Family USS-EE

Patient

One male (USS-EE4) born in 2003.

Brief clinical data

USS-EE4 (*ADAMTS13* genotype: **c.2259delA/c.2259delA**) was born as the second child of bi-ovular twins by a caesarean delivery at 37 weeks of gestation to consanguineous parents (second cousins). Soon after birth, USS-EE4 received an exchange blood transfusion under a diagnosis of DIC. However, the other twin did not have these complications. Since then, USS-EE4 has continued to experience mild thrombocytopenia. At 18 months of age, his platelet count dropped to 11×10^9 L^{-1}, and schistocytes appeared on a blood film when the patient had a rotavirus infection. The patient subsequently experienced repeated episodes of thrombocytopenia and haemolytic anaemia associated with a variety of infectious diseases. At the age of 4 years and 7 months, the patient was admitted to a nearby hospital because of exacerbated asthmatoid bronchitis together with severe thrombocytopenia (4×10^9 L^{-1}). After being diagnosed with ITP, the patient was administered high-dose immunoglobulin therapy with steroid therapy, but there was no clinical improvement (written information from Dr Masahiro Migita). ADAMTS13 analysis showed severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis in USS-EE4 identified a homozygous mutation of **c.2259delA** (exon 19). This patient did not receive prophylactic FFP infusions.

Family USS-FF

Patient

One female (USS-FF3) born in 1991.

Brief clinical data

USS-FF3 (*ADAMTS13* genotype: **p.Q449X/p.Q449X**) was born as the first of two children to non-consanguineous parents [48]. As a newborn, the patient had moderate jaundice that required phototherapy, but no exchange blood transfusion was required. She also had a history of chronic thrombocytopenia as a newborn, but did not receive specific treatment. At 6 years of age, she developed severe thrombocytopenia and haemolytic anaemia, and ADAMTS13 analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis was performed at the laboratory of Dr David Ginsburg, where a homozygous mutation of **p.Q449X** (exon 6) was identified ([40] and written communication with Dr Yoji Sasahara). Since the USS diagnosis was confirmed, the patient has received FFP infusions (5 mL kg^{-1}) every 2 weeks.

Family USS-GG

Patient

One male (USS-GG2) born in 1931.

Brief clinical data

USS-GG2 (*ADAMTS13* genotype: **p.C1024R/p.C1024R**) was born as the fifth of seven children to consanguineous parents (first cousins). The ancestors of this family can be traced back to Kochi on Shikoku Island. The first two siblings died of an unknown aetiology during childhood. Interestingly, USS-GG2 suddenly developed overt TTP with neurological signs at 63 years of age and was admitted to a nearby hospital. Before this, he had never had an episode of anaemia or thrombocytopenia. He was treated with plasma infusions because plasma exchange was not readily available at that hospital. The next day, his neurological signs dramatically improved. He subsequently has experienced repeated episodes of overt TTP, resulting in a clinical diagnosis of CR-TTP, which was treated with biweekly prophylactic FFP infusions (320–480 mL per each). However, at 77 years of age, he had cerebellar bleeding. Thus, he received an ADAMTS13 analysis that showed a significant reduction in ADAMTS13 activity (2.4–3.4% of normal on three different occasions) but no ADAMTS13 inhibitors. An *ADAMTS13* gene analysis revealed that he was a **p.C1024R/p.C1024R** (exon 24) homozygote, confirming the USS diagnosis. Under prophylactic FFP infusions, he was alive until 79 years old, but he suddenly died of stroke in 2011 at the age of 79 (communication with Dr Fumihiro Taguchi, details will be published elsewhere by the physician in charge).

Family USS-HH

Patient

One female (USS-HH4) born in 2003.

Brief clinical data

USS-HH4 (*ADAMTS13* genotype: **p.Q449X/c.4119delG**) was born as the second of two children to non-consanguineous parents. Soon after birth, she developed Coombs-negative haemolytic anaemia that was treated with an exchange blood transfusion. In 2005, she had three episodes of thrombocytopenia and haemolytic anaemia that occurred concomitantly with fever or the chicken pox. Therefore, her ADAMTS13 activity was assayed, and she was determined to have a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis revealed that she was a compound heterozygote with **p.Q449X** (exon 12) from her father and **c.4119delG** (exon 29) from her mother. Although she had a history of severe neonatal jaundice followed by an exchange blood transfusion, she subsequently has only had mild clinical signs and has not received prophylactic FFP infusions. She receives FFP infusions only when her platelet count severely drops.

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Family USS-II

Patient

One female (USS-II3) born in 1977.

Brief clinical data

USS-II3 (*ADAMTS13* genotype: not performed) was born by a caesarean section as the fourth and final pregnancy of her mother at 40 weeks of gestation. Her parents were non-consanguineous. Her mother had previously had two abortions (5 and 3 months of gestation) and a stillbirth (9 months of gestation) before USS-II3 was born. On the second day after birth, USS-II3 was treated with an exchange blood transfusion because of severe jaundice and thrombocytopenia. One month later, the patient was discharged but the thrombocytopenia continued, suggesting ITP. Since then, she has received whole blood transfusions when her platelet counts have dropped to 10×10^3 L^{-1}. At 9 months of age, the patient was clinically diagnosed with TTP. She was administered FFP infusion when severe thrombocytopenia developed. At 10 years of age, she underwent a splenectomy but there was no clinical improvement. The prophylactic FFP infusions have continued. At 21 years of age, she was diagnosed with USS after it was determined that she had a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. She currently receives prophylactic FFP infusions (120 mL) every week.

Family USS-JJ

Patient

One male (USS-JJ3) born in 1980.

Brief clinical data

USS-JJ3 (*ADAMTS13* genotype: **c.1885delT**/**p.C908Y**) was born as the last of four children to non-consanguineous parents. He had no history of exchange blood transfusions as a newborn. At 2 years of age, he suddenly complained of abdominal pain and developed haemolytic anaemia, haematuria, and thrombocytopenia. On this occasion, he was diagnosed with acute renal insufficiency due to diarrhoea-negative atypical HUS at a nearby hospital. Under this diagnosis, he received conservative therapy, including heparin, anti-platelet drugs, and red blood cell transfusion, but no platelet or FFP infusions. Over the next 14 years, he occasionally experienced overt HUS. At 12 years of age, his physician noticed that the FFP infusions were highly effective and improved his clinical manifestations, suggesting a clinical diagnosis of CR-TTP. Since 1996, he has received FFP infusions (160–240 mL per each) when his platelet counts have dropped below 100×10^9 L^{-1}, and has been administered FFP infusions of greater volumes (320–480 mL) during instances of overt TTP. In 1998, he was diagnosed with USS after an ADAMTS13 analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. Furthermore, he was a heavy drinker, which increased the frequency of overt TTP. Under these unhealthy conditions, the prophylactic FFP infusions were sometimes interrupted. Thus, when he was 25 years old, he experienced a cerebral infarction and the prophylactic FFP infusions were re-started. Nevertheless, 1 year later, he had severe renal insufficiency that required haemodialysis. Thus, he currently receives maintenance dialysis therapy and prophylactic FFP infusions of 240 mL per week. In 2009, an ADAMTS13 activity analysis revealed a severe deficiency in ADAMTS13 activity but no ADAMTS13 inhibitors. *ADAMTS13* gene analysis revealed that he was a compound heterozygote with **c.1885delT** (exon 16) and **p.C908Y** (exon 21), but that was not performed to his parents.

Family USS-KK

Patient

One female (KK3) born in 1976.

Brief clinical data

USS-KK3 (*ADAMTS13* genotype: not performed) was born as the second of three children to non-consanguineous parents. She had no history of exchange blood transfusion during the newborn period. At the age of 2, she developed thrombocytopenia and was diagnosed of ITP. She received a steroid therapy for thrombocytopenia at the age of 17 but without improvement, and then received splenectomy. As a university student at the age of 20, she developed thrombocytopenia and haemolytic anaemia after heavily drinking alcohol, and on this occasion she was clinically diagnosed of TTP at Shinshu university hospital in Nagano. A diagnosis of CR-TTP was made by Dr Miha Furlan at University of Bern in 1998, after ADAMTS13 analysis, which showed a severe deficiency of the activity but without its inhibitors (these results were re-confirmed in March 2011 using chromogenic act-ELISA). Her mother and two siblings had a slightly decreased ADAMTS13 activity (25–50%) (communication with Drs Fumihiro Ishida and Hikaru Kobayashi). Now, the patient receives the prophylactic FFP infusions (240 mL per each) every 3 weeks. *ADAMTS13* gene analysis has not been performed.

Family USS-LL

Patient

One female (LL4) born in 1981.

Brief clinical data

USS-LL4 (*ADAMTS13* genotype: **p.C438S**/p.T339R-p.Q448E-p.P618A-**p.G909R**) was born as the last of two children to non-consanguineous parents. She had no history of exchange blood transfusion during the newborn period. At the age of 14, she was diagnosed of HUS of unknown aetiology,

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and received haemodialysis. During 1996–2001, she repeated overt TTP when she had various infectious diseases, and in each occasion she was treated with FFP infusions. In 2002, she was diagnosed of USS after analysing ADAMTS13, showing a severe deficiency of the activity but without its inhibitors in our laboratory. Since then, however, a low-titer ADAMTS13 inhibitor (< 1.4 BU mL^{-1}) was detected on a few occasions, but its clinical significance was not well evaluated. Her parents and elder sister are asymptomatic and have a slightly decreased ADAMTS13 activity (27–57%). The *ADAMTS13* gene analysis in this patient revealed a compound heterozygote of **p.C438S** (exon 12) from her father and **p.G909R** (exon 21) from her mother. She had been treated with FFP infusions on demand. Most recently, she has become pregnant, and her inhibitor titers have remained below 0.5 BU mL^{-1}. Thus, the prophylactic FFP infusions (10 mL kg BW^{-1}) have been started biweekly, and so far no increase of ADAMTS13 inhibitor titer has been observed (communication with Dr Yoshiyuki Ogawa).

Characterisation and allelic numbers of ADAMTS13 gene mutations in Japanese patients with USS

Of our 43 USS-patients, 39 received an *ADAMTS13* gene analysis while it was not performed in four patients (USS-S3, AA3, II3 and KK3). Nine of these 39 USS-patients were homozygous for *ADAMTS13* gene mutations, and 29 were the compound heterozygotes, including one patient (USS-W4) with **p.G550R** mutation on one allele while DCM on the other allele was unidentified. In the remaining patient (USS-X5), two SNPs (p.P475S/p.G1181R) but no DCMs were identified on each allele. Of these 39 USS-patients, five were siblings that each belonged to different families. Thus, the 65 [$2 \times (39 - 5) - 3$] allelic numbers of DCMs in these patients are summarised in Table 3. Interestingly, these mutations are quite different from those reported in the US and Western countries [3,49–66], except for **p.R268P**. However, the **p.R349C** mutation was previously reported in a Chinese USS patient in Hong Kong [67], and **c.330 + 1G > A** was identified in a Korean patient [68]. Thus, it is likely that specific *ADAMTS13* gene mutations are more common among certain ethnicities. In this regard, the mutation of **p.R268P** is quite unique, as the same mutation was reported by Veyradier *et al.* [55] in France, but in a Haitian patient.

