end-stage renal disease (Loirat & Fremeaux-Bacchi, 2011; Noris et al., 2010). Therefore, genetic information in patients with aHUS would be highly valuable for prognosis.

Incomplete penetrance of aHUS in the mutation carriers in the family has previously been reported (Caprioli et al., 2006; Loirat & Fremeaux-Bacchi, 2011; Noris et al., 2010). The present study confirmed this observation in Japanese aHUS patients (Fig. 2). The identified mutations were inherited from the patients' unaffected fathers or mothers. The single exception was a patient (JJ1) whose father (JJ2) had aHUS. None of the proband's siblings with the same mutation developed aHUS. It is likely that mutations do not directly cause an aHUS phenotype but rather modify the phenotype or predispose an individual to aHUS. The environmental factors and/or other genetic variations as a second hit are required for the manifestations of aHUS on one main genetic background (Francis et al., 2012; Pickering et al., 2007). Indeed, the onset of the disease was associated with infection or surgery in 9 out of 10 patients in our study (Table 2).

Patient X1 with the *CFH* p.R1215Q mutation received a live kidney transplantation, but failed three weeks later with recurrent aHUS. This result was consistent with the previous observations that the risk of post-transplant aHUS relapse is rather high in patients with *CFH* mutations (Loirat and Fremeaux-Bacchi, 2011; Noris & Remuzzi, 2009). In contrast, a lower risk of recurrence was reported in patients with the *MCP* mutation (Noris et al., 2010). One of the reasons for this variability is that CFH is a plasma protein synthesized by the liver, whereas MCP is synthesized by each cell locally. Therefore, combined liver–kidney transplantation might sometimes be a better option for CFH-associated patients based on a consideration of the risks/benefits in the individual patient (Saland et al., 2009). However, it should be noted that a relative kidney donor is not recommended, especially for patients with the *MCP* mutation, considering the possibly similar genetic background (Loirat and Fremeaux-Bacchi, 2011). Correspondingly, plasma exchange and plasma infusion may be a better and more logical choice for patients with the *CFH* mutation, but not efficient for correction of the essential defect in patients with MCP mutations, at least in theory.

In summary, the prevalence of genetic variation was evaluated in 10 Japanese aHUS patients. Seven causative or potentially causative mutations were identified in *CFH, C3, MCP,* and *THBD* in 8 patients and another patient was a carrier of CFH autoantibodies. The relationship between the genotype and phenotype was analyzed. Since the phenotype-genotype correlation of aHUS has clinical significance in predicting renal recovery and transplant outcome, a comprehensively accurate assessment of molecular variation would facilitate the clinical management for aHUS patients in Japan.

Conflict of interest

Dr. Fujimura is on the clinical advisory boards for Baxter Bioscience and Alexion Pharmaceuticals. Drs. Matsumoto, Hattori, and Ashida are on the clinical advisory board for Alexion Pharmaceuticals.

Contributions

T. Miyata and Y. Fujimura designed the study. X.P. Fan performed the genetic analysis with the guidance of S. Honda. Y. Yoshida and M. Matsumoto performed the protein analysis and hemolytic assay. Y. Yoshida, M. Matsumoto, Y. Sawada, M. Hattori, S. Hisanaga, R. Hiwa, F. Nakamura, M. Tomomori, S. Miyagawa, R. Fujimaru, H. Yamada, T. Sawai, Y. Ikeda, N. Iwata, O. Uemura, E. Matsukuma, Y. Aizawa, H. Harada, H. Wada, E. Ishikawa, A. Ashida, and M. Nangaku contributed to the sample collection, clinical data acquisition and interpretation of the data. X.P. Fan, T. Miyata, and Y. Fujimura interpreted the data and wrote the manuscript. All authors critically reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary Table 1

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2012.12.006.

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Ticlopidine-associated ADAMTS13 activity deficient thrombotic thrombocytopenic purpura in 22 persons in Japan: a report from the Southern Network on Adverse Reactions (SONAR)

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening generalized disorder. The classic TTP 'pentad' is thrombocytopenia, microangiopathic hemolytic anemia (MAHA), renal impairment, neurological symptoms, and fever (Amorosi & Ultmann, 1966). Laboratory studies identified deficiency of plasma ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type 1 motifs 13) activity (ADAMTS13:AC) among some TTP patients (Furlan *et al*, 1998; Tsai & Lian, 1998). ADAMTS13 cleaves the peptide bond between Thy1605 and Met1606 in the A2 domain of von Willebrand factor (VWF) subunit. VWF is released into the plasma as unusually large VWF multimers (UL-VWFMs), which are degraded into smaller size VWF multimers by ADAMTS13. In the late 1990's, studies in the United States identified 117 cases of TTP that developed after initiation of the thienopyridine, ticlopidine; although at that time, ADAMTS13 activity levels were not widely available (Bennett *et al*, 1999; Steinhubl *et al*, 1999). A study of seven patients in the United States with ticlopidine-associated TTP found that all seven had severe deficiency of ADAMTS13 activity and five had detectable antibodies to ADAMTS13 activity (Tsai *et al*, 2000). We now report on 22 individuals from Japan with ticlopidine-induced TTP and compare these findings to those from the United States. Ticlopidine was the primary anti-platelet agent in Japan from 1989 to 2006.

Since 1998, our laboratory at Nara Medical University has been a nationwide referral centre in Japan for thrombotic microangiopathies (TMAs), including TTP (Fujimura & Matsumoto, 2010). The study protocol was approved by the Ethics Committee of Nara Medical University Hospital. TTP diagnostic criteria were: microangiopathic haemolytic anaemia (haemoglobin ≤ 120 g/l), Coombs test negative, undetectable serum haptoglobin (<1 μmol/l), more than 2 fragmented red cells (schistocytes) in a microscopic field with ×100 magnification, increased serum lactate dehydrogenase (LDH) above institutional baseline, thrombocytopenia (platelet count $\leq 100 \times 10^9$/l), absence of evidence for disseminated intravascular coagulation and no other identifiable cause of TTP. Additional information on fever ≥ 37°C; and central nervous system and renal function data were abstracted. Patients were included if, in addition to criteria for idiopathic TTP, the patient had received ticlopidine prior to TTP onset. Before therapeutic plasma exchange or plasma infusion was initiated, whole blood samples (five ml) were withdrawn from each patient and placed into plastic tubes containing 1/10 volume of 3·2% sodium citrate. Plasma was separated by centrifugation at 3000 g for 15 min at 4°C, kept in aliquots at −80°C until testing, and sent to our laboratory with clinical information. Until March 2005, ADAMTS13:AC was determined by classic VWF multimer (VWFM) assay with a detection limit of 3% of the normal control (Furlan *et al*, 1996; Kinoshita *et al*, 2001). Thereafter, a chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA) with a detection limit of 0·5% of the normal control was developed, and replaced the VWFM assay. Plasma ADAMTS13 inhibitor (ADAMTS13:INH) titres were analysed either by classic VWFM assay or chromogenic ADAMTS13-act-ELISA using heat-inactivated plasmas at 56°C for 30 min.

A total of 22 ticlopidine-associated TTP patients fulfilled the inclusion criteria (Table I). Age at diagnosis ranged from 41 to 89 years, with the median age of onset of 69 years. Females accounted for 45·5% of the cohort. Ticlopidine had been administered for a median of 27·5 d (range, 14–35 d) but was discontinued after a clinical diagnosis of TTP was made. Median values for hemoglobin were 83 (60–146) g/l, platelets 9·5 (3–57) $\times 10^9$/l, and serum creatinine 132·6 (35–380) μmol/l. Abnormal neurological findings were noted in 63·6%. All of the patients had <5% ADAMTS13:AC activity and detectable inhibitors to ADAMTS13 at the time of presentation. ADAMTS13:INH titres were 0·5 to <1·0 Bethesda units (BU)/ml in 4·5% of the patients, 1·0 to <2·0 BU/ml in 13·5%, 2·0 to <5·0 BU/ml in 45·5%, 5·0 to <10 BU/ml in 18·2%, and 4·5% of the patients had ADAMTS13:INH titres of ≥ 10 BU/ml. Mortality during the acute TTP episode was 9·0%. Mortality was highest among persons 60 years of age or older (10·0% vs. 0·0%). Therapeutic plasma exchange was performed in 72·7%, at a median of 3 d after the onset of TTP (range 1–5 d), and the TTP resolved at a median of 8 d (range 3–28 d). Among four patients whose TTP cleared after 20 or more days of therapeutic plasma exchange, ADAMTS13:INH titres were 2,4·4, 17, and 20 BU/ml. Among 12 patients whose TTP resolved with therapeutic plasma exchange at <20 d, none had ADAMTS13:INH titres >4 BU/ml. Both ticlopidine-associated TTP deaths did not receive therapeutic plasma exchange.

To our knowledge, this is the first study to report detailed characteristics of ticlopidine-associated TTP among patients outside of the United States. Our findings, from a cohort of

Table I. Characteristics of ticlopidine-associated thrombotic thrombocytopenic purpura in Japan and United States.

Source	This paper	Bennett *et al* (1999)	Tsai *et al* (2000)	Steinhubl *et al* (1999)
Number of patients	22	98	7	19
Country	Japan	US	US	US
Aetiology	Ticlopidine	Ticlopidine	Ticlopidine	Ticlopidine
% female	45·50%	46·6%	70·0%	30·0%
Median age (years)	69 (41–89)	64·2 (11·1 = SD)	57 (42–89)	62 (38–75)
Platelets <20 × 10^9/l	96·0% (23/24)	71·9%	100·0%	89·4%
Haemoglobin <90 g/l	72·7%	26,9%	42·3%	66·7%
Creatinine >221 μmol/l	18·1%	30·1%	NA	47·0%
Neurological abnormalities	63·6%	73·1%	70·0%	73·7%
Median days ticlopidine (range)	27·5 (14–36)	21 (7–112)	21 (14–56)	21 (14–28)
% with coronary stent	13·6%	42·3%	57·1%	100·0%
% with other Coronary artery disease indication	31·2%	0·0%	14·3%	0·0%
% stroke prevention	55·8%	57·7%	14·3%	0·0%
Survival	91·03%	84·9%	100·0%	78·9%
% Therapeutic plasma exchange (TPE)	63·6%	74·2%	100·0%	68·4%
Survival without TPE	75·0%	42·1%	—	33·3%
Survival with TPE	100·0%	81·7%	100·0%	100·0%
% with ADAMTS13 activity deficiency (<10%)	100·0%	Not available	83·3%	Not available
% with ADAMTS13 inhibitors	100·0%	Not available	100·0%	Not available

ticlopidine-associated TTP patients in Japan, identified severe ADAMTS13 deficiency and antibodies to ADAMTS13 in 100% of these 22 individuals. A decade earlier, severe ADAMTS13 deficiency was reported in 100% of seven patients with ticlopidine-associated TTP in the Uni ted States and antibodies to ADAMTS13 in five of these patients (Bennett *et al*, 1999; Tsai *et al*, 2000). While ticlopidine-induced TTP is undoubtedly a rare disease, it is reassuring that the original observations reported from the United States have been independently replicated in Japan (Bennett *et al*, 1999; Steinhubl *et al*, 1999).

