conventional hemostatic factors. One could, then, notice that the origin of VWF, the substrate of ADAMS13, is indeed transformed hepatic sinusoidal and/or extrahepatic endothelial cells, but not hepatocytes. The procoagulant and anticoagulant proteins synthesized in hepatocytes decrease as liver disease progresses, whereas VWF markedly increases. Under such circumstances, ADAMTS13 deficiency may lead to microcirculatory disturbance not only in the liver, but also in the systemic circulation. The determination of ADAMTS13 and its related parameters will thus be quite useful for better understanding the pathophysiology and for providing appropriate treatments especially in severe liver disease patients. It will be necessary to measure ADAMTS13:AC when patients with unexplained thrombocytopenia in the course of liver disease are encountered. Further investigation will be necessary to clarify potential roles of ADAMTS13:AC in patients with liver disease.

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INTERNAL MEDICINE

CASE REPORT

Influenza A Infection Triggers Thrombotic Thrombocytopenic Purpura by Producing the Anti-ADAMTS13 IgG Inhibitor

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Abstract

A 68-year-old Japanese woman infected with influenza A developed thrombotic thrombocytopenic purpura (TTP) 2 days after having a fever. Routine laboratory tests on admission suggested a diagnosis of disseminated intravascular coagulation. However, ADAMTS13 assays showed an extremely low level of plasma ADAMTS13 activity with a high titer of anti-ADAMTS13 inhibitor (IgG). Despite high-dose methylprednisolone therapy with daily plasma exchange for 3 consecutive days, the patient died of pulmonary congestion complicated by cardiac failure. Our experience here provides the first evidence that influenza A infection is sufficient to trigger TTP by producing the anti-ADAMTS13 IgG inhibitor.

Key words: influenza, TTP

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Introduction

Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening disease, characterized by generalized microvascular occlusion by platelet thrombi (1, 2). TTP was classically identified by a clinical 'pentad' consisting of thrombocytopenia, microangiopathic hemolytic anemia, renal failure, fever, and fluctuating neurological signs (3). More recently, however, TTP, has been associated with a deficiency of plasma ADAMTS13 (a disintegrin-like metalloproteinase with thrombospondin type 1 motifs 13) activity, which is caused by genetic mutations in or acquired autoantibodies to this enzyme.

ADAMTS13 specifically cleaves von Willebrand factor multimers (VWFMs) at the site of the Tyr1605-Met1606 bond of the VWF-A2 domain (4). In the absence of ADAMTS13 activity, therefore, unusually large VWFMs (UL-VWFMs) are produced in the vascular endothelial cells, left uncleaved, and released into the circulation. The accumulated UL-VWFMs in the circulation induce generalized formation of platelet thrombi in the microvasculatures under certain rheological conditions, resulting in TTP. Acquired TTP may develop under various clinical conditions, such as drug use (5), pregnancy, malignancies, and collagen diseases (6), as typically seen in systemic lupus erythematosus. It has also been reported that acquired deficiency of ADAMTS13 activity is closely associated with human immunodeficiency virus (HIV) infection (7) and influenza vaccination with or without adjuvants (8-10), but has not been reported to be associated with influenza infection alone. In this paper, we describe the case of an acquired TTP patient with severe ADAMTS13 activity deficiency due to neutralizing IgG-autoantibodies, manifested as a serious complication from influenza A infection.

Case Report

In early March, 2007, a 68-year-old woman weighing 50 kg visited a local physician with complaints of fever and general malaise. On the following day a diagnosis of influenza A was made from throat swab specimens using the

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Table 1. Laboratory Findings on Admission

Peripheral blood		(Control)
Platelets ($\times 10^6$/L)	6	(140-360)
White blood cell ($\times 10^3$/L)	7200	(3500-9500)
Red blood cell ($\times 10^6$/L)	222	(F360-500)
Hemoglobin (g/dL)	6.6	(F11.5-15.5)
Reticulocyte (‰)	35	(0.5-1.5)
Schistocytes on blood film	+++	(-)
Blood chemistry		
Total protein (g/dL)	5.1	(6.5-8.2)
Total bilirubin (mg/dL)	2.9	(0.2-1.1)
Direct bilirubin (mg/dL)	0.8	(0-0.3)
Aspartate aminotransferase (IU/L)	694	(8-40)
Alanine aminotransferase (IU/L)	399	(3-35)
Lactate dehydrogenase (IU/L)	3060	(119-229)
Blood urea nitrogen (mg/dL)	88	(7-22)
Creatinine (mg/dL)	2.95	(0.4-1.2)
Cytokines		
TNF-α (pg/mL)	3.54	(<1.79)
IL-6 (pg/mL)	12.8	(<2.41)
IL-8 (pg/mL)	28.7	(<8.0)
C-reactive protein (mg/dL)	9.1	(<0.5)
Hemostatic test		
PT (sec)	16.9	(11-12)
A-PTT (sec)	33	(21.0-38.0)
Fibrinogen (mg/dL)	552	(150-400)
Antithrombin (%)	72	(80-130)
Plasminogen (%)	79	(66-130)
α2-Plasmin inhibitor (%)	42.99	(90-140)
FDP-P (ug/mL)	71	(<5)
D-dimer (ug/mL)	23.14	(<1.0)
TAT (ng/mL)	11.8	(<3)
PIC (ug/mL)	2.4	(<1.0)
VWF antigen (%)	184	(50-150)
ADAMTS13		
Activity (%)	<0.5	(50-150)
Antigen (%)	0.1	(50-150)
Inhibitor (Bethesda U/mL)	6.0	(<0.5)

immuno-chromatography method. She received oral administration of oseltamivir (150 mg/day). Previously, she had had surgeries for a gastric cancer (47 y.o.) and uterine myoma (48 y.o.) without transfusion. She had not had either bleeding diathesis or thrombotic tendencies before. Two days later, her general condition worsened, and she was admitted to a nearby hospital, where TTP was suspected from clinical signs and the results of routine laboratory tests. Therefore, she was referred to our hospital for plasma exchange therapy the following day. Laboratory results before the plasma exchange are shown in Table 1. Tests for a series of autoantibodies related to collagen diseases and for antibodies to HIV and hepatitis C were all negative. However, she tested positive for antibodies against both hepatitis B surface and core antigens, but negative for surface antigen. Further, the plasma levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, and C-reactive protein (CRP) were high. The patient had the typical clinical signs of TTP ('pentad'), thus a clinical diagnosis of TTP was made without difficulty; however, retrospectively, it was interesting to note the following laboratory results, which slightly resembled what would be expected for disseminated intravascular coagulation (DIC): 1) prolonged prothombin time (PT), despite normal activated partial prothrombin time (A-PTT); 2) significantly reduced plasma level of antithrombin; 3) increased levels of plasma fibrinogen and two of its degradation products, FDP-P and D-dimer; 4) elevated levels of the DIC markers, thrombin-antithrombin complex (TAT) and plasmin-α2 plasmin inihibitor complex (PIC). Thus, by the diagnostic criteria for DIC from the International Society of Thrombosis and Haemostasis (11), the patient had a DIC score of 4 (non-overt DIC), and by that from the Japanese Ministry of Health and Welfare (12), the patient had a DIC score of 9 (overt DIC). However, ADAMTS13 assays in this patient gave evidence confirming a diagnosis of TTP, showing an extremely low level of plasma ADAMTS13 activity (less than 0.5% of the normal control) with a high titer of anti-ADAMTS13 inhibitor (6.0 Bethesda units/mL), using chromogenic act-ELISA (13). The plasma level of ADAMTS13 antigen was 0.1% by ag-ELISA (14).

