0.153$) or in women ($P = 0.670$) (Fig. 1E).

ABO blood group has a significant influence on VWF:Ag; individuals with blood group O have lower VWF:Ag values than those of non-O groups [8]. We genotyped the ABO blood group by TaqMan assay (Applied Biosystems, Tokyo, Japan), which detects two polymorphisms, c.261Gdel and c.526C > G, on *ABO*. As expected, the subjects of blood group O exhibited a significantly lower VWF:Ag (men, 88 ± 43%; women, 80 ± 28%, mean ± SD) than those of the other blood groups (men A, 110 ± 35%; men B, 112 ± 36%; men AB, 115 ± 44%; women A, 103 ± 37%; women B, 105 ± 36%; women AB, 106 ± 31%) (Fig. 1F). In contrast, the plasma ADAMTS13 activity in men (A, 92 ± 24%; B, 94 ± 24%; AB, 96 ± 24%; O, 93 ± 25%) and women (A, 104 ± 26%; B, 109 ± 28; AB, 109 ± 28%; O, 106 ± 28) was not significantly associated with ABO blood group (Fig. 1G). This is consistent with the finding that ADAMTS13 antigen levels are not associated with ABO blood group in 387 male subjects [12]. The results are also consistent with the fact that VWF [13] but not ADAMTS13 [14] contains ABO blood group-related N-linked oligosaccharides.

In conclusion, this study demonstrated that, in the Japanese general population, the plasma ADAMTS13 activity is lower in men than in women, decreases with age, and is not significantly associated with ABO blood group. The VWF:Ag-to-ADAMTS13 activity ratio is increased with age in both men and women, and this increase may be involved in the prothrombotic state of elderly individuals.

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Disclosure of conflict of interests

The authors state that they have no conflict of interest.

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Polymorphisms and mutations of *ADAMTS13* in the Japanese population and estimation of the number of patients with Upshaw–Schulman syndrome

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Upshaw–Schulman syndrome (USS), also called hereditary thrombotic thrombocytopenic purpura, is an autosomal recessive disease characterized by thrombocytopenia and microangiopathic hemolytic anemia. USS is associated with hereditary severe deficiency of plasma ADAMTS13 activity; patients with USS have homozygous or compound heterozygous mutations in the *ADAMTS13* gene [1–5]. ADAMTS13 is a plasma metalloprotease that regulates platelet aggregation through the cleavage of von Willebrand factor (VWF) multimers. ADAMTS13-deficient plasma derived from patients with USS contains unusually large VWF multimers, which can induce unwanted hyperaggregation of platelets and microvascular thrombi. In this study, we analyzed the relationship between genetic variation of *ADAMTS13* and plasma ADAMTS13 activity in the Japanese general population. In addition, on the basis of the data obtained via our genetic analysis, we estimated the number of patients with USS in Japan.

The population examined is based on the Suita Study [6], an epidemiologic study consisting of randomly selected Japanese residents of Suita City, which is located in the second largest urban area in Japan. Our study protocol was approved by the ethical review committee of the National Cerebral and Cardiovascular Center, and only subjects who provided written informed consent for genetic analyses were included.

To identify common polymorphisms in the population, we first sequenced all 29 exons and exon–intron boundaries of *ADAMTS13* using 346 consecutive subjects, by means of previously described methods [2]. We identified 25 polymorphisms with allele frequencies of the respective minor allele > 0.01, including two in the promoter region, 10 in the exons, and 13 in the introns. Of these, six were missense single-nucleotide polymorphisms (SNPs): p.T339R (c.1016C > G), p.Q448E (c.1342C > G), p.P475S (c.1423C > T), p.P618A (c.1852C > G), p.S903L (c.2708C > T), and p.G1181R (c.3541G > A). Next, we performed TaqMan genotyping assays (Applied Biosystems, Tokyo, Japan) for the missense SNPs, using 3616 subjects whose plasma ADAMTS13 activities had been measured with the FRETS-VWF73 assay [7]. Allele frequencies for the minor alleles were 0.027 for p.T339R, 0.192 for p.Q448E, 0.050 for p.P475S, 0.027 for p.P618A, 0.048 for p.S903L, and 0.022 for p.G1181R. The observed genotypes did not deviate significantly from Hardy–Weinberg equilibrium. The p.T339R and p.P618A SNPs were in absolute linkage disequilibrium ($r^2 = 0.97$), whereas the other missense SNPs were not strongly linked ($r^2 < 0.11$).

The p.Q448E and p.P475S SNPs, but not the other missense SNPs, were significantly associated with plasma ADAMTS13 activity (Fig. 1A). The ADAMTS13 activity (97% ± 25% in men, 111% ± 28% in women, mean ± standard deviation) of p.Q448E heterozygotes (QE) and minor allele homozygotes (EE) was slightly but significantly higher than that of major allele homozygotes (QQ) (91% ± 24% in men, 104% ± 26% in women). In contrast, the ADAMTS13 activity (79% ± 20% in men, 92% ± 24% in women) of p.P475S heterozygotes (PS) and minor allele homozygotes (SS) was significantly lower than that of major allele homozygotes (PP) (94% ± 24% in men, 108% ± 27% in women). The difference in activity was consistent with the observation that the recombinant ADAMTS13-P475S mutant has approximately 70% of the activity of wild-type ADAMTS13 [8]. It is interesting that p.P618A was not associated with plasma ADAMTS13 activity in the present study, whereas the conditioned medium of HEK293 cells expressing the A618 variant showed lower levels of activity (27%) and antigen (14%) than the wild type [9]. PolyPhen-2, a program that predicts damaging missense mutations [10], identified p.T339R and p.P618A as 'possibly damaging' and 'probably damaging', respectively, whereas the other four SNPs were predicted to be 'benign'.