Limitations of our study should be identified. Follow-up ended at the time of hospital discharge, which prevented us from reporting on relapse rates. Ticlopidine is rarely used today, having been replaced by clopidogrel in 1999 because of safety concerns. Our research has shown that clopidogrel, unlike ticlopidine, does not lead to ADAMTS13 antibody formation and deficiency of ADAMTS13 activity and the rare cases of clopidogrel-associated TTP are not responsive to therapeutic plasma exchange. Also, very little is known about TTP associated with prasugrel (the newest thienopyridine), despite 14 cases of prasugrel-associated TTP having been reported to the Food and Drug Administration in 2009 and 2010 (Jacob *et al*, 2012). Careful pharmacovigilance to identify severe adverse drug reactions developing among small numbers of persons can serve as important warning signals for potentially serious adverse drug events internationally.

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Multiple myeloma Leu167Ile (c.499C>A) mutation prevents *XBP1* mRNA splicing

The unfolded protein response (UPR) is a cellular protective mechanism to restore homeostasis of the endoplasmic reticulum (ER) when misfolded proteins accumulate. There are three key players for sensing ER stress in mammalian cells, namely ATF6, PERK and IRE1α (Kim *et al*, 2006). Among these three mediators, IRE1α is the only conserved sensor from yeast to human (Moreno *et al*, 2012). Upon activation, IRE1 splices *XBP1* mRNA to form an active transcription factor (XBP1s). Under unstressed conditions, *XBP1* is constitutively transcribed and translated. However, the XBP1 protein is non-functional due to the presence of a premature stop codon that prevents the translation of the transactivation domain (Tirosh *et al*, 2006). During ER stress, the splicing of 26 nucleotides of the *XBP1* mRNA by IRE1α causes a frame shift, and hence alters the open reading frame. This allows the translation of the transactivation domain, thus forming XBP1s, which possesses transcription activity (Cox & Walter, 1996).

XBP1 is known to play an important role in multiple myeloma (Bagratuni *et al*, 2010). It has been reported that overexpression of XBP1s alone causes a Multiple Myeloma-like syndrome in mice (Carrasco *et al*, 2007). This suggests a possible oncogenic role for XBP1 in the development of the disease. On the contrary, XBP1 downregulation correlates with resistance to the proteasome inhibitor bortezomib, an

ADAMTS13 unbound to larger von Willebrand factor multimers in cryosupernatant: implications for selection of plasma preparations for thrombotic thrombocytopenic purpura treatment

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BACKGROUND: Thrombotic thrombocytopenic purpura (TTP) is characterized by deficient ADAMTS13 activity. Treatment involves plasma exchange (PE). Both fresh-frozen plasma (FFP) and cryosupernatant (CSP) are used, but it remains to be determined which is more effective.

STUDY DESIGN AND METHODS: To analyze the interaction between von Willebrand factor (VWF) and ADAMTS13, we used large-pore isoelectric focusing (IEF) analysis followed by detection with anti-ADAMTS13 monoclonal antibody. FFP, CSP, cryoprecipitate (CP), and purified ADAMTS13 were analyzed for their effects on high shear stress–induced platelet aggregation (H-SIPA).

RESULTS: IEF analysis of normal plasma revealed three groups of ADAMTS13 bands with pI of 4.9 to 5.6, 5.8 to 6.7, and 7.0 or 7.5. Two band groups (pI 4.9-5.6 and 5.8-6.7) were found in plasma of a patient with Type 3 von Willebrand disease, in which VWF is absent, whereas no bands were found in plasma of a patient with congenital ADAMTS13 deficiency. Mixing these plasmas generated the bands at pI 7.0 or 7.5, representing the VWF-ADAMTS13 complex; these bands were absent in CSP. FFP and purified ADAMTS13 down regulated H-SIPA in a dose-dependent manner. However, CP did not inhibit H-SIPA in the initial phase, and the degree of inhibition at the endpoint was almost indistinguishable from those of the other two plasma products.

CONCLUSION: Both plasma products (FFP and CSP) are effective for PE in TTP patients. However, CSP may be more favorable, because it has lower levels of VWF and almost normal ADAMTS13 activity, but lower levels of ADAMTS13 in complex with larger VWF multimers.

Von Willebrand factor (VWF), a multimeric hemostatic glycoprotein, is secreted from vascular endothelial cells into circulation as unusually large VWF multimers (UL-VWFMs).[1] The UL-VWFM is the most biologically active form with regard to platelet (PLT) adhesion properties.[2] Under conditions of high shear stress, UL-VWFMs cause enhanced PLT aggregation and give rise to VWF-rich thrombi in the microvasculature. ADAMTS13 (a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13) down regulates the function of UL-VWFMs by cleaving the VWF A2 domain at the Tyr1605–Met1606 bond, yielding

ABBREVIATIONS: ADAMTS13 = a disintegrin-like and metalloprotease with thrombospondin type-1 motifs 13; CP = cryoprecipitate; CSP = cryosupernatant; dp = depleted; H-SIPA = high shear stress–induced platelet aggregation; IAA = iodoacetamide; IEF = isoelectric focusing; pd = plasma derived; PE = plasma exchange; T3-VWD = Type 3 von Willebrand disease; TBS = Tris-buffered saline; TTP = thrombotic thrombocytopenic purpura; UL-VWFM(s) = unusually large VWF multimer(s); USS = Upshaw-Schulman syndrome.

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a series of smaller molecular forms.[3-5] The proteolytic activity of ADAMTS13 is located in its amino-terminal metalloprotease domain, but optimal enzyme activity requires cooperative interactions with other domains of the ADAMTS13 molecule.[6]

Deficiency of ADAMTS13 activity causes a life-threatening generalized disease, thrombotic thrombocytopenic purpura (TTP), which can be caused either by mutation of the ADAMTS13 gene (Upshaw-Schulman syndrome [USS]) or by acquisition of autoantibodies against the ADAMTS13 enzyme.[7] USS is often treated by prophylactic infusions of fresh-frozen plasma (FFP) as a source of ADAMTS13, but cryoprecipitate (CP) has also been effective.[8,9] On the other hand, for patients with acquired TTP, plasma exchange (PE) is the first-line treatment.[10] For PE treatment, cryosupernatant (CSP) is preferentially used in Canada, but FFP is used in many other countries, including Japan. However, it has not been firmly established which material is more favorable for PE.[11] In this context, immunoprecipitation using anti-VWF revealed that approximately 3% of ADAMTS13 in plasma is bound to VWF, with a stoichiometry of one ADAMTS13 molecule to 250 VWF monomeric subunits.[12] However, the characteristics of the VWF-ADAMTS13 complex in the plasma milieu remain unclear, as does the physiologic relevance of functional differences, if any, between the bound and unbound forms of ADAMTS13.

To address these issues and analyze the VWF-ADAMTS13 complex in the plasma milieu, we employed isoelectric focusing (IEF) analysis using a large-pore agarose-acrylamide composite gel. Using this method, we were able to visualize the VWF-ADAMTS13 complex. We found that in the plasma milieu, ADAMTS13 forms a complex with larger VWFMs, but is less likely to do so with smaller VWFMs (dimers and tetramers); the complex can be separated from its unbound counterpart by cryoprecipitation. Based on these observations, we hypothesize that the bound and unbound forms of ADAMTS13 possess functional differences with respect to the microvascular condition of the patient. Furthermore, we evaluated the functional differences between ADAMTS13 in CSP and CP, by testing their inhibitory effects on high shear stress–induced PLT aggregation (H-SIPA).

MATERIALS AND METHODS

Plasma samples

Anticoagulated blood containing 1/10 volume of 3.8% sodium citrate was collected from normal individuals and from patient with either Type 3 von Willebrand disease (T3-VWD) or USS (Patient USS-EE4). The citrated plasmas were then separated by centrifugation and stored at –80°C until use. T3-VWD plasma had less than 3% of the normal control levels of both VWF antigen and ristocetin cofactor. The USS-EE4 patient had plasma levels of both ADAMTS13 activity and antigen less than 0.5 and 0.1% of the normal control, respectively; the ADAMTS13 gene mutation was identified as 2259delA/2259delA.[8] Informed consent was obtained from all subjects.

Preparation of CSP and CP

FFP was prepared at Nara Red Cross Blood Center and stored in inventory at –30°C. However, FFP preparations beyond 1 year for the inventory were provided to us. These FFP preparations were then kept frozen at –80°C in our institution. Outdated FFP was then thawed overnight at 4°C, followed by centrifugation at 7000 × *g* for 30 minutes at 4°C. After centrifugation, the CSP was separated and kept frozen in aliquots at –80°C. For analysis of ADAMTS13 activity and VWF antigen, the CP was dissolved in one-fifth volume of 20 mmol/L Tris-buffered saline (TBS, pH 7.4) without rinsing.

For H-SIPA, the CP was rinsed with cold TBS containing 0.38% Na_3-citrate, 2 mmol/L benzamidine-HCl, 20 mmol/L 6-amino-*n*-caproic acid, and 0.02% NaN_3 and then centrifuged at 4°C. This procedure was repeated twice. Ultimately, the CP was dissolved in 1/10 volume of TBS containing 0.38% Na_3-citrate and then stored in aliquots at –80°C.

Purified plasma VWF and ADAMTS13

Purification of plasma VWF was performed essentially as previously described:[13] cryoprecipitation of outdated pooled FFP collected from normal volunteers, removal of fibronectin by gelatin-agarose affinity chromatography, precipitation with 40% saturated $(NH_4)_2SO_4$, and finally purification by size-exclusion chromatography in Sepharose 4B gel. Fractions eluted in the anterior half of the void volume of the Sepharose 4B column were pooled; the resulting protein consisted of higher VWF multimers and migrated as a single 250-kDa band on a sodium dodecyl sulfate (SDS)-5% polyacrylamide gel under reducing conditions.[13] After dialysis against TBS, the purified plasma-derived (pd)-VWF was kept frozen in aliquots at –80°C until use.

