specifically cleaves the Y1605-M1606 bond of the VWF-A2 domain. Deficiency of this enzyme results in the accumulation of unusually large VWF multimers in the circulation that can lead to platelet (PLT) aggregation.[14]

During the past decade, assessment of plasma ADAMTS13 activity was performed by an electrophoretic technique with purified VWF as a substrate. This method required a relatively long reaction time in the presence of protein denaturants, such as 1 to 1.5 mol per L urea or guanidine-HCl.[10,11] More recent studies, however, have used *Escherichia coli*–expressed recombinant VWF-A2 polypeptides, tagged with glutathione *S*-transferase (GST)-histidine (His; GST-VWF73-His) or His-T100 (His-VWF188-T100), as the substrate for ADAMTS13 cleavage in the absence of protein denaturants; these changes have dramatically shortened the reaction time.[15-17] In both the classic and the modern assays, however, the principle for determination of ADAMTS13 activity is based on the quantification of residual undigested substrate. A recently introduced FRETS-VWF73 assay with a fluorogenic substrate,[18] unlike other previously reported assays, directly measures the final product generated by ADAMTS13 cleavage. These assays, however, are not sensitive enough to measure the low levels (approx. 5%) of plasma ADAMTS13 activity. Distinction of these levels is critically important to understand the manifestation of the clinical signs of TTP. In addition, this FRETS-VWF73 assay requires a fluorophotometer, which uncommon in routine diagnostic laboratories.

We have developed a highly sensitive enzyme-linked immunosorbent assay (ELISA) measuring ADAMTS13 activity. First, we prepared specific monoclonal antibodies (MoAbs) directed against the decapeptide of the VWF-A2 domain ending with the C-terminal edge residue Y1605, which is generated by ADAMTS13 cleavage. These MoAbs did not react with intact VWF, but did react with the synthetic decapaptide as well as the monomeric or dimeric N-terminal VWF polypeptide (residues 764-1605). Thus, the peroxidase-labeled MoAb could be used as a detection antibody with recombinant VWF73 (residues 1596-1668 of VWF subunit), tagged with both GST and a His tag (GST-VWF73-His),[15] as the enzyme substrate in this novel ELISA. Because this assay would be completed within 3 hours under the hospital environments, it can be introduced for routine laboratory work in transfusion medicine.

MATERIALS AND METHODS

Assays of ADAMTS13 activity and its inhibitors by VWF multimer analysis

We performed a classic VWF multimer assay to screen plasma samples for ADAMTS13 activity and the presence of inhibitors.[10,12] The detection limit of this method for ADAMTS13 activity was 3 percent of the normal levels. The inhibitor titers are expressed as Bethesda units (BUs),[19] where one inhibitor unit is defined as the amount necessary to reduce ADAMTS13 activity to 50 percent of control levels. A titer of greater than 0.5 BUs per mL was considered to be significant. Before assessing the levels of ADAMTS13 inhibitor, test plasma samples were heat-treated at 56°C for 1 hour to kill endogenous ADAMTS13 activity. After centrifugation, the supernatants were examined by these assays.

Patients and their plasma samples

Patient plasma samples were collected from referring hospitals across Japan, along with the patients' clinical information. Those plasma samples that fulfilled the diagnostic criteria for thrombotic microangiopathies (TMAs)[20] were used in this study. Measurement of plasma ADAMTS13 activities and inhibitor titers by VWF multimer analysis categorized these patients into three groups: 1) less than 3 percent of ADAMTS13 activity without the presence of an inhibitor (congenital TTP or USS), 2) less than 3 percent of ADAMTS13 activity with an inhibitor present (acquired TTP), and 3) moderately decreased (≥3%) or subnormal ADAMTS13 activity with or without its inhibitor (TMA). Patients with the clinical manifestations of constitutive TMA, but with normal ADAMTS13 activity, which might be caused by abnormalities in Factor H or CD46, were excluded from this study.

According to these classifications, 81 patients were examined in this study. Of these, 29 had developed acquired TTP, 32 exhibited TMA with measurable ADAMTS13 activity level, and 20 had USS. ADAMTS13 gene analysis confirmed that all USS patients were either compound heterozygotes or homozygotes for gene abnormalities in this protease. In addition, 33 relatives of these USS patients were identified as the carriers of ADAMTS13 gene mutations.[21-24]

Citrated PLT-poor plasma samples prepared from these subjects were frozen on dry ice, sent to our laboratory, and stored at –80°C until use. Normal citrated plasma samples were obtained from 55 healthy individuals (29 female and 26 male, aged 20-40 years) for use as controls and frozen in aliquots at –80°C. Pooled normal plasma samples from these individuals was used as standard plasma in this study. These studies were conducted with the approval of the ethics committee of Nara Medical University.

Preparation and cleavage of GST-VWF73-His fusion protein

Recombinant GST-VWF73-His fusion protein was expressed in *E. coli* inclusion bodies and purified by the method of Kokame and colleagues.[15] Purified GST-VWF73-His was cleaved with plasma ADAMTS13 by the incubation of 540 ng of substrate with 1 μL of normal plasma in

a total volume of 100 μL of reaction buffer (5 mmol/L Tris-HCl, 10 mmol/L $BaCl_2$, and 1 mmol/L PMSF, pH 5.5) at 37°C for 1 hour. Reactions were terminated by the addition of 1 μL of ethylenediaminetetraacetate (EDTA)-2Na (500 mmol/L).

Production of anti-N10 and N15 murine MoAbs

Two synthetic peptides were designed for use as immunogens in the production of MoAbs, a decapeptide (1596-DREQAPNLVY-1605, termed N10) derived from the VWF-A2 domain and a pentadecapeptide (1596-DREQAPNLVYMVTGN-1610, termed N15). Both peptides were conjugated to keyhole limpet hemocyanin (Asahi Techno Glass Corp., Tokyo, Japan). Four 8-week-old Balb/c mice were immunized subcutaneously with 50 μg of each peptide emulsified in complete Freund's adjuvant (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Three immunizations were performed at 2-week intervals. A final booster was given intraperitoneally at a concentration of 10 μg per 100 μL in saline solution at 2 weeks after the last immunization. Mice splenocytes were fused with myeloma cells (P3U1 cell line) according to the method of Köhler and Milstein.[25] Screening of positive hybridomas was performed by ELISA of culture supernatants in polystyrene microtiter plates coated with the recombinant peptides.

Conjugation of horseradish peroxidase to MoAb

Antibodies were purified from culture supernatants of hybridoma cells with a protein A-Sepharose Fast Flow column (Amersham Bioscience, Corp., Piscataway, NJ). Fractions containing immunoglobulin G (IgG) were pooled and dialyzed against PBS. Purified monoclonal IgG was digested with pepsin; the resulting $F(ab')_2$ fragments were purified by the method of Hamaguchi and associates[26] and conjugated to horseradish peroxidase (HRP).[27]

Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with either a Tris-glycine buffer system[28] or a Tris-tricine buffer system.[29] After electrophoresis, separated proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). After blocking nonspecific binding with 3 percent skim milk, PVDF membranes were incubated with monoclonal IgGs. When indicated, the monoclonal IgGs were labeled with HRP; for unlabeled monoclonal IgGs, bound antibody was detected with peroxidase-labeled anti-mouse IgG. Proteins were visualized by chemiluminescence with Western lightning chemiluminescence reagent (Perkin-Elmer Life Sciences, Inc., Boston, MA) and imaged by X-ray autoradiography (Eastman Kodak, Rochester, NY).

ELISA for ADAMTS13 activity with anti-N10 MoAb

One-hundred microliters of GST-VWF73-His solution (250 ng/mL in PBS with 1 percent BSA) was added to each well of microtiter plates coated with an anti-GST polyclonal antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA) and incubated at 37°C for 1 hour. After three washes with PBS-0.05 percent Tween 20 (PBS/T), 100 μL of each sample, plasma diluted 11-fold in reaction buffer (5 mmol/L acetate buffer and 5 mmol/L $MgCl_2$, pH 5.5) was incubated in the wells at 37°C for 1 hour. After washing the wells three times with PBS/T, 100 μL of HRP-conjugated anti-N10 MoAb (No. 146) was added and incubated at 37°C for 1 hour. Wells were then washed three times with PBS/T. One-hundred microliters of HRP substrate (*o*-phenylendiamine-H_2O_2) was added and incubated for 10 minutes. The reaction was terminated with 100 μL of 1 mol per L H_2SO_4; absorbance was then measured at 492 nm. To generate a reference curve, pooled normal plasma serially diluted in heat-immobilized plasma was assessed. To determine sample activity in plasma, the optical density of the test sample was interpolated against the reference curve. The ADAMTS13 inhibitor titer was measured by the combination of this ELISA with a Bethesda method.