As the *ADAMTS13* locus is near (130–190 kb) the *ABO* locus on chromosome 9q34, we compared the frequencies of the SNPs among ABO blood group genotypes (Fig. 1B). The relative frequencies of p.T339R minor allele homozygotes and

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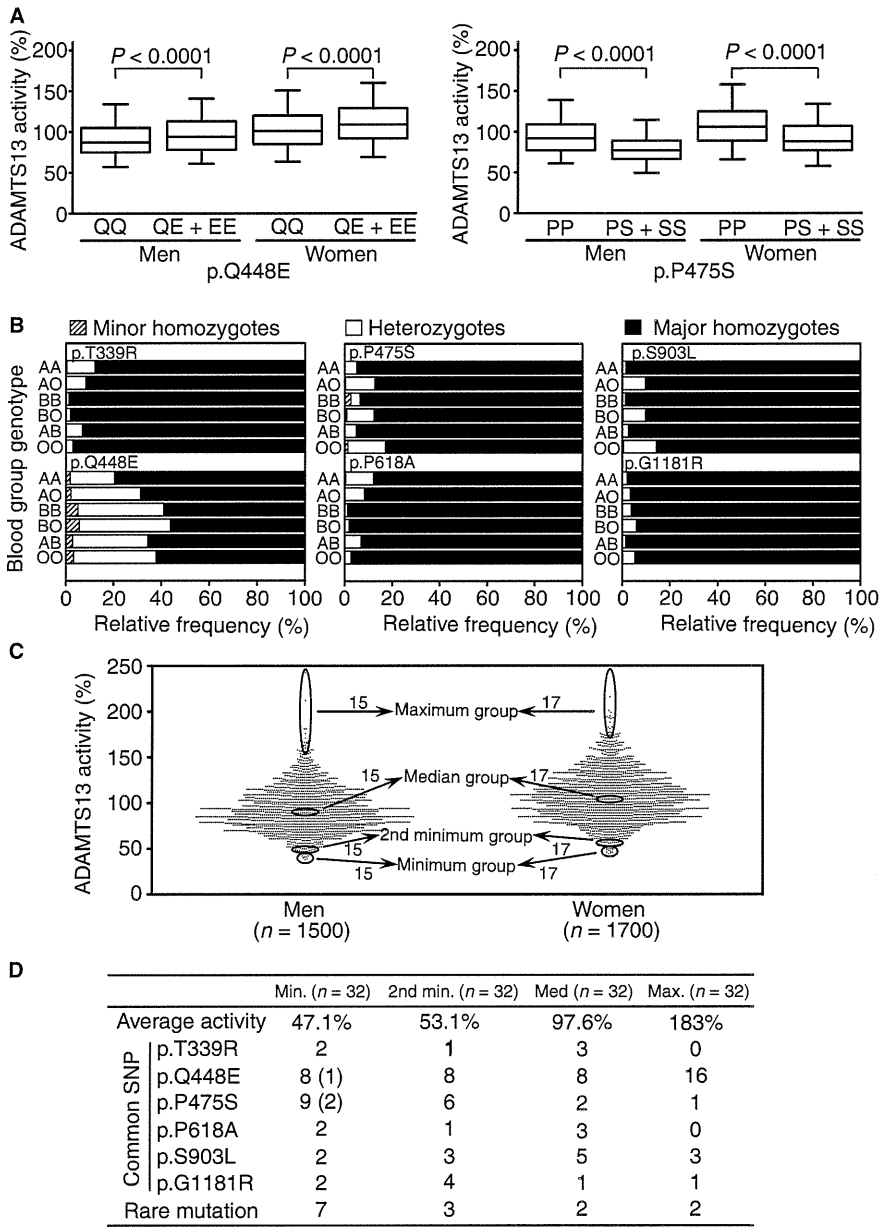

D

		Min. (n = 32)	2nd min. (n = 32)	Med (n = 32)	Max. (n = 32)
Average activity		47.1%	53.1%	97.6%	183%
Common SNP	p.T339R	2	1	3	0
	p.Q448E	8 (1)	8	8	16
	p.P475S	9 (2)	6	2	1
	p.P618A	2	1	3	0
	p.S903L	2	3	5	3
	p.G1181R	2	4	1	1
Rare mutation		7	3	2	2

Fig. 1. *ADAMTS13* variation in a Japanese general population. (A) Box-and-whisker plot (5th–95th percentiles) of plasma ADAMTS13 activity in each genotype of p.Q448E and p.P475S. *P*, Kruskal–Wallis test. (B) The relative frequency of minor homozygotes, heterozygotes and major homozygotes for each genetic polymorphism. (C) Scatter dot plot of plasma ADAMTS13 activity for men and women. On the basis of these activity measurements, 128 subjects were selected for sequencing of *ADAMTS13*. (D) The numbers of minor allele carriers in each group. One in eight p.Q448E carriers and two in nine p.P475S carriers in the minimum group were homozygotes for the respective minor alleles.

heterozygotes were higher for AA, AO and AB than for BB, BO and OO, suggesting that p.T339R is associated with the blood group A allele. The p.P618A SNP, which is tightly associated with p.T339R, exhibited the same pattern. The p.P475S and p.S903L SNPs tended to be associated with the blood group O allele.