After admission, the patient received high-dose methylprednisolone (one gram per day) therapy with concomitant

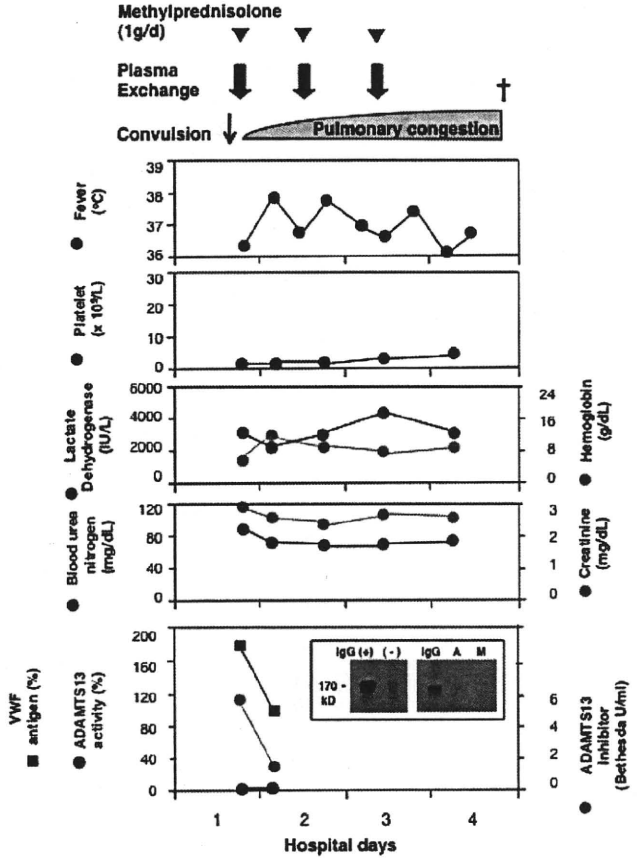

Figure 1. Clinical course of influenza A-associated thrombotic thrombocytopenic purpura (TTP). A 68-year-old Japanese woman infected with influenza A developed TTP 2 days after having a fever. Plasma ADAMTS13 activity was less than 0.5% of normal control, and a high titer of anti-ADAMTS13 inhibitor (6.0 Bethesda units/mL) was measured. Western blot studies using purified plasma-derived ADAMTS13 revealed that the immunoglobulin type of this inhibitor was IgG (inset). As shown in the left panel of inset (bottom figure), heated plasma from the patient with acquired idiopathic TTP and IgG inhibitors against ADAMTS13 (+) displayed a 170 kD-band as a positive control, while that from a normal individual without ADAMTS13 inhibitor (-) showed no band (a negative control). In the right panel of inset, the heated plasma of this patient displayed the band detected by anti-human IgG polyclonal antibody, but not by anti-human IgA, or IgM polyclonal antibody.

daily plasma exchange at a single dose volume of 3,240-3,600 mL for 3 consecutive days. Red blood cell concentrates were also transfused with a total volume of 840 mL, but no infusion of platelet concentrates was performed throughout the clinical course. On the day after admission to our hospital, despite this intensive therapy, the patient developed generalized convulsion, which were controlled by parenteral administration of anti-convulsant (aleviatin). Nevertheless, pulmonary congestion developed, which was presumably induced by hypoproteinemia, and necessitated the use of anti-diuretics. Two days later, the patient's electrocardiogram suddenly showed signs of cardiac ischemia/infarction (elevation of the ST-wave), and 3 hours later she died. Autopsy was not performed.

Western blot studies of the patient's plasma, which had been stored at -80℃, and the purified plasma-derived ADAMTS13, clearly identified that the immunoglobulin type of the inhibitor was IgG (6) (Fig. 1, inset).

Discussion

In 1981, Wasserstein et al (15), described a case of a 50-year-old man with recurrent TTP which manifested solely as aphasia after influenza infection; however, clinical diagnosis of TTP was not made during the acute phase in this patient. During the chronic phase the diagnosis was made by renal biopsy demonstrating the features of chronic renal disease: glomerulus showing thickened capillary wall with numerous 'double contours' as well as several hyaline capillary thrombi, accompanied by microangipathic hemolytic anemia and thrombocytopenia. This paper is the first to describe a close relationship between influenza and TTP, but without addressing pathogenesis.

A relationship between the influenza 'swine-flu' vaccine and an increased risk of Guillain-Barré syndrome was reported in 1991 (16), but the relationship with thrombocytopenia was not shown. In recent years, two groups of investigators (8, 9) have reported that the inactivated human influenza vaccine may induce TTP bouts by boosting the production of anti-ADAMTS13 autoantibodies. To date, however, autoantibody-induced TTP has never been reported in association with the influenza infection itself. In this regard, the present report provides the first evidence that influenza A infection alone may trigger TTP by the production of anti-ADAMTS13 IgG. The question remains: why and how has this effect been hidden to date? Is it a new or extremely rare phenomenon among patients infected with influenza A?

In this regard, we are now particularly interested in influenza-associated encephalopathy (IAE), which has been reported to occur at a frequency of 0.02% among Japanese patients infected with influenza A and/or B. More interestingly, IAE has rarely been reported in the United States or in European countries (17), a fact which we cannot explain. We do, however, note several important phenomena (17). First, IAE develops either on the day that influenza signs appear or on the following day, and is highly heterogeneous in terms of neurological findings. Second, influenza virus can seldom be identified in the cerebrospinal fluid or brain tissues in the affected individuals. Third, influenza infection is often complicated by pneumonia and less commonly by IAE, both of which are occasionally associated with multi-organ failure (MOF). The MOF in these settings has been explained by DIC-like pathogenesis generated by the ex-

tremely high plasma levels of various cytokines, termed a 'cytokine storm,' which injures vascular endothelial cells where platelet thrombi are likely to be formed. In fact, the platelet count is correlated with the outcome of IAE; of the patients with thrombocytopenia (<50,000 platelets/uL), more than 80% died. Fourth, generalized microemboli and hyalinization of the small vessels were observed in autopsies (17). Finally, some patients with IAE have responded well to high-dose steroid therapy with plasma exchange (18). This therapeutic efficacy has been attributed to the resolution of the plasma 'cytokine storm', but the precise mechanism remains undetermined. Thus, we find a high similarity between IAE and influenza A-associated TTP, although they are not identical.

The present case was diagnosed as acquired TTP by severe deficiency of ADAMTS13:AC with anti-ADAMTS13 inhibitor, but also fulfilled the diagnostic criteria for DIC by the Japanese Ministry of Health and Welfare (12). In this regard, we can address the followings: TMAs are often indistinguishable from DIC by clinical signs alone, but now it is well accepted that TMAs are pathologically featured by platelet thrombi and DIC by fibrin thrombi, each formed in microvasculatures. Thus, it is conceivable that TMA is further complicated by DIC and both clinical conditions may co-exist, but its reversal clinical course appears to be less likely.

In our database of Japanese patients (19), 69% (195/284) of patients with acquired idiopathic TTP also had a severe deficiency of ADAMTS13 activity caused by the development of anti-ADAMTS13 autoantibodies (IgG), implying that acquired idiopathic TTP is an autoimmune disease. Consistent with this, the present case report implies that influenza A infection triggers TTP which is almost indistinguishable from acquired idiopathic TTP by producing anti-ADAMTS13 inhibitory IgG. To determine a possible association between influenza-associated TTP and IAE would be of great interest in future studies. Our experience shows the necessity of the analysis of ADAMTS13 activity and its inhibitors to differentiate between clinical conditions and to determine a therapeutic strategy for influenza infection.

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Proteolytic fragmentation and sugar chains of plasma ADAMTS13 purified by a conformation-dependent monoclonal antibody

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ADAMTS13 is a metalloproteinase that specifically cleaves unusually large von Willbrand factor multimers under high-shear stress. Deficiency of ADAMTS13 activity induces a life-threatening generalized disease, thrombotic thrombocytopenic purpura. We established a simple and efficient method to purify plasma ADAMTS13 (pADAMTS13) from cryosupernatant using an anti-ADAMTS13 monoclonal antibody (A10) that recognizes a conformational epitope within the disintegrin-like domain. Using the purified pADAMTS13, the amino acid residues involved in cleavage by thrombin, plasmin and leucocyte elastase were determined, and the carbohydrate moieties of this enzyme was analysed by lectin blots. Purified pADAMTS13 had a specific activity of 300 U/mg (25,057-fold purification) and the pI was 5.1–5.5. Cleavage sites of the purified pADAMTS13 by three proteases were identified; thrombin cleaved the four peptidyl bonds between Arg257–Ala258, Arg459–Ser460, Arg888–Thr889 and Arg1176–Arg1177, plasmin cleaved the three peptidyl bonds between Arg257–Ala258, Arg888–Thr889 and Arg1176–Arg1177, and elastase cleaved the two peptidyl bonds between Ile380–Ala381 and Thr874–Ser875. Lectin blot analysis indicated the presence of non-reducing terminal α2–6 and α2–3-linked sialic acid residues with penultimate β-galactose residues on the N- and O-linked sugar chains of pADAMTS13, suggesting that pADAMTS13 is cleared from the circulation via the hepatic asialoglycoprotein receptor like other plasma glycoproteins.

Keywords: enzyme digestion/lectin blotting/plasma ADAMTS13/purification.

Abbereviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; IEF, Isoelectric focusing; mAb: monoclonal antibody; PVDF, polyvinylidene difluoride, RT, room temperature, TBS, Tris-buffered saline; UL-VWFM, unusually large von Willbrand factor multimers, VWF, von Willbrand factor.