Purification of plasma ADAMTS13 was achieved using anti-ADAMTS13 monoclonal antibody (MoAb) A10 (IgG2b-κ)–coupled beads as recently described.[14] The epitope of A10 resides on the disintegrin-like domain of ADAMTS13.[15] Briefly, the CSP was prepared essentially as described above, from outdated FFP in the presence of two protease inhibitors (2 mmol/L benzamidine-HCl and 20 mmol/L 6-amino-*n*-caproic acid) and 0.02% NaN_3. The CSP was then applied to an A10-coupled column at 4°C and washed extensively. The ADAMTS13 bound to the column was eluted in two steps, first with 10% dimethyl sulfoxide (DMSO) and then with 40% DMSO. The ADAMTS13 eluted with 40% DMSO was pooled and concentrated and then purified by size-exclusion chromatography on a Superdex HR10 column. The purified

pd-ADAMTS13 migrated on a SDS-5% polyacrylamide gel as a single 170-kDa band before reduction and a single 190-kDa band after reduction; specific activity was 300 units/mg.[14] One unit of ADAMTS13 activity was defined as the amount contained in 1 mL of pooled normal plasma.

Assays for ADAMTS13 and VWF

The ADAMTS13 activity and antigen were measured with a chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA)[16] and an in-house sandwich ELISA using two MoAbs, respectively.[17] The VWF antigen was determined with a sandwich ELISA using a rabbit polyclonal anti-human VWF antibody (Dako Cytomation, Kyoto, Japan).[18] A value of 100% of the ADAMTS13 activity and antigen were defined as the amount in the pooled normal human plasmas, which were prepared from a total of 40 normal volunteers, consisting of 10 individuals from each ABO blood group.

Preparation of ADAMTS13-depleted plasma

The ADAMTS13-depleted (dp) plasma was prepared from the whole FFP using an A10-agarose column equilibrated with TBS containing a cocktail of protease inhibitors (5 mmol/L benzamidine, 2 mmol/L phenylmethanesulfonyl fluoride, and 20 mmol/L 6-amino-*n*-caproic acid, each final concentration) at room temperature. Flowthrough fractions were monitored with ADAMTS13 activity and antigen; both the values indicated less than 0.5 and 0.1% of the normal, respectively and were dialyzed with TBS containing 0.38% Na_3-citrate and stored in aliquots at –80°C.

IEF using an agarose-acrylamide composite gel

IEF gel plate was assembled with two glass plates and 1-mm-thick plastic spacers. Four grams of sucrose and 0.3 g of agarose (final 0.75%, agarose IEF, GE Healthcare Bio-Science AB, Uppsala, Sweden) were mixed with 34.2 mL of distilled water. The mixture was dissolved by microwave oven and kept at 56°C. Then, 1.67 mL of 30% acrylamide-bisacrylamide (final 1.25%), 1.67 mL of distilled water, 2.5 mL of 40% carrier ampholyte (Pharmalyte 3-10, GE Healthcare Bio-Science AB), 0.27 mL of ammonium peroxodisulfate (22.8 mg/mL), and 0.01 mL of *N,N,N′,N′*-tetramethylethylenediamine were added to this mixture. The mixture was poured into the IEF gel plate quickly and left for more than 1 hour at room temperature followed by 4°C overnight.

The IEF gel was placed on the Multiphor apparatus (GE Healthcare Bio-Science AB) equilibrated at 10°C. The electrode strips were prepared using 0.5 mol/L acetic acid at the anode and 0.5 mol/L sodium hydroxide at the cathode. The electrical conditions used for IEF were the first 30 minutes at a maximum of 100 V, 5 mA, and 15 W; then 60 minutes at a maximum of 200 V, 10 mA, and 6 W; and finally 90 minutes at a maximum of 1500 V, 15 mA, and 6 W. After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membrane.

Iodoacetamide effect on complex of ADAMTS13 and VWF in plasma milieu

Recent studies have indicated that free thiols exposed in ADAMTS13 play an important role to regulate thiol-disulfide exchange of VWF under a high shear stress. Furthermore, blocking these active thiols decreases ADAMTS13 activity in cleaving UL-VWFM under flow conditions.[19] We evaluated the effect of iodoacetamide (IAA), which blocks the free thiols and prevents the formation of a covalent complex through disulfide bonds. For this experiment, each reagent of ADAMTS13-dp plasma, purified pd-ADAMTS13, and pd-VWF was separately treated with or without 100 mmol/L IAA before mixing for 30 minutes at room temperature. The mixture of these three reagents was exposed to a high shear stress generated by a vortex mixer at 3200 rpm for 5 minutes. The final concentration of each reagent in this mixture (a total of 130 μL) was 60 μg/mL for pd-VWF, 2.3 μg/mL for purified pd-ADAMTS13, and 65 μL for ADAMTS13-dp plasma.

Two-dimensional gel electrophoresis using either polyacrylamide gel electrophoresis or agarose

In some experiments, after IEF the two-dimensional gel electrophoresis was performed using either SDS-5% polyacrylamide gel electrophoresis (PAGE) under reducing conditions or SDS-0.9% agarose gel electrophoresis under nonreducing conditions. The former was used for an analysis of ADAMTS13 antigen and the latter for VWF multimer patterns. In both instances, the separated proteins were electrophoretically transferred to polyvinylidene fluoride membrane or nitrocellulose membrane, and then the blot proteins were immunoreacted with anti-ADAMTS13 MoAb (WH2-11-1, an epitope residing on the fourth thrombospondin Type 1 domain of ADAMTS13)[20] or rabbit polyclonal anti-human VWF antibody and then visualized by chemiluminescent detection kits (Perkin-Elmer Life Science, Inc., Boston, MA).

H-SIPA in the absence of ADAMTS13

To reproduce PLT aggregation assumed to be occurring in TTP patients, H-SIPA at a constant shear rate of 108 dynes/cm^2 was measured with an argon laser-assisted cone PLT aggregometer (Toray Medical, Tokyo, Japan),[21] using a mixture of normal washed PLTs (300×10^9/L, final), ADAMTS13-dp plasma (29% vol/vol, final), and the purified pd-VWF (250% of the normal plasma, final).

For this assay, normal washed PLTs were prepared and suspended in a Hepes-Tyrode buffer (pH 7.3) containing 1.8 mmol/L $CaCl_2$.[22] The mixture with a total volume of

400 μL was preincubated at 37°C for 5 minutes, and then H-SIPA was measured for 6 minutes. The maximum PLT aggregation was seen in the absence of any additives, and the minimum or nonspecific PLT aggregation was determined in the presence of anti-VWF MoAb NMC-4 (10 μg IgG/mL, final), which totally blocks the VWF binding to PLT GPIb.[13]

For assessment of the inhibitory effect of various forms of pd-ADAMTS13 to H-SIPA, they were spiked into the above-mentioned assay mixtures and incubated for 5 minutes at 37°C before measurement. H-SIPA was measured at room temperature and completed within 2.5 hours after blood collection. The inhibition rate of H-SIPA was calculated in the following formula: Inhibition rate (%) = [1 – (% light transmittance of tested sample/% light transmittance of control)] × 100. These data were expressed as the mean ± SD. We calculated the inhibition rate in two points at 140 and 340 seconds after the initiation of H-SIPA. Comparison between these two points was tested for significance using paired t test using computer software (StatView, SAS Institute, Inc., Cary, NC). A p value of less than 0.05 was considered significant.

RESULTS

ADAMTS13 and VWF on IEF agarose-acrylamide composite gels

We detected the purified pd-ADAMTS13 (15 ng) as one band at pI 4.9 to 5.6 (median, 5.4) using anti-ADAMTS13 MoAb (WH2-11-1) on IEF agarose-acrylamide composite gels (Fig. 1, left panel). Next, we analyzed various amounts (1-10 μL) of normal citrated plasma and found that ADAMTS13 antigen in the plasma milieu could also be detected as a major band at pI 4.9 to 5.6, as in the case of purified pd-ADAMTS13. In plasma, however, two additional bands of ADAMTS13 antigen were also detected: one was composed of a cluster of blurred bands at pI 5.8 to 6.7, and the other consisted of two clear bands at pI 7.0 or 7.5. In T3-VWD plasma, two groups of ADAMTS13 bands, pI 4.9 to 5.6 and 5.8 to 6.7, were detected, but the bands at pI 7.0 or 7.5 were totally absent (Fig. 1, right panel). T3-VWD plasma lacks VWF antigen; therefore, the two groups of bands at pI 4.9 to 5.6 and 5.8 to 6.7 appear to exist independently of the presence of plasma VWF. Conversely, we assumed that the bands at pI 7.0 or 7.5 represented a complex with VWF that exists within the plasma milieu. The bands at pI 7.0 or 7.5 were also detected after mixing FFP with 1 mol/L NaCl (final), excluding the possibility that the complex is formed by an ionic linkage (data not shown).

Generation of the pI 7.0 of 7.5 band of ADAMTS13 complex with VWF

Next, we performed the mixing experiments shown in Fig. 2A. T3-VWD plasma spiked with purified pd-VWF yielded a new band at pI 7.5. USS-EE4 plasma initially

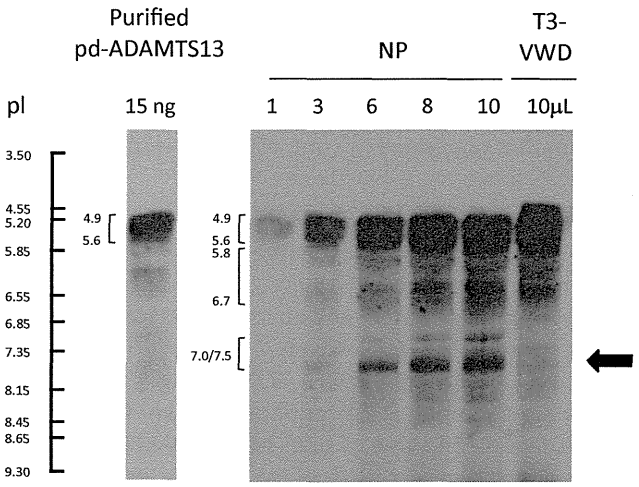

Fig. 1. Separation of plasma ADAMTS13 by IEF in healthy control and patient with T3-VWD. Purified pd-ADAMTS13, normal plasma (NP), and T3-VWD plasma were subjected to IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). Purified pd-ADAMTS13 (15 ng) was detected as one band at pI 4.9 to 5.6 (median, 5.4; left panel). In various amounts (1-10 μL) of NP, ADAMTS13 antigen was detected as a major band at pI 4.9 to 5.6. Two additional groups of bands of ADAMTS13 antigen were also detected: pI 5.8 to 6.7 and pI 7.0 or 7.5. In T3-VWD, the ADAMTS13 band groups of pI 4.9 to 5.6 and 5.8 to 6.7 were detected, but the band of pI 7.0 or 7.5 was barely detectable (right panel). Arrow indicates the VWF-ADAMTS13 complex.

lacked three groups of ADAMTS13 bands (pI 4.9-5.6, 5.8-6.7, and 7.0/7.5), but once that plasma was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When T3-VWD and USS-EE4 plasma samples were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13.