We then utilized the plasma ADAMTS13 activity data to estimate the frequency of hereditary ADAMTS13 deficiency. In the population, 3200 DNA samples (1500 men and 1700 women) were available, from a quantitative standpoint, for sequencing of *ADAMTS13*. We selected 128 subjects according to their plasma ADAMTS13 activity (Fig. 1C): 32 subjects of the 'minimum' group (average activity, 47.1%), consisting of 15 men and 17 women with the lowest activities in each gender; 32 subjects of the 'second minimum' group (53.1%), consisting of 15 men and 17 women with the second lowest activities; 32 subjects of the 'median' group (97.6%), consisting of 15 men and 17 women with median activities; and 32 subjects of the 'maximum' group (183%), consisting of 15 men and 17 women with the highest activities. Each group corresponds to 1% of the population examined. All DNA samples from the four groups were subjected to *ADAMTS13* sequencing, which

revealed that 70 individuals had at least one of the six missense SNPs described above (Fig. 1D). Of these, only p.P475S showed a significant difference in minor allele frequency among four groups ($P = 0.028$, chi-square test). In addition, 14 individuals had rare non-synonymous mutations: seven (p.F324L, p.F418L, p.I673F, p.Q773X, p.Y1074AfsX46, p.R1095Q, and p.S1314L) in the 'minimum' group; three (p.I380T, p.Y1074AfsX46, and p.R1274C) in the 'second minimum' group; two (p.Q723K and p.N1321S) in the 'median' group; and two (p.L19F and p.R268Q) in the 'maximum' group. Of these, p.I673F (c.2017A > T) and p.Y1074AfsX46 (c.3220delTACC) had been identified as causative mutations in patients with USS [11,12]. All of the others were newly identified mutations.

To estimate the number of individuals with a hereditary ADAMTS13 deficiency, we generated several hypotheses: (i) as two individuals in each of the 'median' and 'maximum' groups had rare mutations, two of every 32 people should have a mutation that does not cause a functional defect of ADAMTS13; (ii) thus, five (= 7 – 2) individuals in the 'minimum' group and one (= 3 – 2) individual in the 'second minimum' group should be the heterozygotes carrying a mutation with a functional defect; (iii) other than these six (= 5 + 1) individuals in the 'minimum' and 'second minimum' groups, no individual should have any mutations that confer a functional defect. These hypotheses were consistent with a prediction based on PolyPhen-2: the p.S1314L, p.I380T, p.Q723K, p.N1321S, p.L19F and p.R268Q mutations are 'benign', p.I673F and p.R1274C are 'possibly damaging', and the others are 'probably damaging'. According to the hypotheses, we estimated that six of 3200 individuals were heterozygotes for ADAMTS13 deficiency. This estimation suggested that ~ 1 individual in 1.1×10^6 (= 6/3200 × 6/3200 × 1/4) should be a homozygote or a compound heterozygote for ADAMTS13 deficiency. In Japan, which has a population of approximately 1.3×10^8, ~ 110 individuals may have hereditary ADAMTS13 deficiency or USS. If we adjusted our estimate of ADAMTS13 deficiency from 6/3200 to 7/3200 or 5/3200, the number of patients would be 160 or 80, respectively. The validity of these calculation procedures was confirmed by StaGen Co., Ltd (Chiba, Japan), a company specializing in genetics, statistics, and data analysis.

In conclusion, this study demonstrated that, in the Japanese general population, there are six common missense SNPs: p.T339R, p.Q448E, p.P475S, p.P618A, p.S903L, and p.G1181R. Of these, p.Q448E and p.P475S are significantly associated with plasma ADAMTS13 activity. Allele frequencies of these SNPs correlate with ABO blood group. Finally, we estimated the number of patients with USS in Japan, yielding a figure that corresponds to approximately three times the number of patients already diagnosed as having this condition. Because of insufficient sample sizes, we may have underestimated the prevalence of USS. Further studies are needed to obtain more reliable conclusions.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Quarterly Medical Review

Paradigm shift of childhood thrombotic thrombocytopenic purpura with severe ADAMTS13 deficiency

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In this issue

Summary

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disease with pathological conditions termed thrombotic microangiopathy (TMA). TTP is thought to predominantly affect adults and to rarely occur in children. Currently, TTP is defined by a severe deficiency in the activity of ADAMTS13, a metalloprotease that specifically cleaves unusually large von Willebrand factor multimers under high shear stress. Genetic mutations in and acquired autoantibodies to ADAMTS13 cause congenital TTP (termed Upshaw-Schulman syndrome [USS]) and acquired TTP, respectively. Because of very few overt clinical signs for TTP, USS is often misdiagnosed as chronic idiopathic thrombocytopenic purpura or overlooked during childhood. However, in women with USS, pregnancy can induce thrombocytopenia followed by the development of TTP. Furthermore, early childhood cases of acquired idiopathic TTP have not been characterized. From 1998 to 2008, our institution at Nara Medical University functioned as a TMA referral center in Japan and collected a large dataset on 919 TMA patients (Intern Med 2010;49:7–15). This registry contains 324 patients with a severe deficiency in ADAMTS13 activity, including 41 patients with USS and 283 patients with acquired TTP. Of note, the latter population contains 17 patients who were enrolled as children (≤ 15 years old), including 14 children with idiopathic TTP and three with connective tissue disease-associated TTP. Of the 14 patients with idiopathic TTP, five were very young children (under 2 years old). This study focused on these 58 patients (41 USS and 17 acquired TTP) who were diagnosed with a severe deficiency in ADAMTS13 activity during childhood, causing a paradigm shift in our concept of TTP.

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Thrombotic microangiopathies (TMAs) are pathological conditions that are characterized by organ dysfunction due to platelet thrombi in the microvasculature, consumptive thrombocytopenia, and microangiopathic hemolytic anemia (MAHA). Two of the typical TMA phenotypes are life-threatening generalized diseases, termed thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) [1–4].

A disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13 (ADAMTS13) is a metalloprotease that specifically cleaves the Tyr1605–Met1606 bond in the von Willebrand factor (VWF)-A2 domain [5]. In the absence of ADAMTS13 activity (ADAMTS13:AC), unusually large VWF multimers (UL-VWFMs) are released from vascular endothelial cells (ECs) and improperly cleaved, causing them to accumulate in the circulation and induce the formation of platelet thrombi in the microvasculature under conditions of high shear stress. Currently, a severe deficiency in ADAMTS13:AC, which results either from genetic mutations in the *ADAMTS13* gene or acquired autoantibodies to ADAMTS13, is thought to be a specific feature of TTP but not HUS [6,7].

