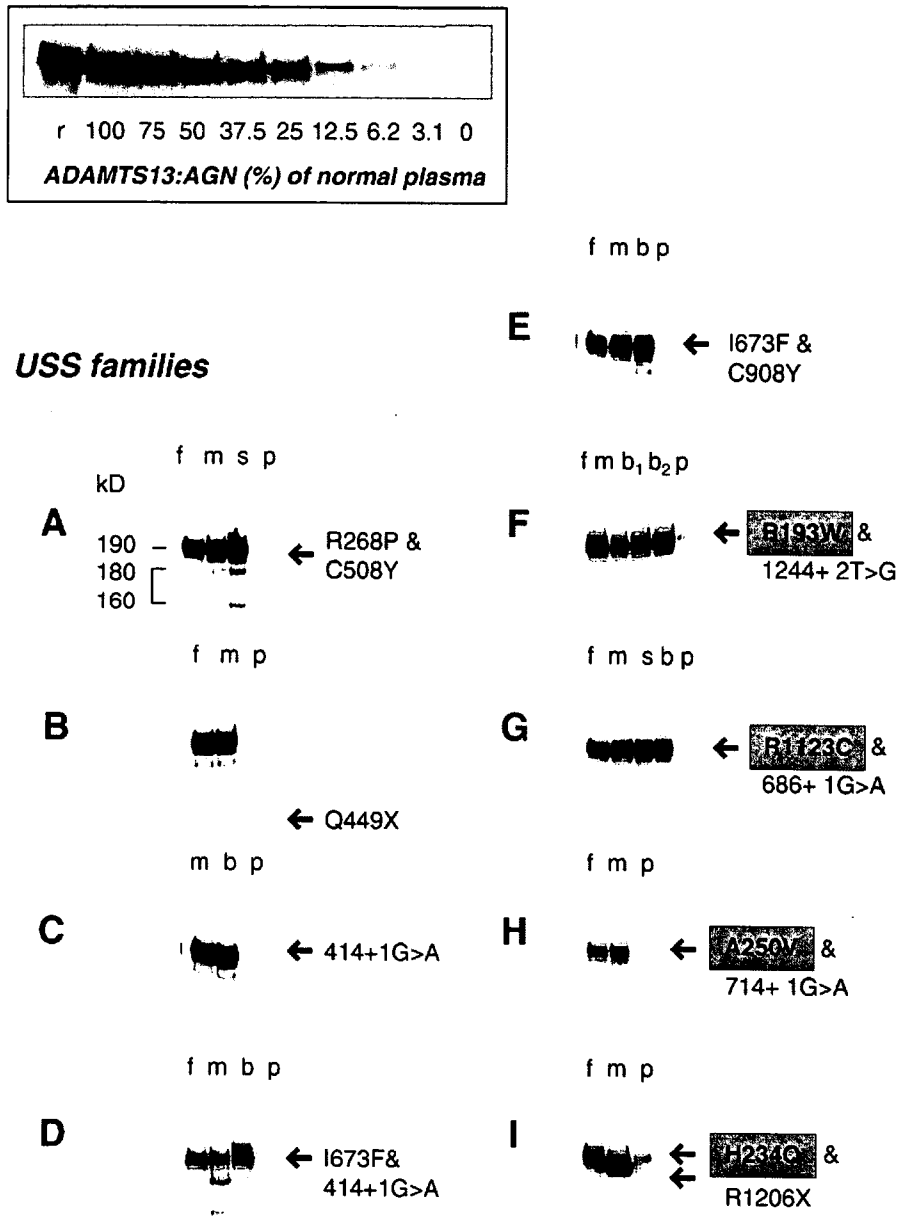


**Figure 1** WB analysis under reducing conditions of plasma ADAMTS13:AGN in 9 families with a history of USS. WB analysis of plasma ADAMTS13:AGN in the members of the nine USS families are shown: f (father), m (mother), b(brother), s (sister), and p (patient). Under reducing conditions, recombinant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

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

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

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

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

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

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

## Discussion

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

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

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

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

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**Abstract**

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

## 1. Introduction

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

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## 2. Materials and Methods

### 2.1. Patients

#### 2.1.1. USS Patients

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

#### 2.1.2. ai-TTP Patients

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

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

### 2.2. Purification of ADAMTS13

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

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

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

### 2.4. Analysis of Plasma ADAMTS13:AG

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

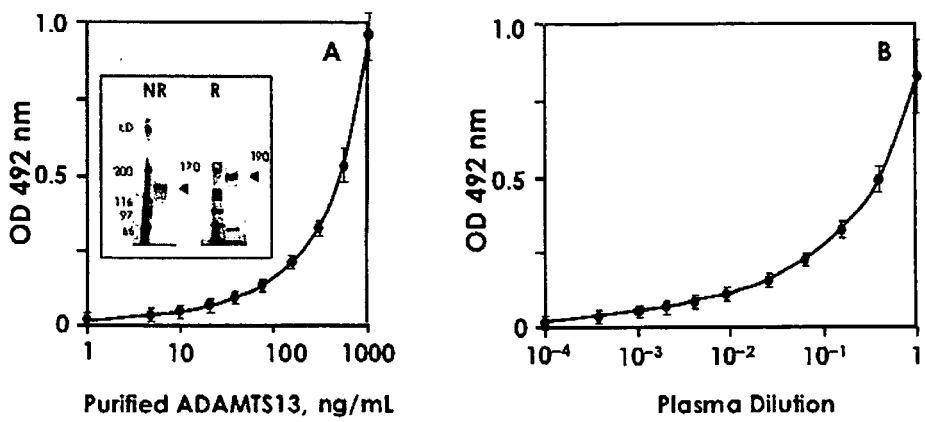


**Figure 1.** Calibration curves for ADAMTS13 antigen (ADAMTS13:AG) obtained with a novel enzyme-linked immunosorbent assay (ELISA) using a neutralizing monoclonal antibody as a capturing antibody. A, Electrophoresis of ADAMTS13 purified from pooled normal human plasma (NHP) revealed a 170-kd band under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

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

### *2.5. Assays for ADAMTS13:AC and ADAMTS13 Inhibitors*

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

### *2.6. Statistical Analysis*

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

## 3. Results

### *3.1. ELISA for ADAMTS13:AG*

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

### *3.2. Measurement of the Plasma Level of ADAMTS13:AG*

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

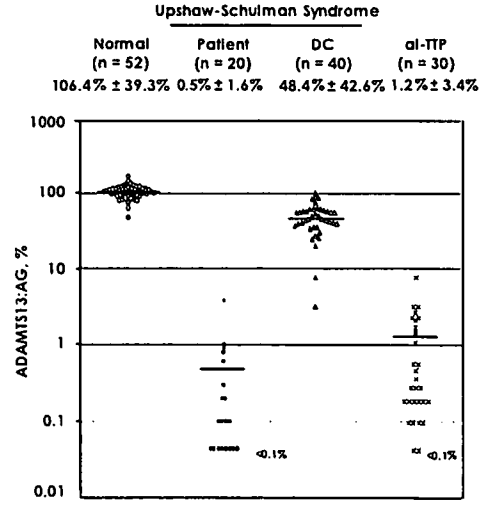


**Figure 2.** Plasma levels of ADAMTS13 antigen (ADAMTS13:AG) measured by the novel enzyme-linked immunosorbent assay. The standard curve in Figure 1B was used to determine the following plasma ADAMTS13:AG levels (mean ± 2 SD): healthy individuals with wild-type ADAMTS13 (Normal), 106.4% ± 39.3% (n =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

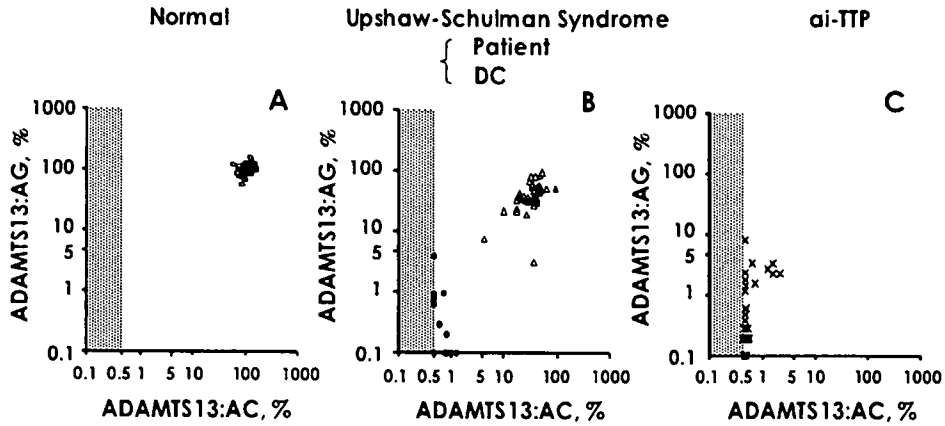


**Figure 3.** Relationship between plasma levels of ADAMTS13 antigen (ADAMTS13:AG) as measured by the novel enzyme-linked immunosorbent assay and ADAMTS13 activity (ADAMTS13:AC). Plasma ADAMTS13:AC (x-axis) and ADAMTS13:AG (y-axis) showed a significantly positive correlation in (A) healthy donors with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