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves von Willbrand factor (VWF)-A2 domain at the peptidyl bond between Tyr1605 and Met1606 (*1–3*). VWF is synthesized exclusively in vascular endothelial cells, and is then either constitutively secreted into subendothelial matrices or released into the circulation as unusually large VWF multimer (UL-VWFM), which is the most biologically active form of the protein and results in excessive platelet aggregation/thrombus formation under high-shear stress generated in the microvasculature (*4–6*). Failure to control VWF activity due to a lack of plasma (p) ADAMTS13 activity typically results in thrombotic thrombocytopenic purpura (TTP), a life-threatening disease characterized by generalized microvascular platelet thrombi (*7–10*). Thus, ADAMTS13 may play a pivotal role in maintaining normal circulation, by controlling the molecular size of UL-VWFM.

In addition to TTP, recent studies indicate that an extremely low ratio of ADAMTS13 to UL-VWFM may cause a variety of clinical issues complicated by platelet thrombi in the microvasculature (*11*), including hepatic veno-occlusive disease (*12*), liver transplantation (*13*), liver cirrhosis (*14*), sepsis-induced disseminated intravascular coagulation (DIC) (*15*) and severe acute pancreatitis (*16*). However, it is poorly understood how ADAMTS13 activity is regulated in these circumstances.

In this regard, Crawley *et al.* (*17*) reported in 2006 that three serine proteases, thrombin, plasmin and factor Xa each cleaves ADAMTS13 to effect a reduction of ADAMTS13 activity *in vitro*. Using purified recombinant ADAMTS13 (rADAMTS13), Lam *et al.* (*18*) subsequently reported that thrombin cleaves the peptide bonds C-terminal to amino acid residues Arg257 and Arg1176. However, human ADAMTS13, as deduced from the cDNA sequence, has 10 potential N-linked and 6 O-linked sugar chains and the structure of the sugar chain moieties depends on the producing cells. This suggests that rADAMTS13 expressed in other types of mammalian cells may have different

carbohydrate moieties than ADAMTS13 expressed in humans, which may result in a distinct proteolytic signature.

In 1996, Furlan *et al.* (*1*) and Tsai *et al.* (*2*) independently reported the partially purified pADAMTS13 with a feature of metalloproteinase and a molecular mass of 200–300 kDa. Using immunoadsorbent columns coupled to anti-ADAMTS13 polyclonal IgG that had been obtained from plasma of TTP patients, Gerritsen *et al.* (*19*) succeeded in purifying pADAMTS13 with heterogeneous molecular sizes (110–150 kDa) by SDS–polyacrylamide gel electrophoretic analysis, but each had the same N-terminal amino acid sequence (AAGGIL–). Almost simultaneously, Fujikawa *et al.* (*20*) reported the purification of an electrophoretically homogeneous pADAMTS13, with a molecular weight of 150 kDa before and 190 kDa after reduction on SDS–polyacrylamide gel, from a commercial factor VIII/VWF concentrate by a series of conventional chromatographic steps. This purification method was artistic, but the starting material was first treated with thrombin to remove fibrinogen, followed by thrombin inactivation by diisopropyl fluorophosphate. It is now known that pADAMTS13 is proteolyzed by thrombin, and a small portion of ADAMTS13 (<10% of the total in the plasma milieu) can co-sediment as a cryoprecipitate, whereas the majority (>90% of the total) remains in the cryosupernatant. Thus, it is important to establish a high-yield purification method for pADAMTS13, and to characterize this enzyme in comparison with rADAMTS13, as both enzymes appear to be under development for therapeutic use.

In this article, we describe a simple and efficient purification method for human pADAMTS13 from cryosupernatant, utilizing an anti-ADAMTS13 mouse monoclonal antibody (mAb). Using the purified pADAMTS13, we determine the peptide bonds cleaved by thrombin, plasmin and leucocyte elastase. Further, the carbohydrate moieties of this purified enzyme identified by lectin blot suggest that ADAMTS13 can be cleared from the circulation by the hepatic asialoglycoprotein receptor, like many other plasma glycoproteins.

Materials and Methods

Plasma and monoclonal antibodies

Outdated fresh frozen plasmas (FFP), anti-coagulated with acid-citrate-dextrose, were kindly provided by the Japan Red Cross Nara Blood Center as the starting materials for purification of ADAMTS13. The anti-ADAMTS13 mouse mAb (A10, IgG1-κ), which recognizes an epitope residing on the disintegrin-like (Dis) domain of ADAMTS13, was previously described (*21*). Most recently, its conformation-dependent epitope was precisely determined to reside on 72 amino acid residues (Tyr305-Glu376) within the Dis domain (*22*). A10-IgG was purified by protein A-Sepharose CL-4B column, and the purified IgG completely inhibited ADAMTS13 activity at a final concentration of 20 μg/ml in the static assay (*21*). The A10-IgG was conjugated to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Another anti-ADAMTS13 mouse mAb (WH2-11-1, IgG1-κ) was kindly supplied by Dr Kenji Soejima of the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) (*23*).

Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, HiTrap DEAE-FF and Superdex 200 HR10/30 were purchased from GE Healthcare Bio-Sciences AB (Piscataway, NJ, USA), and all reagents were analytical grade purchased from Wako Pure Chemicals (Osaka, Japan).

Assay of ADAMTS13 activity and protein concentration

ADAMTS13 activity was measured using a commercial chromogenic ADAMTS13-act-ELISA kit (Kainos, Tokyo) (*24*). In this article, an N-terminal glutathione-*S*-transferase (GST) and C-terminal histidine (His)-tagged fusion protein containing 73 amino acid residues (D1596 to R1668) of human VWF, termed GST-VWF73-His, are used as a substrate. After it is cleaved by ADAMTS13, Tyr-1605 is exposed and is detected by peroxidase-conjugated mAb anti-N10 (IgG) (*24*). Both 1 U (U) and 100% of ADAMTS13 activity were defined as the amount contained in 1 ml of pooled normal plasma. For the standard curve, pooled normal plasma serially diluted with heat-inactivated normal plasma, prepared by incubation at 56°C for 1 h followed by centrifugation, was used.

Protein concentration was estimated by absorption at 280 nm, using an extinction coefficient (E1%) of 10, or by micro BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Purification of pADAMTS13

One liter of FFP was thawed overnight at 4°C, and was then gently mixed with a cocktail of protease inhibitors [5 mM benzamidine–HCl, 1 mM phenylmethanesulphonyl fluoride and 20 mM ε-amino-n-caproic acid (EACA), each final concentration] followed by centrifugation at 7,000 rpm for 30 min at 4°C. After centrifugation, the cryoprecipitate was discarded and the cryosupernatant (~825 ml) was saved. The cryosupernatant was applied to an A10-Sepharose 4B column ($V_t = 50$ ml) at a flow rate of 50 ml/h at 4°C. The column was then washed with five-bed volumes of 20 mM Tris-buffered saline (TBS, pH7.4), five-bed volumes of high-salt TBS (20 mM Tris–HCl, 1M NaCl, pH 7.4), and five-bed volumes of high-salt TBS containing 10% dimethylsulphoxide (DMSO). Major fractions of ADAMTS13 activity were then eluted with high-salt TBS containing 40% DMSO, pooled and dialyzed overnight against 2 l of 20 mM Tris–HCl buffer (pH 7.4) at 4°C. The dialysate was applied to a HiTrap DEAE-FF column ($V_t = 1$ ml) equilibrated with 20 mM Tris–HCl buffer (pH 7.4) at a flow rate of 30 ml/h at room temperature (RT) followed by extensive washing with the same buffer. Bound protein was eluted with 20 mM Tris–HCl buffer (pH 7.4) containing 1 M NaCl, pooled and concentrated using aquacide II (Calbiochem, La Jolla, CA, USA). The protein was then dialyzed against 20 mM imidazole–HCl buffer (pH 6.5) containing 20 mM EACA, 10 mM Na_3citrate, 1 M NaCl and 0.02% NaN_3. The dialysate (~400 μl) was then separated on a Superdex 200 HR10/30 column equilibrated with the same buffer at a flow rate of 0.5 ml/min at RT, and 0.5 ml fractions were collected. The purified protein was exclusively dialyzed against TBS, and kept frozen at −80°C until use in aliquot.

Enzyme stability

pH stability. The purified pADAMTS13 was dialyzed against TBS containing 0.05% Tween-20 (TBST, pH 7.4). The pH of each aliquot of the purified pADAMTS13 was adjusted with 0.1 N HCl or 0.1 N NaOH, and then incubated for 18 h at 4°C. After incubation, 1/22 volume of 1 M Tris–HCl (pH 7.4) was added to each sample to neutralize the pH. Samples were then diluted 11-fold with the reaction buffer (5 mM Na-acetate buffer containing 5 mM $MgCl_2$, pH 5.5) and the residual ADAMTS13 activity was assayed.