TTP was first described in 1924 by Moschcowitz [8], who documented a 16-year-old female who died of multiorgan failure after a clinical disease course of 1 week. An autopsy revealed hyaline membrane thrombi in the small arteries of multiple organs, except for the lung. In 1966, Amorosi and Ultmann [9] examined 16 new patients and reviewed 255 previously documented patients in order to establish a clinical 'pentad', consisting of MAHA, thrombocytopenia, renal failure, fluctuating neurological signs, and fever. Since then, TTP has been considered a life-threatening but rare disease that occurs mainly in adults and presents with predominant neurotropic clinical signs. Because of this classification, the estimated frequency of TTP was low (3.7 per million) [10] before the discovery of ADAMTS13.

On the other hand, in 1955 Gasser et al. [11] described five children who died of acute renal insufficiency, and their autopsies showed prominent necrosis of the renal cortex. This study established the clinical 'triad' for HUS, which consisted of MAHA, thrombocytopenia, and renal insufficiency. In addition, after it was determined that there was a close relationship between HUS and enterohemorrhagic *Escherichia coli* infection, particularly strain O157:H7 that produces a Shiga-like toxin, studies showed that HUS typically affects children with prominent nephrotropic clinical signs [12].

From 1998 to 2008, our institution at Nara Medical University has functioned as a TMA referral center in Japan and collected a large dataset of 919 patients who have TMA but not disseminated intravascular coagulation (DIC) [13]. This registry contains 324 patients with a severe ADAMTS13:AC deficiency (less than 3% of normal), including 41 patients with congenital TTP (Upshaw-Schulman syndrome [USS]) with variable clinical symptoms and 283 patients with acquired TTP. Notably, the latter population includes 17 patients who were diagnosed with TTP as children (≤ 15 years old), including 14 with idiopathic TTP and three with connective tissue disease (CTD)-associated TTP. Surprisingly, the 14 patients with idiopathic TTP included five patients who were very young infants (under 2 years old), which significantly differed from the previous concept of TTP. Therefore, the aim of this study was to characterize these 58 patients (41 USS and 17 childhood TTP) in order to examine the paradigm shift in our understanding of TTP.

Glossary

ADAMTS13	a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13
ADAMTS13:AC	ADAMTS13 activity
ADAMTS13:INH	ADAMTS13 inhibitor
ai-TTP	acquired idiopathic TTP
BU	Bethesda unit
CR-TTP	chronic relapsing TTP
CTD	connective tissue disease
DIC	disseminated intravascular coagulation
EC	endothelial cell
FFP	fresh frozen plasma
HPS	hemophagocytic syndrome
HUS	hemolytic uremic syndrome
ITP	idiopathic thrombocytopenic purpura
MAHA	microangiopathic hemolytic anemia
PE	plasma exchange
PNH	paroxysmal nocturnal hemoglobinuria
SNP	single nucleotide polymorphism
TMA	thrombotic microangiopathy
TTP	thrombotic thrombocytopenic purpura
UL-VWFM	unusually large VWF multimer
USS	Upshaw-Schulman syndrome
VWF	von Willebrand factor
VWF-CP	VWF-cleaving protease
WPBs	Weibel-Palade bodies

Diagnostic criteria for thrombotic microangiopathy and thrombotic thrombocytopenic purpura

As previously described [13], patients were considered to have TMA if they met all of the following criteria:

- MAHA (hemoglobin [Hb] ≤ 12 g/dL), Coombs test negative, undetectable serum haptoglobin (< 10 mg/dL), more than two fragmented red blood cells (RBC) (schistocytes) in a microscopic field with a magnification of 100, and concurrent increased serum lactate dehydrogenase (LDH) above the institutional baseline;
- thrombocytopenia (platelet count ≤ 100×10^9/L);
- a variable degree of organ dysfunction (renal or neurological involvement) without DIC [14,15].

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It is difficult to differentially diagnose HUS and TTP based on routine laboratory data. Therefore, as a rule, the plasma levels of ADAMTS13:AC were determined for all patients who were suspected to have TMA, and patients with a severe ADAMTS13:AC deficiency were classified as having TTP regardless of the clinical signs. This protocol was important because our registry included patients with congenital TTP or an ADAMTS13:AC deficiency (USS), which generally have fewer clinical signs, often isolated thrombocytopenia, than patients with acquired TTP.

Within the large dataset of 324 patients with a severe ADAMTS13:AC deficiency who were enrolled in our registry between 1998–2008 [10], 58 patients were diagnosed with a severe ADAMTS13:AC deficiency during childhood, of which 41 had congenital TTP (USS) and 17 were diagnosed with acquired TTP, including 14 with idiopathic TTP and three with CTD-associated TTP.

Assays for plasma ADMTS13:AC and ADAMTS13:INH

Until March 2005, ADAMTS13:AC was determined with a classic VWFM assay in the presence of 1.5 mol/L urea using purified plasma-derived VWF as a substrate according to the method described by Furlan et al. [16]. In our laboratory, the detection limit of this assay was 3% of the normal control [17].

In 2005, our laboratory developed a novel chromogenic ADAMTS13-act-ELISA using both an N- and C-terminal tagged recombinant VWF substrate (termed GST-VWF73-His). This assay was highly sensitive, and the detection limit was 0.5% of the normal control [18]. Since 2005, the classic VWFM assay was completely replaced with this novel chromogenic act-ELISA. Both assays show a high correlation between the plasma ADAMTS13:AC levels ($R^2 = 0.72$, $P < 0.01$) with similar means ± SD in healthy individuals (102.4 ± 23.0% vs. 99.1 ± 21.5%), as was shown previously [18]. Thus, the results obtained using the chromogenic act-ELISA were used in this study. In addition, we have categorized plasma ADAMTS13:AC levels of < 3%, 3∼ < 25%, and 25∼50% of the normal control as a severe, moderate, and mild deficiency, respectively.