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

### *3.3. Relationship between ADAMTS13:AG and ADAMTS13:AC*

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

## 4. Discussion

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

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

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

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

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

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ORIGINAL ARTICLE

# Prophylactic fresh frozen plasma may prevent development of hepatic VOD after stem cell transplantation via ADAMTS13-mediated restoration of von Willebrand factor plasma levels

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**We initially conducted a multicenter, randomized trial ($n=43$), and subsequently a questionnaire study ($n=209$) of participating hospitals, to evaluate whether infused fresh frozen plasma (FFP) could prevent the occurrence of hepatic veno-occlusive disease (VOD) after stem cell transplantation (SCT). Forty-three patients were divided into two groups: 23 receiving FFP infusions and 20 not receiving it. VOD developed in three patients not receiving FFP. Plasma von Willebrand factor (VWF) antigen levels were lower at days 0, 7 and 28 after SCT in patients receiving FFP than in those not receiving it, whereas plasma ADAMTS13 activity (ADAMTS13:AC) did not differ between them. Plasma VWF multimer (VWFM) was demonstrated to be defective in the high~intermediate VWFM during the early post-SCT phase, but there was a significant increase in high VWFM just before VOD onset. This suggests that a relative enzyme-to-substrate (ADAMTS13/high-VWFM) imbalance is involved in the pathogenesis of VOD. To strengthen this hypothesis, the incidence of VOD was apparently lower in patients receiving FFP infusions than in those not receiving it (0/23 vs 3/20) in the randomized trial. Further, the results combined with the subsequent questionnaire study (0/36 vs 11/173) clearly showed the incidence to be statistically significant (0/59 vs 14/193, $P=0.033$).**

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## Introduction

Hepatic veno-occlusive disease (VOD) is a life-threatening complication that develops in 1–54% of patients undergoing allogenic stem cell transplantation (SCT).[1–6] Hepatic VOD is clinically characterized by hyperbilirubinemia, painful hepatomegaly and fluid retention.[2,3] Histologically, hepatic VOD is marked by fibrosis of sinusoids, necrosis of pericentral hepatocytes and narrowing of central veins with eventual fibrosis.[7] Recent work suggests that the primary site of toxic injury caused by chemotherapy and/or radiation before SCT is the sinusoidal endothelial cells and that this initial insult is followed by a series of biologic processes that ultimately leads to circulatory compromise of centrilobular hepatocytes, fibrosis and obstruction of liver blood flow.[7] However, the precise pathogenesis of hepatic VOD has yet to be clarified.

von Willebrand factor (VWF) is synthesized in vascular endothelial cells and released into the plasma as 'unusually large' VWF multimers (UL-VWFM), which actively interact with platelets.[8,9] In the normal circulation, UL-VWFM are rapidly degraded into smaller VWFM by ADAMTS13 (a disintegrin-like metalloproteinase with thrombospondin type-1 motifs 13),[10,11] which cleaves the Tyr842–Met843 bond within the VWF A2 domain.[12,13] Deficiency of ADAMTS13 caused either by mutations of the *ADAMTS13* gene[10] or by inhibitory autoantibodies against

ADAMTS13[14,15] increases plasma levels of UL-VWFM, which leads to platelet clumping and/or thrombi under high shear stress, resulting in thrombotic thrombocytopenic purpura (TTP).[16]

We previously demonstrated that plasma ADAMTS13 activity (ADAMTS13:AC) is reduced in hepatic VOD patients after SCT compared to non-VOD patients, even before conditioning therapy,[17] and that its activity could, therefore, be a predictor for the development of hepatic VOD. Additionally, ADAMTS13:AC is significantly low in patients with advanced liver cirrhosis[18] and those with alcoholic hepatitis.[19] Furthermore, we have demonstrated a rapid decrease in ADAMTS13:AC in association with early adverse events including ischemia–reperfusion injury and/or acute graft rejection in living donor-related liver transplantation.[20] Our most recent study demonstrates that ADAMTS13 is produced exclusively in hepatic stellate cells (HSCs) located in the space of Disse adjacent to endothelial cells.[21] Considering that hepatic sinusoidal endothelial cell injury is an important causative factor in the development of hepatic VOD[1] and that ADAMTS13:AC is reduced in hepatic VOD,[17] it is of particular interest to evaluate the effect of fresh frozen plasma (FFP) administration as a supplemental source of ADAMTS13 in patients at high risk for post-SCT hepatic VOD.

In this study, we performed a multicenter, prospective, randomized controlled trial, and a subsequent study of participating hospitals by questionnaires to evaluate whether or not the infusion of FFP could prevent the occurrence of hepatic VOD in high-risk patients who had undergone SCT. Towards elucidating the mechanism that underlies the development of VOD, we sequentially determined plasma levels of ADAMTS13:AC, and its substrates, VWF antigen (VWF:AG) and UL-VWFM in a randomized controlled trial.

## Materials and methods

### *Study design*

The study on prevention of hepatic VOD associated with SCT was conducted at 10 hospitals in Osaka, Hyogo and Nara prefecture. The study was performed between April 2001 and March 2003, and was a multicenter, randomized and controlled study. A subsequent study was carried out between January 2004 and December 2006 by questionnaires sent to participating hospitals. With respect to the randomized controlled trial, patients were eligible to participate in this prospective study if they were at high risk for developing VOD after allogenic SCT and fulfilled one of the following criteria: (1) intensified conditioning regimen, (2) second SCT, (3) liver dysfunction and (4) receiving intensified chemotherapy until just before SCT because of the poor situation of the underlying disease. Physicians from the referring hospitals registered patients to our registration office by facsimile, with patient information including age, gender and underlying disease. Patients were divided into a child group (18-year-old or under) and an adult group (over 18-year-old). Patients in each group were then randomly divided into two groups: patients to receive FFP infusion (FFP (+) group) and those not to receive it (FFP (−) group). FFP was infused twice a week during the conditioning regimen and until day 28 after SCT in patients in the FFP (+) group. The volume of FFP infused was based upon body weight and determined as follows: 1 unit (=80 ml) for patients under 10 kg, 2 units for 10–20 kg, 3 units for 20–30 kg, 4 units for 30–40 kg and 5 units for over 40 kg. The amount of FFP infused on this protocol was calculated to contribute about a 10% increase in plasma ADAMTS13:AC, which was enough to eliminate UL-VWFM in patients with congenital TTP, termed Upshaw–Schulman syndrome.[22,23] In our previous study, plasma ADAMTS13:AC was about 20% of the normal control level between pre-conditioning and day 21 in patients with hepatic VOD.[17] We therefore attempted to maintain plasma ADAMTS13:AC at the level of 30% of normal control throughout the study, by FFP infusion. As the half-life of plasma ADAMTS13:AC has been reported to be approximately 3 days,[24] we decided to infuse FFP twice weekly.

A diagnosis of hepatic VOD was made according to the criteria of McDonald *et al.*;[2] VOD patients needed to have at least two of the following clinical features within day 30 after transplantation: (1) jaundice, (2) hepatomegaly and right upper quadrant pain and (3) ascites and/or unexplained body weight gain. Citrated plasma was obtained from patients before the conditioning regimen (pre-SCT) and on days 0, 7, 14, 21 and 28 (post-SCT), and was stored in aliquots at −80°C until use. ADAMTS13:AC, VWF:AG, and UL-VWFM levels were determined using these plasma samples. ADAMTS13 inhibitor was analyzed using plasma from day 28. Other laboratory parameters, including thrombin–antithrombin complex (TAT), D-dimer, thrombomodulin (TM), tissue plasminogen activator/plasminogen activator inhibitor-1 (t-PA/PAI-1) complex and antithrombin (AT), were measured at pre-SCT and on days 0, 7, 14, 21 and 28. The study protocol was approved by the ethics committee of each participating hospital. All patients gave written informed consent before registration in the study.