The *ADAMTS13* gene mutation with the highest frequency in Japan was **p.R193W** ($n = 8$), followed by the remaining alleles in order of descending frequency: **p.Q449X** ($n = 5$), **p.C908Y** ($n = 4$), **c.2259delA** ($n = 4$), etc. The **p.Q449X** mutation was localised to the northern part (Tohoku) of Honshu, **c.2259delA** to Kyushu, **p.C908Y** to western Japan, and **p.R193W** to a relatively wide area across Japan but more frequently in western Japan, suggesting some geographical specificity in these mutations (Fig. 1).

Plasma levels of ADAMTS13 activity, ADAMTS13 inhibitor, and IgG-type anti-ADAMTS13 binding antibody in USS-patients

Most of our USS-patients had the plasma levels of ADAMTS13 activity with a $< 0.5\%$ of the normal (Table 1), but USS-GG2 alone had the ADAMTS13 activity of 2.4–3.4% of the normal, measured in three different occasions, as described above. Further, seven USS-patients (USS-F3, J3, K3, H3, Q1, S3, and LL4) had a trace amount of ADAMTS13 activity (0.6–1.8% of the normal) on some occasions, of whom four patients (USS-J3, K3, Q1, and LL4) had the ADAMTS13 activity below 0.5% of the normal in different occasions. The reason for this slight variation of plasma ADAMTS13 activity in our patients is presently unknown.

As for the ADAMTS13 inhibitors, all of our USS-patients had plasma levels of < 0.5 BU mL^{-1}, with one exception (USS-LL4), who showed the inhibitor titers ranging from < 0.5–1.4 BU mL^{-1}.

In regard to the IgG-type anti-ADAMTS13 binding antibody, 36 of 43 USS-patients did not have it (shown as the titer of 25 or $< 25\times$ in Table 1). However, seven patients (USS-K3, K4, X5, AA3, EE4, II3, and LL4) had the antibody titers ranged from 50 to 400 $\times$ on some occasions. Clinical significance of the IgG-type anti-ADAMTS13 binding antibody is also unclear at moment, but notably six of these seven patients are female.

Table 3 Summary of 65 allelic numbers of ADAMTS13 disease-causing gene mutation out of 69 mutations in 35 Japanese patients with USS (five siblings)

≥ 2 Allelic numbers ($n = 11$)	Allelic numbers	One allelic number ($n = 28$)	
p.R193W	8	p.I178T	p.A606P
p.Q449X	5	p.G227R	p.Q723K
p.C908Y	4	p.H234R	p.G909R
c.2259del A	4	p.H234Q	p.Q929X
c.414 + 1G > A	3	p.A250V	p.W1081X
c.3220delTACC	3	p.R312C	p.R1123C
p.R268P	2	p.C322G/p.T323R/ p.F324L	p.Q1302X
p.Y304C	2	p.R349C	c.372insGT
p.I673F	2	p.G385E	c.1885delT
p.C1024R	2	p.R398C	c.3198delCT
p.R1206X	2	p.C438S	c.4119delG
		p.C508Y	c.330 + 1G > A
		p.G525D	c.686 + 1G > A
		p.G550R	c.1244 + 2T > G

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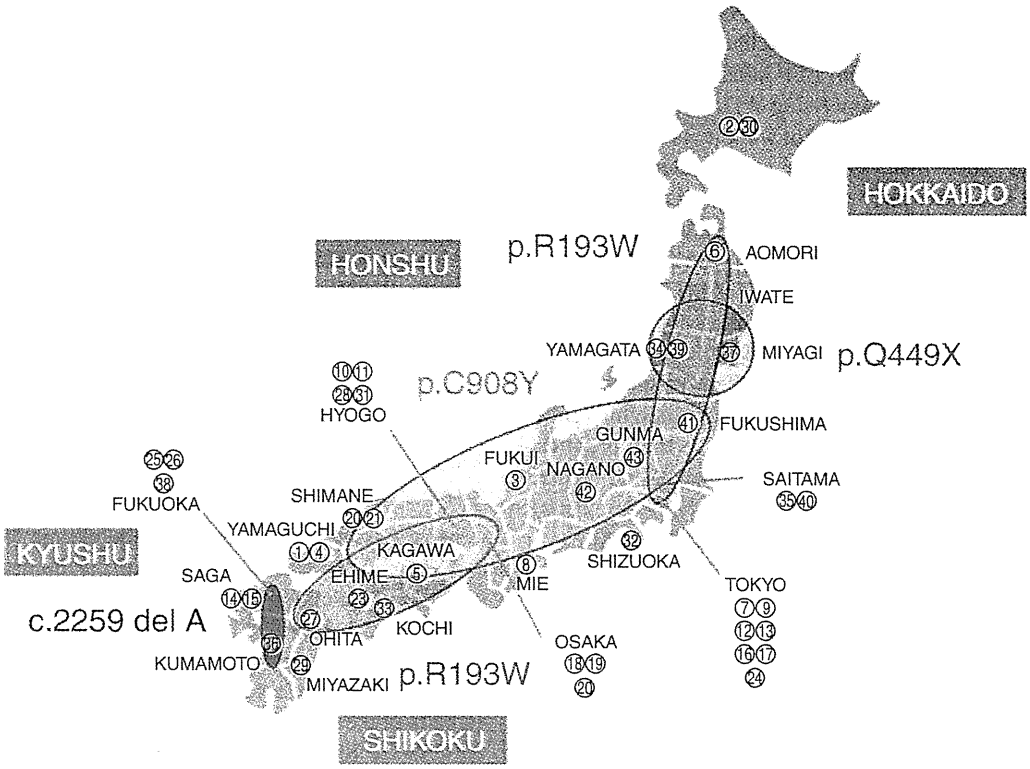

Fig. 1. Geographical distribution of 43 Japanese patients with USS and their *ADAMTS13* gene mutations. Among 43 USS patients, an *ADAMTS13* gene analysis was performed in 39 patients. Nine of the 39 USS patients had homozygous *ADAMTS13* gene mutations, and 29 were the compound heterozygotes, including one patient (USS-W4: patient no 28) with disease-causing mutation (DCM) on one allele while the other was unidentified. In the remaining one patient (USS-X5: patient no 29), two single nucleotide polymorphisms (SNPs), p.P475S and p.G1181R, but not DCMs were identified on each allele. The p.Q449X mutation localised to the northern part (Tohoku) of Honshu, c.2259delA to Kyushu, p.C908Y to western Japan, and p.R193W to a relatively wide area across Japan. Circled numbers indicate the patients shown in Tables 1 and 2.

Discussion

Since ADAMTS13 was originally discovered, one major question has been why USS-patients who consistently lack ADAMTS13 activity do not always experience acute symptoms of overt TTP. Furthermore, symptoms often become evident only when the patients have infections or become pregnant [12,69]. In both instances, vascular endothelial cell injury may be involved, and these cases have been indirectly associated with elevated plasma levels of cytokines or soluble thrombomodulin [70]. Consistent with these observations, studies on two different groups of *ADAMTS13* gene knockout mice revealed that UL-VWFMs were detectable in the blood, although the mice did not exhibit acute symptoms [71,72]. Considering these results, investigators have assumed that a deficiency in ADAMTS13 activity is prothrombotic, but alone is insufficient to provoke acute symptoms. Thus, second hits or triggers must exist. Related to this hypothesis, it has been said that there are two clinical features of USS, termed the 'early-onset' and 'late-onset' phenotypes. To partially address this question, we have extensively analysed the natural histories and *ADAMTS13* genotypes of 43 Japanese patients with USS.

This study has two advantages. One advantage is that Japan basically has four small islands, Hokkaido, Honshu, Shikoku, and Kyushu that make tracing the ancestral roots of a targeted USS family favourable. This is because USS patients tend to live near their parents or healthy relatives to receive medical support when they develop overt signs of TTP. In fact, before ADAMTS13 was discovered in 2001, nine patients were clinically diagnosed with USS or congenital CR-TTP in Japan, and none of these patients have moved to other areas or countries. The other advantage of this study can be attributed to the development of two convenient ADAMTS13 activity assays in our country, FRETS-VWF73 [29] and the chromogenic ADAMTS13-ac-ELISA [28]. Both assays are now used worldwide, and in 1998 Nara Medical University started voluntarily using the VWFM assay to meet the requests of clients across Japan. In 2005, the act-ELISA shortened the time required to diagnose TTP, and more importantly facilitated the identification of new USS-patients in Japan.

Although severe neonatal jaundice that requires exchange blood transfusion has been a hallmark of USS, this clinical sign was only present in 18 of 43 (42%) patients in this study. Because of this just four (/18) physicians correctly diagnosed their patient with USS before the patient reached 6 months of age, whereas 10 (/18) physicians required 6 years to reach a diagnosis of USS. On the other hand, among 25 USS patients without severe newborn jaundice, two (/25) were correctly diagnosed within

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6 months of age, and six (/25) were diagnosed within 6 years. As a whole, 25 of 43 (58%) USS patients were correctly diagnosed before they reached 15 years of age, including 12 females and 13 males, indicating that there is no gender disparity in diagnosing USS during childhood. These 25 patients would be unanimously considered to have the 'early-onset phenotype'. However, the remaining 18 USS patients were diagnosed after 15 years of age. This raises the question of whether these patients were the true 'late-onset phenotype' or not. One particularly interesting result was that 15 (/18) patients were diagnosed between 15 and 45 years of age, and interestingly they were all female. Furthermore, among these 15 female patients, nine were diagnosed in association with pregnancy. The remaining three patients (USS-H3, -BB3, and -GG2) were diagnosed after 45 years of age, and they were all male, which sharply contrasts the previous scenario. Thus, the natural history of these three male patients appeared to be an excellent means to analyse the pathogenesis of the 'late-onset phenotype'. Among these patients, USS-H3 with a p.A250V/c.330 + 1G > A genotype had an episode of thrombocytopenia, but there are few clinical details and the patient died of renal failure in 2002 [42], Thus, no further results on USS-H3 are available.