Effects of heat and metal ions. The purified pADAMTS13 was dissolved in TBST (pH 7.4) or TBST containing 10 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$ or $ZnCl_2$, and incubated for 20 min at various temperatures (from 25 to 65°C). After incubation, samples were chilled on ice, diluted 11-fold in the reaction buffer (pH 5.5) and the residual ADAMTS13 activity was determined. As a control experiment, ADAMTS13 activity was measured at 25°C in the reaction buffer containing 0.9 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$ or $ZnCl_2$ without incubation.

Electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotting onto nitrocellulose or polyvinylidene difluoride (PVDF)

membrane after SDS–PAGE were described previously (*25*). Protein bands in the gels were stained with Coomassie brilliant blue (CBB).

For the immunoblotting studies, blots were incubated with anti-ADAMTS13 mAb A10 (IgG), and the bound antibody was detected using Super Signal Western Blotting Kits (Pierce, Rockford, IL, USA). For the detection of ABO blood group antigens on ADAMTS13, blots were incubated in TBST containing either mAb against blood group A or B (Ortho Clinical Diagnostic Laboratory, Japan) at 1 : 10 dilution for 90 min at RT. HRP-conjugated anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody.

Isoelectric focusing (IEF) was performed according to the manufacturer's instructions using agarose IEF, Pharmalyte TM 3-10 and an IEF calibration kit (Broad pI. pH 3–10) (products of GE Healthcare Bio-Science AB, Sweden). In some experiments, immunoblotting studies were performed after IEF using PVDF membranes, and stained with anti-ADAMTS13 mAb (WH2-11-1).

Lectin blotting

For lectin blotting, pADAMTS13 (0.4 μg/lane) was subjected to SDS–5%PAGE under reducing conditions followed by electrotransfer to PVDF membranes. Each membrane was incubated for 90 min at RT with TBST containing 1–3 μg/ml of biotin- or HRP-conjugated lectin (Seikagaku, Tokyo, EY laboratories, San Mateo, CA, USA), i.e. Concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), *Datura stramonium* agglutinin (DSA), *Sambucus sieboldiamna* agglutinin (SSA), *Agaricus bisporus* agglutinin (ABA), *Maackia amurensis* agglutinin (MAM), *Arachis hypogaea* (peanut) agglutinin (PNA), *Ulex europaeus* agglutinin I (UEA-I) and *Ricinus communis* agglutinin 120 (RCA_{120}). Membranes were washed with TBST and incubated with TBST containing 1 : 1000 diluted HRP-conjugated Streptavidin (Vector Laboratories, Burlingame, CA, USA) where biotinylated lectins were used. After washing with TBST, HRP reaction was performed in a solution containing 50 mM Tris–HCl buffer (pH 7.5) containing 200 mM NaCl, 5 mg/ml DAB and 0.005% H_2O_2 for 2–7 min.

In a separate experiment, the blot was incubated in 100 mM MOPS (3-morpholinopropanesulphonic acid) buffer, pH 6.8, containing 10 mM $CaCl_2$ and 20 mU/ml of *Streptococcus* neuraminidase (Seikagaku) for 1 h at 37°C. The neuraminidase-digested membrane was used for some lectin blot analysis.

Protease digestion and N-terminal amino acid sequencing

The purified pADAMST13 was concentrated by a Microcon-YM10 (Millipore, MA, USA) and aliquots (18 μg ADAMTS13/50–60 μl of TBS) were incubated with 4 U of human thrombin (Calbiochem, CA, USA), 30 mU of human plasmin (Calbiochem, CA, USA) or 1 mU of human leucocyte elastase (Elastin Products Co., MI) at 37°C for appropriate times. Aliquots of the digest were taken at intervals, and kept frozen at −80°C until use. The frozen samples were thawed, diluted 11-fold in reaction buffer and used to assay ADAMTS13 activity. ADAMTS13 activity of pre-incubation samples was defined as 100%. Ten microlitres of each digest were mixed with 2.5 μl of sample buffer (2% SDS, 0.5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8) and heated for 3 min at 95°C. The digests were analysed by SDS–5–20% gradient PAGE under reducing conditions. Proteins were electro-transferred onto PVDF membrane as described (*24*), and the protein bands were stained with Coomassie blue. The protein bands on the membrane were carefully cut out and directly subjected to N-terminal amino acid sequence analysis using an Applied Biosystems Procise protein sequencing system (Model 494 protein sequencer connected to a phenylthiohydantoin analyzer).

Results

Purification of pADAMST13

Using immunoadsorbent chromatography on an A10-Sepharose 4B column, bound ADAMTS13 was eluted with 40% DMSO with a high yield (41.7%) of activity (Table I). After dialysis of pooled fractions, the sample was concentrated using a HiTrap DEAE-FF column ($V_t = 1$ ml) followed by elution with 1M NaCl (see 'Materials and Methods' section). The eluates were concentrated and further separated by size exclusion chromatography on a Superdex 200 HR10/30 column (Fig. 1). A single major protein band with a molecular mass of 170 kDa before and 190 kDa after reduction was detected (inset of Fig. 1, left). Immunoblotting using anti-ADAMTS13 mAb (A10) and N-terminal amino acid sequence analysis confirmed that the major band was pADAMTS13. Some minor bands were identified as immunoglobulin heavy chain by both methods (data not shown). Agarose IEF of the purified pADAMTS13, followed by immunoblotting and staining with anti-ADAMTS13 mAb (WH2-11-1), is shown in the inset of Fig. 1, right. The purified pADAMTS13 had a pI of 5.3 (5.1–5.5), equivalent to ADAMTS13 in the plasma milieu.

From this system, a total of 200 μg of purified pADAMTS13 with a specific activity of 300 U/mg protein (25,057-fold activity purification) was obtained from 825 ml of starting material (cryosupernatant), with an activity yield of 8.5% (Table I). This column had been used >7 times without appreciable reduction of the yield of ADAMTS13 activity.

Effect of pH and metal ions on the heat stability of purified pADAMTS13

pH stability. The activity of purified pADAMTS13 was stable and relatively constant at pH ranges between 6.1 and 8.8 for 18 h storage at 4°C. However, ADAMTS13 activity was almost totally lost below pH 3.1 (Fig. 2A).

Divalent cations and heat stability. As shown in Fig. 2B (upper panel), ADAMTS13 activity in the reaction buffer containing 0.9 mM Ca^{2+} without incubation was enhanced 2.7 times compared to the value determined in the absence of 0.9 mM Ca^{2+}. In contrast, ADAMTS13 activity was markedly decreased in the reaction buffer containing 0.9 mM Zn^{2+} or Ni^{2+}.

Table I. Purification of ADAMTS13 from human plasma.

	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Cryosupernatant	825	701	58,575	0.01	1	100
A10-agarose	88	292	6.1	47.8	3,992	41.7
HiTrap DEAE	12	147	1.4	105.0	8,769	20.9
Superdex 200 HR10/30	2	60	0.2	300.0	25,057	8.5

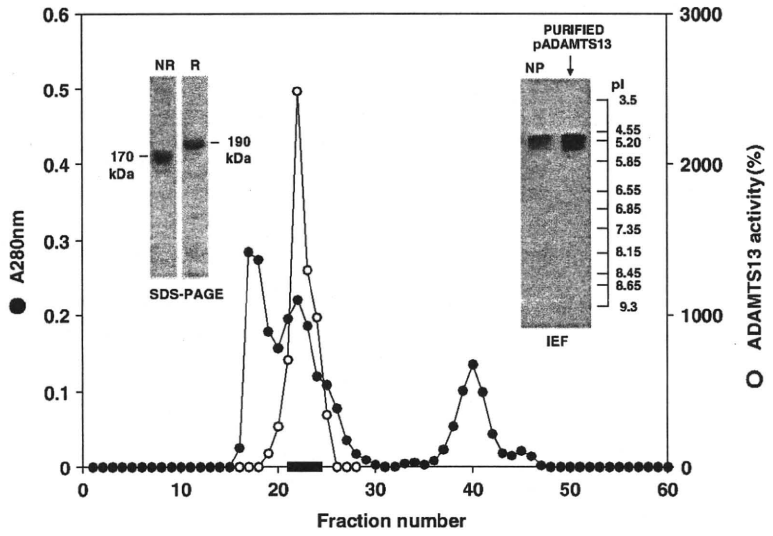

Fig. 1 Gel chromatogram of Superdex 200 HR10/30 as a final step for plasma (p) ADAMTS13 purification. Partially purified pADAMTS13, prepared by immunoadsorbent chromatography and concentrated by HiTrap DEAE gel, was further separated by Superdex 200 HR10/30 column at a flow rate of 0.5 ml/min at RT (see 'Materials and Methods' section for detail). Fractions shown by the black bar were pooled and used as the purified pADAMTS13. The inset (left) shows SDS–5% PAGE of the purified pADAMTS13 under reducing (R) and non-reducing (NR) conditions (stained with CBB). The inset (right) indicates immunoblotting analysis of the purified pADAMTS13 and normal plasma (control), after IEF. ADAMTS13 antigen was detected by anti-ADAMTS13 mAb (WH2-11-1).