The subsequent questionnaire was sent to 10 participating hospitals asking whether or not prophylactic FFP infusion to prevent hepatic VOD had been given in high-risk patients who had undergone SCT, and requesting information on the incidence of hepatic VOD in these patients with and without FFP infusion.

### *Measurements*

ADAMTS13:AC was assayed using a highly sensitive, novel enzyme-linked immunsorbent assay (ELISA), recently developed by our laboratory.[25] The normal level of ADAMTS13:AC using this assay in 55 healthy individuals was 99.1±21.5% (mean±s.d.).[25] The ADAMTS13 inhibitor was evaluated using heat-inactivated plasma at 56°C for 30 min.[14,15] One Bethesda unit of inhibitor was defined as the amount of plasma that reduces ADAMTS13:AC to 50% of the control,[26] and its titer was estimated to be significant in more than 0.5 Bethesda U/ml. The UL-VWFM was evaluated by SDS–0.9% agarose electrophoresis followed by western blotting with luminographic detection.[27,28] Multimers were defined as low molecular

weight (corresponding to bands 1–5), intermediate molecular weight (bands 6–10) and high molecular weight (bands >10).[29] Furthermore, the bands corresponding to higher molecular weight, which could never be detected in pooled normal plasma, were defined as UL-VWFM. VWF:AG was measured by a sandwich ELISA using a rabbit anti-human VWF polyclonal antibody (DakoCytomation, Kyoto, Japan). The value obtained from normal individuals ($n=20$, 13 males and 7 females aged 20–40 years) was 102±33%.[9]

*Statistical analysis*

All experimental data are presented as means±s.d. Paired and unpaired comparisons between the two groups were performed using the Student's *t*-test and Fisher exact test. A two-tailed *P*-value of less than 0.05 was considered statistically significant. Analyses were carried out using the statistical software Statview (version 5.0, SAS Institute, Cary, NC, USA).

## Results

*Randomized control study*

*Patient characteristics.* Of 47 patients enrolled, 15 patients belonged to the child group and 32 belonged to the adult group. Of these, a patient belonging to the FFP (+) group and three patients belonging to the FFP (−) group were excluded from this protocol, because they could not undergo SCT because of poor physical condition. Finally, 43 patients consisting of 15 in the child group and 28 in the adult group were investigated. Twenty-three patients were assigned to the FFP (+) group and the remaining 20 patients to the FFP (−) group. There were no statistically significant differences between the two groups with regard to clinical features and laboratory findings. The clinical characteristics and transplant procedures in patients finally enrolled are shown in Table 1.

*Clinical features of VOD patients with respect to VWF:AG, ADAMTS13:AC and VWF multimers.* Case no. 8 with hepatic VOD was a 53-year-old female diagnosed with leukemic transformation of myelodysplastic syndrome who received a mini-transplant from a human leukocyte antigen (HLA)-matched sibling while she had active disease (Table 1). At day 14 after SCT, this patient developed hyperbilirubinemia (5.1 mg/dl), weight gain and right upper quadrant pain, and was thus diagnosed as having hepatic VOD (Figure 1a). She was treated with steroids from day 14 and FFP since day 28, and she completely recovered at day 40 after SCT. In this case, VWF multimers corresponding to high and intermediate molecular weight, which are usually seen in normal plasma, were lacking pre-SCT before conditioning (Figure 1b). VWF multimers gradually appeared from day 0 to day 7 after SCT. VWF:AG increased from 64% (pre-SCT) to 396% (day 7), and ADAMTS13:AC decreased from 67% (pre-SCT) to 30% (day 7), resulting in an increasing ratio of VWF:AG to ADAMTS13:AC from 1.0 (pre-SCT) to 13.0 (day 7). A week later, VWF:AG decreased to 171%, but ADAMTS13:AC further decreased to 24%, resulting in ratios of VWF:AG to ADAMTS13:AC as high as 7.1 at day 14, when hepatic VOD developed (Figures 1a and b). Thereafter, ADAMTS13:AC increased and VWF:AG remained relatively unchanged. This patient completely recovered by day 40, after the occurrence of hepatic VOD.

Case no. 12 was a 53-year-old male who received a bone marrow transplant from an HLA-matched unrelated donor for refractory acute lymphocytic leukemia (Table 1). At day 28 after SCT, this patient exhibited mild jaundice (1.3 mg/dl), weight gain and painful hepatomegaly, and was then diagnosed as having hepatic VOD (Figure 1c). He was treated with FFP infusions from day 35, but unfortunately died of hepatic failure due to VOD on day 40 after SCT. In this case, VWF multimer patterns were similar to those in case no. 8: VWF multimers of high and intermediate molecular weight were lacking at day 0 (just after SCT), but gradually appeared between days 7 and 21 (Figure 1d). VWF:AG gradually increased from 101% (day 0) to 205% (day 21), and ADAMTS13:AC gradually decreased from 51% (day 0) to 43% (day 21), resulting in increasingly high ratios of 2.0 (day 0) to 4.8 (day 21). A week later, VWF:AG further increased and reached 234%, and ADAMTS13:AC decreased to 32%, resulting in a high ratio of VWF:AG to ADAMTS13:AC of 7.3 at day 28, when hepatic VOD developed (Figures 1c and d).

Case no. 14 was a 41-year-old female with active acute myelocytic leukemia who underwent BMT from an HLA-matched unrelated donor. Soon after SCT, she exhibited weight gain and painful hepatomegaly, and was diagnosed as having hepatic VOD on day 7. This patient was therefore dropped from the study and treated with FFP infusions. She completely recovered from hepatic VOD at day 42 after SCT, and was categorized as a VOD case in the FFP (−) group.

*Comparison of plasma VWF:AG, ADAMTS13:AC and VWF multimers between patients receiving and not receiving FFP infusions.* We next evaluated the effect of FFP on the clinical parameters of ADAMTS13:AC and VWF:AG in patients receiving and not receiving FFP infusions. There were no differences in TAT, D-dimer, TM, PAI-I and AT III between FFP (−) and (+) groups (Table 2). VWF:AG gradually increased over the time period pre-SCT to day 28 post-SCT in patients belonging to the FFP (−) group (Figure 2). In contrast, in patients belonging to the FFP (+) group, the VWF:AG did not increase at days 0 and 7, but gradually increased thereafter between days 14 and 21, and later decreased at day 28. There was a significant difference in VWF:AG at days 0, 7 and 28 between the groups receiving and not receiving FFP (day 0: 96±39 vs 147±78, $P<0.05$; day 7: 103±62 vs 156±83, $P<0.05$; and day 28: 146±82 vs 212±81, $P<0.05$) (Figure 2). On the other hand, ADAMTS13:AC gradually decreased from pre-SCT to day 28 in both groups. No difference in the levels of ADAMTS13:AC was observed between the groups. The ratio of VWF:AG to ADAMTS13:AC gradually increased from pre-SCT to day 14 in patients in the FFP (−) group, but in patients in the FFP (+) group, this ratio did not increase at days 0 and 7 but gradually increased later at day 14 (Figure 2). Plasma inhibitor of ADAMTS13 at day 28 was detected only in one patient