However, two other males, USS-BB3 and USS-GG2, had received annual health examinations during adulthood, and there were no apparent abnormalities until sudden and overt TTP developed at 55 and 63 years of age, respectively. This may indicate that the clinical signs of TTP were very mild during their childhood and adulthood, and any symptoms might have been attributed to isolated mild thrombocytopenia. Interestingly, these two elderly men carried two different homozygous *ADAMTS13* gene mutations, p.R193W/p.R193W and p.C1024R/p.C1024R, respectively. We previously reported that the p.R193W protein was present in the plasma of patient USS-Z3 [12,73]. In this study we also determined that the p.C1024R protein was present in the plasma of patient USS-GG2 (data not shown). Furthermore, *in vitro* expression studies using HeLa cells that were transfected with either of these two mutant gene plasmids showed that each protein was consistently secreted into the culture medium but had much reduced activity compared to the wild-type protein ([39] and unpublished data). Consistent with these observations, the ADAMTS13 activity of patient USS-GG2 was mildly reduced (2.4–3.4% of the normal) on three different occasions. As for the homozygous p.R193W/p.R193W mutation, we identified another female patient (USS-Z3) who was correctly diagnosed with USS at 27 years of age as a result of pregnancy-associated TTP at 25 years of age. Her past history was well recorded, and indicated that she had mild jaundice as a newborn and thus did not receive an exchange blood transfusion. However, she was diagnosed with ITP with isolated thrombocytopenia at 7 years of age. Taken together, these results indicate that the phenotype of the homozygous p.R193W/p.R193W mutation is mild. Therefore, patients carrying this mutation would presumably have mild thrombocytopenia during childhood, as shown in USS-Z3, unless they are exposed to strong stimuli such as a cytokine storm during influenza virus infection. However, after adolescence the gender disparity apparently determines the fate of these USS-patients. Pregnancy undoubtedly is a strong inducer of overt TTP in female USS-patients, although the pathogenesis is not fully elucidated. However, it is now well established that plasma VWF levels remarkably increase as gestation progresses, along with the appearance of UL-VWFMs, which are accompanied by reduced ADAMTS13 activity due to consumption, even in normal pregnant women [74,75]. Thus, in pregnant USS women, an enormous excess of the substrate (larger VWFM) relative to the ADAMTS13 enzyme is the most plausible pathogenic mechanism.

As a consequence, our studies here have re-confirmed that pregnancy, influenza infection, and DDAVP administration can be the strong triggers inducing overt TTP in USS-patients. Besides, now it is indicated that the aging, interferon therapy, and heavily drinking alcohol could be additional modifiers aggravating clinical signs of USS-patients.

Given that the p.R193W mutation is a frequent DCM for USS in Japan, male patients carrying this mutation might not exhibit clinical signs of thrombosis at a younger age. However, as they age, multi-factorial endogenous and exogenous causes mentioned above would facilitate thrombotic events, leading to brain infarctions and chronic renal failure as a result of microcirculation disturbances. We speculate that thrombotic events in the brain or kidney, which still have an unknown pathogenesis, might result from *ADAMTS13* gene abnormalities. Our examination of the natural history in this large cohort of USS-patients with *ADAMTS13* mutations may shed light on these important diseases. Thus, here we emphasise again an importance of the assay for ADAMTS13 activity as a routine test to make and/or exclude a diagnosis of USS, when physicians meet the patients with thrombocytopenia of unknown aetiology, not only in childhood but also in adulthood.

Acknowledgements

This work was supported in part by research grants from the Ministry of Health, Labor, and Welfare of Japan, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. Y. Fujimura dedicates this paper to the late Professors Theodore S. Zimmerman and Hiromu Fukui, who both encouraged me to be involved in the research on VWF and its clinical application.

Disclosure of Conflict of Interests

YF is a clinical advisory board for Baxter Bioscience.

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INTERNAL MEDICINE

□ CASE REPORT □

H1N1 Influenza (Swine Flu)-Associated Thrombotic Microangiopathy with a Markedly High Plasma Ratio of von Willebrand Factor to ADAMTS13

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Abstract

We describe an 18-year-old woman infected with H1N1 influenza followed by thrombotic microangiopathy. During the acute phase, her plasma levels of von Willebrand factor (VWF) were remarkably elevated, whereas those of ADAMTS13 were reduced without its inhibitors, generating a markedly high ratio of VWF to ADAMTS13 in circulation. A retrospective analysis established the following hypothesis: an influenza-mediated cytokine storm induced an enhanced release of unusually large VWF multimers (UL-VWFM) from vascular endothelial cells, generating platelet thrombi in microcirculatures under high shear stress. Plasma exchange removed UL-VWFM and cytokines, and rescued her life. This report sheds a light on a hitherto unrecognized influenza complication.

Key words: swine influenza, TMA, cytokine storm, UL-VWFM, ADAMTS13

(Intern Med 50: 643-647, 2011)
(DOI: 10.2169/internalmedicine.50.4620)

Introduction

Thrombotic microangiopathies (TMAs) are pathological conditions, characterized by organ dysfunction due to platelet thrombi in the microvasculature, consumptive thrombocytopenia, and microangiopathic hemolytic anemia (MAHA). Two typical phenotypes of TMAs are thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) (1). But, other diseases such as HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet syndrome) associated with pregnancy-induced hypertension are also included in a category of TMA.

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, member 13) is a metalloprotease which specifically cleaves the Tyr1605-Met1606 bond in the von Willebrand factor (VWF) A2 domain (2). In the absence of ADAMTS13 activity (ADAMTS13: AC), unusually large VWF multimers (UL-VWFM) released from vascular endothelial cells (EC) are not appropriately cleaved, accumulate in circulation, and induce generalized formation of platelet thrombi in the microvasculature under conditions of high shear stress. This results in TTP, a life-threatening disease. Currently, a severe deficiency of ADAMTS13: AC due to the genetic mutations or acquired autoantibodies is thought to be a specific feature for TTP (3).

The accumulation of UL-VWFM in the circulation is alternatively achieved under conditions of an extremely low enzyme-to-substrate (E/S) ratio, when the plasma level of UL-VWFM is substantially higher than that of ADAMTS13, which is mainly produced in the liver (4). This scenario often occurs when there are high plasma levels of cytokines such as interleukin (IL)-6 and its receptor complex, IL-8, and tumor necrosis factor (TNF)-α. This condition, termed cytokine storm, which stimulates the release of UL-VWFM from vascular EC (5), can develop in the context of connective tissue disease, organ transplantation, and sepsis. In these clinical settings, a pathological diagnosis of TMA is often used in clinical practice (6).

In 1981, Wasserstein et al (7) described the first case of

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Received for publication October 1, 2010; Accepted for publication December 6, 2010
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recurrent TTP in association with influenza. The pathological diagnosis was made by renal biopsy findings with features of chronic renal disease: glomeruli with thickened capillary walls and numerous 'double contours,' as well as several hyaline capillary thrombi, accompanied by MAHA and thrombocytopenia. However, there have been no subsequent reports of influenza-induced TTP described since, except for cases of influenza vaccine-associated TTP (8, 9). We previously identified a 68-year-old woman with influenza A-associated TTP who had severe ADAMTS13: AC deficiency due to the production of neutralizing IgG-inhibitors (10), but we suggested that the frequency of this phenomenon is far less common.

Here, we describe an 18-year-old woman with H1N1 influenza who developed TMA with a mild reduction of plasma ADAMTS13 activity (ADAMTS13: AC) without ADAMTS 13-neutralizing autoantibodies (inhibitors) (ADAMTS13: INH), and was successfully treated with plasma exchange (PE). A retrospective analysis of plasma VWF and VWF multimers may address how influenza-induced TMA, a hitherto unrecognized complication, developed in this patient and why PE was effective.

Case Report

Clinical course: An 18-year-old woman presented to our institution, Matsudo City Hospital, with gross hematuria and massive epistaxis in August, 2009. Two days earlier she visited a nearby clinic complaining of fever and chills, where she was diagnosed with influenza and received a prescription for zanamivir 20 mg daily for 2 days. At age 4 she had an episode of atypical hemolytic uremic syndrome (HUS) associated with influenza A infection which was successfully treated by three courses of PE but she had no subsequent episodes.

On physical examination, the patient was afebrile (37.2 ℃). Her heart rate was 85 beats per minute and regular, blood pressure 121/78 mmHg, respiration rate 18 breaths per minute and regular. She was alert, without any disturbances in consciousness. There were no apparent signs of bruising. The results of laboratory testing on hospital day (HD) 1-3 are shown in Table 1. Of note, prothrombin time (PT) and activated prothrombin time (A-PTT) on HD1 were within the normal ranges, but two markers of disseminated intravascular coagulation (DIC), fibrinogen degradation products (FDP) and D-dimer, were slightly increased. Thus, by the diagnostic criteria for DIC from the International Society of Thrombosis and Haemostasis (11) and from the Japanese Ministry of Health and Welfare (12), the patient had a DIC score of 4 (non-overt DIC) and 7 (overt DIC), respectively. On the other hand, the patient's plasma level of VWF antigen (VWF: Ag) was increased (191% of normal), and that of ADAMTS13: AC determined by chromogenic act-ELISA (13) was significantly reduced (37% of normal) with a marginal level of ADAMTS13: INH (0.7 Bethesda U/mL). These results suggested that a diagnosis of TMA was preferable to TTP. Thus, on admission a differential diagnosis of DIC or TMA was not readily suggested. In fact, through the following clinical course, plasma levels of fibrinogen, PT, and A-PTT were within the normal range, however the high plasma levels of VWF: Ag and low levels of ADAMTS13: AC were consistently noted during the acute phase. Together with these results, the characteristic clinical features, such as MAHA, thrombocytopenia and renal dysfunction, a diagnosis of influenza-associated TMA rather than DIC was made. The H1N1 influenza infection was confirmed on HD 2 by genotyping.

The timing of various therapeutic interventions and relevant laboratory data trends are shown in Fig. 1A. The patient was initially treated with an infusion of 480 mL of fresh frozen plasma (FFP). Since there was no appreciable clinical improvement, we next initiated PE, which was performed on a total of 4 days (HD 2-4 and HD 6), with a single daily plasma dose of 3,000-4,200 mL. Red blood cell concentrates (RCC) were also infused with a total volume of 420 mL on HD 5, but no platelets were administered. Since no distinct anti-ADAMTS13: INH were detected, steroid therapy was not initiated. Platelet counts began to increase on HD 5 and reached the normal range on HD 9, along with the patient's clinical condition and laboratory data for MAHA and renal function. Renal biopsy performed on HD 18 showed signs of vascular endothelial cell injury, namely focal segmental endocapillary proliferative glomerulonephritis with focal segmental double contours (figure not shown).