Anti-ADAMTS13 murine MoAb

Two anti-ADAMTS13 murine MoAbs, termed A10 and C7, were produced in our laboratory as previously reported.[13] Of these, A10 completely inhibited plasma ADAMTS13 activity at a final concentration of 50 μg IgG per mL by VWF multimer assay, and the epitope was shown residing on a disintegrin-like domain of ADAMTS13 molecule.[13]

RESULTS

Epitope mapping of anti-N15 and N10 MoAbs

By ELISA-based screening assay, we obtained three MoAb clones specific for peptide N15 and 26 MoAb clones against peptide N10. The reactivity of these MoAbs was also tested by Western blotting (WB) with two GST fusions of VWF, GST-VWF73-His (uncleaved) and GST-VWF10 (cleaved by ADAMTS13; Fig. 1). Clones of anti-N15 MoAbs immunoreacted solely with the intact GST-VWF73-His (35 kDa) and did not react with cleaved GST-VWF10 (30 kDa). In contrast, the clones of anti-N10 MoAbs reacted with the GST-VWF10, but only barely with GST-VWF73-His. We chose two anti-N15 clones (No. 22 and 73) and three anti-N10 clones (No. 116, 146, and 229) for further characterization.

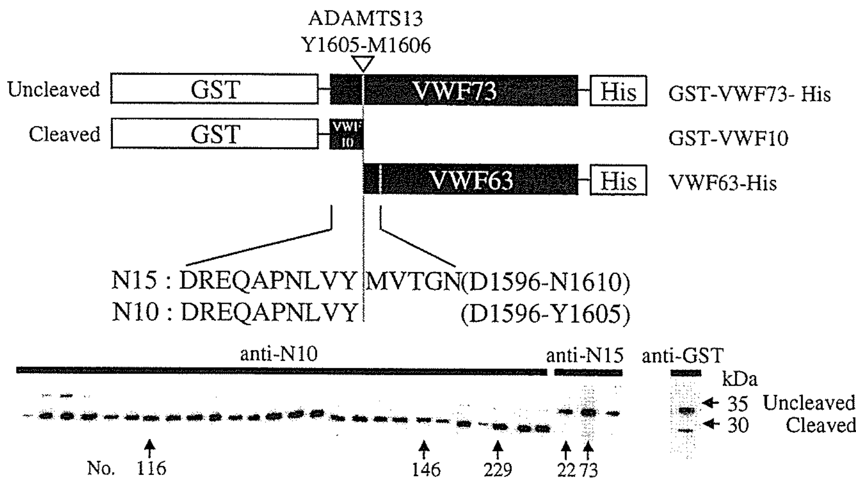

Fig. 1. Production of anti-N10 and anti-N15 murine MoAbs. The schematic structures of the GST-VWF73-His (uncleaved) and GST-VWF10 (cleaved by ADAMTS13) fusions are shown in the top panel. Anti-N10 MoAbs (26 clones), anti-N15 MoAbs (3 clones), and a polyclonal anti-GST antibody serving as a control were used for WB. The anti-N15 clones immunoreacted with the uncleaved GST-VWF73-His band (35 kDa), but not with the cleaved GST-VWF10 (30 kDa). In contrast, the anti-N10 MoAbs reacted with the GST-VWF10, but only minimally with GST-VWF73-His.

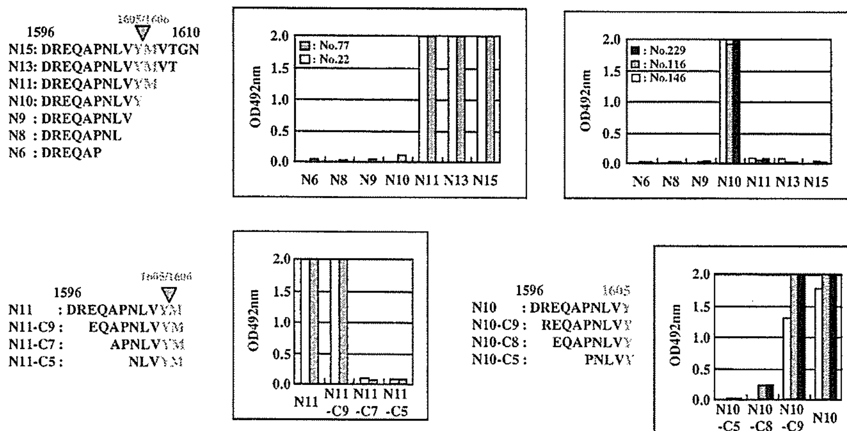

Fig. 2. Epitope mapping of anti-N15 (left) and anti-N10 (right) MoAbs by antigen-immobilized ELISA. For epitope mapping, we tested the immunoreactivities of anti-N15 and anti-N10 MoAbs against a panel of synthetic peptides by ELISA. The immunoreactivities of N15 against the C-terminal truncated peptides are shown in the top panel. The anti-N15 MoAbs reacted with N15 to N11, but lost any interaction upon deletion of M1606. In contrast, the anti-N10 MoAbs specifically reacted with N10 only. In the bottom panel, the reactivity of the anti-N15 and anti-N10 MoAbs with N-terminally deleted N11 and N10 peptides were shown. The anti-N15 MoAbs completely lost their immunoreactivity with the deletion of the four N-terminal residues (D1596 to Q1599) from the N11 peptide. The anti-N10 MoAbs lost their immunoreactivity upon deletion of the two N-terminal residues (D1596 and R1597).

To determine the precise epitopes recognized by these MoAbs, we tested the immunoreactivity of anti-N15 and anti-N10 MoAbs for a panel of synthetic peptides by ELISA (Fig. 2). The two anti-N15 MoAbs shared epitopes within amino acid residues 1596-DREQAPNLVYM-1606 of the VWF-A2 subunit, which required the presence of the two adjacent residues (1605-YM-1606). In contrast, three anti-N10 MoAbs consistently recognized epitopes on the 1597-REQAPNLVY-1605 peptide. For the anti-N10 MoAbs, the presence of Y1605 as the C-terminal residue was an absolute requirement; neither of these MoAbs reacted with the N11 or N9 peptides. Deletion of the two N-terminal residues (D1596 and R1597) from the N10 peptide resulted in an almost complete loss of immunoreactivity with the three anti-N10 MoAb clones. Thus, the epitopes recognized by the anti-N10 MoAbs consistently reside within the 1597-REQAPNLVY-1605 peptide. Hereafter, two MoAbs, one anti-N10 (No. 146) and one anti-N15 (No. 22), were used for further studies.

Immunoreactivity of anti-N15 and anti-N10 MoAbs with VWF fragments generated by ADAMTS13 cleavage

Cleavage of purified VWF by rADAMTS13 generated two major fragments with molecular weights of 340 kDa (C-terminal dimer of VWF subunits of residues 1606-2813) and 280 kDa (N-terminal dimer of VWF subunits of residues 764-1605), analyzed by a SDS-5 percent PAGE under nonreducing conditions. Reducing conditions, however, displayed two distinct bands of 140 kDa (a monomer of VWF residues 764-1605) and 176 kDa (a monomer of VWF residues 1606-2813; Fig. 3). We analyzed these rADAMTS13-cleaved VWF fragments by WB with the anti-N10 and N15 MoAbs.

The anti-N10 MoAb reacted exclusively with the 280-kDa fragment and did not react with the 340-kDa fragment under nonreducing conditions. Under reducing conditions, the anti-N10 MoAb reacted with the 140-kDa fragment, but not with the 176-kDa fragment.