ADAMTS13 (pI 7.5) is a noncovalent complex with VWF in the plasma milieu

We next evaluated the effects of IAA, which blocks free thiols and prevents the formation of disulfide bond–mediated covalent complexes, under high shear stress in a vortex mixer. As shown in Fig. 2B, the band at pI 7.5, representing the VWF-ADAMTS13 complex, was generated irrespective of the presence of IAA. When pd-VWF was spiked into this mixture, the density of the band at pI 7.5 increased. These results indicate that in our experiments, formation of the VWF-ADAMTS13 complex does not depend upon disulfide bond bridges.

ADAMTS13 is present in plasma in complex with a large VWFM

As shown in Fig. 3, IEF gel analysis of normal plasma revealed ADAMTS13 as three groups of bands (pI 4.9-5.6,

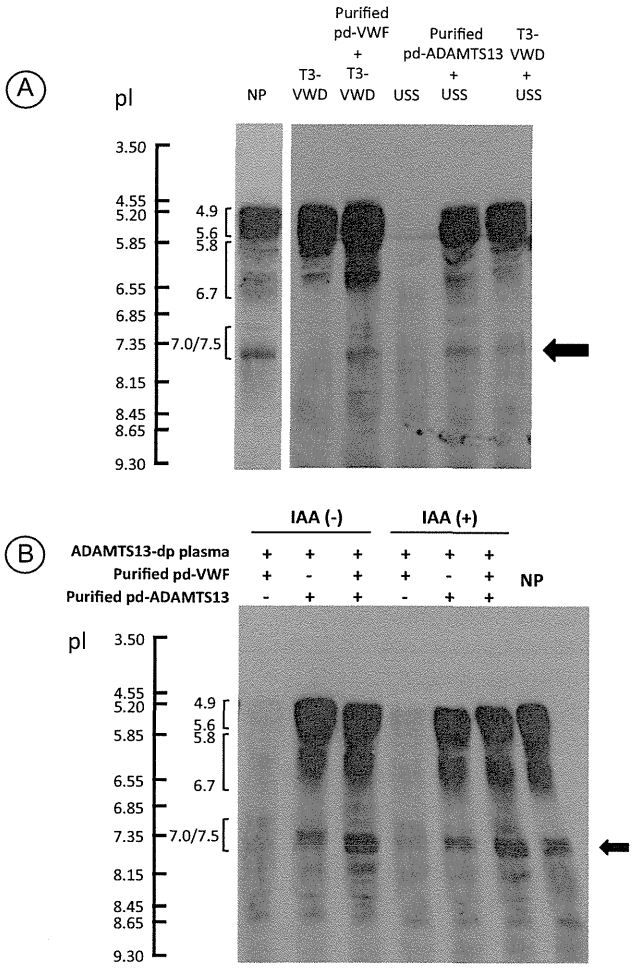

Fig. 2. Spiking experiments and effect of IAA treatment on VWF-ADAMTS13 complex formation. (A) VWF-ADAMTS13 complex formation was analyzed by IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). Plasma of T3-VWD spiked with the purified pd-VWF generated a new band at pI 7.5. When plasma from a patient with USS was spiked with purified pd-ADAMTS13, the band at pI 7.5 clearly appeared. When plasmas from T3-VWD and USS patients were mixed together, the band at pI 7.5 also appeared, confirming that it represents a complex of VWF and ADAMTS13. (B) Purified pd-VWF (3 μg), pd-ADAMTS13 (200 ng), and ADAMTS13-dp plasma were treated for 30 minutes at room temperature with 100 mmol/L IAA. ADAMTS13-dp plasma was mixed with purified pd-VWF (final concentration, 60 μg/mL) and/or pd-ADAMTS13 (final concentration, 2.3 μg/mL). Mixtures were exposed to high shear stress in a vortex mixer at maximum speed (3200 rpm) for 5 minutes. The VWF-ADAMTS13 complex, represented by the band at pI 7.5, is generated irrespective of the presence of IAA. Purified pd-VWF spiked into this mixture increased the density of this band. Arrow indicates the VWF-ADAMTS13 complex.

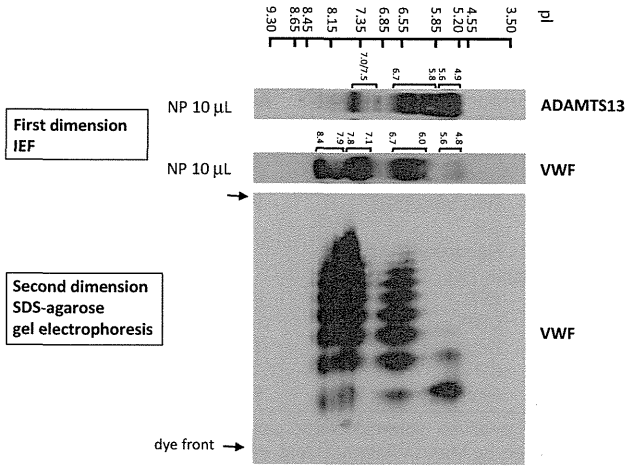

Fig. 3. Two-dimensional analysis of VWF in NP. Ten microliters of NP was subjected to IEF and immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1; upper panel) and polyclonal anti-VWF antibody (middle panel). IEF gel of NP was subjected to a second dimension of electrophoresis on a SDS-0.9% agarose gel and immunoblotting with anti-VWF polyclonal antibody. In the lower panel, the upper arrowhead indicates the start point of two-dimensional SDS-0.9% agarose gel electrophoresis; the lower arrowhead indicates the position of the dye front at the termination of electrophoresis. VWF antigen in NP was separated into four series of pI bands (4.8-5.6, 6.0-6.7, 7.1-7.8, and 7.9-8.4) by IEF (middle panel), whereas ADAMTS13 antigen was separated into three series of pI bands (4.9-5.6, 5.8-6.7, and 7.0/7.5; upper panel). Two-dimensional analysis of normal plasma by IEF followed by SDS-0.9% agarose gel electrophoresis (lower panel) revealed that plasma ADAMTS13 is primarily in complex with larger VWF multimers and to a lesser extent with smaller VWF multimers.

5.8-6.7, and 7.0/7.5; also see Fig. 1), and VWF antigen was largely separated into four series of bands: pI 4.8 to 5.6 (trace), 6.0 to 6.7, 7.1 to 7.8, and 7.9 to 8.4 (Fig. 3, middle).

Since two ADAMTS13 bands with pI 4.9 to 5.6 and 5.8 to 6.7 were seen in T3-VWD plasma (Fig. 1), both bands appeared to be present in plasma irrespective of the presence of VWF. Further, two-dimensional analysis of normal plasma (IEF gel followed by SDS-0.9% agarose gel electrophoresis) confirmed that ADAMTS13 forms a complex with a larger VWFM with pI 7.1 to 7.8, but less likely with a smaller VWFM (dimers and tetramers) with pI 4.8 to 5.6 (Fig. 3, lower panel).

Amounts of ADAMTS13 and VWF in CP and CSP

Gill and colleagues[23] reported that the level of VWF antigen in plasmas from normal individuals with blood group O is significantly lower than that in plasmas with non-O blood groups. Further, Feys and colleagues[12] indicated that ADAMTS13 is bound to VWF with a stoichiometry of one ADAMTS13 molecule to 250 VWF

TABLE 1. ADAMTS13 activity and VWF antigen levels in plasma products*

Plasma products	All (n = 120)	Blood type A (n = 30)	O (n = 30)	B (n = 30)	AB (n = 30)	Overall p value
ADAMTS13 activity (%)						
FFP	81 ± 16	84 ± 17	77 ± 15	80 ± 16	83 ± 13	NS
CSP	71 ± 14	72 ± 14	69 ± 17	70 ± 12	72 ± 13	NS
Recovery (%)†	92.7 ± 3.7	92.9 ± 3.6	95.2 ± 2.1	91.4 ± 3.9	91.2 ± 3.5	<0.01[a]
CP	5.6 ± 2.8	5.4 ± 2.4	3.5 ± 1.6	6.5 ± 2.9	6.9 ± 2.8	<0.01[a]
Recovery (%)†	7.3 ± 3.7	7.1 ± 3.6	4.8 ± 2.1	8.6 ± 3.9	8.8 ± 3.5	<0.01[a]
VWF antigen (%)						
FFP	124 ± 46	121 ± 49	80 ± 24	144 ± 32	150 ± 38	<0.01[b]
CSP	16 ± 7	15 ± 7	11 ± 3	19 ± 5	19 ± 6	<0.01[c]
Recovery (%)†	14.0 ± 2.6	12.7 ± 2.5	14.6 ± 2.2	14.6 ± 2.9	14.2 ± 2.4	<0.05[d]
CP	98 ± 35	100 ± 37	64 ± 18	112 ± 22	116 ± 32	<0.01[e]
Recovery (%)†	86.0 ± 2.6	87.3 ± 2.5	85.4 ± 2.2	85.4 ± 2.9	85.8 ± 2.4	<0.05[d]

* Overall p values were calculated using the Kruskal-Wallis H test. Significant differences between four groups (overall $p < 0.05$) were further analyzed by Mann-Whitney U test with Bonferroni correction.
† Recovery was calculated as the level in CP or CSP divided by the total (sum of levels in CP and CSP).
[a] $p < 0.01$ between O and B, AB, <0.05 between O and A.
[b] $p < 0.01$ between O and A, B, AB, <0.05 between A and B.
[c] $p < 0.01$ between O and A, B, AB, <0.01 between A and B, <0.05 between A and AB.
[d] $p < 0.05$ between A and O, B, AB.
[e] $p < 0.01$ between O and A, B, AB.
NS = no significant difference ($p \geq 0.05$).