Sequential VWF multimer analysis during the clinical course: A retrospective analysis of VWFM by vertical SDS-agarose gel electrophoresis (14) was performed using plasma samples stored at -80℃. As shown in Fig. 1B, plasma levels of VWF: Ag were significantly elevated during the acute phase (HD 1-7) with a uniquely and dynamically changed multimer profile. On HD 1 the plasma VWF: Ag level was elevated but lacked High molecular weight (HMW) forms. We identified an extremely high level of plasma VWF: Ag on HD 4 and 7 along with the appearance of UL-VWFM. In sharp contrast, on HD 3 and 6, UL-VWFM and HMW-VWFM were undetectable. During the recovery stage (HD 9-23) the VWFM pattern normalized and became almost indistinguishable. Plasma levels of the cytokines IL-6, IL-8, and TNF-α, were also analyzed and found to be remarkably elevated on admission.

Discussion

Although this patient had a history of atypical HUS at age 4, she did not have any further episodes during the subsequent 14 years. It is currently recognized that there is a late-onset form of congenital ADAMTS13: AC deficiency called Upshaw-Schulman syndrome (15). However, this possibility was excluded by the measurement of ADAMTS13: AC on this occasion. Another possibility which remains is that the patient has TMA-predisposing genetic defects in complement regulatory proteins, such as factor H, factor I,

Table 1. Laboratory Findings

Hospital days	1	2	3	Normal range
Peripheral blood				
Platelet count (x10^9/L)	8	7	20	(130-369)
WBC (× 10^6/L)	5200	6000	7300	(3500-9100)
RBC (× 10^{12}/L)	3.91	3.44	2.56	(3.76-5.00)
Hb (g/dL)	11.0	9.7	7.1	(11.3-15.2)
Schistocytes on blood smear	++	++	++	(-)
Blood chemistry				
Total protein (g/dL)	7.1	7.4	6.5	(6.7-8.3)
Total bilirubin (mg/dL)	3.92	4.57	2.44	(0.2-1.2)
AST (IU/L)	123	136	70	(0-31)
ALT (IU/L)	15	19	16	(0-41)
LDH (IU/L)	3241	3974	2293	(119-234)
BUN (mg/dL)	28.2	29.8	28.3	(5.0-21.0)
Creatinine (mg/dL)	0.96	1.15	1.45	(0.4-1.0)
Antinuclear antibody (titer)	40	ND	ND	(<40)
Anti-DNA antibody (RIA: IU/mL)	<2.0	ND	ND	(<6.0)
Coagulation				
PT (sec)	13.9	11.1	13.9	(11.5-15.5)
A-PTT	35.0	30.9	26.7	(29.5-39.5)
Fibrinogen (mg/dL)	ND	352.5	357	(200-400)
Fibrin degradation products (μ g/mL)	20	38.1	7.5	(0-10)
D-dimer (μ g/mL)	17.4	18.1	2.9	(0-1.0)
VWF:Ag	191	ND	187	(50-150)
Cytokines				
TNF- α (pg/mL)	5.7	ND	ND	(0.6-2.8)
IL-6 (pg/mL)	7.6	ND	ND	(<4.0)
IL-8 (pg/mL)	18.7	ND	ND	(<2.0)
C-reactive protein (mg/dL)	3.2	2.0	2.0	(0-0.3)
ADAMTS13				
:AC (%)	37	ND	48	(50-150)
:INH (Bethesda U/mL)	0.7	ND	0.6	(<0.5)
Urinalysis				
Specific gravity	>1.030	1.020	1.015	(1.005-1.039)
Protein	2+	3+	3+	(-)
Sugar	-	-	-	(-)
Ketones	-	+/-	-	(-)
Blood	2+	3+	3+	(-)
Sediment				
RBC/HPF	>100	4-6/EF	4-6/EF	(<1-2)
WBC/HPF	4-6	3-6/SF	10-20/EF	(<2-5)

ND: not determined

factor B, membrane cofactor protein (CD46), or most recently, thrombomodulin, an anticoagulant glycoprotein (16). However, if she had such an underlying defect, she would likely have severe renal impairment, whereas her renal dysfunction was marginal and transient; thus, it is less likely she had such genetic defects.

We have recently proposed a model for the pathogenesis of TMA, in which an extremely low circulating E/S (ADAMTS13/VWF) ratio is sufficient to cause TMA under certain rheological conditions, such as high shear stress (6). This hypothesis was drawn from previous reports (5), in which VWF release from vascular EC is upregulated in vitro by cytokines such as IL-6 (and its receptor complex), IL-8, and TNF-α, and plasma ADAMTS13: AC is thereby compensatively consumed. This further aggravates the low E/S (ADAMTS13/UL-VWFM) ratio while generating platelet thrombi in the microcirculation. Influenza viremia stimulates monocytes to release cytokines, which can result in severe cytokinemia, or cytokine storm.

To test this hypothesis, here we performed a retrospective analysis of cytokine and VWFM levels in this patient. The plasma levels of IL-6, IL-8, and TNF-α, were indeed markedly increased. Most interestingly, however, a striking change was seen in the plasma levels of VWF: Ag, which were significantly elevated during the acute phase (HD 1-7) with a uniquely and dynamically changed multimeric pattern (Fig. 1B). In particular, the selective absence of UL- and HMW-VWFM under these circumstances might be attributable to the reduced production or increased removal from and/or degradation in the circulation. The former mechanism is less likely, because the cytokinemia observed in influenza patients during the acute phase continuously stimulates VWF release from vascular EC. Furthermore, to cleave the newly-released UL-/HMW-VWFM, plasma ADAMTS13: AC is vigorously consumed. The mild reduction of ADAMTS13: AC seen here, therefore, appears to be a reflection of this reaction. As for the latter mechanism, the circulating UL-/HMW-VWFM is consumed when it func-

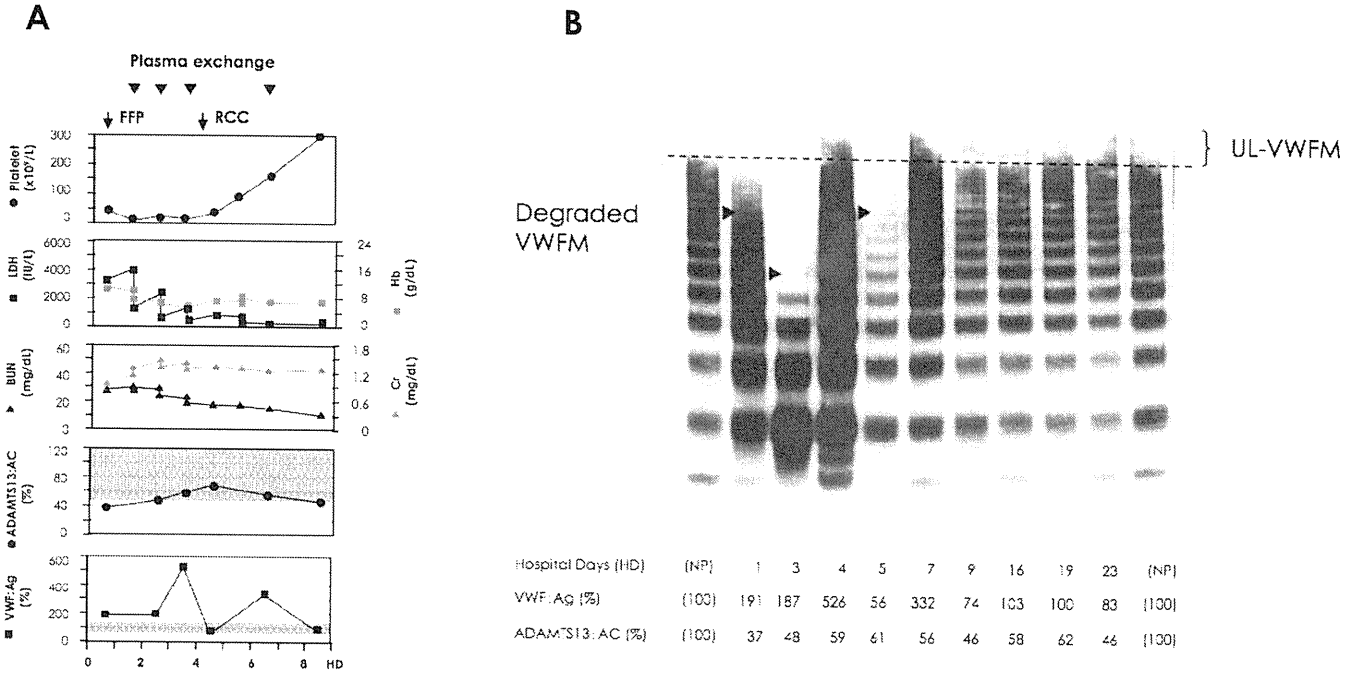

Figure 1. Clinical course and VWF multimer analysis. A: Clinical course and laboratory findings are shown in the left panel. On admission, plasma ADAMTS13:AC was 37% of normal, and the anti-ADAMTS13 inhibitor titer was 0.7 Bethesda U/mL. Therapeutic plasma exchange, each 3,000 - 4,200 mL, was performed on 4 occasions as indicated by the arrows, with a concomitant improvement of laboratory findings: lactate dehydrogenase (LDH), blood urea nitrogen (BUN), hemoglobin (Hb), and creatinine (Cr). Fresh frozen plasma (FFP) and red blood cell concentrates (RCC) were also administered. Note that plasma levels of ADAMTS13: AC were around the lower limit of normal (shadow area), but those of VWF:Ag were consistently high. HD denotes hospital day. B: As shown in the right panel, a retrospective analysis of VWF multimer patterns during the clinical course was performed. Note that plasma levels of VWF:Ag were remarkably high during the acute phase corresponding to hospital day (HD) 1-7, while ADAMTS13:AC levels were relatively low. We observed the presence UL-VWFM only on HD 4 and 7. In contrast, plasma UL- and HMW-VWFM were undetectable on HD 3 and 5. NP denotes plasma from a healthy control.

tions as a molecular glue that facilitates platelet hyperaggregation or thrombi formation. These explanations may address the co-existence of undetectable UL-/HMW-VWFM and a mild reduction of ADAMTS13: AC in the same patient. In line with this scenario, during the recovery stage on HD 9-23 the VWFM patterns became normal and almost indistinguishable from each other.

If the extensive analyses on VWF and ADAMTS13 had not been performed, the patient in this study might have had a diagnosis of flu-associated DIC, according to the previous diagnostic criteria (11, 12). In fact, TMAs are pathologically featured by platelet thrombi and DIC by fibrin thrombi, each formed in microvasculatures. Thus, it is conceivable that TMA is further complicated by DIC and both clinical conditions may co-exist, but its reversal clinical course appears to be less likely. Thus, the most important finding in this study is the markedly high plasma ratio of VWF to ADAMTS13, as seen in swine flu patients, that may induce microcirculatory disturbance by platelet thrombi under high shear-stress, a hitherto unrecognized flu complication.