Since 2005, ADAMTS13:INH has also been evaluated with the chromogenic act-ELISA by means of the Bethesda method [19]. Prior to this inhibition assay, the tested samples were heat-treated at 56 °C for 60 min to eliminate endogenous enzymatic activity. The ADAMTS13:INH assay consists of two steps. In the 1st step, the test or control plasma is heat-inactivated, mixed with an equal volume of intact normal pooled plasma, and incubated for 2 hours at 37 °C. After the incubation, the residual enzyme activity is measured. One Bethesda unit is defined as the amount of inhibitor that reduces the enzymatic activity by 50% of the control value, and values greater than 0.5 U/mL are considered significant.

Pathogenesis of thrombotic thrombocytopenic purpura

ADAMTS13-producing cells

ADAMTS13 is a metalloproteinase that consists of 1427 amino acids and a multi-domain structure, including a signal peptide, short propeptide, metalloproteinase domain, disintegrin-like domain, thrombospondin-1 (TSP1) domain, cysteine-rich domain, spacer domain, seven additional TSP1 repeats, and two CUB domains [20]. The *ADAMTS13* gene is located on chromosome 9q34, and initial northern blotting studies indicated that ADAMTS13 mRNA is exclusively expressed in the liver [20]. Subsequent immunological studies with *in situ* hybridization analyses indicated that ADAMTS13 is unambiguously produced in hepatic stellate cells (Itoh cells) [21]. However, ADAMTS13 was also identified in platelets [22], vascular ECs [23], and kidney podocytes [24]. Therefore, an outstanding and important question is which organ is most responsible for maintaining the plasma levels of ADAMTS13:AC. In this regard, we have two observations that suggest that the liver is the major ADAMTS13-producing organ. Childhood patients with advanced billiary cirrhosis due to bile duct atresia often showed pathological features of TMA with low plasma levels of ADAMTS13:AC (20–30%), but these clinical signs disappeared and plasma ADAMTS13:AC rapidly recovered to normal levels after a successful liver transplantation [25]. Adulthood patients with cirrhosis that was largely related to hepatitis C infection tended to have lower plasma ADAMTS13:AC levels that correlated with their clinical severity, and the lowest values were approximately 20–30% of the normal levels [26].

Cleavage of unusually large von Willebrand factor multimer

Although the mechanism by which TTP develops in the absence of ADAMTS13:AC has not been fully elucidated, accumulating evidence has provided a hypothesis as illustrated in *figure 1* [27]. In this proposed model, UL-VWFMs are produced exclusively in vascular ECs and stored in an intracellular organelle termed Weibel-Palade bodies (WPBs) and then released into the circulation upon stimulation. Under physiological conditions, epinephrine acts as an endogenous stimulus but other stimuli are largely unknown. In contrast, under non-physiological conditions, DDAVP (1-deamino-8-D-arginine vasopressin), hypoxia, and several cytokines such as interleukin (IL)-2, IL-6, IL-8, and tissue necrotizing factor (TNF)-α act as stimuli that up-regulate VWF release. Once ECs are stimulated, UL-VWFMs and P-selectin, both stored in WPBs, move to the membrane surface of ECs, where P-selectin anchors UL-VWFMs on the EC surface [28]. Under these circumstances, high shear stress generated in the microvasculature induces a change in the UL-VWFM molecule that alters its conformation from a

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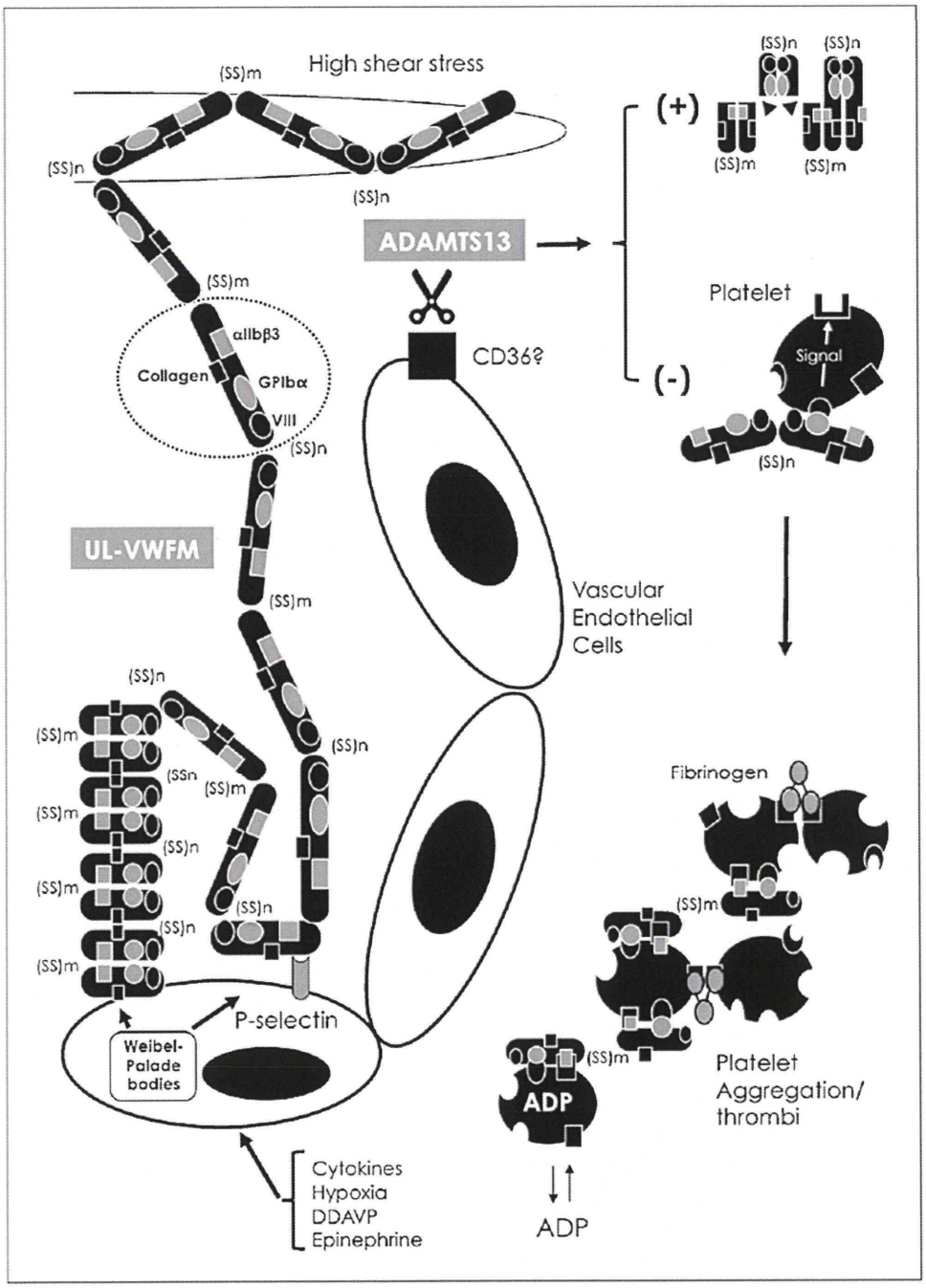