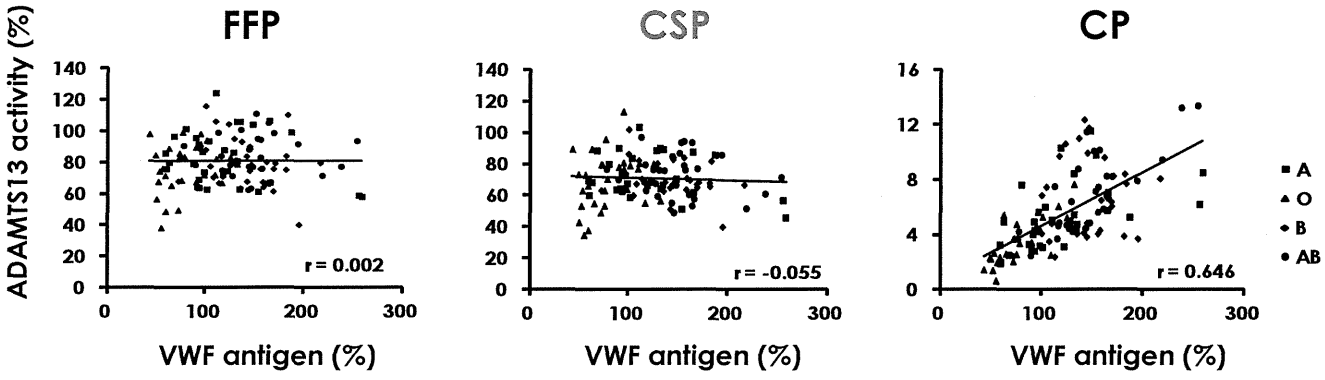

Fig. 4. Correlation between VWF antigen and ADAMTS13 activity in FFP, CSP, and CP. The correlation between ADAMTS13 activity and VWF antigen in FFP, CSP, and CP was analyzed. In FFP and CSP, there is no correlation between ADAMTS13 activity and VWF antigen. On the other hand, a significant correlation was observed between the two variables in CP ($r = 0.646$, $p < 0.01$).

monomeric subunits. Taking these two reports together, it is conceivable that the amount of a complex of ADAMTS13 and VWF in CP could be influenced by the ABO blood groups.

We analyzed ADAMTS13 activity and VWF antigen in FFP, CSP, and CP from 120 normal volunteers, with 30 individuals of each ABO blood type (A, B, O, and AB). The recovery rates of ADAMTS13 activity and VWF antigen in CP or CSP were expressed as the level in CP or CSP divided by the sum of the levels in both (CP + CSP). As summarized in Table 1, a mean of 7.3% (range, 4.8%-8.8%) of plasma ADAMTS13 activity was recovered in CP, whereas a mean of 92.7% (range, 91.2%-95.2%) remained in CSP. The amounts of ADAMTS13 remaining in CP from A, O, B, and AB plasmas were 5.4 ± 2.4, 3.5 ± 1.6, 6.5 ± 2.9, and 6.9 ± 2.8%, respectively; the amount of ADAMTS13 activity in CP was significantly lower in blood group O than in other blood groups. On the other hand, a mean of 86.0% (range, 85.4%-87.3%) of plasma VWF antigen was recovered in CP, whereas a mean of 14.0% (range, 12.7%-14.6%) remained in CSP. The amounts of VWF antigen in FFP, CP, and CSP from blood group O were significantly lower than in samples from other blood groups. The recovery rate of VWF antigen in CP was significantly higher in blood group A than in other blood groups.

As shown in Fig. 4, we analyzed the correlation between the levels of ADAMTS13 activity and VWF antigen in three plasma preparations with the Spearman rank test. We did not find a significant correlation between VWF antigen in FFP and ADAMTS13 activity in either FFP or CSP. In contrast, we did observe a significant correlation between ADAMTS13 activity in CP and VWF antigen in

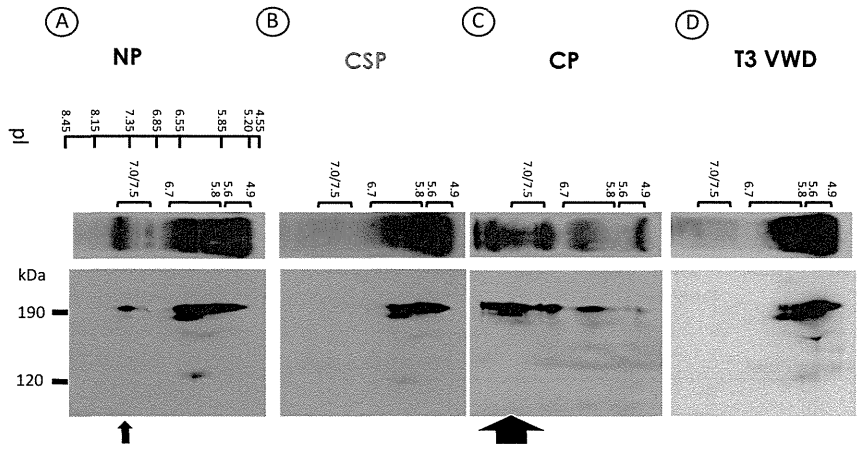

Fig. 5. ADAMTS13 in plasma fractions separated by IEF followed by SDS-PAGE. Normal plasma (NP), CSP, CP, and T3-VWD plasmas were subjected to IEF (upper panel). ADAMTS13 separated by IEF was subjected to a second dimension of electrophoresis by SDS-5% PAGE under reducing conditions and then to immunoblotting with anti-ADAMTS13 MoAb (WH2-11-1). (A) All three groups of ADAMTS13 bands (4.9-5.6, 5.8-6.7, and 7.0/7.5) in NP appeared as a 190-kDa by SDS-5% PAGE. Arrow indicates the VWF-ADAMTS13 complex. (B, D) The ADAMTS13 band at pI 7.0 or 7.5 was completely absent in CSP, almost indistinguishable to the case in T3-VWD plasma. (C) In CP, two faint bands with pI ranges of 4.9 to 5.6 and 5.8 to 6.7 and several strong bands with pI beyond 7.0 were detected. Arrow indicates the VWF-ADAMTS13 complex.

FFP ($r = 0.646$, $p < 0.01$; Fig. 4, right). These results indicate that the decreased level of ADAMTS13 activity in CP of blood group O was correlated with the low level of VWF antigen.

Cryoprecipitation efficiently removes the VWF-ADAMTS13 complex from plasma

To determine whether cryoprecipitation can remove the VWF-ADAMTS13 complex from plasma, we performed two-dimensional analysis (IEF followed by SDS-5% PAGE) under reducing conditions. As shown in Fig. 5A, all three groups of ADAMTS13 bands (pI 4.9-5.6, 5.8-6.7, and 7.0/7.5) in normal plasma migrated mainly as a 190-kDa band in SDS-5% PAGE, indicating that all three groups of bands included ADAMTS13. In CSP, however, the pI 7.0 or 7.5 band of ADAMTS13 was totally absent, almost indistinguishable from the case of T3-VWD plasma (Figs. 5B and 5D). Furthermore, when CSP was spiked with purified pd-VWF, a new band with pI 7.5 was generated (data not shown), indicating that ADAMTS13 in CSP can bind to higher molecular weight VWFMs and form a complex, as is the case in FFP.

In CP, we observed two faint bands with pI ranges of 4.9 to 5.6 and 5.8 to 6.7, and also several strong bands with pI greater than 7.0 (Fig. 5C).

Down regulation of H-SIPA with purified pd-ADAMTS13, CP, and CSP

In H-SIPA using a mixture of normal washed PLTs, ADAMTS13-dp plasma, and purified pd-VWF, maximum PLT aggregation (approx. 70% light transmittance) was achieved in the absence of ADAMTS13 (Fig. 6A). Under this condition, purified pd-ADAMTS13 spiked into the mixture inhibited H-SIPA in a dose-dependent manner at ranges of 5% to 20% of ADAMTS13 activity, but this effect reached a plateau (approx. 20% light transmittance) at the ranges from 50% to 500% of ADAMTS13 activity (Fig. 6A). Addition of NMC-4 almost totally blocked the PLT aggregation (approx. 3% light transmittance).

Further, ADAMTS13 in both FFP and CSP from normal individuals inhibited H-SIPA in a dose-dependent manner at the ranges of 5% to 20% of ADAMTS13 activity (Figs. 6B and 6C), comparable to the effect of purified pd-ADAMTS13.

On the other hand, ADAMTS13 in CP did not clearly inhibit H-SIPA at the initial phase before 140 seconds, even at 20% of ADAMTS13 activity. However, at the later phase of H-SIPA, the aggregation curves were uniformly reversed at a final concentration of 5% to 20% of ADAMTS13 activity. Consequently, the maximum PLT aggregation at the endpoint at 340 seconds was almost indistinguishable from that of FFP or CSP. Thus, the inhibition rates (%) in CP at two time points (140 and 340 sec) with two different final concentrations (5 and 20%) of ADAMTS13 activity were measured in each three times at the same occasion, and the results were the following: $20.5 \pm 14.0\%$ (at 140 sec) versus $46.9 \pm 11.3\%$ (at 340 sec; $p = 0.012$) in the presence of 5% ADAMTS13 activity and $57.7 \pm 5.9\%$ (at 140 sec) versus $85.7 \pm 2.7\%$ (at 340 sec; $p = 0.004$) in the presence of 20% ADAMTS13 activity (figure not shown).