From another point of view, the desialyation of VWF by influenza viral neuraminidase may play a role. The carbohydrate- and sialic acid-rich VWF, once desialylated by neuraminidase, can bind to platelet glycoprotein Ib and induce platelet aggregation (17, 18). This was left unaddressed here since the level of sialyation in the patient's VWF was not assessed; future studies should address this possibility.

In conclusion, we propose that TMA is induced by influenza infection through the following mechanism. First, the release of UL-VWFM from vascular EC is enhanced by stimulation with cytokinemia induced by influenza infection. Second, elevated levels of plasma UL- and HMW-VWFM mediate platelet hyperaggregation and thrombi formation in the microvasculature. Third, the formed platelet thrombi can cause dysfunction in various organs, typically renal insufficiency. PE is a highly effective treatment for influenza-induced TMA by reducing plasma levels of cytokines and UL-/HMW-VWFM and through replenishing VWFM and ADAMTS13.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors would like to thank Dr. Reinhard Schneppenheim

for his valuable suggestion before preparing this manuscript.

Financial Support: This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Ministry of Health, Labor, and Welfare of Japan.

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SAGE-Hindawi Access to Research
International Journal of Hepatology
Volume 2011, Article ID 759047, 12 pages
doi:10.4061/2011/759047

Review Article

Determination of ADAMTS13 and Its Clinical Significance for ADAMTS13 Supplementation Therapy to Improve the Survival of Patients with Decompensated Liver Cirrhosis

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Received 25 February 2011; Accepted 8 April 2011

Academic Editor: Deepak Amarapurkar

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The liver plays a central role in hemostasis by synthesizing clotting factors, coagulation inhibitors, and fibrinolytic proteins. Liver cirrhosis (LC), therefore, impacts on both primary and secondary hemostatic mechanisms. ADAMTS13 is a metalloproteinase, produced exclusively in hepatic stellate cells, and specifically cleaves unusually large von Willebrand factor multimers (UL-VWFM). Deficiency of ADAMTS13 results in accumulation of UL-VWFM, which induces platelet clumping or thrombi under high shear stress, followed by sinusoidal microcirculatory disturbances and subsequent progression of liver injuries, eventually leading to multiorgan failure. The marked imbalance between decreased ADAMTS13 activity (ADAMTS13 : AC) and increased production of UL-VWFM indicating a high-risk state of platelet microthrombi formation was closely related to functional liver capacity, hepatic encephalopathy, hepatorenal syndrome, and intractable ascites in advanced LC. Some end-stage LC patients with extremely low ADAMTS13 : AC and its IgG inhibitor may reflect conditions similar to thrombotic thrombocytopenic purpura (TTP) or may reflect "subclinical TTP." Hence, cirrhotic patients with severe to moderate deficiency of ADAMTS13 : AC may be candidates for FFP infusion as a source of ADAMTS13 or for recombinant ADAMTS13 supplementation. Such treatments may improve the survival of patients with decompensated LC.

1. Introduction

The liver is a major source of clotting and fibrinolytic proteins and plays a central role in thromboregulation [1–4]. Liver diseases, hence, impact on both primary and secondary hemostatic mechanisms. Because the hemostatic system is normally in a delicate balance between pro-hemostatic and antihemostatic processes, advanced liver cirrhosis (LC) patients experience multiple changes in the hemostatic system that may lead to either bleeding or thrombosis [1–4]. Despite clinical evidence of increasing bleeding tendency in LC patients, many facts indicate local and systemic hypercoagulability including portal or hepatic vein thrombosis, pulmonary embolism, and deep vein thrombosis, which are closely related to microcirculatory disturbances [4]. Deficiency of anticoagulant proteins and high levels of several procoagulant factors may favor hypercoagulability [4], but the mechanisms underlying this disorder have not been fully elucidated.

ADAMTS13 (**a** **d**isintegrin-like **a**nd **m**etalloproteinase with thrombospondin type-1 motifs **13**) is a metalloproteinase that specifically cleaves multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 residues in the A2 domain [5, 6]. 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 and to 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 (Upshaw-Schulman syndrome, (USS)) [5–8] or acquired autoantibodies against ADAMTS13 [9, 10], is thought to be a specific feature of thrombotic thrombocytopenic purpura (TTP) [5–12].

In 2000, we demonstrated that a decreased plasma ADAMTS13 : AC in patients with cirrhotic biliary atresia can be fully restored after liver transplantation, indicating that the liver is the main organ producing ADAMTS13 [13]. One year later, northern blot analysis showed that the 4.6-kilobase ADAMTS13 mRNA was highly expressed in the liver [7, 14, 15], and subsequently both *in situ* hybridization and immunohistochemistry clearly indicated that ADAMTS13 is produced exclusively in hepatic stellate cells (HSCs) [16]. Platelets [17], vascular ECs [18], and kidney podocytes [19] have also been implicated as ADAMTS13-producing cells, but the amount produced by these cell types in the liver appears to be far less than that produced by HSC.

Mannucci et al. [20] originally reported a reduction of the ADAMTS13 : AC in advanced LC. Since HSCs were shown to be the major producing cells in the liver [16], much attention has been paid to the potential role of ADAMTS13 in the pathophysiology of liver diseases associated with sinusoidal and/or systemic microcirculatory disturbance [21–35]. ADAMTS13 : AC significantly decreased in patients with hepatic veno-occlusive disease (VOD) [22, 23], alcoholic hepatitis [24–27], liver cirrhosis [29, 30], and those undergoing living-donor-related liver transplantation [31–33] and partial hepatectomy [34]. Furthermore, hepatitis C virus- (HCV-) related LC patients with ADAMTS13 inhibitor (ADAMTS13 : INH) typically developed TTP [35]. Once patients with LC develop a decompensated condition, the risk of early mortality sharply increases for specific life-threatening complications such as ascites, hepatic encephalopathy, sepsis, hepatorenal syndrome, or hepatopulmonary syndrome [36].

In this paper, we will focus on the importance of ADAMTS13 determination for a better understanding of pathophysiology and/or for possible therapeutic approaches of ADAMTS13 supplementation to improve survival in patients with advanced LC.

2. Hepatic Microcirculation and Hypercoagulable State in LC

Hepatic microcirculation compromises a unique system of capillaries, called sinusoids, which are lined by three different cell types: sinusoidal endothelial cells (SECs), HSC, and Kupffer cells [37]. The SEC modulates microcirculation between hepatocytes and the sinusoidal space through the sinusoidal endothelial fenestration. The SEC has tremendous endocytic capacity, including VWF and the extracellular matrix, and secretes many vasoactive substances [37]. The HSC is located in the space of Disse adjacent to the SEC and regulates sinusoidal blood flow by contraction or relaxation induced by vasoactive substances [38]. Kupffer cells are intrasinusoidally located tissue macrophages and secrete potent inflammatory mediators during the early phase of liver inflammation [37]. Intimate cell-to-cell interaction has been found between these sinusoidal cells and hepatocytes [37, 38]. In LC, a sinusoidal microcirculatory disturbance occurs when the normal hepatic structure is disrupted by fibrin deposition [39] or by impaired balance between the action of vasoconstrictors and vasodilators in hepatic vascular circulation [37]. Studies have shown that cirrhotic liver exhibits a hyperresponse to vasoconstrictors, including catecholamine, endothelin, and leukotrienes D_4 [37].

Vascular endothelial cells play a pivotal role in hemostasis and thrombosis [5, 6]. VWF is a marker of endothelial cell activation (damage) and plays an essential role in hemostasis [5, 6]. In the normal state, VWF immunostaining is usually positive in large vessels but negative in the SEC [40]. On the occurrence of liver injury accompanied by a necroinflammatory process, the SEC becomes positive for VWF, presumably in association with the capillarization of hepatic sinusoids [39]. Subsequently, platelets adhere to subendothelial tissue mediated by UL-VWFM [5, 6]. ADAMTS13 then cleaves UL-VWFM into smaller VWF multimers [5, 6]. This interaction of ADAMTS13 and UL-VWFM is, indeed, the initial step in hemostasis [5, 6].

In patients with LC, circulating plasma VWF levels are extremely high [41, 42]. In liver tissue from cirrhotics [43] and even from the early stages of alcoholic liver diseases [44], VWF immunostaining shows positive cells predominantly at the scar-parenchyma interface, within the septum, and in the sinusoidal lining cells. Actually, portal or hepatic vein thrombosis is often observed in advanced LC routinely screened with Doppler ultrasound [45], and, in cirrhotic liver removed at transplantation, intimal fibrosis suggesting hepatic and portal vein thrombosis was frequently observed [46]. An autopsy series revealed microthrombi in one or multiple organs in one-half of cirrhotics [47]. Such a hypercoagulable state in liver diseases may be involved in hepatic parenchymal destruction, the acceleration of liver fibrosis and disease progression [4], leading to hepatorenal syndrome, portopulmonary hypertension, and spontaneous bacterial peritonitis [48].

Systemically, deficiency of anticoagulant proteins (antithrombin, protein C, and protein S) and the high levels of several procoagulant factors (factor VIII and VWF) may contribute to hypercoagulability in patients with LC [4]. Locally, the SEC dysfunction could lead to the development of a hypercoagulable state at the hepatic sinusoids corresponding to the site of liver injury, even in the face of a systemic hypocoagulable state [4]. Considering that ADAMTS13 is synthesized in HSC and its substrate, UL-VWFM, is produced in transformed SEC during liver injury, decreased plasma ADAMTS13 : AC may involve not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver diseases, finally leading to multiorgan failure. Based on these findings, it is of particular interest to evaluate the activity of plasma ADAMTS13 : AC in LC patients.

3. Cleavage of UL-VWFM by ADAMTS13

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 [49]. UL-VWFMs are produced exclusively in vascular ECs and stored in an intracellular organelle termed Weidel-palade bodies (WPBs) and then released into the circulation upon stimulation. Under physiological conditions, epinephrine acts as an endogenous stimulus, but under nonphysiological conditions, DDAVP (1-deamino-8-D-arginine vasopressin), hypoxia, and several cytokines such as interleukin IL-2, IL-6, IL-8, and tumor necrosis factor- (TNF-) α act as stimuli that upregulate 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 ECs surface [50]. Under these circumstances, high shear stress generated in the microvasculature induces a change in the UL-VWFM from a globular to an extended form [51]. The ADAMTS13 protease efficiently cleaves the active extended form of UL-VWFM between the Tyr1605 and Met1606 residues in the A2 domain [52]. 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 [53]. ADAMTS13 may more efficiently cleave newly released UL-VWFMs that exist as solid-phase enzymes 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 [54]. When ADAMTS13 activity is reduced, UL-VWFM interacts more intensively with platelet GPIb and generates signals that further accelerate platelet activation [5, 6]. A series of these reactions leads to platelet microaggregates and thrombocytopenia. However, little information has been available on the cleavage of the UL-VWFMs by ADAMTS13 in the sinusoidal microcirculation in LC.