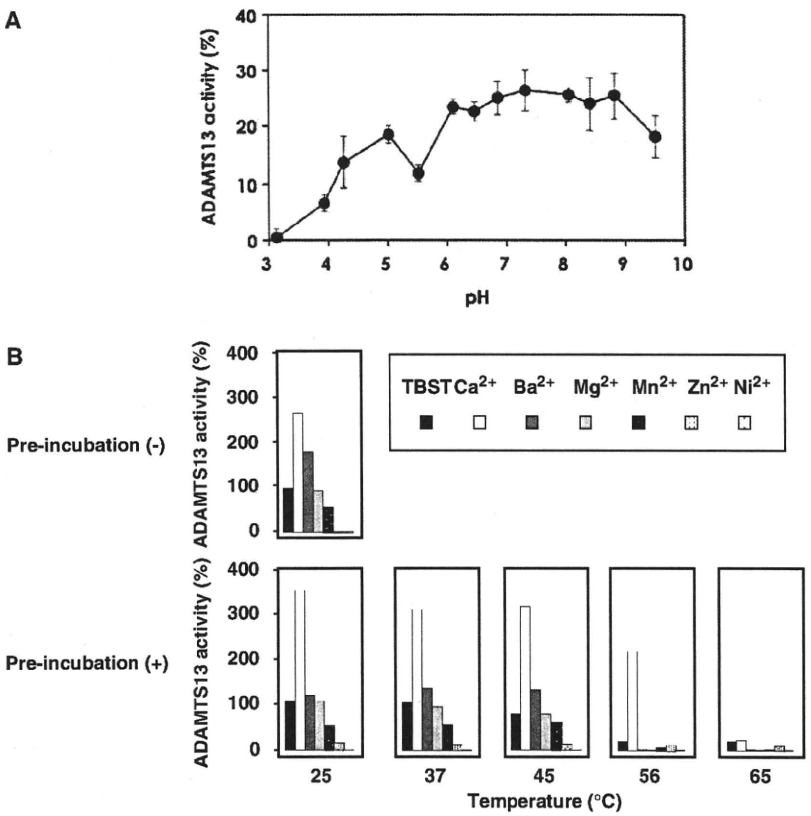

Fig. 2 pH stability, and effects of temperature and divalent metal ions on purified pADAMTS13 activity. (A) The purified pADAMTS13 was dialyzed against 20 mM TBS containing 0.05% Tween-20 (TBST, pH 7.4), and the pH of each aliquot was adjusted from 3.1 to 9.5 with 0.1 N HCl or NaOH, followed by incubation for 18 h at 4°C. After incubation, each sample was neutralized with 1 M Tris–HCl buffer (pH 7.4), diluted and the residual ADAMTS13 activity was determined. (B) The purified pADAMTS13 dissolved in TBST (pH 7.4) or TBST containing 10 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ or $NiSO_4$, was incubated for 20 min at various temperatures from 25 to 65°C. In either before incubation (upper panel) or after incubation (lower panel), samples were diluted, and the residual ADAMTS13 activity was determined. ADAMTS13 activity in TBST incubated at 25°C was arbitrarily defined as 100%.

Next, ADAMTS13 activity was measured after 20-min incubation of purified protein under various temperatures and in the presence of six different divalent cations. The activity of pADAMTS13 in the presence of 10 mM Ca^{2+} was enhanced 3–5-fold as compared with control experiments in TBST alone at temperatures between 25 and 56°C. However, the activity in the presence of 10 mM Ca^{2+} was decreased from 364% at 25°C to 23% at 65°C. In the presence of 10 mM Ba^{2}, the ADAMTS13 activity was enhanced 1.1- to 1.4-fold as compared with control experiments in TBST alone at temperatures between 25 and 56°C. Mn^{2+} had an inhibitory effect on the enzyme activity, but Mg^{2+} was equivalent to the enzyme activity in TBST alone. Both Ni^{2+} and Zn^{2+} had a strong inhibitory effect on the ADAMTS13 activity. Perhaps the most striking result was obtained in the experiment at 56°C for 20 min, where the purified pADAMTS13 containing 10 mM Ca^{2+} maintained 60% of its original activity, whereas enzyme containing other divalent cations almost completely lost activity (Fig. 2B, lower panel), indicating that Ca^{2+} provided thermal resistance to this enzyme. Further, no significant loss of enzyme activity was observed during storage in the presence of 10 mM Ca^{2+} for 2 weeks at 37°C (data not shown).

Cleavage sites of thrombin, plasmin and leucocyte elastase

As shown in Fig. 3, the purified 190-kDa pADAMTS13 was digested with thrombin, plasmin and leucocyte elastase at 37°C for various time intervals. The residual ADAMTS13 activity was measured in each sample, and the degradation of ADAMTS13 antigen was monitored by SDS–5–20% gradient PAGE under reducing conditions. In each instance, the major 190-kDa band was gradually degraded into several fragments of smaller molecular mass. Thrombin digestion resulted in fragments with molecular mass of 37, 40, 48, 70, 100 and 170 kDa (Fig. 3A, left). Plasmin digestion produced 36, 40, 70, 100, 140 and 180 kDa bands (Fig. 3B, centre). Digestion with leucocyte elastase gave 38, 41, 63, 114, 130 and 160 kDa bands (Fig. 3A, right).

In terms of the residual ADAMTS13 activity, 20% of the original ADAMTS13 activity remained at 5 h after thrombin digestion, but the activity was completely lost at 20 h, with a concomitant disappearance of the 190-kDa band (Fig. 3B, left). Similarly, 30–50% of the original activity was maintained at 30 min after plasmin digestion (Fig. 3B, centre) and at 120 min after elastase digestion (Fig. 3B, right) with a loss of the 190-kDa band.

The aforementioned peptides were sequenced, and the N-terminal amino acid sequences are shown in Table II. In summary, thrombin cleaves at least four peptidyl bonds, between Arg257–Ala258, Arg459–Ser460, Arg888–Thr889 and Arg1176–Arg1177, and plasmin cleaves three peptidyl bonds between Arg257–Ala258, Arg888–Thr889 and Arg1176–Arg1177. Interestingly, thrombin and

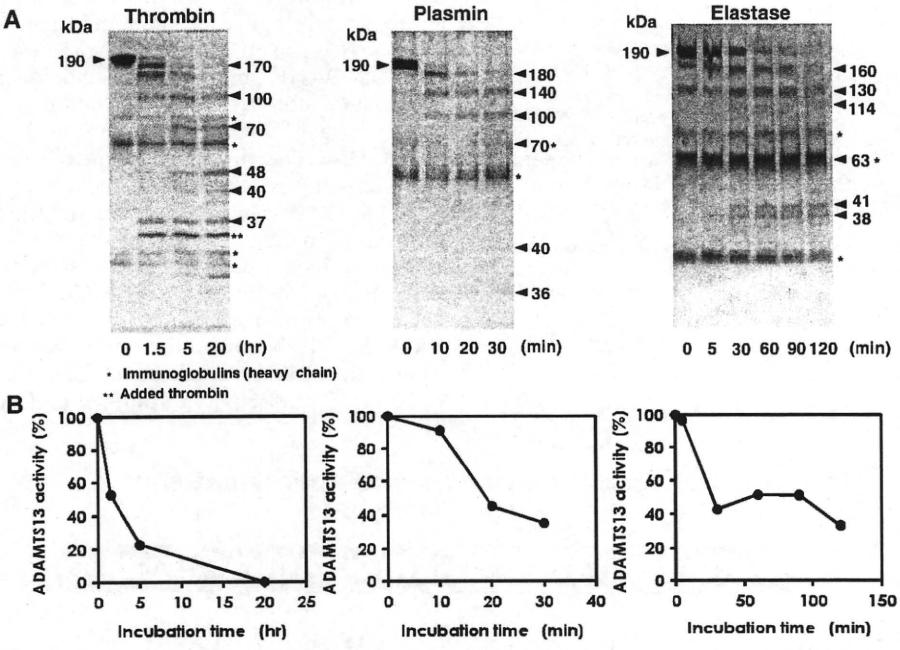

Fig. 3 Proteolytic fragmentation of the purified pADAMTS13 by thrombin, plasmin or leucocyte elastase, and residual activity. (A) The purified pADAMST13 was dissolved in TBS (pH 7.4), and incubated at 37°C for various time intervals with human thrombin (left), plasmin (middle) or leucocyte elastase (right), as described in 'Materials and Methods' section. Aliquots of each mixture were taken at intervals, and kept frozen at −80°C until use. The frozen samples were thawed, and the residual ADAMTS13 activity was determined by simultaneous analysis on SDS–5–20% gradient PAGE under reducing conditions. The indicated arrows were subjected to N-terminal sequencing after electro-blotting onto PVDF membranes (see the results in Table I). (B) Residual ADAMTS13 activity of each digest at various time intervals is shown. The ADAMTS13 activity determined by prior enzyme digestion was arbitrarily defined as 100%.

plasmin both cleaved three peptidyl bonds, just after the Arg residues at positions 257, 888 and 1176. In contrast, leucocyte elastase cleaved two peptidyl bonds between Ile380–Ala381 and Thr874–Ser875 (Table II). Fig. 4 depicts the putative cleavage sites on ADAMTS13 by thrombin, plasmin and leucocyte elastase.