**Table 1** Clinical characteristics of patients with SCT

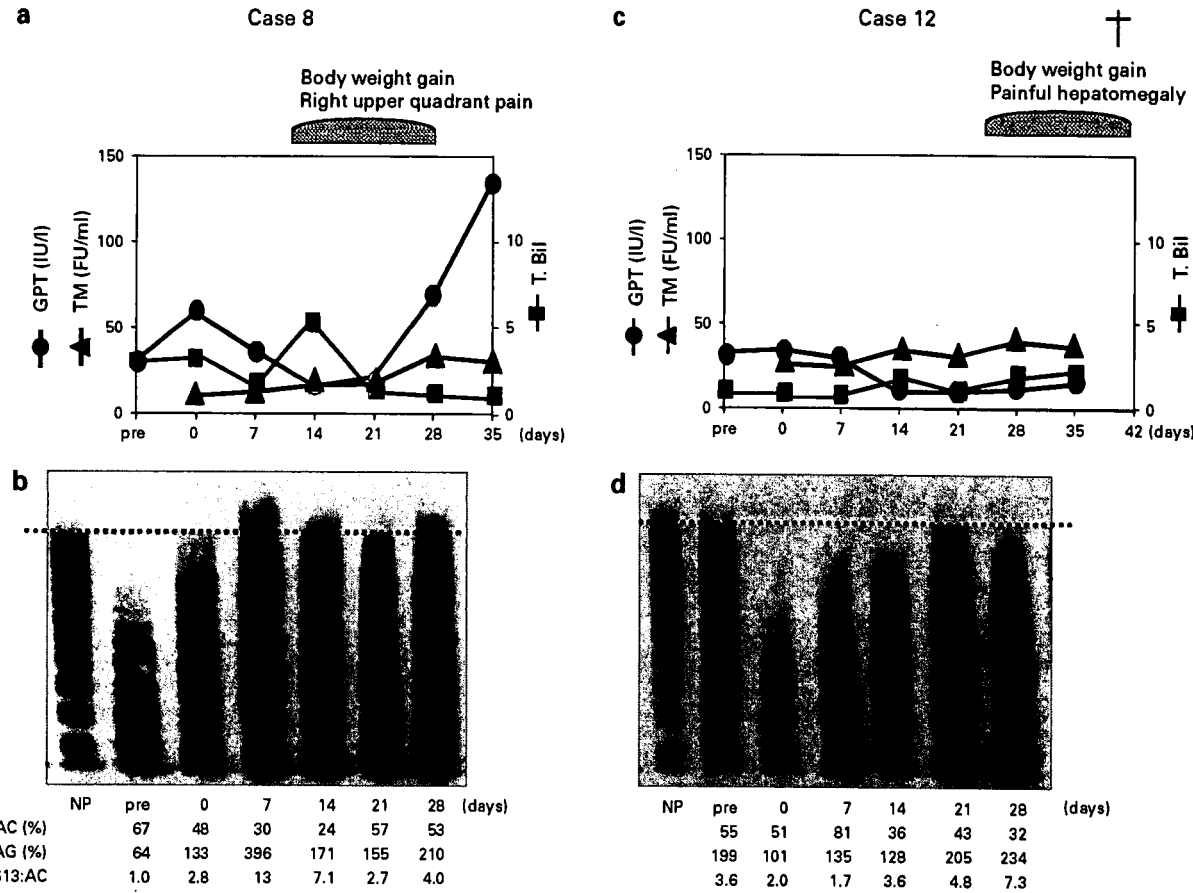
| Case number | Age (years) | Gender | Underlying disease | Disease state | Transplant type | Related (R) or unrelated donor (N) | Conditioning regimen | GVHD prophylaxis | Acute GVHD grade |
|---|---|---|---|---|---|---|---|---|---|
| *FFP (−)* | | | | | | | | | |
| 1 | 15 | F | ALL | CR2 | CBSCT | U | Flu/LPAM/TBI | CsA | I |
| 2 | 10 | M | ALL | CR2 | BMT | R | LPAM/TBI | FK/MTX | I |
| 3 | 13 | M | ALL | Refractory | PBSCT | R | BU/Cy/TEPA | – | I |
| 4 | 9 | M | NK-leukemia | CR1 | PBSCT | R | VP16/Cy/TBI | FK | II |
| 5 | 14 | M | CAEBV | Refractory | PBSCT | R | Flu/Cy/TBI | FK | II |
| 6 | 17 | M | AML | Refractory | BMT | U | Cy/Flu/TBL | FK/sMTX | II |
| 7 | 1 | M | ALL | CR1 | CBSCT | U | BU/Cy/VP16 | CsA/MTX | 0 |
| 8 | 53 | F | MDS | Non-CR | Mini-PBSCT | R | Bu/Flu | CsA/MTX | II |
| 9 | 64 | M | HD | Refractory | Mini-CBSCT | U | Flu/TBI | FK | NE |
| 10 | 47 | M | ALL | Refractory | BMT | U | Cy/TBI | CsA/MTX | II |
| 11 | 32 | F | AML | CR2 | CBSCT | U | Flu/TBI | FK | II |
| 12 | 52 | M | ALL | Refractory | BMT | U | Bu/Cy | CsA/MTX | 0 |
| 13 | 39 | M | CML | BC | BMT | R | Bu/Cy/TBI | CsA/MTX | I |
| 14 | 41 | F | AML | Refractory | BMT | U | Bu/Cy/TBI | CsA/MTX | I |
| 15 | 27 | M | ALL | CR1 | CBSCT | U | Flu/TBI | FK | I |
| 16 | 26 | M | ALL | Refractory | Mini-BMT | U | Flu/LPAM | CsA/MTX | II |
| 17 | 43 | M | NHL | Refractory | PBSCT | U | Bu/Cy/TBI | CsA/MTX | 0 |
| 18 | 28 | M | AML | Refractory | BMT | U | Flu/Bu/TBI | CsA/MTX | III |
| 19 | 51 | M | MDS | Non-CR | BMT | U | Bu/Cy/TBI | CsA/MTX | III |
| 20 | 25 | M | AML | CR1 | CBSCT | U | Flu/TBI | CsA/MTX | NE |
| *FFP (+)* | | | | | | | | | |
| 21 | 5 | M | AML | CR2 | Mini-CBSCT | U | Cy/TBI | CsA | I |
| 22 | 5 | M | AML | CR2 | BMT | U | Cy/TBI | FK/MTX | I |
| 23 | 3 | F | MDS | Non-CR | BMT | R | Flu/LPAM/TBI | CsA | II |
| 24 | 1 | M | MDS | Non-CR | PBSCT | R | Bu/Cy/CA | CsA | III |
| 25 | 7 | M | ALL | CR2 | BMT | U | LPAM/TBI | FK/MTX/PSL | II |
| 26 | 3 | M | AML | Refractory | BMT | R | VP16/Cy/Flu/TBI | FK/sMTX | I |
| 27 | 1 | M | WAS | Non-CR | CBSCT | U | Bu/Cy/Flu | CsA/MTX | 0 |
| 28 | 6 | M | ALL | CR2 | BMT | U | CA/Cy/TBI | CsA | II |
| 29 | 54 | F | ALL | Refractory | CBSCT | U | Cy/TBI | CsA/MTX | II |
| 30 | 35 | F | NHL | Refractory | PBSCT | R | VP16/Cy/TBI | CsA/MTX | II |
| 31 | 37 | F | AML | CR2 | CBSCT | U | Flu/TBI | FK | NE |
| 32 | 34 | M | AML | Refractory | CBSCT | U | Cy/TBI | CsA | IV |
| 33 | 47 | F | AML | CR2 | Mini-CBSCT | U | Flu/TBI | FK/MTX | NE |
| 34 | 45 | F | NHL | Refractory | CBSCT | U | Flu/TBI | FK | IV |
| 35 | 43 | F | AML | CR1 | PBSCT | R | Bu/Cy | CsA/MTX | I |
| 36 | 38 | M | MDS | Refractory | CBSCT | U | Flu/LPAM/TBI | FK/MTX | NE |
| 37 | 34 | F | MDS | Refractory | BMT | U | Bu/Cy/TBI | FK/MTX | I |
| 38 | 40 | M | CML | CP2 | CBSCT | U | Flu/TBI | FK | II |
| 39 | 50 | M | ALL | Refractory | CBSCT | U | Flu/TBI | FK | II |
| 40 | 43 | M | AML | Refractory | PBSCT | R | Bu/Cy/TBI | FK/MTX | NE |
| 41 | 19 | M | AA | Severe | BMT | R | Flu/Cy/TBI | FK/MTX | II |
| 42 | 53 | M | CML | CP1 | Mini-PBSCT | R | Flu/Bu | CsA/MTX | 0 |
| 43 | 51 | M | CML | Refractory | CBSCT | U | Flu/TBI | CsA | NE |

Abbreviations: AA = aplastic anemia; ALL = acute lymphoblastic leukaemia; BC = blast crisis; BMT = bone marrow transplantation; BU = busulfan; CA = cytosine arabinoside; CAEBV = chronic active EBV infection; CBSCT = cord blood stem cell transplantation; CML = chronic myeloblastic leukemia; CR1 = 1st complete remission; CR2 = 2nd complete remission; CsA = cyclosporin; Cy = cyclophosphamide; FK = tacrolimus; Flu = fludarabine; HD = Hodgkin's disease; LPAM = melphalan; MDS = myelodysplastic syndrome; MTX = methotrexate; NE = not evaluated; NHL = non-Hodgkin lymphoma; PBSCT = peripheral blood stem cell transplantation; PSL = predonisolone; SCT = stem cell transplantation; TBI = total body irradiation; VP16 = vepeside; WAS = Wiskott–Aldrich syndrome.
The patients with shaded area developed hepatic VOD.

overall (patient no. 26), who belonged to the FFP (+) group and exhibited an inhibitor level of 1.3 Bethesda U/ml.