4. Assays for Plasma ADAMTS13 : AC and ADAMTS13 : INH

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. [55], and the detection limit of this assay was 3% of the normal control in our laboratory [56]. In 2005, we 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 [57]. Plasma ADAMTS13 : AC levels highly correlated between VWFM assay and ADAMTS13-act-ELISA (mean $\pm$ SD, 102 $\pm$ 23% versus 99.1 $\pm$ 21.5%, $r^2 = 0.72$, $P < .01$) [57]. No interference of the ADAMTS13-act-ELISA occurred even in the presence of hemoglobin, bilirubin, or chylomicrons in the samples, thus enabling distinction from the FRETS-VWF73 assay [58]. Because of its high sensitivity, easy handling, and lack of interference from plasma components, the ADAMTS13-act-ELISA would be recommended for routine laboratory use.

The ADAMTS13 : INH has also been evaluated with the chromogenic act-ELISA by means of the Bethesda method [59]. Prior to the assay, the test samples were heat-treated at 56°C for 60 min to eliminate endogenous enzyme activity, mixed with an equal volume of intact normal pooled plasma, and incubated for 2 hours at 37°C. The residual enzyme activity is measured after incubation. One Bethesda unit is defined as the amount of inhibitor that reduces activity by 50% of the control value, and values greater than 0.5 U/mL are significant.

5. Thrombocytopenia, Determination of ADAMTS13 : AC, and Its Clinical Significance in LC

5.1. Thrombocytopenia. It is well accepted that thrombocytopenia gradually progresses as functional liver capacity decreases [30, 60] (Figure 2(a)). The pathogenesis of thrombocytopenia in LC includes splenic sequestration in portal hypertension [61], impaired platelet production due to decreased synthesis of thrombopoietin in the liver [62] or due to myelosupression resulting from HCV infection [63], folic acid deficiency, or ethanol chronic consumption [64], which has a negative effect on megacaryocytopoiesis. However, our recent studies have provided evidence that in patients with advanced LC, elevated plasma levels of UL-VWFM enhance high-shear stress-induced platelet aggregation, resulting in thrombocytopenia [30].

5.2. ADAMTS13 : AC. Our study showed that ADAMTS13 : AC decreased with increasing severity of cirrhosis [30] (Figure 2(b)). The values determined by act-ELISA correlated well with those of the classical VWFM assay and also closely correlated with ADAMTS13 antigen de-termined by the antigen-ELISA. These results confirmed that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity [30] (Figures 2(b) and 2(c)), which are consistent with findings described by Feys et al. [29]. In contrast, Lisman et al. showed that both ADAMTS13 activity and antigen levels were highly variable; however, they did not distinguish between patients with varying degrees of cirrhosis [28]. It is unclear why they reached different conclusions from ours. One possible explanation relates to different etiologies: a majority of our patients developed cirrhosis secondary to HCV infection, whereas in their study one-half of the patients suffered from alcohol abuse-related cirrhosis. Further, the techniques used to determine ADAMTS13 : AC differed between our study [55–57] and theirs [65]. It is assumed that the collagen binding assay they used can be highly influenced by the increased amount of VWF : Ag in tested cirrhotic plasmas [29], because the substrate in this assay is intact multimeric VWF. In this regard, our act-ELISA is performed using VWF73-based fusion protein, termed GST-VWF73-His, which is readily cleaved by ADAMTS13 without any protein denaturant, and therefore the increased amount of VWF : Ag in tested plasmas does not interfere with the assays [57].

As shown in Figure 3, ADAMTS13 : ACs were significantly lower in LC patients with hepatic encephalopathy (Figure 3(a)), hepatorenal syndrome (Figure 3(b)), and

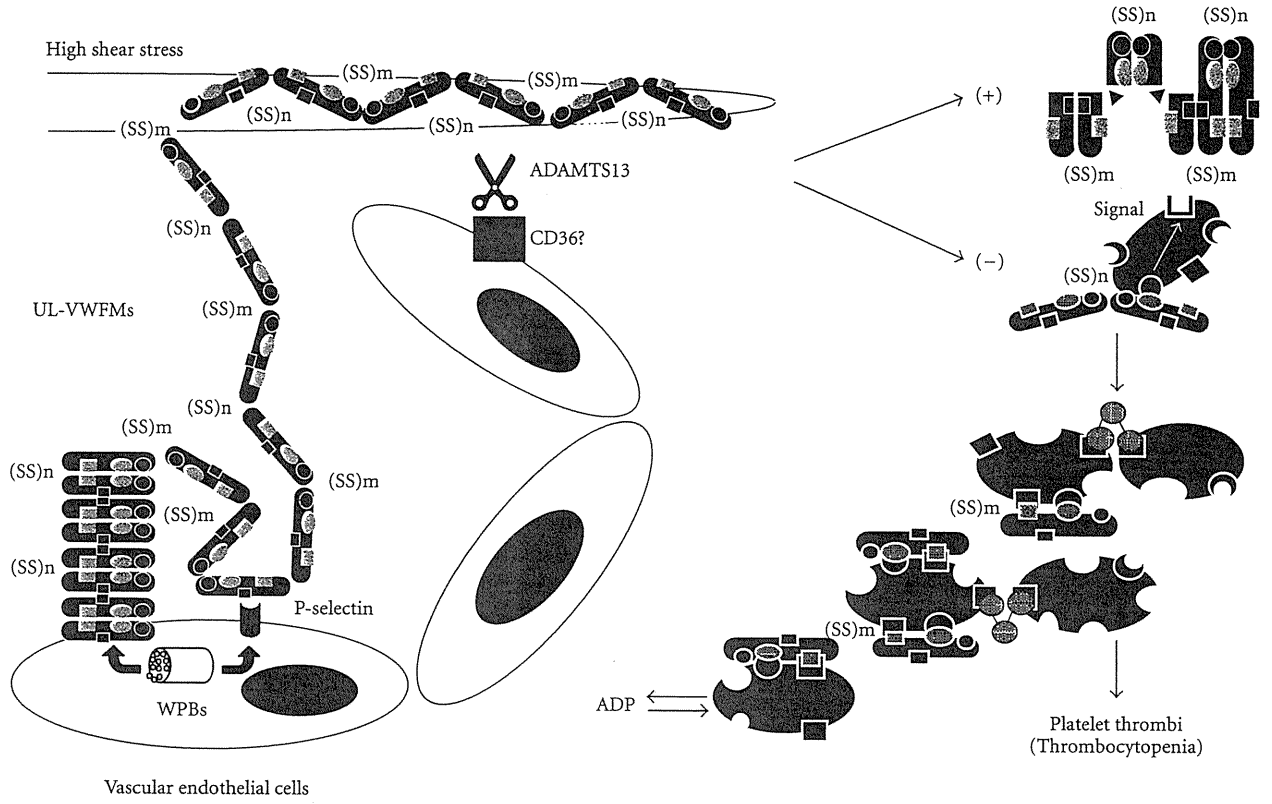

FIGURE 1: Proposed mechanism of platelet thrombi under high shear stress in the absence of ADAMTS13 : AC. Unusually large von Willebrand factor multimers (UL-VWFMs) are produced in vascular endothelial cells (ECs) and stored in Weidel-palade bodies (WPBs). UL-VWFMs are released from WPBs into the circulation upon stimulation by cytokines, hypoxia, DDAVP, and epinephrine. P-selectin that comigrates from WPBs anchors UL-VWFMs on the vascular EC surface. Under these circumstances, high shear stress changed the molecular conformation of UL-VWFMs from a globular to an extended form, allowing ADAMTS13 to access this molecule. In the absence of ADAMTS13 : AC, UL-VWFMs remain uncleaved, allowing them to excessively interact with platelet glycoprotein (GP)Ibα and activate platelets via intraplatelet signaling, which result in the formation of platelet thrombi. (Partially modified from Fujimura et al., [49]).

severe esophageal varices than those without [30]. Moreover, patients with refractory ascites had lower ADAMTS13 : AC levels than patients without ascites or those with easily mobilized ascites (Figure 3(c)). A multivariate analysis using all significant baseline parameters determined by the univariate analysis, excluding the Child-Pugh score, showed spleen volume, blood ammonia, and serum creatinine independently correlated with ADAMTS13 : AC. As a second step, the three parameters that contribute to the Child-Pugh classification (total bilirubin, albumin, and prothrombin time) were replaced by the Child-Pugh score. As a result, the Child-Pugh score and spleen volume were independently selected, indicating that ADAMTS13 : AC is closely related to the severity of liver disease and splenomegaly in cirrhotic patients [30].

5.3. VWF : Ag and VWF Multimer Patterns. Plasma levels of VWF : Ag substantially increase as liver diseases progress (Figure 2(d)) [30], as previously reported [41, 42]. This is presumably attributed to sinusoidal and/or extrahepatic endothelial damage induced by endotoxin and cytokines [41, 42, 66, 67]. The VWF : RCo was higher (Figure 2(e)) [30], but the ratio of VWF : RCo/VWF : Ag was lower in LC patients than that in healthy subjects. These findings suggest that increased VWF : Ag appears less functional in LC patients [30], which are consistent with previous reports [28]. Nevertheless, our study has clearly shown that the ratio of VWF : RCo/ADAMTS13 : AC progressively increases with the worsening of chronic liver diseases (Figure 2(f)), further intensifying an enhanced thrombogenesis with the progression of liver dysfunction and thrombocytopenia [30].