FIGURE 1

Proposed mechanism of platelet thrombi under high shear stress in the absence of ADAMTS13:AC

Unusually large von Willebrand factor multimers (UL-VWFMs) are produced in vascular endothelial cells (ECs) and stored in Weibel-Palade bodies (WPBs). UL-VWFMs are released from WPBs into the circulation upon stimulation by cytokines, hypoxia, DDAVP, and epinephrine. P-selectin that co-migrates from WPBs anchors UL-VWFMs on the vascular EC surface. Under these circumstances, high shear stress changes the molecular conformation of UL-VWFM from a globular to an extended form, allowing ADAMTS13 to access this molecule. In the absence of ADAMTS13:AC, UL-VWFMs are left uncleaved, allowing them to excessively interact with platelet glycoprotein (GP) Ibα and activate platelets via intraplatelet signaling, which results in the formation of platelet thrombi (dotted circle indicates a VWF subunit, which contains a set of binding domains with factor VIII, subendothelial collagen, platelet GPIbα, and integrin αIIbβ3).

globular to an extended form, allowing ADAMTS13 to cleave UL-VWFM. In this context, it has been postulated that multiple exocites within the disintegrin-like/TSP1/cysteine-rich/spacer (DTCS) domains of ADAMTS13 play an important role in interacting with the unfolded VWF-A2 domain [29]. Furthermore, although a direct link to TTP pathogenesis had not been shown, in 1994 Tandon et al. [30] reported that approximately 80% of patients with acquired TTP had autoantibodies to CD36. Recently, Davis et al. [31] showed that recombinant (r) ADAMTS13 specifically binds to rCD36 *in vitro*. Thus, it is possible that ADAMTS13 more efficiently cleaves newly released UL-VWFMs that exist as solid-phase enzymes

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anchored to the vascular EC surface by binding to CD36 because CD36 is a receptor for TSP1, which is a repeated domain within the ADAMTS13 molecule.

In 2001, we clearly showed that pre-existing UL-VWFMs in the plasma of USS patients began to disappear within 1 hour and completely disappeared 24 hours after ADAMTS13 was replenished with infusions of fresh frozen plasma (FFP) as shown in *figure 2* [32]. Retrospectively, these results indicated that exogenous ADAMTS13 could efficiently cleave both UL-VWFMs that pre-existed in the circulation and the newly produced molecules at the EC surface. Related to this phenomenon, Zhang et al. [33] recently analyzed the crystal structure of the VWF-A2 domain and found that the ADAMTS13 cleavage site within this domain is not exposed to the outer surface of the molecule, indicating that the enzyme cannot readily access this site. More recently, Zanardelli et al. [34] proposed that the '2-site initial interaction mechanism between VWF and ADAMTS13', in which a binding site in the VWF C-terminal domains (D4CK) is constitutively exposed, allows this domain to interact with the ADAMTS13 C-terminal domains [TSP1(5-8)/CUB]. Under high shear stress, the '2-site initial interaction' may help expose this binding site within the VWF-A2 domain and favor the correct positioning of the ADAMTS13 spacer domain. Once the higher-affinity interaction between the spacer domain and the VWF-A2 domain is achieved, the metalloproteinase domain of ADAMTS13 can access and cleave the Tyr1605–Met1606 bond within the VWF-A2 domain.