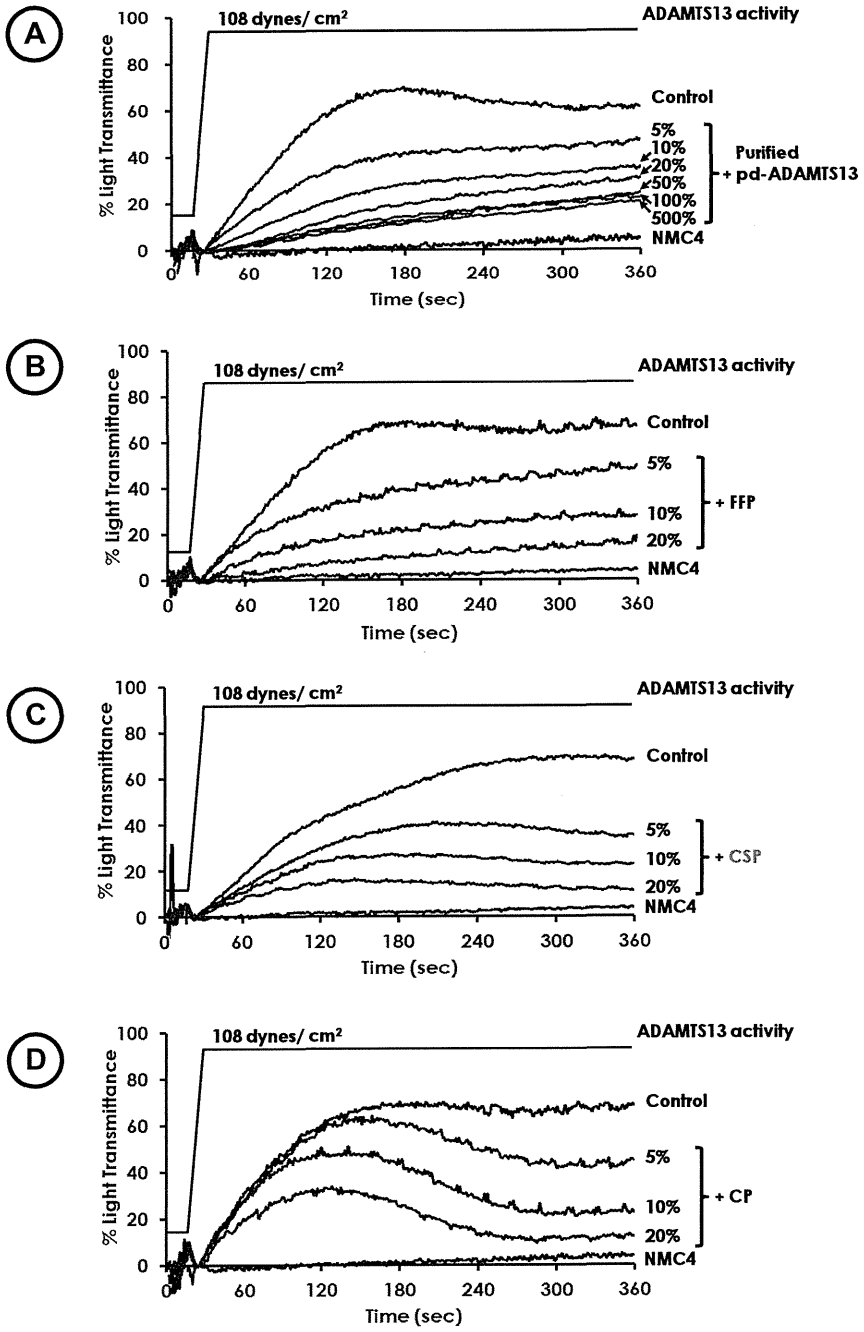

Fig. 6. Inhibitory effect of ADAMTS13 on H-SIPA. (A) The purified pd-ADAMTS13 inhibits H-SIPA in a dose-dependent manner at ranges of 5% to 20% of ADAMTS13 activity in the mixture. The inhibition reaches a plateau (approx. 20% light transmittance) at ranges of 50% to 500% of ADAMTS13 activity. (B, C) ADAMTS13 in both FFP and CSP from normal individuals exhibited dose-dependent inhibition of H-SIPA at the ranges of 5% to 20% of ADAMTS13 activity. (D) ADAMTS13 in CP did not clearly inhibit H-SIPA at the initial phase at less than 10% of ADAMTS13 activity. The inhibition of PLT aggregation was found at the ranges of 5% to 20% of ADAMTS13 activity; at the later phase of H-SIPA the maximum aggregation at the endpoint was almost the same as in FFP and CSP.

DISCUSSION

Using IEF analysis with a large-pore agarose-acrylamide composite gel, we have shown that ADAMTS13 in the plasma milieu is present in a complex with larger VWFMs, but is less likely to form complexes with smaller VWFMs (dimers and tetramers). Thus, cryoprecipitation followed by centrifugation could efficiently separate the two forms of ADAMTS13, with the VWF-bound ADAMTS13 in CP and the free ADAMTS13 and ADAMTS13 bound to smaller VWFMs in CSP. In support of these results regarding coprecipitation of ADAMTS13 and VWF, the ADAMTS13 activity levels we observed were closely correlated with VWF antigen levels in CP ($r = 0.646$), but not in either CSP ($r = -0.055$) or FFP ($r = 0.002$; Fig. 4). In addition, we observed no difference among blood groups with respect to the recovery rate of VWF antigen in CP, but the VWF antigen level in CP was lower in blood group O than in the other blood groups. As a result, both the ADAMTS13 activity and the VWF antigen levels in CP were significantly lower in blood group O than in non-O blood groups (Table 1). Further, we determined that approximately 95% of the original ADAMTS13 activity in FFP is recovered after cryoprecipitation; approximately 93% of the recovered ADAMTS13 activity remained in CSP, whereas 7% was found in CP. This relative distribution of ADAMTS13 in FFP and CSP was consistent with previous reports.[24-26]

Evidence that the pI 7.0 or 7.5 band is a complex of VWF and ADAMTS13 is clearly provided by the following observations: 1) plasmas from both VWF antigen–defective T3-VWD and ADAMTS13 antigen–defective USS patients lacked the bands at pI 7.0 or 7.5; 2) an equal mixture of plasmas from T3-VWD and USS generated the bands at pI 7.0 or 7.5; and 3) CSP prepared from normal plasma lacked the bands at pI 7.0 or 7.5, whereas CSP spiked with purified VWF regenerated these bands. On the other hand, we assume that the proteins in the two other band groups (pI 4.9-5.6 and 5.8-6.7) are less involved

in complex formation with VWF, because both band groups are present in T3-VWD plasma. Furthermore, because pd-ADAMTS13 purified from pooled normal plasmas has only one band with pI 4.9 to 5.6,[14] the pI 5.8 to 6.7 band might represent a complex with proteins other than VWF. This speculation originates from the observation that ADAMTS13 can bind in vitro to a soluble form of CD36[27] and Lys-plasminogen.[28]

In a previous study, immunoprecipitation method using anti-VWF was used to show that approximately 3% of the total in plasma ADAMTS13 is bound to VWF.[12] By contrast, in our IEF gel analysis, coupled with densitometry, we observed that the amount of VWF-bound ADAMTS13 in plasma appeared to be much lower than the amount of unbound ADAMTS13, but greater than the 3% of total ADAMTS13 (Fig. 1).[12] This discrepancy might be attributable to differences in the experimental designs employed in these studies.

The mechanism by which ADAMTS13 binds to VWF in the plasma milieu is a critical issue that remains to be addressed. Because Fujikawa and coworkers[29] succeeded in purifying ADAMTS13 from a commercial concentrate of Factor VIII and VWF, prepared from CP, such concentrates might contain the VWF-ADAMTS13 complex itself. After extensive fractionation, including fibrin-clot formation, ammonium sulfate precipitation, and sequential chromatography, the purified ADAMTS13 described in that study was free of VWF. However, in our experience, the VWF-ADAMTS13 complex in CP is not readily dissociated by size-exclusion chromatography in the presence of either 0.15 or 1 mol/L NaCl (data not shown). In fact, when we treated CP with 1 mol/L NaCl for 1 hour at room temperature before IEF, the bands at pI 7.0 or 7.5 persisted, indicating that no dissociation of VWF-ADAMTS13 complex had taken place under conditions of high ionic strength. These results may indicate that VWF binding to ADAMTS13 is independent of ionic strength. In this regard, McKinnon and colleagues[30] reported that the N-linked glycans of VWF exert a modulatory effect on the interaction with ADAMTS13 and that removal of the N-linked glycans from VWF increased its affinity for ADAMTS13 under static conditions. Furthermore, Yeh and coworkers[19] recently reported that ADAMTS13 possesses a disulfide bond–reducing activity that regulates shear-induced thiol-disulfide exchange. Therefore, one of the mechanisms underlying formation of VWF-ADAMTS13 complexes might involve disulfide-bond formation between ADAMTS13 and VWF. To address this issue, we investigated whether IAA, a blocker of free thiols, might prevent the formation of a disulfide bond–mediated covalent complex under high shear stress. We observed, however, that VWF-ADAMTS13 complex formation was unaffected by IAA treatment, suggesting that in CP, the amount of VWF-ADAMTS13 complex formed in a thiol-dependent fashion is marginal. This finding rules out a major role for disulfide bonds, but otherwise we have not elucidated the binding mechanism of VWF and ADAMTS13; this issue remains to be addressed in future studies.

PE is a first-line treatment for acquired TTP. For this purpose, either FFP or CSP is commonly used,[31] but the results regarding CP have been controversial.[32] At least one case of congenital TTP (USS) has been successfully treated with CP.[9] Therefore, it is important to determine whether there is a functional difference between bound and unbound ADAMTS13 and whether any such difference has physiologic relevance. Here we have clearly shown that CSP contains the unbound or less bound ADAMTS13, whereas CP contains much more bound ADAMTS13 and lower levels of its unbound counterpart. An authoritative determination regarding which form of ADAMTS13 more efficiently down regulates H-SIPA will be crucial in establishing the optimal treatment modality for TTP patients.

In most acquired TTP patients, plasma ADAMTS13 activity is less than 5% of normal. As a consequence, UL-VWFMs are not cleaved after secretion from endothelial cells and remain anchored to the cell surface in long strings.[33] Circulating PLTs adhere to these long strings, resulting in occlusive PLT thrombi. However, smaller VWFMs do not induce this spontaneous adhesion and aggregation of PLTs. Consequently, increased fluid shear stress is required to induce PLT aggregation in vitro.[2]

To reproduce the PLT aggregation generated in the microvasculature of ADAMTS13 activity–deficient TTP patients, here we employed an H-SIPA assay system that uses a mixture of washed normal PLTs and ADAMTS13-dp plasma spiked with purified pd-VWF to mimic TTP plasmas. In this assay, the purified pd-ADAMTS13 inhibited H-SIPA in a dose-dependent manner, reaching a plateau of 20% ADAMTS13 activity at final pd-ADAMTS13 concentrations up to 500%. Under the same experimental conditions, FFP, CSP, and CP inhibited H-SIPA in a dose-dependent manner to the same extent at the end points; in CP, however, the aggregation inhibition curves were different, and in fact no distinct inhibition was observed at the initial phase of PLT aggregation. This might be simply explained by the fact that the VWF concentration in the H-SIPA reaction mixtures was much higher than in CSP or FFP. Alternatively, the binary complex of ADAMTS13 and larger VWFMs might modulate a different phase of H-SIPA than unbound ADAMTS13, because CSP spiked with purified VWF readily generates the ADAMTS13–larger VWFM complex with pI 7.0 or 7.5. The larger VWFM is required in the earliest phase of PLT thrombi formation and high shear stress, but in the later phase the ADAMTS13–larger VWF complex embedded in the thrombi may play a role in regulating the size of the thrombi to prevent microvascular occlusion. Further studies are required to determine the functional differences between ADAMTS13 in CSP and CP.

In conclusion, our results indicated that both plasma products of FFP and CSP are effective in treatment of TTP. However, CSP may be more favorable for PE in acquired TTP patients: relative to FFP, CSP has a lower level of VWF and a comparable ADAMTS13 activity, but lower amounts of ADAMTS13–larger VWFM complex.

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CONFLICT OF INTEREST

YH, MH, AI, and KS have no conflict of interest. MM is a member of the clinical advisory board for Alexion Pharma. YF is a member of the clinical advisory boards for Baxter BioScience and Alexion Pharma.