With regard to VWF multimers, the higher molecular weight multimer showed greater degradation than in healthy controls, thus maintaining normal enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity [29]. We showed that there were three different VWFM patterns in LC patients with lower ADAMTS13 : AC (<50 % of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and UL-VWFM in 16% (Table 1) [30]. UL-VWFM-positive patients showed the lowest ADAMTS13 : AC and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia. In addition, LC patients with UL- and normal-VWFM had higher levels of VWF : RCo

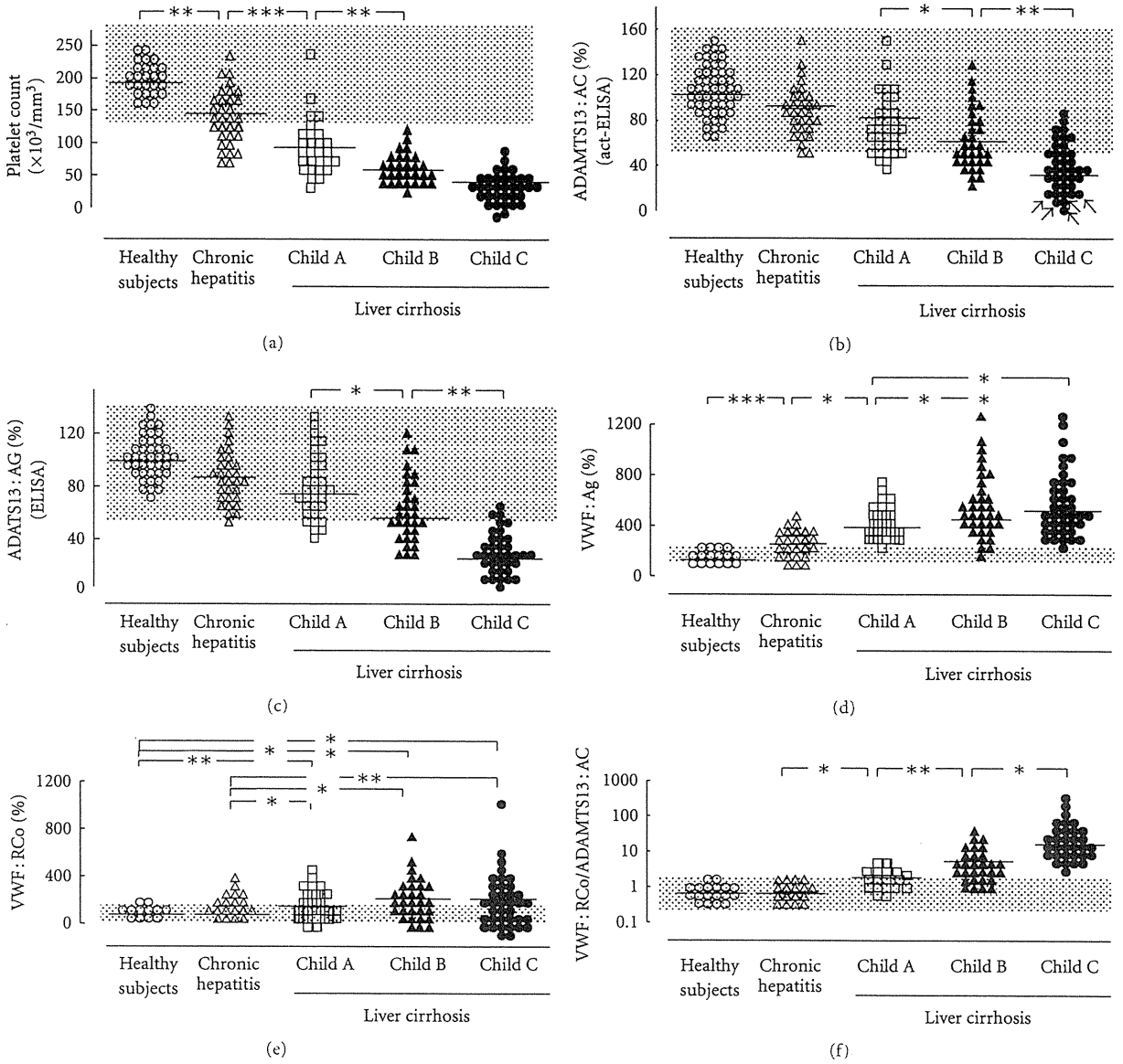

FIGURE 2: Platelet counts and plasma levels of ADAMTS13 : AC and its related parameters in patients with chronic liver diseases. Platelet counts decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (a). Plasma ADAMTS13 : AC determined by ELISA progressively decreased with worsening cirrhosis (b). Arrows indicate patients whose plasma ADAMTS13 : AC was extremely low (< 3% of normal control by VWFM assay). The ADAMTS13 : AG levels determined by ELISA also decreased with increasing cirrhosis severity (c), which highly correlated with ADAMTS13 : AC measured by the act-ELISA ($r = 0.715$, $P < .001$). The VWF : Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (d). The VWF : RCo is higher in liver cirrhosis patients than that in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within liver cirrhosis (e). The VWF : RCo relative to ADAMTS13 : AC progressively increased with worsening chronic liver disease (f). Open circles: normal controls; open triangles: chronic hepatitis; open squares: cirrhosis with Child A; closed triangles: cirrhosis with Child B; closed circles: cirrhosis with Child C. Shaded area shows normal range. ADAMTS13 : AC : ADAMTS13 activity, ADAMTS13 : AG = ADAMTS13 antigen. VWF : Ag = von Willebrand factor antigen, VWF : RCo = von Willebrand factor ristocetin cofactor activity; $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ significantly different between the two groups. (Partially modified from Uemura et al., [30]).

and Child-Pugh score and lower values of cholinesterase and hemoglobin than those with degraded-VWFM [30] (Table 1). The pattern, therefore, appears to shift from degraded- to normal-VWFM, and finally to UL-VWFM as functional liver capacity and renal function deteriorates, indicating that advanced LC may be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombotic events [30].

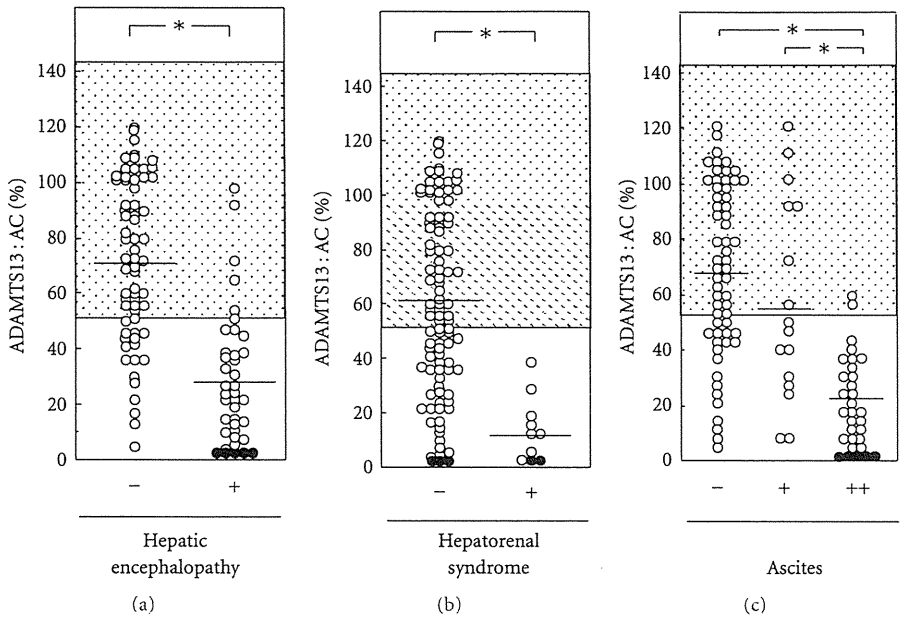

FIGURE 3: Relationship of ADAMTS13 : AC to the presence or absence of hepatic encephalopathy, hepatorenal syndrome, and ascites in patients with liver cirrhosis. The ADAMTS13 : AC was significantly lower in LC patients with hepatic encephalopathy (a) and hepatorenal syndrome (b) than that those without. Moreover, patients with refractory ascites had lower ADAMTS13 : AC than those without ascites or those with easily mobilized ascites (c). Closed circles indicate patients whose plasma ADAMTS13 : AC was extremely low (< 3% of normal control by VWFM assay). ADAMTS13 : AC: ADAMTS13 activity; $^{*}P < .001$ significantly different between the two groups. (Partially modified from Uemura et al., [30]).

TABLE 1: Comparison of clinical parameters among cirrhotic patients according to VWF multimer patterns.

Variables	VWF multimer pattrens			a versus b	a versus c	b versus c
	Degraded[a] ($n = 15$)	Normal[b] ($n = 26$)	Unusually large[c] ($n = 8$)			
ADAMTS13 : AC (%) (ELISA)	47 ± 24	44 ± 13	26 ± 14	n.s.	$P < .05$	$P < .01$
VWF : RCo (%)	110 ± 92	196 ± 134	216 ± 110	$P < .05$	$P < .05$	n.s.
Child-Pugh score	8.6 ± 2.5	10.9 ± 2.1	12.4 ± 1.7	$P < .01$	$P < .005$	n.s.
Serum albumin (g/dL)	3.07 ± 0.54	2.85 ± 0.54	2.59 ± 0.25	n.s.	$P < .05$	n.s.
Cholinesterase (IU/L)	126 ± 62	78 ± 64	60 ± 36	$P < .05$	$P < .02$	n.s.
Total cholesterol (mg/dL)	142 ± 51	93 ± 45	88 ± 40	$P < .01$	$P < .03$	n.s.
Hemoglobin (g/dL)	11.0 ± 1.7	9.3 ± 2.0	8.9 ± 1.7	$P < .02$	$P < .02$	n.s.
Serum creatinine (mg/dL)	1.06 ± 0.72	1.11 ± 0.79	2.43 ± 2.16	n.s.	$P < .05$	$P < .03$
Blood urea nitrogen (mg/dL)	22 ± 17	30 ± 21	74 ± 62	n.s.	$P < .01$	$P < .01$
Blood ammonia (μg/dL)	87 ± 50	100 ± 39	144 ± 53	n.s.	$P < .05$	$P < .05$

VWF: von Willebrand factor; ADAMTS13 : AC : ADAMTS13 activity; ELISA : enzyme-linked immunosorbent assay; VWF : RCo: VWF ristocetin cofactor activity; n.s.: not significant. (Partially modified from Uemura et al., [30]).