In contrast, the anti-N15 MoAb did not react with VWF either before or after cleavage by rADAMTS13 in either nonreducing or reducing conditions. These results

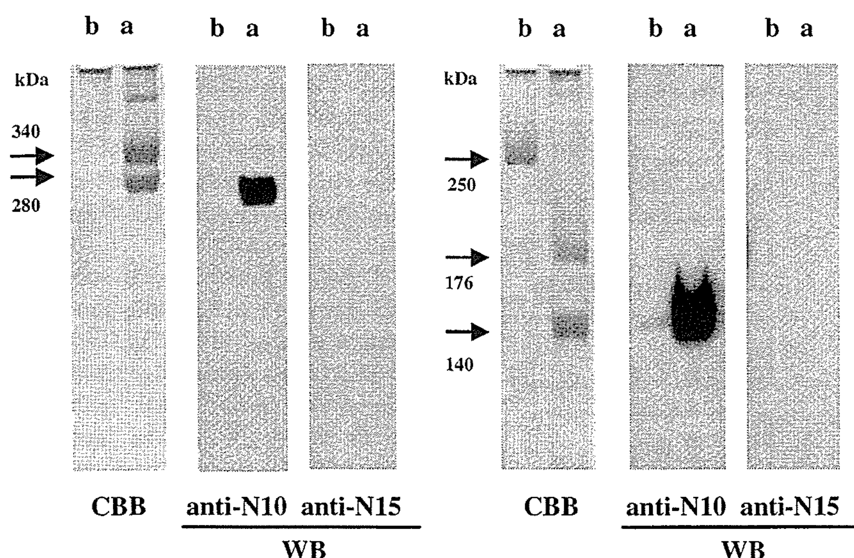

Fig. 3. Immunoreactivity of anti-N10 and anti-N15 MoAbs against VWF fragments generated by ADAMTS13 cleavage. Cleavage of purified VWF by recombinant ADAMTS13 generated two major fragments with molecular weights of 340 kDa (C-terminal dimer of VWF subunit) and 280 kDa (N-terminal dimer of VWF subunit) by a SDS-5 percent PAGE under nonreducing conditions (left). Under reducing conditions (right), two bands corresponding to the N-terminal 140-kDa fragment and the C-terminal 176-kDa fragment could be seen. WB analysis of these VWF fragments before (b) and after (a) cleavage by recombinant ADAMTS13 was performed with the anti-N10 (No. 146) and anti-N15 (No. 22) MoAbs. The anti-N10 MoAb reacted exclusively with the 280-kDa fragment, not the 340-kDa fragment, under nonreducing conditions. Under reducing conditions, the anti-N10 MoAb reacted with the 140-kDa fragment, but not with the 176-kDa fragment or the uncleaved 250-kDa VWF. In contrast, the anti-N15 MoAb did not react with VWF either before or after cleavage by rADAMTS13, under either nonreducing or reducing conditions.

indicate that the epitope recognized by the anti-N15 MoAb on VWF subunit is cryptic before rADAMTS13-mediated cleavage, but no longer exists after cleavage of the 1605-YM-1606 bond. After SDS-1.2 percent agarose gel electrophoresis of normal plasma, WBs with either the anti-N10 or the anti-N15 MoAbs could not detect the VWF multimeric patterns (data not shown).

Establishment of ELISA for ADAMTS13 activity

When ELISA was performed with pooled normal plasma, as described under Materials and Methods, reactivity measured at OD492 nm increased proportionally to the amount of plasma added (Fig. 4A). This additive effect, however, was not seen when with plasma sample derived from patients with USS or acquired TTP. The reactivity of pooled normal plasma was completely blocked by 10 mmol per L EDTA, but was not abolished by the addition of a protease inhibitor cocktail (effective against a broad range of serine proteases, cysteine proteases, aminopeptidases, and acid proteases; Sigma-Aldrich, Inc., St. Louis, MO).

The optimal conditions for this ELISA were determined with pooled normal plasma. We varied the following conditions in the reaction mixture (Fig. 4B): 1) incubation time for enzymatic cleavage, 2) species of divalent metal ions present, 3) NaCl concentration (ionic strength), 4) Mg^{2+} concentration, 5) urea concentration, and 6) pH. The ELISA was most efficient when performed for 1 hour in 5 mmol per L acetate buffer (pH 5.5) containing 5 mmol per L $MgCl_2$ in the absence of urea. A detection limit of 0.5 percent ADAMTS13 activity was estimated by the intersection point of the curve of minus 2.6 standard deviations (SDs) for the standards measured in 10 replications and the zero standards of plus 2.6 SDs (data not shown). To evaluate the precision of this assay, we measured the ADAMTS13 activities present in three different plasma samples in eight replications (within-run assay). The mean ± SD values of three samples were 4.4 ± 0.4, 52.0 ± 2.1, and 103.2 ± 7.4 percent (n = 8). The coefficient of variation values of three samples were 9.7, 3.9, and 7.1 percent (n = 8), respectively (data not shown).

Effect of antibodies in the novel ELISA

Under the optimal conditions determined above, we examined the effect of antibodies against VWF and ADAMTS13 in this novel ELISA (Fig. 5A). Pooled undiluted normal plasma was incubated for 2 hours at 37°C with varying concentrations of each purified IgG; the residual activity of ADAMTS13 in each mixture was then determined. The purified IgG from normal subjects did not exhibit significant inhibition at any concentration tested, but samples from patients with acquired TTP showed a dose-dependent inhibition, completely abrogating ADAMTS13 activity at a final concentration of 500 µg per mL. Anti-ADAMTS13 MoAb A10 (20 µg IgG/mL, final) totally blocked ADAMTS13 activity in this novel ELISA, as shown in the VWF multimer assay. The anti-N15 MoAb displayed total inhibition of plasma ADAMTS13 activity at a concentration of 20 µg per mL. These results indicate that this novel ELISA can specifically recognize the cleavage of the peptide bond between Y1605 and M1606 of the VWF-A2 domain.

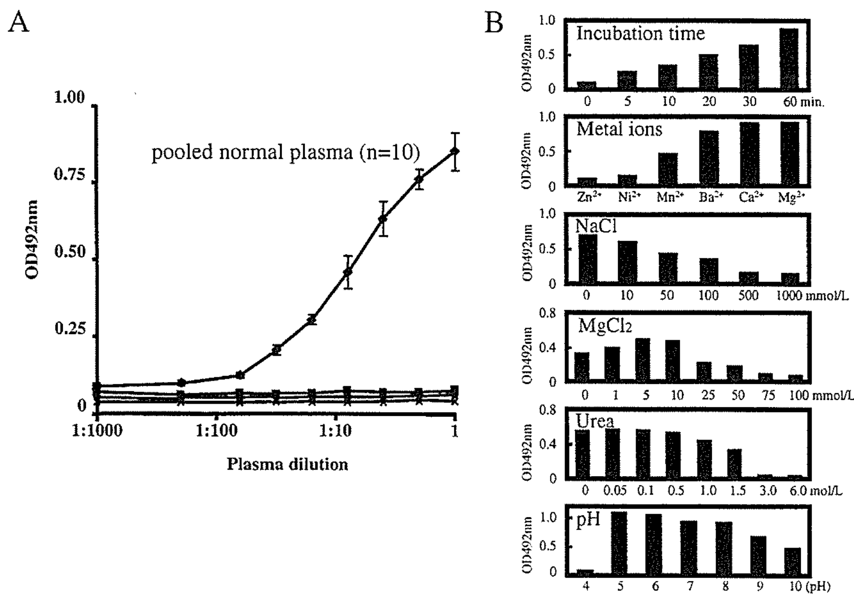

Fig. 4. Establishment of ELISA for ADAMTS13 activity. (A) In this ELISA, intensity at OD492 nm increased in proportion to the normal plasma (◆) concentration. This correlation was not observed for plasma samples from patients with USS (●) or acquired TTP (■) or in the presence of 10 mmol/L EDTA (×). The values for normal plasma shown are the mean and SD (n = 10). (B) Optimal conditions for this ELISA were determined with pooled normal plasma by varying the following conditions: 1) incubation time for enzymatic cleavage, 2) the species of divalent metal ions included (5 mmol/L, final concentration), 3) NaCl concentrations (ionic strength), 4) Mg^{2+} concentrations, 5) urea concentrations, and 6) pH.

Measurement of plasma ADAMTS13 inhibitory activity

We also determined plasma ADAMTS13 inhibitory activity in samples from patients with acquired TTP with this ELISA. We observed a good correlation between the activities of anti-ADAMTS13 inhibitors determined by this ELISA and those given by the VWF multimer assay (Fig. 5B). The detection limit of VWF multimer and ELISA methods were 0.5 and 0.1 BUs per mL, respectively. A significant positive correlation with a coefficient of 0.99 was observed for 38 independent samples.

Levels of plasma ADAMTS13 activity

For 20 patients with USS who consistently exhibited plasma levels of ADAMTS13 activity less than 3 percent of normal levels by a classic VWF multimer assay, the value determined with this novel ELISA was less than 0.5 percent of normal levels in 16 patients. In the remaining 4 patients, the values ranged from 0.6 to 1.3 percent. USS carriers (n = 33) exhibited activities averaging 34.3 ± 12.3 percent (mean ± SD; Fig. 6). We have reported a very rare individual, the father of a patient with USS, who carries two ADAMTS13 gene mutations (R268P/P475S). He consistently exhibited very low levels of plasma ADAMTS13 activity (4.5-7%) by a classic VWF multimer assay.[21] He is now 38 years old, but so far he has no episode of thrombocytopenia or TTP. By our novel ELISA, this individual also showed low plasma ADAMTS13 activity (4.2%).