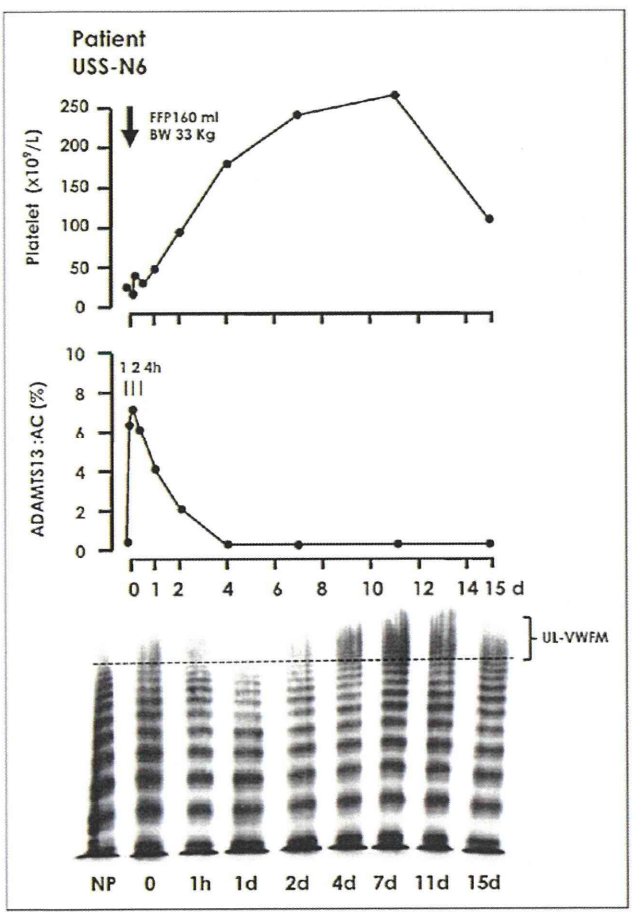

FIGURE 2

Effect of fresh frozen plasma (FFP) infusion on platelet counts, ADAMTS13:AC, and VWFM patterns in patient USS-N6

A total of 160 mL of FFP was transfused into female patient USS-N6 (BW 33 kg). As shown in the top panel, her platelet counts increased from 23 × 10^9/L before the FFP infusion to 251 × 10^9/L at 11 days after the infusion. The middle panel shows the plasma levels of ADAMTS13:AC that were re-examined by the chromogenic act-ELISA using deep-frozen plasma samples. Note that 4 days after the infusion, the plasma ADAMTS13:AC decreased to the pre-infusion level (< 0.5%). In the lower panel, the pre-existing UL-VWFM levels before the FFP infusion rapidly disappeared 24 hours after infusion, and 4 days later, UL-VWFMs re-appeared in the plasma. It should be noted that the platelet count began to decrease concomitantly with the re-appearance of UL-VWFMs (cited from [32] with a slight modification).

Anti-ADAMTS13 autoantibodies

Soejima et al. [35] were the first to report that the cysteine-rich and spacer domains of ADAMTS13 are a major binding site for ADAMTS13 autoantibodies in acquired TTP. Subsequently, Klaus et al. [36] showed that there are multiple antibody binding sites within the ADAMTS13 molecule. Now it is accepted that anti-ADAMTS13 neutralizing autoantibodies target epitopes within the spacer domain [37]. More recently, Pos et al. [38] identified three amino acids, Arg660, Tyr661, and Tyr665, within the spacer domain of ADAMTS13 that are critical for the binding of both the VWF-A2 domain and anti-ADAMTS13 autoantibodies.

Upshaw-Schulman syndrome (congenital TTP/deficiency in ADAMTS13:AC)

Background

The classic hallmarks of USS are repeated childhood episodes of chronic thrombocytopenia and MAHA that are reversed by infusing fresh frozen plasma (FFP). The most striking clinical feature is severe neonatal jaundice with a negative Coombs test that requires exchange blood transfusion therapy. Although USS is now defined as a congenital ADAMTS13:AC deficiency due to genetic mutations, there was a lengthy history that led to this conclusion, as has been described in detail in previous publications [39]. In fact, the term USS had almost been embedded in 1997, when the assay for VWF-cleaving protease (VWF-CP) activity (now ADAMTS13;AC) was established. This is because the pathogenic features that were initially postulated for the disease, such as a defect in 'platelet-stimulating factor', 'decreased plasma fibronectin

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level', or 'lack of thrombopoietin', have been entirely excluded by subsequent investigations. Instead, the practical diagnostic term 'chronic relapsing TTP' (CR-TTP) has long been used. This term was coined by Moake et al. [40], who found that UL-VWFMs were present in the plasma of 4 CR-TTP patients during the remission phase, but disappeared during the acute phase. In 1997, Furlan et al. [41] showed that four CR-TTP patients, different from those of Moake et al. [40], lacked VWF-CP activity, but did not address ADAMTS13:INH. Retrospectively, however, each two CR-TTP patients, reported by Moake et al. [40] and Furlan et al. [41], were congenital TTP, and the remaining two each were acquired TTP. Under these circumstances, we re-visited the term USS [17], which included analyzing three Japanese patients with USS, and found that they uniformly had a severe deficiency in VWF-CP activity (determined by VWFM assay in the presence of 1.5 mol/L urea) in the absence of its inhibitors. The parents of these patients were asymptomatic with a moderately decreased activity (17–60% of normal), except for one carrier who had very low VWF-CP activity (5.6% of normal). Later, this carrier was shown to have a unique single nucleotide polymorphism (SNP), a P475S mutation in the *ADAMTS13* gene in one allele, which is very common in Japanese people (9.6% of normal individuals are heterozygous for the P475S mutation) [42]. However, Levy et al. [43] provided solid evidence that linked congenital TTP or USS and *ADAMTS13* gene mutations. Since this discovery, approximately 100 patients have been identified worldwide [44], but the precise incidence is completely unknown because USS is an extremely rare disease.