We further investigated VWF multimer patterns in patients from the FFP (−) and (+) groups because VWF multimers of high and intermediate molecular weight were specifically lacking pre-SCT in case 8 and at day 0 post-SCT in case 12, but thereafter gradually increased in these cases (Figures 1b and d). Representative VWF multimer patterns are shown in Figure 3. In patients from the FFP (−) group, VWF multimers corresponding to high and/or intermediate molecular weight were less or absent at pre-SCT and on days 0 and 7 in case 1 (Figure 3a); at pre-SCT and on days 0, 14 and 21 in case 5 (Figure 3b); and at pre-SCT and on days 0, 7 and 21 in case 10 (Figure 3c). In contrast, in patients from the FFP (+) group, no apparent changes in VWF multimer patterns were found throughout SCT, including during the preconditioning period when they compared them to VWF multimer levels found in normal control plasma.

*The incidence of hepatic VOD occurrence by randomized controlled trial.* Out of the 20 patients belonging to the




| NP | pre | 0 | 7 | 14 | 21 | 28 (days) |
|---|---|---|---|---|---|---|
| ADAMTS13:AC (%) | 67 | 48 | 30 | 24 | 57 | 53 |
| VWF:AG (%) | 64 | 133 | 396 | 171 | 155 | 210 |
| VWF:AG/ADAMTS13:AC | 1.0 | 2.8 | 13 | 7.1 | 2.7 | 4.0 |

| NP | pre | 0 | 7 | 14 | 21 | 28 (days) |
|---|---|---|---|---|---|---|
| | 55 | 51 | 81 | 36 | 43 | 32 |
| | 199 | 101 | 135 | 128 | 205 | 234 |
| | 3.6 | 2.0 | 1.7 | 3.6 | 4.8 | 7.3 |

**Figure 1** von Willebrand factor (VWF) multimeric analysis in patients with hepatic veno-occlusive disease (VOD). Patient no. 8 (left panel) was diagnosed with hepatic VOD on day 14 (**a**). VWF multimer analysis showed absence of high and intermediate molecular weight VWF multimer (VWFM) before stem cell transplantation and on day 0. At day 7, VWF:AG was increased and unusually large VWF multimers (UL-VWFM) were detected (**b**). Patient no. 12 (right panel) was diagnosed with hepatic VOD on day 28 (**c**). VWF multimer analysis showed absence of high and intermediate molecular weight VWFM on days 0, 7 and 14. VWF:AG was increased at day 21 (**d**). The multimers over the dotted line indicate UL-VWFM, which can be never detected in normal plasma.

FFP (−) group, three (15%) patients (patient no. 8, 12 and 14) developed hepatic VOD. In contrast, none of the 23 patients in the FFP (+) group did so (Table 3). The incidence of VOD was clearly higher in the FFP (−) group than in the FFP (+) group, but the difference between the FFP (−) group and (+) group did not reach statistical significance ($P = 0.092$) because of the small number of patients in the study.

As for risk factors for the development of hepatic VOD, all three patients with VOD came from the 29 patients who had been treated with intensified chemotherapy until just before SCT. Both case 8 and 12 came from the 11 patients with liver dysfunction, and case 14 was one of 21 patients who received an intensified conditioning regimen. There was thus no relationship between these risk factors for developing hepatic VOD. Further, we compared ADAMTS13:AC and VWF:AG among four criteria of risk factors as described in Materials and methods. However, no significant difference was found in either ADAMTS13:AC and VWF:AG among these criteria (data not shown).

*The incidence of VOD occurrence by subsequent questionnaire study*

According to the questionnaire, two out of 10 participating hospitals gave prophylactic FFP infusions to prevent hepatic VOD in high-risk patients who had undergone SCT, but others did not. Hepatic VOD developed in 11 of 173 high-risk patients who did not receive prophylactic FFP infusions (Table 3). In contrast, hepatic VOD never occurred in 36 high-risk patients who had prophylactic FFP infusion. Based on the result under this questionnaire, the difference in the incidence of hepatic VOD did not reach statistical significance between patients receiving and not receiving prophylactic FFP infusions ($P = 0.120$). However, when we analyzed all the patients who underwent SCT between 2001 and 2006, the incidence of hepatic VOD was significantly lower in patients receiving prophylactic FFP infusions (0/59) than in those who did not (14/193) (0 vs 7.3%, $P = 0.033$) (Table 3).

## Discussion

In this study, we performed a randomized controlled trial, and a subsequent study by questionnaire, to evaluate whether prophylactic FFP infusions could prevent the occurrence of hepatic VOD in high-risk patients receiving SCT. As for the incidence of hepatic VOD in the randomized controlled study, 3 (15%) of 20 patients belonging to the FFP (−) group developed hepatic VOD,

**Table 2** Differences in clinical parameters between patients with and without FFP infusions

| | *WBC* ($\times 10^9$/l) | *RBC* ($\times 10^{12}$/l) | *Hb* (g/l) | *Ret* (‰) | *Plt* ($\times 10^9$/l) | *T.Bil* (mg/dl) | *GOT* (IU/l) | *GPT* (IU/l) | *LDH* (IU/l) | *ALP* (IU/l) | *BUN* (mg/dl) | *Cre* (mg/dl) | *CRP* (mg/dl) | *TAT* (ng/l) | *D-dimer* (ng/ml) | *TM* (FU/ml) | *tPA/PAI-1* (ng/ml) | *AT* (%) | *VWF: AG (%)* | *ADAMTS13: AC (%)* | *VWF:AG/ ADAMTS13: AC* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pre* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 7.9±19.9 | 2.9±0.5 | 95±19 | 14±15 | 96±91 | 1.1±1.5 | 30±30 5 | 37±41 | 336±444 | 414±396 | 11±5.0 | 0.6±0.3 | 3.4±5.6 | 2.9±3.2 | 2.2±3.2 | 15±9 | 11±10 | 95±17 | 121±44 | 65±26 | 2.1±1.0 |
| FFP (+) | 4.2±4.4 | 3.2±0.7 | 101±23 | 9.9±8.2 | 101±78 | 0.6±0.3 | 30±2 | 48±60 | 211±60 | 441±315 | 10±3.9 | 0.5±0.3 | 1.1±2.6 | 5.6±10.1 | 0.7±0.5 | 11±8 | 22±22 | 101±16 | 103±59 | 63±26 | 1.9±1.4 |
| *Day 0* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 1.3±1.8 | 2.7±0.5 | 89±15 | 5.2±7.1 | 48±40 | 1.2±1.2 | 18±10 | 27±30 | 206±75 | 354±326 | 13±7.7 | 0.5±0.3 | 2.5±4.4 | 7.1±8.2 | 3.5±6_3 | 14±8 | 14±12 | 104±23 | 147±78 | 55±23 | 2.9±1.8 |
| FFP (+) | 0.5±0.7 | 3.0±0.6 | 93±18 | 2.6±3.8 | 53±62 | 0.6±0.3 | 20±12 | 34±48 | 179±52 | 297±144 | 12±6.3 | 0.4±0.3 | 2.4±5.1 | 4.8±7.9 | 1.1±1.1 | 8.9±7 | 20±14 | 100±18 | 96±39 | 62±24 | 1.9±1.2 |
| *Day 7* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 0.2±0.7 | 2.9±0.5 | 91±13 | 3.5±5.8 | 24±10 | 1.0±1.0 | 23±29 | 28±44 | 175±78 | 339±269 | 15±5.2 | 0.5±0.3 | 3.2±3.2 | 6.4±9.1 | 1.7±2.4 | 11±7 | 15±10 | 87±16 | 156±83 | 54±20 | 3.5±3.1 |
| FFP (+) | 0.1±0.2 | 2.9±0.6 | 89±15 | 2.3±3.5 | 23±19 | 0.8±0.5 | 17±10 | 25±18 | 170±55 | 280±119 | 14±6.7 | 0.4±0.2 | 5.8±8.8 | 8.1±24 | 1.1±0.9 | 8.7±6 | 13±6.5 | 85±16 | 103±62 | 55±23 | 2.0±1.2 |
| *Day 14* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 1.3±2.2 | 2.9±0.5 | 91±14 | 3.9±5.2 | 32±23 | 1.3±1.4 | 46±83 | 56±62 | 199±88 | 339±187 | 25±14 | 0.7±0.4 | 6.1±7.5 | 4.8±7.9 | 2.3±2.4 | 15±11 | 18±13 | 80±17 | 165±73 | 42±15 | 4.5±2.5 |
| FFP (+) | 2.3±2.9 | 3.0±0.4 | 91±14 | 19±53 | 39±32 | 1.6±3.2 | 54±76 | 73±71 | 360±172 | 381±175 | 22±19 | 0.5±0.4 | 6.1±7.7 | 4.9±4.4 | 3.1±3.2 | 16±15 | 23±14 | 86±24 | 169±91 | 49±26 | 3.8±2.4 |
| *Day 21* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 3.5±2.2 | 3.0±0.5 | 93±15 | 5.4±5.4 | 40±30 | 1.1±1.6 | 40±31 | 78±76 | 266±120 | 414±214 | 20±9.1 | 0.5±0.2 | 1.3±1.8 | 5.6±9.0 | 1.3±0.9 | 17±10 | 19±15 | 102±21 | 157±68 | 53±17 | 3.2±1.7 |
| FFP (+) | 3.2±2.5 | 3.1±0.5 | 93±14 | 33±54 | 52±51 | 2.1±4.6 | 86±75 | 92±81 | 424±339 | 500±385 | 30±30 | 0.6±0.6 | 3.8±4.9 | 7.7±14 | 1.5±1.6 | 16±12 | 39±53 | 90±22 | 178±87 | 51±20 | 4.3±4.3 |
| *Day 28* | | | | | | | | | | | | | | | | | | | | | |
| FFP (−) | 3.7±3.4 | 2.9±0.5 | 91±15 | 13±8.6 | 50±48 | 1.2±2.0 | 53±55 | 96±92 | 251±92 | 441±359 | 22±11 | 0.7±0.4 | 2.2±3.9 | 6.9±11 | 1.2±1.0 | 20±11 | 21±20 | 105±27 | 212±81 | 57±22 | 4.4±2.9 |
| FFP (+) | 3.5±2.0 | 2.9±0.6 | 88±17 | 39±57 | 45±42 | 2.3±3.8 | 51±45 | 83±80 | 317±164 | 545±393 | 29±33 | 0.7±0.7 | 2.3±3.5 | 4.6±4.9 | 1.9±2.8 | 16±13 | 28±19 | 93±23 | 146±82 | 49±23 | 3.8±3.9 |