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RATIO OF VON WILLEBRAND FACTOR PROPEPTIDE TO ADAMTS13 IS ASSOCIATED WITH SEVERITY OF SEPSIS

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ABSTRACT—Von Willebrand factor (VWF)–cleaving protease (ADAMTS13) cleaves ultralarge VWF (ULVWF) secreted from endothelium and by which is regulating its physiologic function. An imbalance between ULVWF secretion and ADAMTS13 level occurs in sepsis and may cause multiple organ dysfunction. We evaluated the association between the VWF-propeptide (VWF-pp)/ADAMTS13 ratio and disease severity in patients with severe sepsis or septic shock. In 27 patients with severe sepsis or septic shock and platelet count less than 120 000/μL, we measured plasma VWF, VWF-pp, and ADAMTS13 levels on hospital days 1, 3, 5, and 7. The VWF-pp/ADAMTS13 ratio was increased greater than 12-fold in patients with severe sepsis or septic shock on day 1 and remained markedly high on days 3, 5, and 7 compared with normal control subjects. The VWF-pp/ADAMTS13 ratio significantly correlated with Acute Physiology and Chronic Health Evaluation II score on days 1 and 5; Sepsis-related Organ Failure Assessment score on days 1, 3, and 5; maximum Sepsis-related Organ Failure Assessment score and tumor necrosis factor α level on days 1, 3, 5, and 7; and creatinine level on days 1, 5, and 7. Patients with greater than stage 1 acute kidney injury had significantly higher VWF-pp/ADAMTS13 ratio than patients without acute kidney injury. In summary, the VWF-pp/ADAMTS13 ratio was associated with disease severity in patients with severe sepsis or septic shock and may help identify patients at risk for multiple organ dysfunction by detecting severe imbalance between ULVWF secretion and ADAMTS13 level.

KEYWORDS—Sepsis, von Willebrand factor propeptide, ADAMTS13, multiple organ dysfunction

INTRODUCTION

Severe sepsis and septic shock result from the systemic host response to infection, including inflammation, coagulation, and changes in the vascular endothelium. Vascular endothelial activation, dysfunction, and injury facilitate leukocyte and platelet aggregation and aggravate inflammation and thrombosis (1). Von Willebrand factor (VWF) is a key marker of endothelial changes (2).

Von Willebrand factor is a multimeric glycoprotein that circulates in plasma and functions as a bridge between the subendothelial matrix and platelets. The subunit precursor proVWF (350 kd) is synthesized in the endothelium and contains signal peptide, VWF propeptide (VWF-pp), and VWF subunit. The proVWF is dimerized through disulfide bonds after removal of signal peptide in the endoplasmic reticulum. The proVWF dimers are transported to the Golgi apparatus, VWF-pp is cleaved, and additional disulfide bonds form between proVWF dimers to yield ultralarge VWF (ULVWF; size, >20,000 kd). Ultralarge VWF condenses into tubules and forms Weibel-Palade bodies. Ultralarge VWF and VWF-pp are stored in Weibel-Palade bodies in equimolar amounts on a subunit basis (3, 4).

Several inflammatory mediators, such as thrombin, histamine, and proinflammatory cytokines, including tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8), activate endothelial cells and induce Weibel-Palade body exocytosis (5, 6), causing cell surface expression of ULVWF and release of VWF-pp into the bloodstream. Because longer VWF is more active, and ULVWF causes spontaneous platelet aggregation and thrombosis, it is immediately cleaved by VWF cleaving protease after secretion, which is also known as a disintegrin-like and metalloprotease with thrombospondin type 1 motif, member 13 (ADAMTS13). This cleavage results in smaller and less adhesive plasma forms of VWF (7). In the absence of ADAMTS13, secreted ULVWF strings that are bound to endothelium are not cleaved but adhere to platelets, which bind to leukocytes and cause thrombosis and inflammation (8, 9).

Because an appearance of ULVWF in plasma has been demonstrated in patients with inadequate function of ADAMTS13, as in thrombotic thrombocytopenic purpura (TTP) or sepsis (10, 11), it may suggest an imbalance between ULVWF secretion and ADAMTS13 function. Plasma ULVWF may be a good marker to detect this imbalance, but it is technically difficult to determine ULVWF and quantify it. Furthermore, plasma ULVWF often cannot be detected in patients having

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this imbalance and developing organ failure, as in chronic relapsing TTP (12).

The mean or median levels of ADAMTS13 are decreased to 20% to 43% normal in sepsis (13–15). However, ADAMTS13 level less than 10% normal is enough to prevent the clinical manifestation of primary thrombotic microangiopathy in patients with congenital ADAMTS13 deficiency (16). This suggests that patients with sepsis have a high enough ADAMTS13 level to prevent thrombotic microangiopathy, but it may not be high enough to cleave all ULVWF secreted from endothelium during sepsis. Furthermore, multiple organ dysfunction in children with thrombocytopenia was resolved by restoring ADAMTS13 activity by plasma exchange (17). Therefore, both decreased ADAMTS13 level and the imbalance between ULVWF secretion and ADAMTS13 activity may cause microvascular thrombosis formation in sepsis. If so, it may be clinically relevant to measure the imbalance between ULVWF secretion and ADAMTS13 activity.

The VWF-pp is secreted in equimolar amounts to the total subunits of secreted ULVWF and more rapidly cleared from the circulation than VWF (half-life: VWF-pp, 3 h; VWF, 12 h) (5). Therefore, we hypothesized that VWF-pp level may reflect ULVWF secretion and that the VWF-pp/ADAMTS13 ratio may be a sensitive and real-time measure of imbalance between ULVWF secretion and plasma ADAMTS13 level. Higher VWF-pp/ADAMTS13 ratio may reflect insufficient control of VWF multimer size, and this may accelerate microvascular thrombus formation, inflammation, and organ failure.

The purpose of this study was to investigate whether the VWF-pp/ADAMTS13 ratio is associated with disease severity in patients with severe sepsis or septic shock. Although there have been several previous studies about VWF, VWF-pp, and ADAMTS13 levels in patients with severe sepsis or septic shock, limited information is available about the time course of these levels simultaneously measured. We determined the time course of the levels of VWF, VWF-pp, and ADAMTS13 during the early phase of sepsis.

MATERIALS AND METHODS

Patients

From January 2008 to December 2009, all patients treated at the intensive care unit of the Department of Emergency and Critical Care Medicine, Nara Medical University Hospital, was considered for the study. Inclusion criteria for the study were (i) severe sepsis or septic shock as defined by published guidelines (18) and (ii) platelet count less than 120 000/μL. Exclusion criteria were (i) patients younger than 18 years, (ii) pregnancy, (iii) medical history of chronic renal failure (stage 5 chronic kidney disease) (19) or chronic liver disease (20), (iv) cardiopulmonary arrest, (v) other hematologic disorders that may lower the platelet count such as TTP, and (vi) malignancy. There were 27 patients included in the study. This study protocol was approved by the institutional review board of Nara Medical University Hospital. Written informed consent was obtained from enrolled patients or family members.

Evaluation

Clinical information was collected including age, sex, diagnosis, serum creatinine level, and survival status at 28 days after admission. Survivors were defined as patients who were alive 28 days after admission, and nonsurvivors were patients who died within 28 days after admission. The severity of disease and organ failure were assessed with Acute Physiology and Chronic Health Evaluation II (APACHE II) score (21) and Sepsis-related Organ Failure Assessment (SOFA) score (22) at days 1 (on admission), 3, 5, and 7 after admission. Maximum SOFA (Max SOFA) score was defined as the Max SOFA score during the clinical course at any time on day 28 or less. Acute kidney injury (AKI) stage was assessed by the criteria of the Acute Kidney Injury Network Working Group (23).

Assays

Citrated blood samples were obtained from patients who met the inclusion criteria on admission to the intensive care unit (day 1) and days 3, 5, and 7. Blood samples were centrifuged at 1,500*g* for 10 min in a cooled centrifuge immediately after drawing, and aliquots of plasma were stored at −80°C until assayed. Blood samples were obtained from 15 healthy volunteers (nine men and six women; age range, 23–55 years [mean, 40 years]) and pooled for ADAMTS13, VWF-pp, and VWF assays as the normal controls being 100%.

Activity of ADAMTS13 was assayed using a commercial kit (Kainos Laboratories, Inc, Tokyo, Japan). The plasma level of VWF-pp was measured with an enzyme-linked immunosorbent assay kit (Sanquin, Amsterdam, the Netherlands). Levels of IL-6 (R&D Systems Inc, Minneapolis, Minn), TNF-α (R&D Systems Inc), and VWF (Dako, Glostrup, Denmark) were measured.

Data analysis

Data analysis was performed with statistical software (SPSS, Inc, Armonk, NY; and GraphPad, San Diego, Calif). Data are reported as mean ± SD or median with interquartile range. The Shapiro-Wilk test was used to evaluate normality of data. Groups were compared with *t* test or Mann-Whitney *U* test, and the relation between two variables was evaluated with Spearman rank correlation. Statistical significance was defined by $P \leq 0.05$ for 2-sided tests.

RESULTS

Most patients were men, and the most common diagnosis was intra-abdominal infection (Table 1). Most patients (20 of 27 patients) were survivors; one patient with acute abdomen died on day 6, and the other 26 patients completed blood collection until day 7. All measurements did not differ between male and female patients. There were no differences between survivors and nonsurvivors in APACHE II score, SOFA score, serum creatinine level, platelet count, and fibrin degradation product level (data not shown).

In patients with severe sepsis and septic shock, the mean VWF level was high on day 1, and there was no significant change in mean VWF level from day 1 to day 7 (Table 2). The VWF level did not differ between survivors and nonsurvivors (data not shown). The level of VWF did not correlate with any clinical scores or laboratory markers (data not shown).

The mean VWF-pp level was high on day 1, remained high but decreased significantly from day 1 to day 3, and remained high from day 3 to day 7 (Table 2). There were no differences in mean VWF-pp level between survivors and nonsurvivors (data not shown). The levels of VWF-pp were correlated significantly with SOFA score on days 1, 3, and 5; with Max SOFA at days 5 and 7; and with TNF-α level on day 1 (Table 3).

The mean level of ADAMTS13 was significantly lower in patients on day 1 than normal controls, and the mean level of ADAMTS13 increased in patients significantly from day 1 to day 3 and from day 3 to day 5 (no difference between values on days 5 and 7) (Table 2). The mean level of ADAMTS13 was significantly higher in survivors than in nonsurvivors on days 1, 5, and 7 but not on day 3 (Table 2). The levels of ADAMTS13 correlated negatively with APACHE II score on days 1 and 5; SOFA score on day 5; Max SOFA score on days 1, 3, and 5; TNF-α on day 5; and IL-6 and creatinine levels on day 7 (Table 3).