ADAMTS13 gene knock-out humans and mice

Although USS patients consistently lack ADAMTS13:AC, they do not always have acute symptoms, and symptoms often become evident only when the patients have infections or become pregnant. In both instances, vascular EC injuries might be involved, and these cases have been indirectly shown to have elevated plasma levels of cytokines or soluble thrombomodulin [45]. However, studies on *ADAMTS13* gene knock-out mice [46,47] showed that UL-VWFMs were detectable in the blood, although the mice did not have acute symptoms. Considering these results, investigators have assumed that an ADAMTS13:AC deficiency is prothrombotic but alone is insufficient to provoke acute symptoms. Therefore, second hits or triggers must exist. However, the lack of symptoms in knock-out mice sharply contrasts the clinical symptoms of USS. For example, USS patients, but not mice, were reported to have acute clinical aggravation soon after receiving infusions of DDAVP [48,49]. However, it is still controversial whether mice have a receptor to DDAVP. Furthermore, there are striking differences between humans and mice during pregnancy. In our studies, nine USS females had a history of pregnancy and all had thrombocytopenia during the 2nd–3rd trimesters. When this thrombocytopenia was not well managed, they developed clinical signs of TTP and the fetus died in many cases [50]. However, this disease course was not found in knock-out mice.

Natural history of 41 Upshaw-Schulman syndrome patients in Japan

USS is inherited in an autosomal recessive fashion, indicating that the female-to-male ratio in the patient population should be one-to-one. However, in our registry of 41 USS patients from 36 families (Table I), the female-to-male ratio was 25-to-16 with an apparent female predominance. Furthermore, all patients had a severe ADAMTS13:AC deficiency (under 3% of normal), except for one USS-GG2 patient (ADAMTS13:AC 2.4–3.4%).

Although severe neonatal jaundice is a typical sign of early-onset bouts of USS, our analysis indicates that such cases represent a relatively small number (16/41, 39%) of patients. Thirty-two patients (32/41, 78%) had repeated episodes of thrombocytopenia during childhood, but many USS patients were primarily misdiagnosed with idiopathic thrombocytopenic purpura (ITP) or Evans syndrome. Therefore, the age at which these patients were diagnosed with CR-TTP or USS was widely distributed from 1 month to 63 years. Sixteen patients (16/41, 39%) were diagnosed with TTP beyond childhood.

Of particular interest, pregnant women with USS inevitably have thrombocytopenia during the 2nd–3rd trimester when the plasma VWF levels rapidly increase with the appearance of UL-VWFM. *Figure 3* presents data for two female patients who were siblings in an USS-L family and were diagnosed based on their precise natural history around pregnancy followed by an examination of ADAMTS13:AC and ADAMTS13:INH [50]. Furthermore, an *ADAMTS13* gene analysis gave a solid diagnosis of USS. As illustrated in these two cases, USS is thought to have two clinical phenotypes, the early-onset and late-onset types. However, generally we cannot find any clear differences in the plasma ADAMTS13:AC levels between these two phenotypes, even when examined by the sensitive act-ELISA.

Regarding severe renal complications in USS patients, we know that two patients thus far have received hemodialysis for chronic renal insufficiency. One patient, USS-C3 (male, born in 1972), was diagnosed with USS at 8 years of age, and then given prophylactic FFP infusions that were continued for the rest of his life. However, his renal function deteriorated yearly and he began to receive hemodialysis when he was 24 years old. During the clinical course of his disease, he experienced chronic heart failure and died of a sudden cardiac attack at 38 years of age. The other patient, USS-JJ3 (male, born in 1980), was diagnosed with USS at 16 years of age, after which he received prophylactic FFP infusions. However, his renal function deteriorated and he has been receiving hemodialysis since he was 26 years old.

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TABLE I

Registration of 41 Japanese patients with Upshaw-Schulman syndrome (USS)

No	Patient	Year of birth	Sex	Exchange blood transfusion during newborn period	Thrombocytopenia during childhood	ADAMTS13:AC (%)	Disease-causing ADAMTS13 gene mutations		Age of TTP diagnosis	Prophylactic FFP infusion		Remarks	Ref.
											From when		
1	A4	1999	M	+	+	< 0.5	C-Hetero	p.R268P/ p.C508Y	4 m	+	4 m		[53]
2	B3	1986	F	+	+	< 0.5	Homo	p.Q449X	2 m	+	11 m		[53]
3	C3	1972	M	–	+	< 0.5	Homo	c.414+1G>A	8 y	+	8 y	Dead (chronic heart failure at the age of 36)	[54]
4	D4	1978	F	+	+	< 0.5	C-Hetero	c.414+1G>A/ p.I673F	4 y	+	4 y		[54]
5	E4	1985	M	+	+	< 0.5	C-Hetero	p.I673F/ p.C908Y	5 y	–	–		[54]
6	F3	1993	M	+	+	0.6	C-Hetero	p.R193W/ p.1244+2 T>G	2.5 y	–	–		[54]
7	G3	1987	F	+	+	< 0.5	C-Hetero	c.686+1G>A/ p.R1123C	14 y	–	–		[54]
8	H3	1951	M	–	–	0.6	C-Hetero	p.A250V/ c.330+1G>A	51 y	+	50 y	Dead (renal failure at the age of 51)	[51]
9	I4	1972	M	–	+	< 0.5	C-Hetero	p.H234Q/ p.R1206X	2 y	+	2 y		[55]
10	J3	1977	F	–	+	< 0.5–0.8	C-Hetero	p.R312C/ c.3198del CT	3 y	+	22 y		[56]
11	J4	1979	M	–	+	< 0.5	C-Hetero	p.R312C/ c.3198del CT	5 y	–	–		[56]
12	K3	1976	F	–	+	< 0.5–0.7	C-Hetero	p.Y304C/ p.G525D	27 y	+	27 y		[50]
13	K4	1978	F	+	+	< 0.5	C-Hetero	p.Y304C/ p.G525D	25 y	+	25 y		[50]
14	L2	1967	F	–	–	< 0.5	C-Hetero	p.R125VfsX6/ p.Q1302X	25 y	–	–		[50]
15	L3	1972	F	–	+	< 0.5	C-Hetero	p.R125VfsX6/ p.Q1302X	25 y	–	–		[50]