Of the 61 patients with acquired TTP, 29 patients had less than 3 percent of normal plasma ADAMTS13 activity, while 32 patients exhibited greater than 3 percent by classic VWF multimer assay. In these groups, the levels of plasma ADAMTS13 activity measured by the novel ELISA were 0.7 ± 0.5 and 13.8 ± 10.3 percent, respectively. In normal individuals, plasma level of ADAMTS13 activity measured under these conditions averaged 99.1 ± 21.5 percent (26 male, 97.1 ± 18.1%; 29 female, 100.1 ± 24.4%; Fig. 6).

The ADAMTS13 activities measured by either ELISA or the classic VWF multimer assay were compared for the three groups of USS patients and carriers, patients with acquired TTP, and normal individuals (Fig. 7). The regression line for the three groups were $y = 0.67x + 3.51$, $y = 1.15x + 1.40$, and $y = 0.90x + 10.05$, respectively. The correlation coefficients for the three groups were $r = 0.82$, $r = 0.79$, and $r = 0.85$, respectively.

DISCUSSION

We have developed a convenient and highly sensitive ELISA measuring ADAMTS13 activity. We prepared mouse MoAbs that recognized the C-terminal edge residue Y1605 of the VWF-A2 domain that is exposed by ADAMTS13 cleavage. Two synthetic peptides, N15 and N10, derived from the VWF-A2 domain were prepared and used as immunogens. We have obtained a number of MoAb clones specific for both peptides and used one MoAb from each group for further studies. Anti-N15 MoAb reacted with both the N15 peptide and GST-VWF73-His, but did not react with N10, GST-VWF10, or the 250-kDa VWF subunit, indicating that the epitope includes the 1605-YM-1606 bond within the VWF-A2 domain, which is cryptic in the full-length VWF subunit. In contrast, the anti-N10 MoAb reacted with the N10 peptide, GST-VWF10, the dimer of the N-terminal VWF subunit, and the monomer of the N-terminal 140-kDa band, but did not react with other synthetic peptides, GST-VWF73-His, or the undigested 250-kDa VWF subunit. Studies with a panel of N10-related

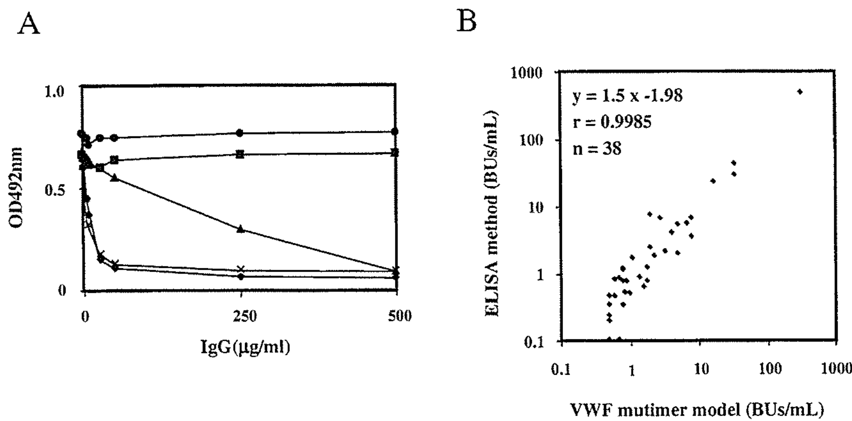

Fig. 5. Effect of antibodies in this ELISA and levels of plasma anti-ADAMTS13 inhibitor. Pooled undiluted normal plasma was incubated for 2 hours at 37°C with varying concentrations of purified IgGs. The residual activity of ADAMTS13 in this mixture was then determined. (A) Purified IgG from normal subjects (■) and normal mouse IgG (●) did not exhibit any significant inhibition at any of the concentrations tested; samples from patients with acquired TTP (▲) demonstrated a dose-dependent inhibition. Of two anti-ADAMTS13 MoAbs generated in this study, both the A10 antibody (×) and the anti-N15 MoAb (No. 73; ◆) completely inhibited ADAMTS13 activity at a concentration of 20 μg/mL. (B) The measurements of anti-ADAMTS13 inhibitor concentration by this ELISA and the VWF multimer assay correlated well. The inhibitor detection limit for the VWF multimer and ELISA methods were 0.5 and 0.1 BUs per mL, respectively. A significant positive correlation with a coefficient of 0.99 was observed for 38 independent samples.

synthetic peptides indicated that the crucial epitope for anti-N10 MoAb requires Y1605 to be the C-terminal residue, but also recognizes a conformation created by the nanopeptide sequence (1597-REQAPNLVY-1605). This recognition pattern allows the use of this MoAb in our novel ELISA, as the C-terminal Y1605 residue is exposed by the cleavage of full-length VWF by ADAMTS13.

When we performed an ELISA with GST-VWF73-His as a substrate and HRP-labeled anti-N10 MoAb as a second antibody, reactivity increased in proportion to the amount of enzyme added. Next we optimized reaction conditions of this ELISA, because ADAMTS13 requires the presence of divalent cations for its activity. In our novel ELISA, Mg^{2-} was most efficient for expressing the proteolytic activity. This result appeared to be slightly different from previous studies, because Ba^{2-} was optimal in the VWF multimer assay with native VWF as substrate,[30] whereas Ca^{2+} was most efficient in FRETS-VWF73 assay. This inconsistency is apparently caused by the difference of substrate species and reaction conditions, including protein denaturants and pH. Thus, we have included 5 mmol per L $MgCl_2$ in the reaction mixture of our assay. We confirmed the specificity of this novel ELISA by inhibition with EDTA and with a variety of well-characterized MoAbs. As a result, this novel ELISA is superior to previously reported assays.

The first advantage of this assay is the increased sensitivity. The lower limit of this assay was determined to be 0.5 percent of the normal control, in contrast to that of conventional assays at 3 to 5 percent. The sensitivity of this assay provided us with new information about USS and acquired TTP. For example, the novel ELISA demonstrated that 16 of 20 USS patients had plasma levels of ADAMTS13 activity below 0.5 percent of the normal control. The remaining 4 patients, however, had the values of 0.6 to 1.3 percent. Interestingly, an asymptomatic carrier with R268P/P475S gene mutations, the father of a USS patient, showed 4.2 percent of the activity by this ELISA. This may indicate that the plasma level of ADAMTS13 activity between 1.3 and 4.2 percent is a range essentially important to regulate the manifestation of clinical signs of TTP, unless other precipitating factors are present. Prophylactic infusions with a small amount of fresh-frozen plasma (approx. 5 mL/kg, every 2-3 weeks) to USS patients, which is effective at preventing the clinical manifestations, may support our speculation. Further, in patients with acquired TTP with less than 3 percent of ADAMTS13 activity by a VWF multimer assay, this ELISA showed that 23 of 29 had ADAMTS13 activity below 0.5 percent, and the remaining six patients had the values of 0.6 to 2.6 percent.

The second advantage of this assay is the sensitivity of measurement for inhibitors of ADAMTS13. The existence of inhibitors of ADAMTS13 is the key to diagnosing acquired TTP. The levels of plasma inhibitors against ADAMTS13 are also an indicator of the efficacy of therapy, typically plasma exchange or corticosteroids, in patients with acquired TTP. It is difficult, however, to determine accurately the low titers of plasma inhibitors of ADAMTS13 with conventional methods. The detection limit for inhibitors in this novel ELISA was calculated at 0.1 BU per mL. Inhibitor levels under 0.5 BU per mL, the lower limit of a VWF multimer assay, were detected in five patients with acquired TTP with this ELISA. Further investigation will be required to identify the significance of low titer inhibitors.

The third advantage of this ELISA is convenience of its performance in hospital environments, because it does not require any special technique or instrument except for standard ELISA equipment routinely used in many laboratories. As a consequence, we have established a convenient and highly sensitive MoAb-based ELISA to measure

ADAMTS13 activity. The values determined by this method correlated well with those determined by classic VWF multimer assay. Because this novel assay utilizes MoAbs, it may be possible to develop a rapid, automated assay to assess ADAMTS13 activity based on this technology.