Abbreviations: ALP = alkaline phosphate; AT = antithrombin; BUN = blood urea nitrogen; Cre = creatine; CRP = C reactive protein; FFP = fresh frozen plasma; GOT = glutamic oxaloacetic transaminase; GPT = glutamic-pyruvate transaminase; Hb = hemoglobin; LDH = lactic dehydrogenase; Plt = platelet; RBC = red blood cell; Ret = reticulocyte; TAT = thrombin–antithrombin complex; T.Bil = total bilirubin; TM = thrombomodulin; t-PA/PAI-1 = tissue plasminogen activator/plasminogen activator inhibitor-1; VWF:AG = von Willebrand factor antigen; WBC = white blood cell.
Values are mean ± s.d.
The values in shaded area denote statistically significant differences between FFP (−) and FFP (+) groups ($P<0.05$).

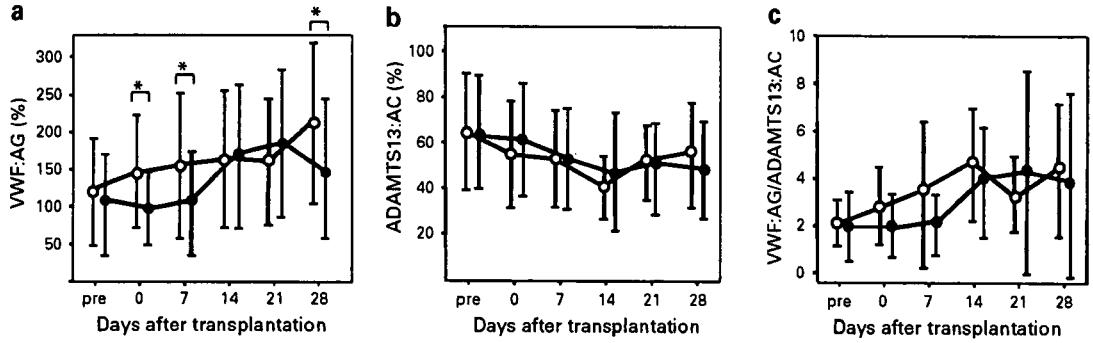



**Figure 2** Changes in von Willebrand factor (VWF):AG, ADAMTS13:AC and VWF:AG/ADAMTS13:AC after stem cell transplantation. Open circles represent patients from the fresh frozen plasma (FFP) (−) group. Closed circle represents patients from FFP (+) group. (**a**) VWF:AG in patients from the FFP (−) group was significantly higher than in patients from FFP (+) group on days 0, 7 and 28. (**b**) No difference in the level of ADAMTS13:AC was observed between the groups. (**c**) The ratio of VWF:AG/ADAMTS13:AC in FFP (−) group was higher than in FFP (+) group, but this difference was not statistically significant. $*P<0.05$.

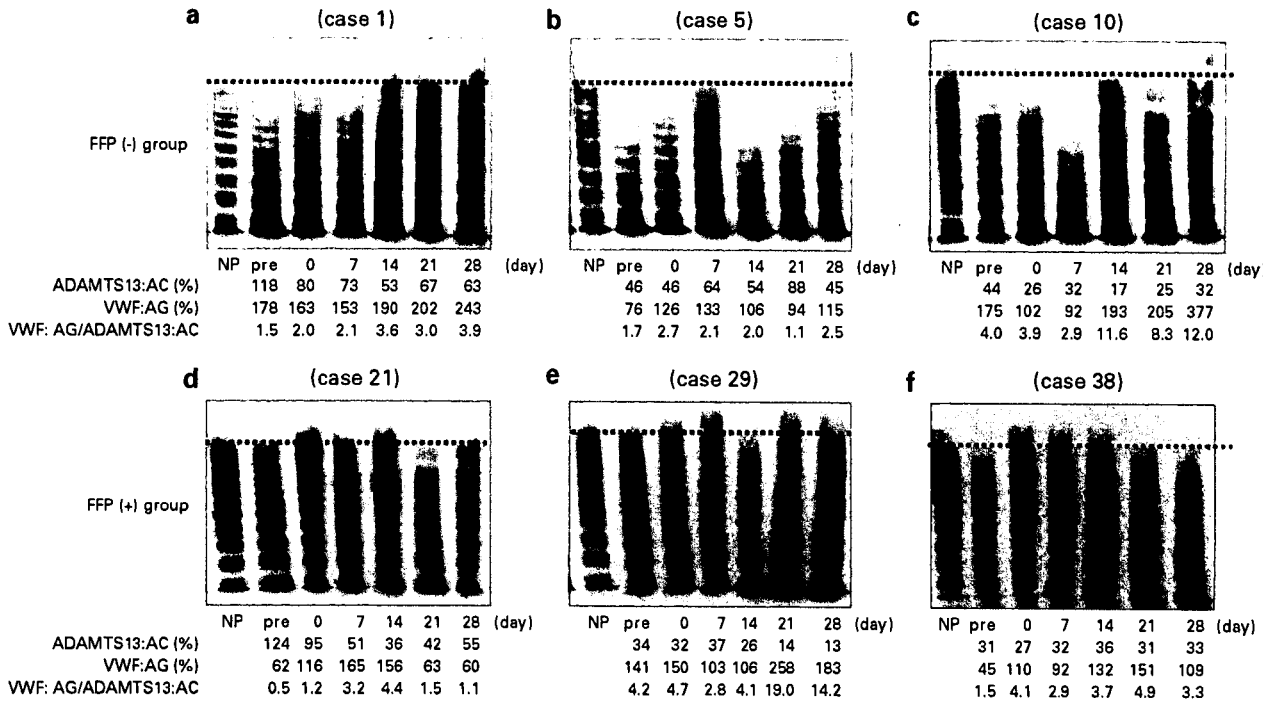



**Figure 3** von Willebrand factor (VWF) multimer analysis in patients without hepatic veno-occlusive disease (VOD). VWF multimer analyses of six representative cases, three fresh frozen plasma (FFP) (+) and three FFP (−), without hepatic VOD are shown. In patients from FFP (−) group, VWF multimers of high and intermediate molecular weight were less or absent before stem cell transplantation (SCT) and on days 0 and 7 in case 1 (**a**); at pre-SCT and on days 0, 14 and 21 in case 5 (**b**) and at pre-SCT and on days 0, 7 and 21 in case 10 (**c**). In contrast, in patients from FFP (+) group (case 21, 29, 38), no apparent changes in VWF multimer patterns were found throughout SCT including the preconditioning period, when compared to patterns found in normal control plasma (**d**, **e**, **f**). The multimers over the dotted line indicate unusually large VWF multimers (UL-VWFM), which can never be detected in normal plasma.

whereas none of the 23 patients belonging to the FFP (+) group did so. The incidence between patients receiving and not receiving FFP did not reach statistical significance because of the small numbers of enrolled patients. However, when analysis involving all the patients examined both the randomized controlled trial and the subsequent study by questionnaire, the incidence of hepatic VOD was significantly lower in patients receiving prophylactic FFP infusion than in those not doing so (Table 3). These results suggest that prophylactic FFP infusions may be instrumental in preventing the development of hepatic VOD after SCT.