Lectin blotting analysis of purified pADAMTS13 and detection of ABO blood group antigens on ADAMTS13

Carbohydrates on the purified pADAMTS13 were surveyed by lectin blotting analysis (Fig. 5A). Major 190 kDa and minor 170-kDa bands positively reacted to Con A, LCA and DSA. Since Con A and LCA show specific affinity to mannosyl residues and DSA to tri- or tetra-blanched poly-*N*-acetyllactosamine repeats, the presence of high-mannose or complex-type Asn-linked sugar chains is likely. PNA, which has specific affinity to Galβ1-3GalNAc structures, did not bind to purified pADAMTS13 before neuraminidase treatment, but did bind following this treatment. Since NeuNAc-Galβ1-3GalNAc structures are often found in Ser/Thr-linked sugar chains, it is likely that pADAMTS13 contains this type of Ser/Thr-linked sugar chain. SSA, which specifically binds to α2–6-linked sialic acid but not to α2–3-linked sialic acid, bound to pADAMTS13, whereas MAM, which is specific to α2–3-linked sialic acid in Asn-linked sugar chains, reacted only very weakly with pADAMTS13. These results indicate the presence of α2–6 (and partially α2–3-linked) sialic acid residues at the non-reducing terminus. The reactivity of RCA_{120} was also strongly enhanced after neuraminidase digestion, indicating the presence of β1–4-linked Gal residues penultimate to the sialic acid.

Further, the purified pADAMTS13 neither reacted to anti-blood groups A and B antibodies (Fig. 5B) nor UEA-I (anti-H) lectin (data not shown), indicating the absence of ABO-blood group antigens in this enzyme; in contrast, these antigens were found in human VWF.

Table II. The N-terminal amino acid sequence of ADAMTS13 peptides generated by the cleavage of thrombin, plasmin or elastase.

Protease	Fragment (kDa)	N-terminal sequence	Amino acid residue number
Thrombin	170	AAGGILHLE	N-terminal sequence
	100	AGLAxSP	R257-A258
	70	AGLAxSP	R257-A258
	48	SSPGGASF	R459-S460
	40	TGAQAAH	R888-T889
	37	RLLPGPQENS	R1176-R1177
Plasmin	180	AAGGILxL	N-terminal sequence
	140	AAGGILxL	N-terminal sequence
	100	AAGGILxL	N-terminal sequence
	70	AGLAxSP	R257-A258
	40	TGAQAA	R888-T889
	36	RLLPGPQE	R1176-R1177
Elastase	160	AAGGILxL	N-terminal sequence
	130	AAGGILxL	N-terminal sequence
	114	AAGGILxL	N-terminal sequence
	63	AAVHGR	I380-A381
	41	AAGGILxL	N-terminal sequence
	38	SAGEKAP	T874-S875

x, means unknown.

Discussion

Here, we have established a purification method for pADAMTS13 using an immunoadsorbent column containing an anti-ADAMTS13 mAb (A10-IgG). This method is unique, because A10 has recently been shown to bind to a conformational epitope consisting of 72 amino acid residues (Tyr305-Glu376) within the Dis domain of ADAMTS13 (*21*, *22*). The bound protein was eluted with 40% DMSO dissolved in a neutral buffer (pH 7.4) with a high yield (41.7%) of enzyme activity. In the two subsequent chromatographic steps (anion exchange and size exclusion columns), however, the activity yield was reduced by approximately half each, and the final activity yield was ~8.5%. The purified pADAMTS13 was confirmed by SDS–PAGE analysis as a homogeneous band before and after reduction. In addition, the protein had a single N-terminal amino acid sequence of AAGGIL–. The pI of the purified enzyme was 5.3 (5.1–5.5). These data indicated that the purified pADAMTS13 was comparable to ADAMTS13 in the plasma milieu.

The purified pADAMTS13 was stable in buffers with pH ranging from 6.1 to 8.8, but the activity decreased under acidic pH, suggesting that pADAMTS13 bound to the A10-column was not efficiently eluted under acidic conditions. At temperature of 25–45°C, the activity of purified pADAMTS13 was

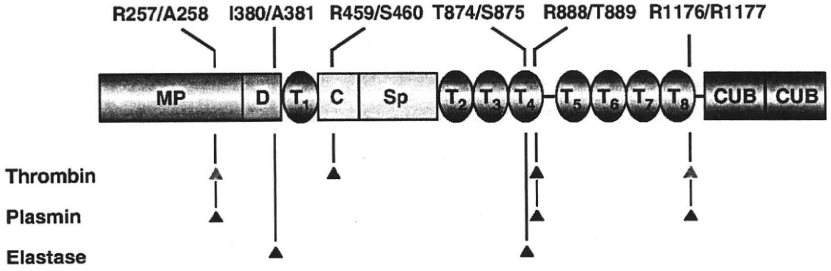

Fig. 4 Sites of pADAMTS13 cleavage by thrombin, plasmin and leucocyte elastase. A Schematic diagram of the identified pADAMTS13 cleavage sites for thrombin, plasmin and leucocyte elastase is shown. Note that thee of the four thrombin cleavage sites are identical to those of plasmin, but are different from of the two leucocyte elastase sites. MP, metalloproteinase; D, disintegrin-like; T, TSP type1; C, cystein-rich; Sp, spacer; CUB, complement C1r/C1s sea urchin epidermal growth and bone morphogenic protein 1.

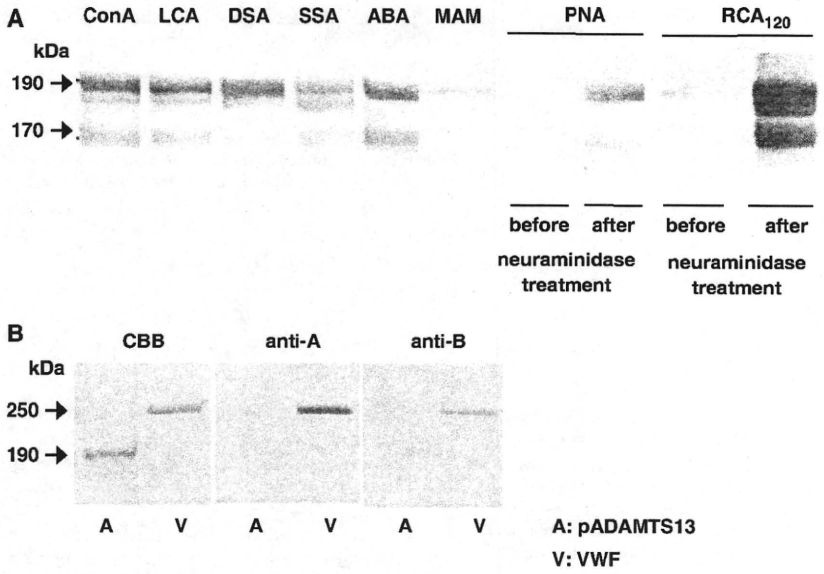

Fig. 5 Lectin blotting analysis of purified pADAMTS13 and detection of ABO blood group antigens on ADAMTS13. (A) Purified pADAMTS13 (0.4 μg) was subjected to SDS–5%PAGE under reducing conditions and transferred to a PVDF membrane. In some experiments, the blot proteins were treated with neuraminidase and then reacted with various lectins as described in 'Materials and Methods' section. Two protein bands, a major band of 190 kDa and a minor band of 170 kDa, are observed. Both bands positively reacted to Con A, LCA and DSA. PNA did not bind to purified pADAMTS13 before neuraminidase treatment, but did following neuraminidase treatment. (B) VWF (0.3 μg/lane) or pADAMTS13 (0.3 μg/lane) purified from pooled plasmas was subjected to SDS–5%PAGE under reducing conditions and transferred to a PVDF membrane as describe above. Protein bands on the membrane were stained with CBB. Reactivity to blood groups A or B antigen was clearly shown in VWF, but not in pADAMTS13.

enhanced by ~3-fold in the presence of 10 mM Ca^{2+}, but strongly inhibited in the presence of 10 mM Zn^{2+} and Ni^{2+}. This result appears to be in good agreement with that of Anderson *et al.* (*26*), who showed that Zn^{2+} at final concentrations of 1–3 mM enhanced the enzymatic activity of rADAMTS13 by the classic VWF multimer assay, but Zn^{2+} at higher concentrations (4–5 mM) inhibited activity. Gardner *et al.* (*27*) recently reported that rADAMTS13, extensively dialyzed against 0.15 M NaCl, 20 mM Tris–HCl (pH 7.8), requires pre-incubation with Ca^{2+} for 40–50 min to restore full enzyme activity. Furthermore, the activity of EDTA-treated enzyme, extensively dialyzed against 0.15 M NaCl, 20 mM Tris–HCl (pH 7.8) containing 5 mM Ca^{2+}, could be fully restored in a Zn^{2+}-dependent manner. These results indicated that dialysis can remove all functional Ca^{2+}, but does not remove the active site-bound Zn^{2+}. In addition, we have demonstrated here that 10 mM Ca^{2+} provides thermal resistance to pADAMTS13 activity, but it remains unaddressed how this happens in relation to the recently identified putative Ca^{2+} binding sites (Glu184 and Asp 187 of ADAMTS13).