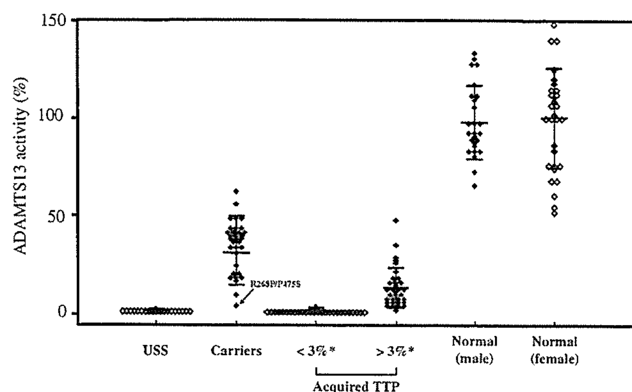

Fig. 6. Plasma levels of ADAMTS13 activity in normal subjects and patients with TTP. Plasma levels of ADAMTS13 activity in 16 patients with USS, determined by the novel ELISA, were less than 0.5 percent of normal levels; in the remaining four patients, activities ranged from 0.6 to 1.3 percent of normal. USS carriers (n = 33) exhibited an average activity of 34.3 ± 12.3 percent (mean ± SD). Of 61 patients with acquired TTP, 29 patients had less than 3 percent and 32 patients displayed greater than 3 percent of normal plasma ADAMTS13 activity by the VWF multimer method. In these two groups, the levels of plasma ADAMTS13 activity by the novel ELISA were 0.7 ± 0.5 and 13.8 ± 10.3 percent, respectively. In normal individuals, our novel ELISA gave a mean plasma ADAMTS13 activity of 99.1 ± 21.5 percent (26 male, 97.9 ± 18.1%; 29 female, 100.1 ± 24.4%).

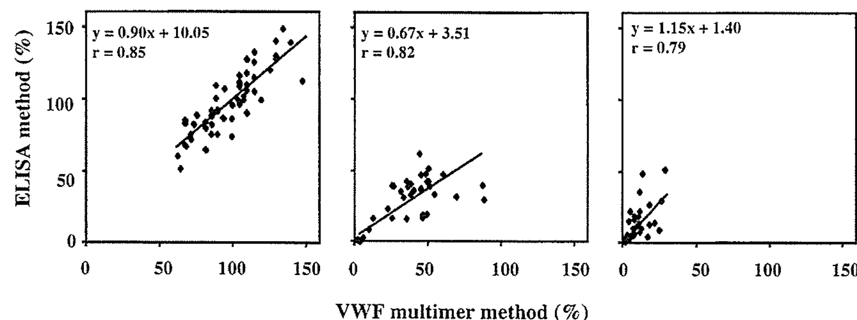

Fig. 7. Correlation of ADAMTS13 activity between ELISA and VWF multimer method. ADAMTS13 activities, measured by ELISA or the VWF multimer method, were compared for samples divided into three groups of patients with USS and carriers (middle, n = 53), patients with acquired TTP (right, n = 61), and normal individuals (left, n = 55). Significant positive correlations were observed in all three groups.

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Thrombotic thrombocytopenic purpura associated with pegylated-interferon alpha-2a by an ADAMTS13 inhibitor in a patient with chronic hepatitis C

A deficiency of ADAMTS13 leads to platelet clumping and/or thrombi formation, finally resulting in thrombotic thrombocytopenic purpura (TTP). In this study, a 62-year-old male with chronic hepatitis C developed TTP a month after long-term pegylated-interferon (PEG-IFN) treatment. The observed low level of activity of plasma ADAMTS13 following PEG-IFN treatment was shown to gradually increase with the improvement of TTP, while the titer of an inhibitory anti-ADAMTS13 IgG antibody decreased concomitant with the increase in ADAMTS13 activity. Serial determination of ADAMTS13 activity and its inhibitor may provide useful information for the diagnosis and treatment of IFN-associated TTP, as well as its pathogenesis.

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening disease, characterized by thrombocytopenia, microangiopathic hemolytic anemia, fluctuating neurological signs, renal dysfunction, and fever.[1] Recent studies have indicated that most TTP patients have deficient activity in von Willebrand factor-cleaving protease (VWF-CP/ADAMTS13), which specifically cleaves the multimeric von Willebrand factor (VWF) between Tyr1605 and Met1606 within the VWF A2 domain.[2,3] Deficiency of ADAMTS13 increases the plasma levels of ultra-large VWF multimer (UL-VWFM), which, under high shear stress, leads to platelet clumping and/or thrombi formation, finally resulting in TTP.[2-5] Various clinical conditions can induce TTP, including such conditions as infection, malignancies, autoimmunity, pregnancy, stem cell transplantation, and certain drugs such as interferon (IFN).[3-10] IFN is an essential component for treating hepatitis C virus (HCV)-related chronic hepatitis.[11] A number of adverse reactions to IFN have been reported, including pancytopenia, psychological symptoms, renal dysfunction and immunological disorders,[12] but it is clear that severe thrombocytopenia is one of the most important life-threatening adverse effects. Some of the cases were diagnosed as idiopathic thrombocytopenic purpura[13] and others were diagnosed as TTP.[8-10]

We here report on a patient with HCV-related chronic hepatitis who developed TTP a month after a full treatment course with pegylated-IFN (PEG-IFN). We demonstrate that TTP in this patient was caused by the absence of ADAMTS13 activity due to the presence of an inhibitory anti-ADAMTS13 immunoglobulin G (IgG) antibody.

A highly sensitive ELISA assay was used for measuring plasma ADAMTS13 activity and its inhibition, as recently described.[14] The inhibitor titer against ADAMTS13 activity was expressed in Bethesda units.[15] IgG was purified using a protein A column.

The patient was a 62-year-old male who had a history of blood transfusion in 1984 and was clinically diagnosed as having hepatitis C virus (HCV)-related chronic hepatitis on May, 1999. The circulating HCV was Ib in genotype and high in RNA concentration (1100×10^3 copies/mL, Amplicore HCV monitor assay: Roche Diagnostic System, USA). Liver biopsy was not performed because of the patient's request. Throughout a 5 year follow-up period serum transaminase levels were stable and within twice of the normal range. The patient received a series of 180 μg PEG-IFNα-2a every week for 48 weeks (from September 4, 2004 to July 28, 2005). Before beginning the PEG-IFN therapy, laboratory data revealed a normal range in trasnaminases, serum albumin (4.4 g/dL, normal range 3.8–5.1 g/dL), ZTT (13.7 U, normal range 4-15 U), and platelet count (168×10^9/L, normal range 130-369$\times 10^9$/L). No clinical findings indicative of liver cirrhosis were observed from the physical findings, abdominal ultrasonography, or gastro-fiberscope. During the IFN treatment period, transaminase levels had been within normal ranges and peripheral blood analyses showed mild pancytopenia without apparent adverse effects. Platelet count was 114×10^9/L two weeks after the final administration of IFN on August 11, 2005. Two weeks later on August 25 (a month after the final administration of IFN), the patient was admitted to our hospital because of high-grade fever and petechiae on the legs, which had been noticed three days before admission. Laboratory data demonstrated severe thrombocytopenia (6×10^9/L), anemia (Hb 10.0 g/dL, normal range: 13.0-18 g/dl) with numerous schistocytes, and elevations of lactate dehydrogenase (LDH)(1072 IU/l, normal range: 123-220 IU/L), creatinine (1.6 mg/dL, normal range: 0.6-1.1 mg/dl), and indirect bilirubin (1.6 mg/dl, normal range: 0.1-0.6 mg/dl). Reticulocyte count was 89×10^9/L (normal range: 30-94$\times 10^9$/L). Urinalysis demonstrated albuminemia, hematuria, and many hyaline and granular casts. Direct Coombs test and antinuclear antibody was negative. Lupus anticoagulant and anti-cardiolipin antibodies were also negative. Coagulation tests such as prothrombin time, activated partial thromboplastin time, fibrinogen, and fibrinogen degradation product were normal. Serum HCV-RNA was undetectable by RT-PCR. Magnetic resonance imaging examination demonstrated multiple small infarctions in the cerebellum. On bone marrow examination, findings showed a normal number of megakaryocytes without hemophagocytic reticulosis. Plasma ADAMTS activity on admission showed a marked decrease to less than 0.5% of normal. An inhibitor against ADAMTS13 was detected in both the patient's plasma (1.6 Bethesda units/ml) and purified IgG (0.19 Bethesda units/ mg IgG), thus confirming the diagnosis of acquired TTP caused by ADAMTS13 inhibitor (IgG autoantibody).