In the patients who developed VOD (cases 8 and 12) in the randomized controlled study, VWF:AG progressively increased and ADAMTS13:AC gradually decreased, resulting in ratios of 13.0 at day 7 in case 8, and 4.8 at day 21 in case 12, which were notably higher than that found in normal subjects (1.0). Hepatic VOD developed a week later, when the ratio of VWF:AG to ADAMTS13:AC showed values as high as 7.1 in case 8, and 7.3 in case 12. An inverse correlation between decreased ADAMTS13:AC and increased VWF:AG was thus seen in our VOD patients. These findings were consistent with previous

**Table 3** The incidence of VOD occurrence in SCT patients with and without FFP infusion

| | *FFP (−)* | *FFP (+)* | P-*value* |
|---|---|---|---|
| Randomized study | 3[a]/20[b] (15%) | 0/23 (0%) | 0.092 |
| Study by the questionnaire | 11/173 (6.4%) | 0/36 (0%) | 0.120 |
| Total | 14/193 (7.3%) | 0/59 (0%) | 0.033 |

Abbreviations: FFP = fresh frozen plasma; SCT = stem cell transplantation; VOD = veno-occlusive disease.
[a]Number of patients with hepatic VOD.
[b]Number of high-risk patients to lapse into hepatic VOD.

findings in pathological conditions including liver cirrhosis, chronic renal insufficiency, acute inflammatory states and major surgery.[18] Furthermore, our previous study[17] demonstrated that the mean value of ADAMTS13:AC pre-SCT was 32% in seven patients with hepatic VOD. In this study, ADAMTS13:AC was, however, 67 and 55% pre-SCT in cases 8 and 2, respectively. The activity thereafter decreased from 67 to 24% on day 14 in case 8, and from 55 to 32% on day 28 in case 12. From these results, there appeared to be a possibility that hepatic VOD could develop even in the patient whose plasma ADAMTS13:AC pre-SCT did not drop below 30%, indicating that the imbalance of VWF:AG to ADAMTS13:AC before and throughout SCT may be more important for the development of hepatic VOD than the decrease of ADAMTS13:AC.

Surprisingly, VWF multimers corresponding to high and intermediate molecular weight, which are usually seen in normal plasma, were absent before SCT in case 8 and on day 0 in case 12 (Figures 1b and d), but thereafter gradually appeared. These results suggest that the initial absence of high and intermediate VWF multimers at preconditioning or just after SCT, and subsequent appearance of these VWF multimers in combination with the increase in VWF:AG and decrease in ADAMTS13:AC may play an important role in the development of VOD after SCT. It remains unclear why high and intermediate VWF multimers were lacking at preconditioning and/or during the early period after SCT but thereafter gradually appeared in VOD patients. We speculate, however, that the target lesion caused by intensive chemotherapy and/or total body irradiation given in the setting of SCT is to the sinusoidal endothelial cells. Indeed, chemotherapy including cyclophosphamide and busulfan before SCT is a regimen associated with a high incidence of hepatic VOD,[4] and total body irradiation causes radiation-induced liver disease.[30] The amount of VWF released from injured endothelial cells may be increased at first, but may decrease thereafter because the endothelial cells are extensively damaged. After SCT, as damaged endothelial cell gradually regenerate, the release of VWF may increase, resulting in the appearance of high and intermediate VWF multimers. Under these circumstances, plasma ADAMTS13 may be consumed to degrade the large amounts of VWF derived from damaged hepatic endothelial cells. Moreover, ADAMTS13 that is exclusively generated in the HSCs may be decreased owing to the liver injury itself. Our previous report that plasma ADAMTS13:AC is significantly reduced in patients with hepatic VOD even before and throughout SCT[17] supports the hypothesis that ADAMTS13:AC decreases may be an indicator of impending development of VOD. This imbalance of decreased activity of ADAMTS13 vs 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, especially in zone 3 of the hepatic lobule where hepatocytes are easily damaged by hypoxia.[1] Whether or not patients develop VOD after SCT may depend upon the degree of imbalance between VWF:AG and ADAMTS13:AC at preconditioning and/or just after SCT.

We then compared clinical parameters between patients receiving and not receiving FFP infusions to evaluate the effect of FFP on the prevention of VOD. VWF:AG was significantly lower at days 0, 7 and 28 in patients receiving FFP than in those not doing so. The reciprocal relationship between gradually decreased ADAMTS13:AC and gradually increased VWF:AG after SCT was seen in the FFP (−) group, but not in the FFP (+) group (Figures 2a and b). No difference in the levels of ADAMTS13:AC was found between the two groups (Figure 2b). The ratio of VWF:AG to ADAMTS13:AC tended to be lower at days 0 and 7 in the FFP (+) group (Figure 2). These results indicated that the FFP infusions can suppress the increase in plasma VWF:AG in the early stages after SCT. Furthermore, in the FFP (−) group, high and/or intermediate molecular weight VWF multimers were lacking in the early stages and even in the later stage after SCT (Figure 3). In the FFP (+) group, however, no apparent changes in VWF multimer patterns were found throughout SCT (Figure 3). These results indicate that the supplementation of ADAMTS13 achieved by FFP administration may suppress the increase in VWF:AG that is extensively released from damaged endothelial cells after chemotherapy and/or total body irradiation. ADAMTS13 may be consumed to degrade a large amount of UL-VWFM released from damaged endothelial cells throughout SCT. We therefore were unable to observe any increase in ADAMTS13:AC supplemented by the administration of FFP in patients with FFP infusion. From this vantage point, FFP administration as a source of ADAMTS13 affords a compellingly effective means of preventing hepatic VOD.

In conclusion, hepatic VOD developed only in patients not receiving FFP infusions, probably because of increased VWF production relative to decreased ADAMTS13:AC throughout SCT, although other mechanisms may have played a role. Prophylactic FFP infusions should therefore be considered in patients at high risk of developing of hepatic VOD after SCT.

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Brief report

# Functional imaging of shear-dependent activity of ADAMTS13 in regulating mural thrombus growth under whole blood flow conditions

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**The metalloprotease ADAMTS13 is assumed to regulate the functional levels of von Willebrand factor (VWF) appropriate for normal hemostasis in vivo by reducing VWF multimer size, which directly represents the thrombogenic activity of this factor. Using an in vitro perfusion chamber system, we studied the mechanisms of ADAMTS13 action during platelet thrombus formation on a collagen surface under whole blood flow conditions. Inhibition studies with a function-blocking anti-ADAMTS13 antibody, combined with immunostaining of thrombi with an anti-VWF monoclonal antibody that specifically reflects the VWF-cleaving activity of ADAMTS13, provided visual evidence for a shear rate–dependent action of ADAMTS13 that limits thrombus growth directly at the site of the ongoing thrombus generation process. Our results identify an exquisitely specific regulatory mechanism that prevents arterial occlusion under high shear rate conditions during mural thrombogenesis. (Blood. 2008; 111:1295-1298)**



## Introduction

The adhesive protein von Willebrand factor (VWF) plays a major role in platelet thrombogenesis, a process crucial for hemostasis. However, the excessive function of VWF is thought to increase the risk of fatal arterial thrombosis.[1,2] The thrombogenic activity of VWF is strictly dependent upon its multimeric structure, which is thought to be regulated in vivo by the metalloprotease ADAMTS13 through its cleavage of the A2 domain of the VWF subunit.[3,4] Indeed, patients with congenital deficiency of ADAMTS13 suffer repeated thrombotic complications attributed to excessive function of the ultra-large VWF (ULVWF) multimer, which is not found in normal blood circulation.[3-6] This concept was recently confirmed by knock-out mouse studies, in which ADAMTS13$^{-/-}$ mice exhibited enhanced thrombogenicity in the ex vivo or in vitro experimental blood flow conditions tested.[7,8]

The mechanisms by which ADAMTS13 regulates VWF remain poorly understood. However, recent studies showing that ADAMTS13 under flow conditions can rapidly cleave ULVWF secreted from and anchored to cultured endothelial cell layers[9,10] have raised the possibility that blood flow is critical in activating ADAMTS13.[11] Indeed, the VWF-cleaving activity of ADAMTS13 cannot be reproduced in vitro under static conditions unless the substrate VWF molecule is somewhat modified (eg, denatured by guanidine-HCl or urea).[3,4] Further, the question arises of whether ADAMTS13, in addition to its known action on ULVWF freshly released from endothelial cells, might also act directly at the local sites of thrombus generation to regulate thrombus growth.