Crawley *et al.* (*17*) showed that the three serine proteases, thrombin, plasmin and coagulation factor Xa, down-regulate ADAMTS13 activity by proteolysis. Lam *et al.* (*18*) identified the peptidyl bonds between Arg257–Ala258 and Arg1176–Arg1177 within ADAMTS13 as targets of thrombin. They predicted additional possible thrombin cleavable sites at Arg287, Arg393, Arg415, Arg910 and Arg968 of ADAMTS13 based on primary sequence analysis. In addition to the two thrombin cleavage sites reported by Lam *et al.* (*18*), we report two novel thrombin targets using the purified pADAMTS13: the peptidyl bonds between Arg459–Ser460 and Arg888–Thr889, neither of which was predicted. Of particular interest was the observation that plasmin cleaved the purified pADAMTS13 at the Arg257–Ala258, Arg888–Thr889 and Arg1174–Arg1177 sites, the same as thrombin.

Ono *et al.* (*15*) reported that ADAMTS13 antigen bands with lower molecular weights were found in plasmas of patients with sepsis-induced DIC that might have derived from digestion by bacterial proteases or leucocyte elastase. In this study, we therefore subjected the purified pADAMTS13 to leucocyte elastase digestion, and observed that the two peptidyl bonds between Ileu380–Ala381 and Tyr874–Ser875 were cleaved by this enzyme. These cleavage sites were different from those of thrombin or plasmin, and therefore also exclude the possibility that the three common cleavage sites of thrombin and plasmin were artificially generated during the purification process. According to the partial crystal structure of ADAMTS13 (*28*), Ileu380–Ala381 and Arg459–Ser460 were located on the surface exposure part between the Dis and thrombospondin1-1 domains and of the loop of the cystein-rich domain, respectively (Fig. 6).

Lam *et al.* (*18*) further reported that thrombin-cleaved rADAMTS13 remained partially active against intact VWF, and fully active against a recombinant VWF-A2 fragment (termed VWF115). However, the binding affinity of thrombin-treated rADAMTS13 for intact VWFM was significantly reduced compared

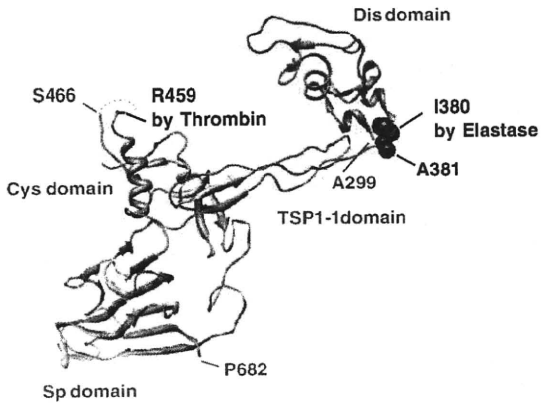

Fig. 6 Partial crystal structure of ADAMTS13 and location of the Ileu380–Ala381 and Arg459–Ser460 peptidyl bonds on ADAMTS13. Ribbon structure of ADAMTS13 (residues 299–682) was drawn with the UCSF Chimera package (*34*) using Protein Data Bank (PDB) entry as 3GHM. I380 and A381 are shown by space-filling model. Cleavage sites of I380–A381 by elastase and R459–S460 by thrombin (existing within the unsolved region) were located on the surface exposure part between the disintegrin-like and thrombospondin1-1 domains and of the loop of the cystein-rich domain, respectively. Dotted line indicates the unsolved region. Dis, disintegrin-like; TSP, thrombospondin1; Cys, cystein-rich; Sp, spacer domains.

to VWF115. As part of this study, we therefore also determined that >30% of the original pADAMTS13 activity against the GST-VWF73-His substrate remained during the incubation period (0.5–5.0 h) in each enzyme digestion.

ADAMTS13 has 10 putative *N*-glycosylation sites and *O*-fucosylated sugar chains in at least six thrombospondin type 1 repeats. Both the *N*- and *O*-glycans were reported to be prerequisite for enzyme secretion using rADAMTS13 (*29*, *30*). We here demonstrated by lectin blotting analysis that the purified pADAMTS13 possesses α2–6 (and partially α2–3-linked) sialic acid residues at the non-reducing terminus, and the presence of β1–4- and β1–3-linked galactose residues penultimate to sialic acid, through the observation that the reactivities of RCA_{120} and PNA were strongly enhanced after neuraminidase digestion. Since almost all sugar chains on pADAMTS13 are capped by sialic acid with no exposure of galactose residues, a clearance mechanism via hepatic asialogrycoprotein receptor might be involved, as is the case for other plasma glycoproteins (*31*, *32*). These sialo-sugar chains may also protect ADAMTS13 from proteolytic cleavage. In addition, neither blood group A nor B antigens were detected on the purified pADAMTS13, as previously reported by other investigators (*33*).

This study may in part contribute to understanding how ADAMTS13 activity is modulated by proteases generated in various clinical settings, and also to preparing the heat-treated pADAMTS13 concentrates.

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Conflict of interest

None declared.

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ORIGINAL ARTICLE

A second national questionnaire survey of TMA

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Abstract A second questionnaire survey of Japanese patients with thrombotic microangiopathy (TMA) was carried out to investigate the frequency, laboratory abnormalities and outcome in 2004 and 2005. The first and second surveys evaluated 397 patients including 19 with familial TMA and 378 with acquired TMA. The patients with acquired TMA included 165 with *Escherichia coli* O-157 infection-related TMA (O-157 TMA), 70 with ADAMTS13-related TMA (ADAMTS13 TMA) and 38 with other types of TMA (other TMA). The rate of ADAMTS13 TMA was significantly higher in patients with collagen diseases than in patients with all other underlying diseases ($p < 0.001$). The treatment of acquired TMA included plasma exchange (PE), steroids, antiplatelet agents, and anticoagulants, PE was carried out in 91.4% of patients with ADAMTS13 TMA, 68.4% of patients with other TMA and 12.7% of patients with O-157 TMA. The efficacy of PE and steroid therapy tended to be higher in patients with ADAMTS13 TMA than in those with other TMA. The complete remission rate was the highest and the mortality rate was the lowest in the patients with O-157 TMA. The mortality rate tended to be lower in patients with ADAMTS13 TMA than in those with other TMA. However, not all of the patients in our study were examined for ADAMTS13 at the time that this questionnaire survey was conducted.

Keywords TMA · TTP · HUS · ADAMTS13

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1 Introduction

Thrombotic microangiopathies (TMAs) are defined by the association of acute mechanical hemolytic anemia, thrombocytopenia, and visceral ischemic manifestations related to the formation of platelet thrombi in the microcirculation [1]. Clinically, TMA includes mainly thrombotic thrombocytopenic purpura (TTP) [2–4] and the hemolytic uremic syndrome (HUS) [5] characterized by multivisceral ischemia, fever and renal ischemia. In 1982, Moake et al. [6] demonstrated the presence of "unusually large multimers of Von Willebrand factor" (UL-VWF) in the plasma of patients suffering from chronic relapsing TTP. UL-VWF produced in and then quickly released from vascular endothelial cells is found in the plasma of patients

with both familial and non-familial TTP [6, 7]. These UL-VWF are thought to interact with circulating platelets, thus resulting in platelet clumping due to elevated shear stress [7]. Platelet-rich microthrombi in the small vessels are the pathologic hallmark of this disease [8].

ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type I domain 13), which was identified in 2001 [9–11], is a zinc metalloprotease that specifically cleaves UL-VWF at the Tyr (1605)-Met(1606) boundary located in the A2 region of VWF [12, 13]. TTP was previously a life-threatening syndrome, although the survival rate has increased from 20 to 80% since the development of plasma exchange (PE) [14] and it recently has reached about 90% [15]. The mainstay of treatment is therapeutic PE, both to remove the causative antibodies to ADAMTS13, UL-VWF and cytokines, and to replace ADAMTS13 and normal size VWF [16]. The current guidelines [17] for thrombotic microangiopathy (TMA) recommend that PE should be initiated within 24 h of diagnosis.

Two questionnaire surveys for Japanese patients with TMA were carried out in 2004 [18, 19] and 2005. In the present study, the frequency, underlying diseases, symptoms, laboratory abnormalities, treatments, efficacy of treatment and outcome were analyzed from all of the data collected in the two questionnaire surveys conducted in 2004 and 2005.

2 Materials and methods

One hundred and eighty-five patients and two hundred and twelve patients diagnosed with TMA between 1 January 1999 and 31 December 2003 and between 1 January 2004 and 31 December 2004 were examined by a national questionnaire survey. The questionnaire was mainly of a selective type, and the contents of the questionnaire were about age, sex, underlying disease(s), acute symptoms, laboratory data including ADAMTS13, treatment, outcomes, etc. The first questionnaire in 2004 was sent to 994 departments of hematology in Japanese hospitals or institutes. A total of 429 hospitals responded and 73 had 185 cases of TMA.

In 2005, the second questionnaire concerning the number of patients was sent to 3,301 departments of rheumatism-collagen disease, internal medicine, pediatrics, urology and emergency and dialysis-renal centers in Japanese hospitals. A total of 2,275 departments responded and 457 cases of TMA (familial TMA: 20 cases, acquired TMA: 437 cases) were reported. The next questionnaire was sent to the hospitals where the 457 patients had received their diagnosis and treatment. In this case, 146 hospitals responded and 212 cases of TMA were included in the analysis. The hospital name was made anonymous. Finally, 397 cases in total (185 cases in 2004 and 212 cases in 2005) were analyzed.

Derangement, lethargy, behavioral disorders, convulsions, stupor, coma and other neurological abnormalities were considered to be neurological symptoms. Creatinine levels >1.3 mg/dl indicated renal injury. A body temperature >37.5°C was considered as a fever. Cough, sputum, and other symptoms of bronchitis and pneumonia were considered to be respiratory symptoms.

TMA patients were classified into 4 groups: those with ADAMTS13-related TMA (ADAMTS13 TMA), where the ADAMTS13 level was less than 20% or when patients were positive for an inhibitor of ADAMTS13; *Escherichia coli* O-157 infection-related TMA (O-157 TMA), in which TMA was caused by an O-157 infection; other TMA, the cause of which, was not known; or those in which TMA was not measured (NM TMA). In these patients, the ADAMTS13 level was not measured, and the disease was not induced by an O-157 infection.

The study protocol was approved by the Human Ethics Review Committees of Keio University School of Medicine and Mie University School of Medicine.

2.1 Statistical analysis

The data are expressed as the medians (25–75 percentile). Differences between the groups were examined for significance using the Chi-squared test for independence. A p value of less than 0.05 was considered to indicate a significant difference. All statistical analyses were performed using the SPSS II software package (SPSS Japan, Tokyo).

3 Results

The patients included 19 with familial TMA and 378 with acquired TMA. In the patients with familial TMA, the ADAMTS 13 level was markedly reduced in 13 of the patients (ADAMTS13 TMA), but not in 2 of the patients (other TMA). The level of ADAMTS13 was not measured in the other 4 patients (NM TMA). In the patients with acquired TMA, 165 cases of TMA were caused by O-157 infection (O-157 TMA), and 70 were due to ADAMTS13 (ADAMTS13 TMA), 38 were due to other causes (other TMA), and 105 were NM TMA (Table 1). There tended to be more females than males among those with ADAMTS13 TMA and O-157 TMA within those with acquired TMA. There were more patients from 0 to 15 years in the O-157 TMA group, while those with acquired TMA not due to an O-157 infected were generally between the ages of 31 and 65 (Fig. 1). O-157 infection was the most frequent underlying disease in patients with TMA, while collagen disease was the second, the presence of a malignant tumor and transplantation were the third, and drug-induced TMA was

Table 1 Subjects

	Number			Sex (F:M)		
	First	Second	Total	First	Second	Total
Familial	13	6	19	9:4	2:4	11:8
ADAMTS13 TMA	8	5	13	7:1	2:3	9:4
Other TMA	1	1	2	0:1	0:1	0:2
NM TMA	4	0	4	2:2	0:0	2:2
Acquired	172	206	378	92:79[a]	133:73	225:152[a]
ADAMTS13 TMA	35	35	70	20:15	21:14	41:29
O-157 TMA	66	99	165	40:25[a]	69:30	109:55[a]
Other TMA	22	16	38	11:11	5:11	16:22
NM TMA	49	56	105	21:28	38:18	59:46

ADAMTS13 TMA ADAMTS13 activity markedly decreased, *other TMA* ADAMTS13 activity did not markedly decrease, *NM TMA* ADAMTS13 activity was not measured, *O-157 TMA* O-157 related TMA

[a] 1 patient is not described

the fourth, etc. (Table 2). In patients with collagen diseases, the rate of acquired ADAMTS13 TMA (47.6%) was significantly higher than that of other TMA ($p < 0.01$). With regard to the underlying disease, the rate of ADAMTS13 TMA was significantly higher in patients with collagen diseases than in those with all other types of underlying disease ($p < 0.001$). In patients with an underlying O-157 infection, the rate of ADAMTS13 TMA was 0%. In patients with familial TMA, icterus neonatorum was observed in most patients (68.8%). The acute symptoms reported are shown in Table 3. The incidence of neurological symptoms was significantly lower in patients with O-157 TMA than in those with all other types of acquired TMA ($p < 0.001$) and their incidence tended to be higher in patients with ADAMTS13 TMA than with other TMA ($p = 0.089$). In patients with acquired TMA, the frequency of renal dysfunction was significantly higher in patients with other TMA than in those with ADAMTS13 TMA ($p < 0.001$). A fever was observed in 70.7% of patients with acquired TMA. Respiratory symptoms were not regularly associated with TMA although they occurred with significantly lower frequency in patients with O-157 TMA than in those with all other types of acquired TMA ($p < 0.001$).

The laboratory data are shown in Table 4. A decreased platelet count and a decreased hemoglobin level, and an increase in total bilirubin (T-bil) and lactate dehydrogenase (LDH) were frequently observed in each type of TMA. The platelet count (median value) was significantly lower in patients with familial TMA than in those with acquired TMA ($p < 0.05$), and tended to be lower in patients with ADAMTS13 TMA than in those with other TMA ($p < 0.061$). However, the platelet count was significantly higher in patients with O-157 TMA than in those with all other types of acquired TMA ($p < 0.001$).

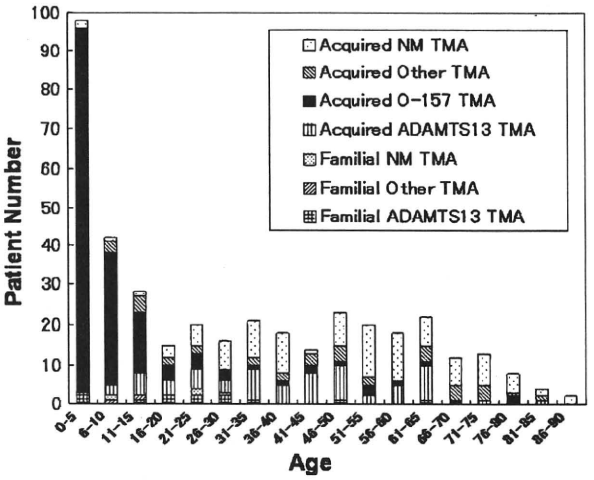

Fig. 1 The age of patients at the onset of TMA

The hemoglobin level was usually less than 13.0 g/dl and typically was between 5.0 and 10.0 g/dl. It was significantly lower in patients with acquired TMA than those with familial TMA ($p < 0.05$), and significantly lower in patients with O-157 TMA than in all patients with other types of acquired TMA ($p < 0.01$). The T-bil level was significantly lower in patients with O-157 TMA than in those with all other types of acquired TMA ($p < 0.01$). The LDH level was significantly higher in patients with other TMA than those with ADAMTS13 TMA ($p < 0.05$) and in patients with O-157 TMA compared to those with all other types of acquired TMA ($p < 0.001$). The levels of fibrin and fibrinogen degradation products (FDP) were slightly increased in most TMA patients, although the fibrinogen level was reduced in a few TMA patients. In those with acquired TMA, the frequency of positivity for antinuclear antibodies was higher in those with ADAMTS13 TMA