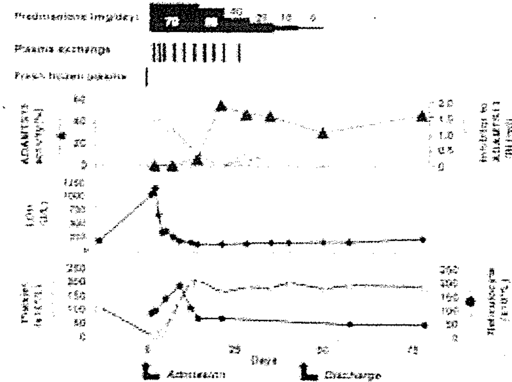

Figure 1. The clinical course of a patient with chronic hepatitis C who developed thrombotic thrombocytopenic purpura after pegylated-interferon treatment

The clinical course is shown in Figure 1. Six ml/kg of fresh frozen plasma (FFP) and 1 mg/kg of prednisolone were administered on the first hospital day. After the sec-

ond hospital day, plasma exchange (PE) was performed using 47 ml/kg/day FFP three times per week and periodically continued until the 23rd day (total 10 times). Prednisolone was tapered and stopped by the 56th day. The patient responded well to the PE and steroid treatment: platelet count immediately increased and LDH promptly decreased, both parameters of which normalized by the 9th day. Hb reached a nadir (7.9 g/dL) on day 4 after admission, then gradually increased to 9.5 g/dL, and serum creatinine normalized by the 14th day. The ADAMTS13 activity still remained below the detection limit on day 7, when platelet count recovered to 86×10^9/L and LDH was markedly decreased by 297 IU/L. Thereafter, ADAMTS13 activity gradually increased to 7% accompanied by the normalization of platelet count and LDH on the 14th day. The inhibitor of ADAMTS13 gradually decreased with concomitant increase of ADMTS13 activity, and became less than 0.5 Bethesda units/ml on the 21st day, when the ADMTS13 activity had recovered to 56%, and remained between 31% and 48% after discharge on the 41st day.

We demonstrated the absence of ADAMTS13 activity due to the presence of an anti-ADAMTS13 antibody in a case of TTP occurring a month after a series of PEG-IFN therapy for chronic hepatitis C. To date, patients who developed TTP during IFN therapy have been reported; however, the plasma ADAMTS13 activity has not been determined.[8,9] In our case, ADAMTS13 activity was dramatically decreased, reaching an undetectable level, and thereafter gradually increased with the improvement of TTP. It is notable that the activity of ADAMTS13 remained below the detection limit on day 7 after treatment by PE, when the platelet count recovered to 86×10^9/L and the LDH markedly decreased, suggesting rapid consumption of the enzyme to cleave a large amount of UL-VWFM during the initial treatment period for TTP. The antibody against ADAMS13 contained within the IgG fraction detected at the occurrence of TTP gradually decreased with concomitant increase of ADAMTS13, and fell to less than 0.5 Bethesda units, thus indicating a close relationship between the presence of an ADAMTS13 inhibitor and the development of TTP. This is the first patient associated with IFN therapy for whom serial determination of ADAMTS13 activity and its inhibitor was assessed.

We previously encountered a patient with HCV-related liver cirrhosis who was complicated by fetal TTP following the development of an ADAMTS13 inhibitor.[16] This case showed advanced liver cirrhosis and tense ascites without IFN therapy. In advanced cirrhotics, the levels of VWF antigen were remarkably high,[17] and plasma activity of ADAMTS13 was low, most likely because of the consumption of the enzyme used for the degradation of increased levels of VWF,[17] and/or the decreased production or secretion of ADAMTS13 that is exclusively produced in the hepatic stellate cells.[18] The imbalance of an increased amount of VWF over a deficiency of ADAMTS13 activity may, therefore, result in TTP in advanced cirrhotics, which certain triggers, including infection or endotoxemia, may precipitate. The presence of an ADAMTS13 inhibitor may enhance the situation.

In contrast, the present case of IFN treatment for 48 weeks showed chronic hepatitis, but not liver cirrhosis. Interestingly, IFN-induced thrombotic microangiopathy (TMA) occurred after much longer IFN administration,[9] indicating that higher cumulative doses of IFN may be one of the risk factors for the development of IFN-induced TTP. In our case, TTP onset may be associated with IFN therapy, but there is no direct evidence to prove the development of TTP by IFN itself. It is unclear whether anti-ADAMTS13 antibodies could be present before IFN therapy, but there remains the possibility that IFN might induce autoimmune reactions, resulting in the generation of autoantibodies against ADAMTS13.[12]. We have shown that an ADAMTS13 inhibitor in the plasma was detected in 13 (48%) of 27 patients with autoimmune-associated TTP, and in all (100%) of 7 patients with ticlopidine-associated TTP.[6] Furthermore, irrespective of IFN therapy, HCV infection itself might contribute to the development of TTP. HCV infection was, indeed, confirmed in all 4 patients with chronic hepatitis including our present case who developed TTP after IFN therapy[8,9] and in five of 10 patients (50%) who developed TMA after living-donor liver transplantation.[19]?

Pegylated-IFN is currently widely used for the treatment of patients with chronic hepatitis C, because its tolerability and efficacy are superior to conventional IFN.[20] Discontinuation of therapy because of thrombocytopenia was required in 2% of the patients receiving 180ug of PEG-IFN-α2a for the treatment of chronic hepatitis C that displayed cirrhosis or bridging fibrosis.[20] Due attention should be paid to the development of TTP during IFN therapy, especially in the case of PEG-IFN, which has a longer action than conventional IFN.

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Key words: TTP, peginterferon, ADAMTS13, autoantibody

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Successful Treatment of Primitive Neuroectodermal Tumor-associated Microangiopathy with Multiple Bone Metastases

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We report here a 16-year-old male with primitive neuroectodermal tumor (PNET)-associated probable microangiopathy with multiple bone metastases. Laboratory findings excluded the possibility of amegakaryocytic or immune thrombocytopenia and/or disseminated intravascular coagulation. He was first treated with plasma-exchange (PE), followed by platelet transfusions, steroid pulse therapy and combined chemotherapy. PE and steroid pulse therapy reduced his plasma CRP level. Combined chemotherapy drastically increased his platelet count until it had almost normalized without further transfusion. The plasma level of von Willebrand factor-cleaving protease (ADAMTS13) activity measured before PE was not severely deficient (48% of normal) and an unusually large von Willebrand factor multimer (UL-VWFM) was detected. We consider that this therapeutic strategy has the following benefits: (1) reduction of plasma levels of factors that are harmful to both platelet activation and endothelial cell injury; and (2) the safe transfusion of platelet concentrate in thrombotic microangiopathy. This strategy should be confirmed in further cases.

Key words: PNET – microangiopathy – chemotherapy – ADAMTS13 – UL-VWFM

INTRODUCTION

Malignancy-associated thrombotic microangiopathy (TMA), characterized by thrombocytopenia and microangiopathic hemolytic anemia, is a rare but life-threatening complication of sarcoma and its treatment remains controversial. Recent studies, however, have indicated that such patients usually have normal plasma von Willebrand factor-cleaving protease (ADAMTS13) activity (1,2), and that platelet transfusions are generally contra-indicated in these patients because transfusions have been associated with disease exacerbation (3,4). We report here a case of PNET-associated probable TMA that was successfully treated by platelet transfusion after extensive plasmapheresis (PE) followed by chemotherapy.