To address these issues, we analyzed the role and mechanisms of ADAMTS13 action in mural platelet thrombogenesis on a collagen-coated glass surface in an in vitro perfusion chamber system. Our visual evidence demonstrates that ADAMTS13 cleaves VWF and down-regulates mural thrombus growth at the site of ongoing thrombus generation in a shear rate–dependent manner under whole blood flow conditions.

## Methods

### Blood collection

The present work was approved by the institutional review board of Nara Medical University, and informed consent was obtained in accordance with the Declaration of Helsinki. Using 200 μM argatroban as an anticoagulant, blood was collected from 10 nonsmoking healthy volunteers who had not taken any medications in the previous 2 weeks.

### Monoclonal antibodies

A function-blocking anti-ADAMTS13 monoclonal antibody (A10), which completely inhibits plasma ADAMTS13 activity at the concentration of 20 μg/mL,[12] was used as a divalent $(ab')_2$ fragment in inhibition studies. An anti-VWF monoclonal antibody (N10) was used that reacts with an epitope within the VWF A2 domain (10-amino acid VWF peptide; $D^{1596}$REQAPNLVY$^{1605}$) only after cleavage by ADAMTS13 exposes the epitope; thus, reactivity of antibody N10 specifically reflects the VWF-cleaving activity of ADAMTS13, as described.[13]

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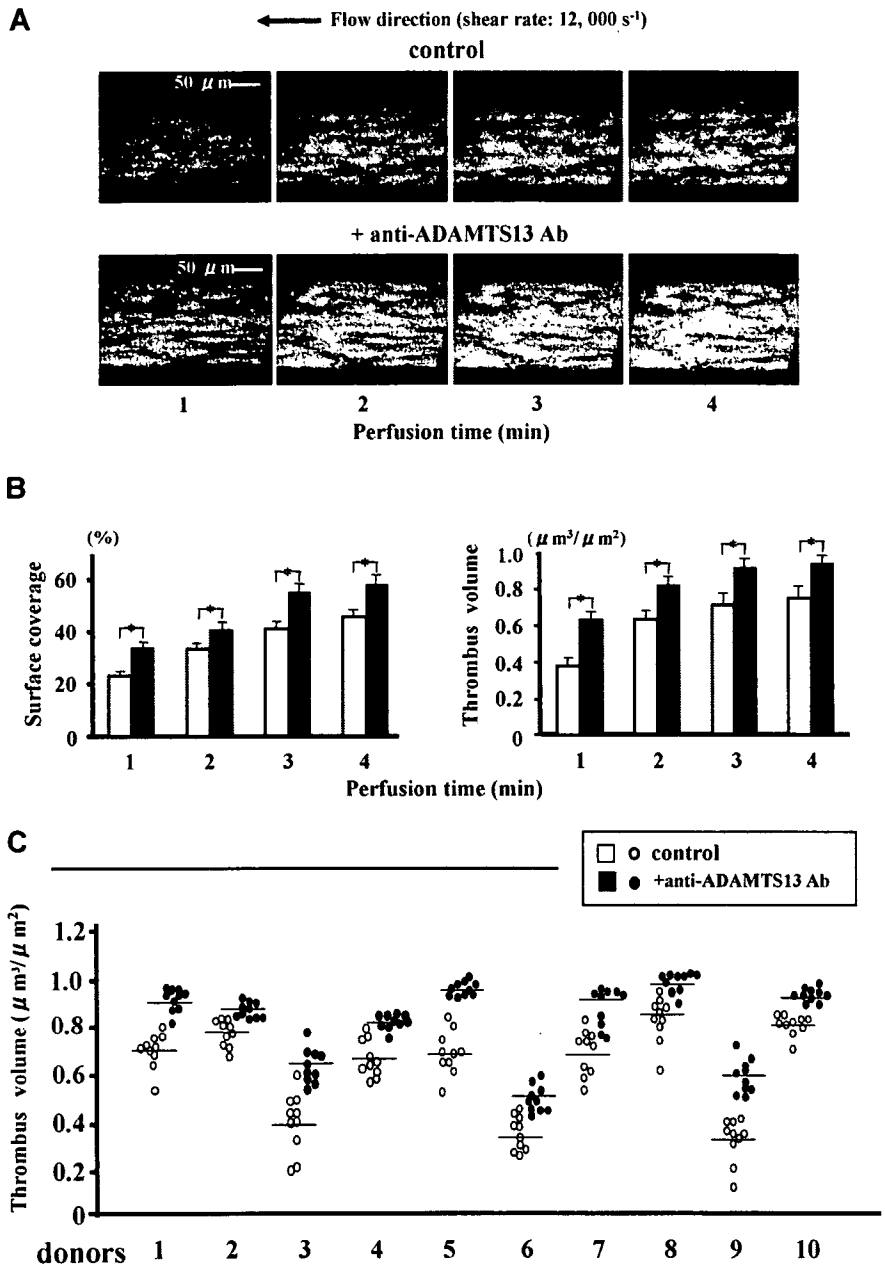


**Figure 1. Effects of a function-blocking anti-ADAMTS13 monoclonal antibody (A10) on mural thrombus generation under very high shear rate conditions.** Whole blood from healthy volunteers containing DiOC6 (1 μM)-labeled platelets, anticoagulated with argatroban, was perfused over a type I collagen-coated glass surface under very high shear rate (12 000 $s^{-1}$) with anti-ADAMTS13 antibody A10 or with control mouse IgG (each 20 μg/mL). (A) Time-course changes of 3-dimensional images of thrombi (original magnification, ×600), which were constructed by the image-analyzing system of confocal laser scanning microscopy (CLSM) based on successive horizontal slices at identical portions, are representative of 10-pair flow experiments using blood from 10 independent donors. (B) Statistical analyses corresponding to the above images; bars represent mean (+ SD) surface coverage or total thrombus volume in 10 areas (each 133 × 100 μm) randomly selected in each perfusion using a single donor blood (donor number 1 in panel C). Note that thrombus generation is significantly (*; $P < .01$) accelerated in the presence of the anti-ADAMTS13 antibody. (C) Thrombus volume at 3 minutes' perfusion in 10-pair flow experiments using 10 independent donors; data points represent values of 10 areas randomly selected in each perfusion with (●) or without (○) anti-ADAMTS13 antibody, and transverse lines indicate mean values for each group. Note also that thrombus volumes generated in the presence of anti-ADAMTS13 antibody are significantly ($P < .01$; asterisks not included in the figure) greater than control thrombi in all 10-pair experiments.

### In vitro perfusion studies

Thrombus generation on a type I collagen-coated (Sigma-Aldrich, Tokyo, Japan) glass surface was studied under various shear rates in a parallel plate flow chamber system as described.[14-17] Surface coverage and volume of thrombi generated at the indicated time points during whole blood perfusion were evaluated based on images obtained by confocal laser scanning microscopy (CLSM; FV300; Olympus, Tokyo, Japan), as described.[15-17] Immunohistochemical staining of thrombi using anti-VWF antibodies was performed as described.[15-17] Briefly, thrombi on a glass surface were fixed with paraformaldehyde and incubated with a mixture of anti–whole VWF rabbit polyclonal antibody (30 μg/mL; DAKO Cytomation, Kyoto, Japan) and N10 antibody (60 μg/mL) or with the negative control IgG mixture (rabbit; 30 μg/mL, mouse; 60 μg/mL; DAKO Cytomation) for 90 minutes at 37°C. Samples were then stained with a mixture of fluorescein isothiocyanate (FITC)–conjugated anti–rabbit IgG (3.3 μg/mL; BioSource International, Camarillo, CA) and Cy3-conjugated anti–mouse IgG (3.3 μg/mL; Sigma-Aldrich) as secondary fluorescent antibodies for 90 minutes at 37°C, and viewed by CLSM. These conditions were determined in preliminary experiments to confirm the sufficient infiltration of both primary and secondary fluorescent antibodies into thrombi.

## Results and discussion

To address the potential role of ADAMTS13 in the ongoing process of mural thrombus generation, we compared the size of thrombi generated in the presence or absence of a function-blocking antibody against ADAMTS13 in a perfusion chamber system, using blood from the same donor. This relatively simple experimental approach is able to precisely evaluate ADAMTS13 function in uniform blood conditions, avoiding the individual heterogeneity of sample blood conditions including VWF and platelets that might otherwise seriously affect the size of thrombi generated in this type of flow experiment.