6. Mechanism of Decreased ADAMTS13 : AC in LC Patients

The mechanism responsible for the decrease in ADAMTS13 : AC in advanced LC may include enhanced consumption due to the degradation of large quantities of VWF : AG [20], inflammatory cytokines [68, 69], and/or ADAMTS13 plasma inhibitor [9, 10]. It is controversial whether ADAMTS13 deficiency is caused by decreased production in the liver; Kume et al. reported that HSC apoptosis plays an essential role in decreased ADAMTS13 : AC using dimethylnitrosamine-treated rats, but not carbon tetrachloride- (CCl_4-) treated

animals [70], whereas Niiya et al. found upregulation of ADAMTS13 antigen and proteolytic activity in liver tissue using rats with CCl_4-induced liver fibrosis [71]. We observed the inhibitor of ADAMTS13 in 83% of patients with severe to moderate ADAMTS13 deficiency, but its inhibitory activity was in a marginal zone between 0.5 and 1.0 BU/mL in most cases except in cases of a TTP patient (2.0 BU/mL) and a patient with severe ADAMTS13 deficiency (3.0 BU/mL) [30]. Interestingly, IgG-type autoantibodies specific to purified plasma derived-ADAMTS13 were detected by Western blotting only in five end-stage cirrhotics with severe ADAMTS13 deficiency (<3%) corresponding to TTP [30]. One patient showed an apparent TTP [35], while the other four cirrhotics did not show apparent clinical features of TTP but had complications of hepatorenal syndrome, spontaneous bacterial peritonitis (SBP), marked inflammation together with cytokinemia, and advanced hepatocellular carcinoma (HCC) [30]. Various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP [72]. In advanced LC patients, endotoxemia is frequently detected [42, 73], and SBP sometimes occurs [74]. HCC is highly complicated as the cirrhotic stage progresses [75], suggesting a high-risk state of platelet microthrombi formation. Some end-stage LC patients with extremely low ADAMTS13 : AC and its IgG inhibitor may reflect conditions similar to TTP or may reflect "subclinical TTP" [21]. Further studies will be necessary to clarify whether inhibitors other than the IgG inhibitor might be involved in cirrhotics with lower ADAMTS13 : AC.

Alternatively, cytokinemia [25, 68, 69, 76] and endotoxemia [25, 77] are additional potential candidates for decreasing plasma ADAMTS13 : AC. Recent investigations demonstrated that IL-6 inhibited the action of ADAMTS13 under flow conditions and both IL-8 and TNF-α stimulated the release of UL-VWFM in human umbilical vein endothelial cells *in vitro* [68]. It remains to be clarified whether IL-6 directly hampers the cleavage of UL-VWFM or downregulates gene expression of ADAMTS13 with modification of promoter activity. IFN-γ, IL-4, and TNF-α also inhibit ADAMTS13 synthesis and activity in rat primary HSC [69]. In addition, ADAMTS13 deficiency associated with inflammation promoted formation of UL-VWFM [78], and intravenous infusion of endotoxin to healthy volunteers caused a decrease in plasma ADAMTS13 : AC together with the appearance of UL-VWFM [77]. In patients with alcoholic hepatitis, especially in severe cases complicated by LC, ADAMTS13 : AC concomitantly decreased, and VWF : Ag progressively increased with increasing concentrations of these cytokines from normal range to over 100 pg/mL [25]. Plasma endotoxin concentration inversely correlated with ADAMTS13 activity and was higher in patients with UL-VWFM than that those without [25]. From these results as well as our own, marked cytokinemia and/or enhanced endotoxemia may be closely related to decreased ADAMTS13 : AC and the appearance of UL-VWFM [25]. It will be necessary to clarify what types of inhibitor may be involved in association with inflammatory cytokines and endotoxin.

7. Typical TTP in Patients with Liver Diseases

We previously encountered a patient with HCV-related LC who was compromised by fatal TTP [35]. This case showed advanced LC and rigid ascites. As reported in the literature, since 1979, there have been 13 patients with liver diseases who developed TTP [35, 79–90]. Five of them were treated with IFN therapy, but the remaining 8 were not. Three of them showed evidence of autoimmune hepatitis, one of which was complicated by systemic lupus erythematosus (SLE). The remaining 4 patients had HCV-related LC, hepatitis B virus- (HBV-) related LC, alcoholic LC, or haemochromatosis. IFN may be able to induce autoimmune reactions, resulting in the generation of autoantibodies against ADAMTS13, although this phenomenon has yet to be confirmed. On the other hand, irrespective of IFN therapy, HCV infection and/or advanced LC itself may contribute to the development of TTP.

There is general consensus that the overall prevalence of serum non-organ-specific autoantibodies is significantly higher in patients with HCV (about one third of all cases) than that in both healthy subjects and patients with HBV [91–93], but not alcoholic liver injury. In addition, HCV infection was confirmed in five of 10 patients (50%) who developed thrombotic microangiopathy (TMA) after living-donor liver transplantation [94]. In our study, the etiology of our five end-stage LC patients with IgG-type autoantibodies was HCV in 2, HBV in 1, PBC in 1, and cryptogenic in 1, but none of the patients displayed alcohol-abuse-related cirrhosis [30]. Nevertheless, the diagnosis of TTP may be hampered by clinical features accompanying hepatic failure similar to the pentad of typical TTP (fever, thrombocytopenia, renal failure, fluctuating neurological signs, and microangiopathic hemolytic anemia) [11, 12].

8. Possible Therapeutic Approaches of ADAMTS13 Supplementation for Patients with Decompensated LC

Fresh frozen plasma (FFP) infusion is commonly used to correct the prolonged prothrombin time in patients with advanced chronic liver disease, but exact indication for its use has not been clearly defined [95]. The aim of FFP infusions is usually either to improve the coagulopathy before invasive procedures or to control ongoing bleeding from various sites in patients with vitamin K-unresponsiveness prolonged prothrombin time. The mean prothrombin time was improved by the infusion of 2–6 units of FFP, but only 12.5% of the retrospective study group and 10% of the prospective study groups showed reversal of their coagulopathy, and higher volume (6 or more units) may be more effective but rarely is employed [96]. However, attention should be directed to complications including the risk of infection, allergic reaction, and acute volume expansion leading to heart failure or pulmonary edema [95, 96].

With regard to FFP infusion as a unique source of ADAMTS13, we clearly showed that preexisting UL-VWFMs

in the plasma of USS patients began to diminish within 1 hour and completely diminished 24 hours after ADAMTS13 was replenished with infusions of FFP [97]. Retrospectively, these results indicated that exogenous ADAMTS13 could efficiently cleave both UL-VWFMs that preexisted in the circulation and the newly produced molecules at the ECs surface. Advanced LC is known to be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombi [30]. In our study, UL-VWFM-positive patients showed the lowest ADAMTS13 : AC and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia, and the VWFM patterns appeared to shift from degraded to normal VWFM and finally to UL-VWFM as functional liver capacity and renal function deteriorated (Table 1). From these results, it may be reasonable to assume that advanced LC patients with severe to moderate deficiency of ADAMTS13 : AC (<3% to ~25% of normal control) could be candidates for FFP infusion as a source of ADAMTS13. It is necessary to evaluate the effectiveness of FFP administration to patients with ADAMTS13 : AC levels from 25% to 50%.

Alternatively, our recent study demonstrated that plasma ADAMTS13 : AC is reduced in VOD patients after stem cell transplantation (SCT) (12–32% of normal) compared to non-VOD patients (57–78% of normal), even before any conditioning regimen and throughout SCT, and that the activity might thus be a predictor for the development of hepatic VOD [22]. A multicenter, prospective, randomized controlled study revealed that prophylactic FFP infusion may be instrumental in preventing the development of hepatic VOD after SCT [23]. The imbalance caused by decreased ADAMTS13 : AC versus increased production of VWF : Ag before and during the early stage after SCT would contribute to a microcirculatory disturbance that could ultimately lead to VOD [23]. The supplementation of ADAMTS13 by prophylactic FFP infusion may suppress the increase in VWF : AG that is extensively released from damaged SEC. Furthermore, we first reported in 2006 that a significant reduction of ADAMTS13 : AC with a concomitant appearance of UL-VWFM was consistently observed in patient plasma soon after liver transplantation [31]. These changes were closely related to liver-graft dysfunction, ischemia-reperfusion injury, and acute rejection. The ADAMTS13 : AC often decreased to less than 10% of normal controls, concurrent with severe thrombocytopenia. The organ dysfunction appeared to be restricted to the liver graft, indicating that a decrease of plasma ADAMTS13 : AC coupled with the appearance of UL-VWFM was attributed to a mechanism of "local TTP" within the liver graft [21, 31]. It is, therefore, extremely important to monitor plasma ADAMTS13 : AC in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation. This is because the infusions of platelet concentrate under conditions of an imbalance of decreased ADAMTS13 : AC to enhanced UL-VWFM production might further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFM, leading to graft failure via the "local TTP" mechanism [21, 31]. FFP infusion as ADAMTS13 replacement therapy may improve both liver dysfunction and thrombocytopenia in liver transplant patients. From this point of view, we are particularly interested in conducting clinical trials with recombinant ADAMTS13 preparations not only in patients with advanced LC but also in patients with VOD and liver transplantations.

9. Conclusion and Future Perspectives

The introduction of ADAMTS13 to the field of hepatology not only enabled us to confirm the diagnosis of TTP early but also provided novel insight into the pathophysiology of liver diseases. Some diseases were shown to be TTP itself, but others did not show any apparent clinical features of TTP, even in the presence of extremely decreased ADAMTS13 : AC and increased UL-VWFM corresponding to TTP. Such TTP-like states, but without disseminated intravascular coagulation, might be "subclinical TTP" as seen in advanced liver cirrhotics [30] and SAH patients [24–27] or "local TTP" as shown in patients with hepatic VOD after SCT [22, 23] and patients with adverse events after living-donor liver transplantation [31, 32]. Essentially, one would be unable to detect such TTP-like phenomena without the determination of ADAMTS13 : AC, because the interaction of ADAMTS13 and UL-VWFM is the initial step in hemostasis, and their abnormalities do occur in the absence of apparent imbalance in other hemostatic factors and/or irrespective of the presence or absence of abnormal conventional hemostatic factors. The origin of VWF, the substrate of ADAMS13, indeed may be transformed hepatic sinusoidal and/or extrahepatic endothelial cells, but not hepatocytes. The procoagulant and anticoagulant proteins synthesized in hepatocytes decrease as liver disease progresses, whereas VWF markedly increases. Under such circumstances, ADAMTS13 deficiency may lead to a microcirculatory disturbance not only in the liver, but also in the systemic circulation. The determination of ADAMTS13 and its related parameters thus will be quite useful for improved understanding of the pathophysiology and for providing appropriate treatments especially in severe liver disease patients. It will be necessary to measure ADAMTS13 : AC when patients with unexplained thrombocytopenia are encountered in the course of liver disease. When "subclinical or local TTP" status would be confirmed, FFP infusion as ADAMTS13 replacement therapy may improve both liver dysfunction and thrombocytopenia. Further investigation will be necessary to define candidates for ADAMTS13 supplementation therapy and to evaluate its potential therapeutic efficacy in advanced LC patients.

Acknowledgments

The authors sincerely thank Hiromichi Ishizashi, Ayami Isonishi, Seiji Kato, Tomomi Matsuyama, Chie Morioka, and Masatoshi Ishikawa for their great help in the assay of ADAMTS13 activity, VWF antigen, and UL-VWFM. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Science (to M. Uemura, Y. Fujimura, S. Ko, and M. Matsumoto) and from