CASE REPORT

The patient was a 16-year-old male (body weight, 60 kg) who had complained of a high fever and fatigue beginning in June 2002. He was admitted to a nearby hospital on 14 June and received penicillin injections for three days. Suspicion of meningitis, sepsis, viral infection and immunologic diseases was excluded by negative results of leucocytosis in the cerebrospinal fluid, culture of blood and cerebrospinal fluid and antibodies against certain viruses and nucleus. A lytic area in the right eighth rib was then noted on radiography. Bone scintigraphy showed multiple hot lesions on 18 June. Bone marrow examination performed on 19 June was normal without invasion of malignant cells. Pathological examination using biopsy specimens together with the demonstration of EWS-FLI1 translocation confirmed a diagnosis of a PNET (Fig. 1). On 25 June, he developed slight bilirubinemia (1.2 mg/dl) and thrombocytopenia ($94 \times 10^9/l$), which then rapidly progressed together with hemolytic signs consisting of rouleaux formation and poikilocytosis of erythrocytes in the peripheral blood, and microscopic hematuria. Normoblasts and immature myeloid cells in the peripheral blood were also found as leucoerythroblastic features. Because of this complex clinical picture, he was transferred to our hospital on 8 July. On admission, he had anemia (Hb 105 g/l) (normal range: 135–176), thrombocytopenia ($26 \times 10^9/l$), and high serum levels of CRP

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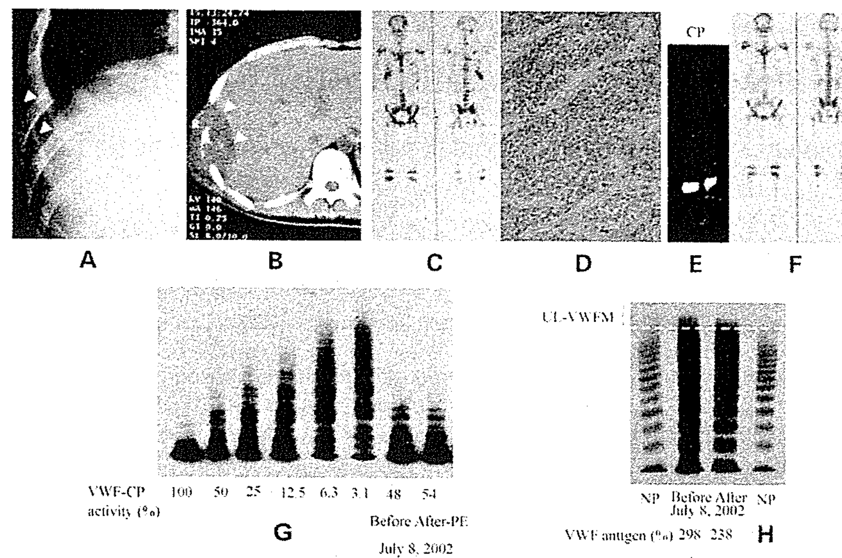

Figure 1. Results of clinical and diagnostic images. Plain radiograph (A) and CT (B) showing a lytic lesion and large expansive tumor in the right eighth rib. Bone scintigram (C) showing multiple areas of abnormal uptake in the skull, spine, pelvis, ribs, shoulders and knees. Histology of the biopsy specimen (D) was compatible with primitive neuroectodermal tumor. Demonstration of EWS-FLI1 translocation (E) by reverse transcription of polymerase chain reaction; c, positive control; p, patient. Bone scintigram, 10 months after diagnosis (F), did not show any abnormal uptake. Assay of plasma VWF-cp activity before and after plasma exchange (PE) on 8 July 2002 (G). Detection of unusually large von Willebrand factor multimers (UL-VWF) and VWF antigen level in the patient plasma before and after PE on 8 July 2002 (H). Note that the plasma VWF antigen level decreased after PE.

(251 mg/l), LDH (1787 U/l) (normal range: 106–211 U/l), GOT (49 U/l) (normal range: 12–32 U/L), and ALP (2,100 U/l) (normal range: 200–760 U/l). Other laboratory findings were as follows: BUN 6.78 μmol/l (normal range: 2.85–7.12 μmol/l), creatinine 53.04 μmol/l (normal range: 26.52–79.56 μmol/l), and total bilirubin 18.8 μmol/l (normal range: 5.1–18.8 μmol/l). His blood type was O-Rho (D) positive and both direct and indirect Coombs tests were negative. Antiplatelet antibody determined by mixed passive hemaglutination assay was negative. He had never previously undergone chemotherapy or blood transfusion. Coagulation screening tests including the levels of antithrombin (86%) and fibrinogen (5.91 g/l) were within normal ranges; however, his serum FDP level had increased slightly to 43.7 μg/ml. Plasma ADAMTS13 activity determined by the multimer assay was not immediately available. Based on these clinical and laboratory findings, we suspected that the patient had PNET-associated TMA rather than immune thrombocytopenia or disseminated intravascular coagulation (DIC). Because of his extremely poor general condition, surgical and/or chemotherapeutic approaches were thought to be inadvisable.

Thus, we prepared a protocol consisting an initial plasma exchange (PE) followed by transfusion of a single-donor platelet concentrate (PC) supplied by the Japan Red Cross Blood Center. This regimen was repeated for five consecutive days, together with steroid pulse therapy (methyl prednisolone 1 g/d for 3 days). Using this approach, PC was transfused without any appreciable adverse reactions. The expected rise in platelet count was identified 1 h after each infusion. It then decreased to the pre-infusion level ($23-33 \times 10^9$/l) over the next few days. Bone marrow examination on the fourth hospital day (11 July) demonstrated a normal nuclear cell count (137×10^9/l), of which malignant cells accounted for 29.5%. Meanwhile, the megakaryocyte count was normal or had increased slightly (200/μl), supporting the concept of enhanced consumption of newly-produced platelets. After sequential PE, a marked decrease in the CRP level was observed, and the general condition of the patient appeared to improve. However, the LDH level remained elevated and even increased slightly while the anemia worsened, indicating invasive expansion of tumor cells. Thus, on 13 July, we started combined chemotherapy, consisting of vincristine (VCR), adriamycin (ADR), and cyclophosphamide (CPA), that resulted in a dramatic increase in the platelet count with a concomitant decrease in LDH. Furthermore, the anemia ceased to progress, with no red blood cell transfusion required throughout this clinical course. Partial response was confirmed by resection of the right eighth rib after chemotherapy. Total body and local irradiation was performed after the Hi-MEC regimen, resulting in an absence of abnormal accumulation on bone scintigraphy 10 months after diagnosis. However, 13 months after diagnosis, recurrences in the right hip joint and orbit were detected and the patient died of disease. Survival after diagnosis was 23 months.

Before PE, his plasma VWF antigen level was elevated (298%) and an unusually-large VWF multimer (UL-VWFM) was present (Fig. 2).

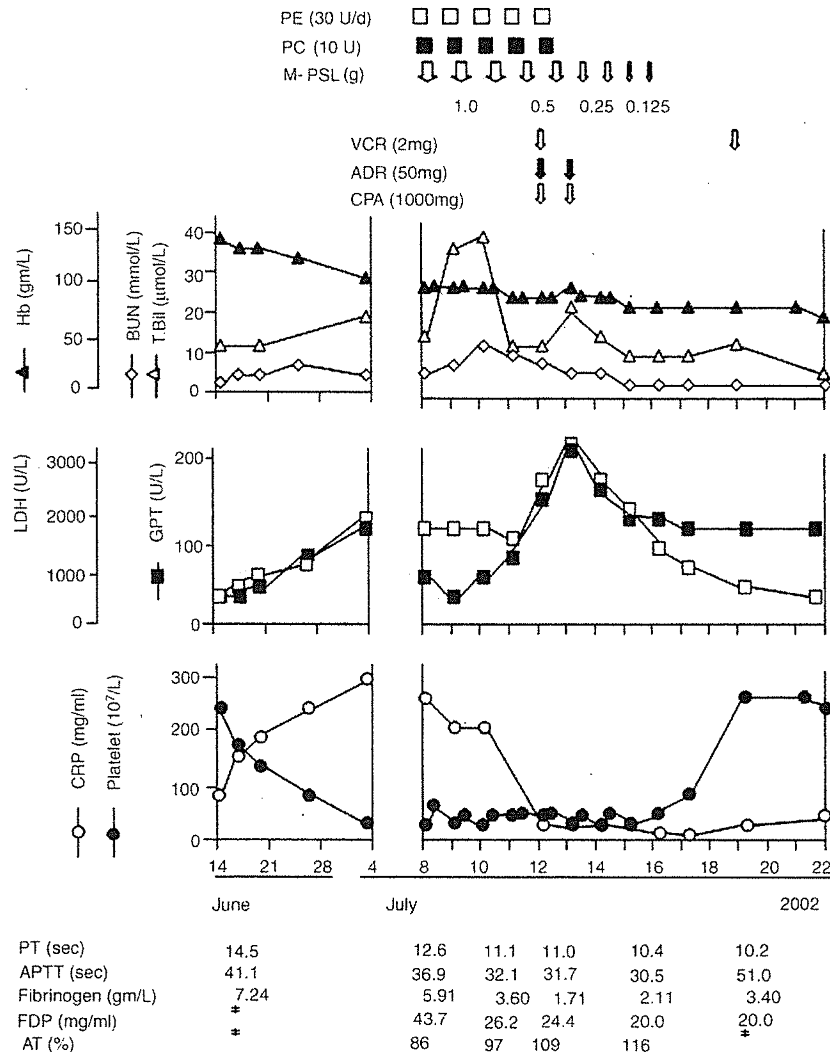

Figure 2. Time course of laboratory parameters and therapeutic regimen. PE, plasma exchange; PC, platelet concentration; m-psl, methyl prednisolone; VCR, vincristine; ADR, adriamycin; CPA, cyclophosphamide; Hb, hemoglobin; BUN, blood urea nitrogen; T.Bil, total bilirubin; LDH, lactate dehydrogenase; GPT, glutamic-pyruvic transaminase; CRP, C-reactive protein; PT, prothrombin time; APTT, activated partial thromboplastin time, FDP; fibrinogen degradation products; AT, antithrombin.