The mean VWF-pp/ADAMTS13 ratio was 12-fold greater in patients on day 1 than normal control subjects, and the mean ratio decreased significantly in patients from day 1 to day 3

TABLE 1. **Clinical and laboratory findings on admission in patients with severe sepsis or septic shock***

	Total	Male (n = 16)	Female (n = 11)
Age, y	70 ± 16	71± 14	68 ± 19
APACHE-II score	21.0 ± 7.3	22.0 ± 7.4	24.0 ± 4.6
SOFA score	11.1 ± 3.3	12.3 ± 3.2	11.3 ± 2.8
SIRS score†	20	12	8
Survivors	20 (74)	13 (81)	7 (64)
Diagnosis			
Intra-abdominal infection	18 (67)	11 (69)	7 (64)
Urinary tract infection	3 (11)	1 (6.3)	2 (18)
Pneumonia	2 (7)	2 (13)	0
Burn wound sepsis	2 (7)	0	2 (18)
Necrotizing fasciitis	1 (4)	1 (6.3)	0
Descending mediastinitis	1 (4)	1 (6.3)	0
Acute Kidney Injury > stage 1	19 (70)	11 (69)	8 (73)
Platelet count, /μL	8.3 ± 3.0	9.1 ± 3.3	7.2 ± 2.2
Creatinine, mg/dL	1.7 ± 1.0	1.9 ± 1.0	1.4 ± 1.0
ADAMTS13, %	24.9 ± 8.5	25.9 ± 9.5	23.5 ± 7.4
von Willebrand factor propeptide, %	293.8 ± 153.8	294.6 ± 142.8	292.7 ± 175.7
von Willebrand factor, %	212.3 ± 86.3	225.2 ± 81.4	194.7 ± 83.8

ADAMTS13, von Willebrand factor propeptide, and von Willebrand factor are expressed as a percentage of normal controls. Data are reported as mean ± SD or number (%).
*n = 27 patients.
†Systemic inflammatory response syndrome score >3.

and remained markedly increased compared with controls at days 5 and 7 (Table 2). The VWF-pp/ADAMTS13 ratio correlated significantly with APACHE II score on days 1 and 5; with SOFA score on days 1, 3, and 5; and with Max SOFA score and TNF-α level on days 1, 3, 5, and 7 (Table 3). The IL-6 and TNF-α levels in patients on days 1 and 3 were markedly greater than the upper limit of normal (Table 2).

Nineteen patients with severe sepsis or septic shock developed AKI of greater than stage 1 within 48 h after admission (Table 1). The mean levels of VWF and ADAMTS13 did not differ between patients with or without AKI, but patients with AKI had significantly greater mean levels of VWF-pp (AKI, 338% ± 143%; no AKI, 190% ± 134%; $P \leq 0.02$) and VWF-pp/ADAMTS13 ratio (AKI, 15% ± 7%; no AKI, 7% ± 6%; $P \leq 0.001$) on day 1. The VWF-pp level correlated significantly with serum creatinine level on day 1, and the VWF-pp/ADAMTS13 ratio correlated significantly with serum creatinine level on days 1, 5, and 7 (Table 3).

DISCUSSION

A decreased level of ADAMTS13 on admission had been described previously in patients with sepsis (24) and correlated with AKI (11), APACHE II score, and poor prognosis (13). The present results confirmed that decreased ADAMTS13 levels correlated with disease severity scores including APACHE II and Max SOFA on the same days of observation including the day on admission (Table 3). The finding that means ADAMTS13 level was significantly lower in nonsurvivors than survivors on days 1, 5, and 7 (Table 2) suggests that ADAMTS13 level may be a prognostic marker for survival during the early phase of sepsis.

The cause of the decreased ADAMTS13 levels in sepsis is controversial. Possible mechanisms for the decrease include consumption because of excess substrate and proteolytic degradation by thrombin, plasmin, and neutrophil protease (11, 25). In addition, infusion of endotoxin or desmopressin into healthy volunteers may increase plasma VWF and VWF-pp levels and may decrease ADAMTS13 activity (26, 27); this suggests that ADAMTS13 may be consumed mainly by excessive ULVWF released by endotoxin or desmopressin, or secretion of ADAMTS13 may be inhibited. Greater duration or intensity of stimulation to endothelium, causing ULVWF secretion with proinflammatory cytokines such as TNF-α (28), may induce greater imbalance between ULVWF secretion and plasma ADAMTS13 level, resulting in larger VWF molecules in plasma and a prothrombotic condition.

What can be used to estimate the extent of the imbalance between ULVWF secretion and plasma ADAMTS13 level? The appearance of ULVWF in plasma may be a good marker for the imbalance between ULVWF secretion and plasma ADAMTS13 level (14). However, ULVWF can be detected only by time-consuming immunoblotting after electrophoresis, and it is difficult to quantify ULVWF reproducibly (11, 15). Furthermore, ULVWF is very adhesive to platelets and can cause spontaneous platelet aggregation, associated consumption, and decreased levels of ULVWF. The disappearance of ULVWF may be observed in some patients with chronic TTP during acute episodes (12). In addition, some studies show no correlation between ULVWF and decreased levels of ADAMTS13 (11, 29).

The ratio of VWF level to ADAMTS13 activity is reported to be more useful than VWF multimer analysis (ULVWF detection) alone for the diagnosis of highly prothrombotic states induced by the imbalance between VWF secretion and ADAMTS13 (15). However, plasma VWF level may not reflect ULVWF secretion accurately because VWF may be affected by ABO blood group antigens; in addition, secreted plasma VWF can be consumed at the endothelial injury site, especially during inflammation, by binding to the subendothelial matrix, endothelium, platelets, or white blood cells (9). An increased plasma level of VWF on admission is reported to be associated with an increased risk of death from severe sepsis (30); yet, the present study showed that markedly increased VWF levels in patients with severe sepsis or septic shock were not associated with disease severity during the first 7 days and showed increasing tendency despite resolution of clinical symptoms, consistent with other studies (15, 24). Thus, plasma VWF level did not likely reflect ULVWF secretion rate in the present study.

In contrast with VWF, the VWF-pp is not affected by ABO antigen and does not bind to the vascular wall; consequently, plasma level of VWF-pp may more accurately reflect ULVWF

Table 2. **Levels of VWF, VWF-pp, ADAMTS13, and inflammatory markers in patients with severe sepsis or septic shock***

		Patients with severe sepsis or septic shock			
		Day			
Variables	Control subjects	1	3	5	7
VWF, %	96 ± 14	212 ± 86	228 ± 85	240 ± 85	252 ± 112
VWF-pp, %	96 ± 16	294 ± 154	240 ± 115†	219 ± 117	228 ± 162
ADAMTS13, %					
All patients	100 ± 10	25 ± 8.5‡	30 ± 9‡	33 ± 11‡	33 ± 11
Survivors	NA	27 ± 8.6	31 ± 8.7	35 ± 9.4	36 ± 10
Nonsurvivors	NA	19 ± 5.4	27 ± 8.2	25 ± 10	24 ± 9.4
P	NA	0.03§	NS	0.03§	0.02§
VWF-pp/ADAMTS13 ratio	0.97 ± 0.18	12.9 ± 7.2‖	8.9 ± 5.1‖	7.7 ± 6.0	7.9 ± 7.1
IL-6,¶ pg/mL	<2.4	1,220 (362–3,610)	206 (58–1,050)	115 (29–338)	75 (20–446)
TNF α (pg/mL)**	<1.8	5.8 (3.3–21.3)	3.4 (2.4–5.8)	2.5 (1.2–4.0)	2.0 (1.4–3.3)

Data are reported as mean ± SD or median (interquartile range). VWF, VWF-pp, and ADAMTS13 are expressed as a percentage of normal controls.
*n = 27 patients (day 1) or 26 patients (days 3, 5, and 7) with sepsis or septic shock; 15 normal control subjects.
†VWF-pp: difference between days 1 and 3, $P \leq 0.05$.
‡ADAMTS13: difference between normal controls and patients on day 1, $P \leq 0.001$; difference between days 1 and 3, $P \leq 0.01$; difference between days 3 and 5, $P \leq 0.05$.
§ADAMTS13: difference between survivors and nonsurvivors, $P \leq 0.05$.
‖VWF-pp/ADAMTS13 ratio: difference between normal controls and patients on day 1, $P \leq 0.001$; difference between days 1 and 3, $P \leq 0.01$.
¶Upper limit of normal, 2.41 pg/mL.
**Upper limit of normal, 1.79 pg/mL.
NS indicates not significant ($P > 0.05$); NA, not applicable.

secretion induced by endothelial activation than VWF (5). In the present study, increased plasma VWF-pp level was associated with SOFA score and TNF-α on day 1 (Table 3), suggesting that VWF-pp may be a better marker of acute endothelial activation than VWF in the early phase of sepsis. The marked increase in VWF-pp level on admission significantly

Table 3. **Relation between VWF-pp, ADAMTS13, and clinical scores and markers in patients with severe sepsis or septic shock**

	Day 1		Day 3		Day 5		Day 7	
	*r**	*P* ≤	*r**	*P* ≤	*r**	*P* ≤	*r**	*P* ≤
VWF-pp								
APACHE II	0.32	NS	0.16	NS	0.45	0.05	0.07	NS
SOFA	0.51	0.007	0.47	0.02	0.55	0.01	−0.62	NS
Max SOFA	0.25	NS	0.38	NS	0.44	0.03	0.59	0.003
TNF-α	0.47	0.02	0.54	NS	0.38	NS	0.44	NS
IL-6	0.20	NS	−0.11	NS	0.25	NS	0.42	NS
Creatinine	0.59	0.001	0.34	NS	0.32	NS	0.18	NS
ADAMTS-13								
APACHE II	−0.54	0.004	−0.30	NS	−0.68	0.001	−0.34	NS
SOFA	−0.32	NS	−0.30	NS	−0.57	0.007	−0.13	NS
Max SOFA	−0.53	0.005	−0.42	0.03	−0.47	0.02	−0.29	NS
TNF-α	−0.07	NS	−0.37	NS	−0.40	0.05	−0.43	NS
IL-6	−0.04	NS	−0.36	NS	−0.39	NS	−0.45	0.05
Creatinine	−0.33	NS	−0.22	NS	−0.35	NS	−0.47	0.02
VWF-pp/ADAMTS-13 ratio								
APACHE II	0.45	0.03	0.15	NS	0.69	0.001	0.19	NS
SOFA	0.65	0.001	0.41	0.04	0.68	0.001	−0.61	NS
Max SOFA	0.45	0.03	0.41	0.04	0.52	0.007	0.63	0.001
TNF-α	0.44	0.03	0.57	0.002	0.44	0.03	0.59	0.007
IL-6	0.12	NS	0.04	NS	0.48	0.02	0.70	0.001
Creatinine	0.76	0.001	0.29	NS	0.49	0.02	0.48	0.02

*Spearman rank correlation (ρ).
NS indicates not significant ($P > 0.05$).