DISCUSSION

The diagnosis of TMA was based on schistocytosis and evidence of hemolysis. In our case, an elevated level of LDH was evident; however, schistocytosis was not tested in our hospital.

Thrombocytopenia occurred as a result of low platelet production and/or increased breakdown. In our case, a normal or slight increase in the production of platelets in the bone marrow was confirmed during hospitalization. Therefore, the increased breakdown of platelets was assumed. Spherocytes in the peripheral blood, which are characteristic in immune and hereditary hemolysis, were not found before transition. Considering these data along with the negative results on direct and indirect Coombs tests, the possibility of immune hemolysis was considered highly improbable and DIC was also excluded by the absence of signs indicating decreased ATIII.

Though a high CRP value persisted, severe infection, including meningitis, sepsis and viral infection were excluded by intensive examination. High fever and leucocytosis were thus considered characteristic symptoms of PNET and not owing to infection. Splenomegaly as a sign of increased breakdown of platelets was not confirmed by CT. There was no history of blood transfusion.

Invasion of malignant cells as confirmed by bone marrow examination occurred between 11 June and 19 July, and thrombocytopenia with hemolytic anemia occurred concomitant with this invasion, though multiple bone metastases had

already been confirmed by bone scintigraphy. Therefore the formation of bone metastases is insufficient to explain thrombocytopenia in this case. We consider that thrombocytopenia was probably as a result of malignant tumor-associated TMA.

Detection of UL-VWFM, released from endothelial cells and cleaved by ADAMTS13, and subnormal activity of ADAMTS13, reported as a marker to differentiate between TTP and HUS (3), were also confirmed later. Detection of UL-VWFM suggests injury of the endothelial cells or obstruction of cleavage by ADAMTS13. In our case, ADAMTS13 activity was subnormal, which agreed with the findings in the majority of TMA reported (1). Therefore, detection of UL-VWFM suggested injury of endothelial cells (2). Invasion of malignant cells, synchronously occurring, may have caused endothelial cell injury. Histological examination may help clarify the mechanism.

Cytokines have recently been reported to mediate UL-VWFM release from vascular endothelial cells (5). Furthermore, it was proposed that cytokines that injure vascular endothelial cells may interfere with the efficient supply of ADAMTS13 (5). Thus, cytokines may be another cause of TMA.

Systemic chemotherapy is usually indicated except in cases of chemotherapy-induced TMA. However, low platelet count made the initiation of this therapy inadvisable. Thus, transfusion of platelets was performed after PE to prevent adverse reaction. It has been proposed that PC transfusion is contra-indicated in TMA because uncleaved UL-VWFM induces platelet aggregation under high shear stress and exacerbates thrombosis (3). However, after removal of factors including UL-VWFM and cytokines from the circulation by PE, PC transfusion was performed safely and quickly resulted in raising the platelet count over the short time. However, the basic conditions, for example, expansion of tumor cells, may gradually lower the platelet count again. Thus, treatment of the tumor itself is necessary. Combined chemotherapy after PE dramatically improved TMA in our case. This also supports the hypothesis that malignant cells were related to injury of the endothelial cells. Prognosis of TMA depends on the chemosensitivity of the tumor itself. Further experience is necessary to confirm this regimen.

The present findings may improve our understanding the reason why malignancy-associated TMA responds poorly to PE therapy alone, as has been commonly accepted.

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Conflict of interest statement

None declared.

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REGULAR ARTICLE

Decreased ADAMTS13 activity in plasma from patients with thrombotic thrombocytopenic purpura

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Abstract The ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13) activity was measured by a fluorescence resonance energy transfer (FRET) assay in the plasma of healthy volunteers and thrombotic thrombocytopenic purpura (TTP) patients to examine its usefulness in the diagnosis of TTP.

The plasma levels of the ADAMTS13 activity did not show a normal distribution. Its median value was 107% (range: 55–170%) in healthy volunteers, but was significantly lower in patients with TTP (acquired or familial) and in patients with hematopoietic stem cell transplantation. However, it was not significantly lower in patients with antiphospholipid syndrome (APS). The ADAMTS13 activity by a FRET assay was closely correlated with that by the ADAMTS13 multimer method ($r=0.816$; $p<0.001$). In 18 patients with less than 10% of ADAMTS13 activity by FRET assay, less than 10% of that by multimer assay was 16, thus suggesting a good correlation for a low level of ADAMTS13.

These findings suggest that the ADAMTS13 FRET assay correlates well with the ADAMTS13 multimer method and it is therefore useful for making a diagnosis of TTP.
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Introduction

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

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

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

Materials and methods

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

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

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

Fluorescent assay to measure the ADAMTS13 activity

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

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

Table 1 Subjects

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

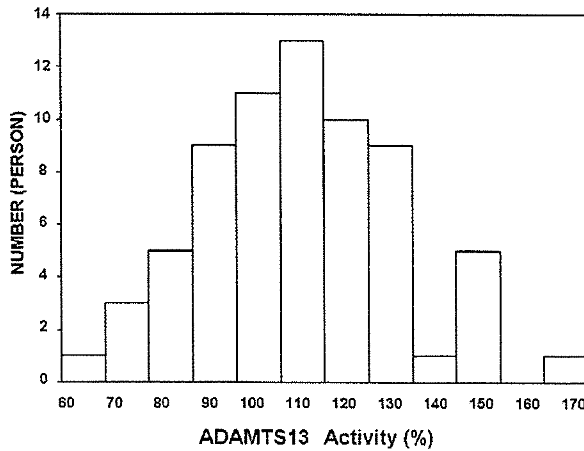

Figure 1 ADAMTS13 activity in healthy volunteers.

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

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

Statistical analysis

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

Results

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

Table 2 TTP patients

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

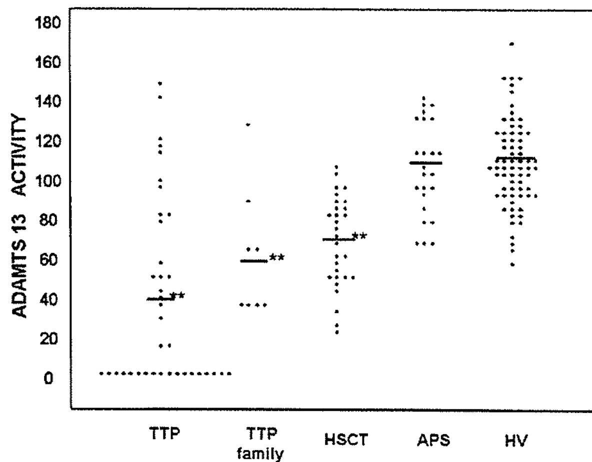

Figure 2 ADAMTS13 activities in TTP, TTP family, HSCT, APS and HV HSCT; hematopoietic stem cell transplantation, APS; antiphospholipid syndrome, HV; healthy volunteer. **$p<0.01$.

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

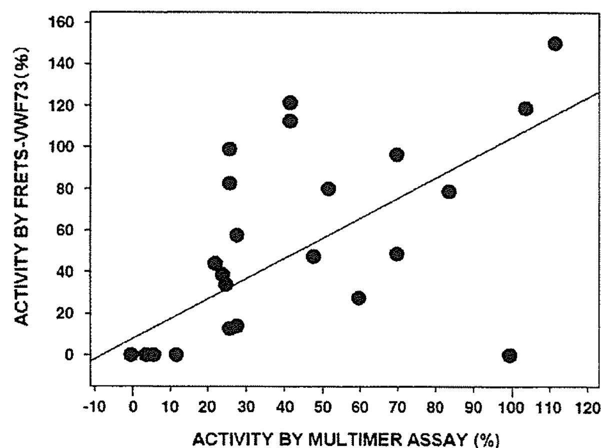

Figure 3 Correlation of ADAMTS13 activity between the FRET assay and multimer assay.

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

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

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

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

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

Discussion

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

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

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

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

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

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

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