autoantibodies against ADAMTS13 [7, 8], results in the accumulation of "unusually large" VWF multimers (UL-VWFM) in plasma; this, in turn, leads to platelet clumping and/or thrombi under high shear stress and subsequent microcirculatory disturbances.

In 2000, we reported that predominantly decreased ADAMTS13 activity (ADAMTS13:AC) in sick children with advanced cirrhotic biliary atresia could be fully restored after living donor liver transplantation, indicating that the liver is the major organ producing ADAMTS13 [9]. In 2001, three other groups indicated that ADAMTS13 mRNA was exclusively expressed in the liver by northern blot analysis [5, 10, 11]. Subsequently, we were able to demonstrate that ADAMTS13 was produced exclusively in hepatic stellate cells (HSCs) using both in situ hybridization and immunohistochemistry [12]. Platelets [13], vascular endothelial cells [14], and kidney podocytes [15] were also shown to be ADAMTS13-producing cells, but the relevance to the pathogenesis of thrombo-regulation in each organ remained unclear.

Since HSCs are the major ADAMTS13-producing cells in human liver [12], we will review the potential functional role of ADAMTS13 in association with the pathogenesis of liver diseases.

2 Hepatic microcirculation and hypercoagulability in liver diseases

Hepatic microcirculation compromises a unique system of capillaries, called sinusoids, which are lined by three different cell types: sinusoidal endothelial cells (SEC), HSC, and Kupffer cells [16]. The SEC modulates microcirculation between hepatocytes and the sinusoidal space through the sinusoidal endothelial fenestration. The SEC has tremendous endocytic capacity, including for VWF and the extracellular matrix, and secretes many vasoactive substances [16]. The HSC is located in the space of Disse adjacent to the SEC, and regulates sinusoidal blood flow by contraction or relaxation induced by vasoactive substances [17]. Kupffer cells are intrasinusoidally located in tissue macrophages, and secrete potent inflammatory mediators during the early phase of liver inflammation [16]. Intimate cell to cell interaction has been found between these sinusoidal cells and hepatocytes [16, 17].

Vascular endothelial cells play a pivotal role in hemostasis and thrombosis [3, 4]. VWF is a marker of endothelial cell activation (damage), and plays an essential role in hemostasis [3, 4]. In the normal state, VWF immunostaining is usually positive in large vessels, but negative in the SEC [18]. On the occurrence of liver injury accompanied by a necroinflammatory process, the SEC becomes positive for VWF, presumably in association with the capillarization of hepatic sinusoids [19]. Subsequently, platelets adhere to subendothelial tissue mediated by UL-VWFM [3, 4]. ADAMTS13 then cleaves UL-VWFM into smaller VWF multimers [3, 4]. This interaction of ADAMTS13 and UL-VWFM is, indeed, the initial step in hemostasis [3, 4]. Recent work has further shown that recombinant ADAMTS13 binds to recombinant CD36 and platelet membrane CD36 in vitro, demonstrating a role for this protein in localizing ADAMTS13 to endothelial cells expressing CD36, where ADAMTS13 regulates the cleavage of VWF [20].

In patients with fulminant hepatic failure and liver cirrhosis, circulating plasma VWF antigen (VWF:AG) levels are extremely high [21–23]. Many fibrin thrombi were found in the hepatic sinusoids in acute liver failure, suggesting a role for intravascular coagulation in the pathogenesis of hepatic necrosis [24]. In cirrhotic liver tissue [25] and even tissue from patients in early stages of alcoholic liver diseases [26], VWF immunostaining shows positive cells predominantly at the scar–parenchyma interface, within the septum, and in the sinusoidal lining. Portal or hepatic vein thrombosis is often observed in advanced cirrhosis [27, 28] and microthrombi formation was found in one or multiple organs in half of autopsied cirrhotics [29]. This hypercoagulable state in liver diseases may be involved in hepatic parenchymal extinction, the acceleration of liver fibrosis, and disease progression.

Considering that ADAMTS13 is synthesized in HSC [12] and its substrate, UL-VWFM, is produced in transformed SEC during liver injury [18], decreased plasma ADAMTS13:AC may involve not only sinusoidal microcirculatory disturbances, but also subsequent progression of liver diseases, eventually leading to multiorgan failure. Based on these findings, it is of particular interest to evaluate plasma ADAMTS13:AC in liver disease patients.

3 ADAMTS13 assays

The classic VWF multimer assay used to be the gold standard method for evaluating plasma ADAMTS13:AC; however, its major disadvantage was that it took several days to provide results [7]. In this regard, the discovery of a minimum 73 amino acid residue sequence within the VWF-A2 domain (VWF73) by Kokame et al. [30], which was prerequisite for the rapid cleavage by ADAMTS13, provided a breakthrough in developing novel methods to assay ADAMTS13:AC. Indeed, a convenient fluorescence method based on FRET-VWF73 is now widely used as the gold standard second generation method [31]. However, the sensitivity of FRET-VWF73 remains approximately 3% of the normal control, and the presence of hemoglobin, bilirubin, and/or chylomicron in samples significantly

influences the results [32]. To solve these problems, a unique method for determining ADAMTS13:AC, termed ADAMTS13-act-ELISA, was developed in our laboratory as a third generation method [33]. This assay was established after production of a novel murine monoclonal antibody to ADAMTS13, termed N-10, which specifically recognizes the Y1605 residue of the VWF-A2 domain, generated by ADAMTS13 cleavage [33]. The lower limit of this assay is 0.5% of the normal control. Developing an automated more rapid assay for ADAMTS13:AC and its usage in hospitals is urged to prevent unnecessary or harmful infusions of platelet concentrates to patients with masked thrombocytopenia, such as "subclinical TTP".

4 The physiological significance of ADAMTS13 in liver diseases

4.1 Liver cirrhosis

Sinusoidal microcirculatory disturbance in liver cirrhosis occurs when the normal hepatic structure is disrupted by fibrin deposition [19] or by impaired balance between the action of vasoconstrictors and vasodilators in hepatic vascular circulation [16]. Studies have shown that cirrhotic liver exhibits a hyperresponse to vasoconstrictors, including catecholamine, endothelin, and leukotrienes D_4 [16]. Now it is well-accepted that thrombocytopenia gradually progresses as functional liver capacity decreases (Fig. 1a). Previously, thrombocytopenia in liver cirrhosis has been speculated to be associated with hypersplenism [34] and decreased synthesis of thrombopoietin in the affected liver [35]. Our recent studies, however, have provided evidence considering that UL-VWFM accumulated in plasmas with far advanced cirrhotic patients enhances high shear-stress-induced platelet aggregation, resulting in thrombocytopenia [36].

Mannucci et al. [37] originally reported a significant reduction of plasma ADAMTS13:AC in advanced cirrhotics. Recently, we showed that ADAMTS13:AC decreased with increasing severity of cirrhosis [36] (Fig. 1b). The values determined by act-ELISA correlated well with those of the classical VWFM assay, and also closely correlated with ADAMTS13 antigen determined by the antigen-ELISA. These results confirmed that both ADAMTS13 activity and antigen decreased with increasing cirrhosis severity [36] (Fig. 1b, c). Our results are consistent with findings described by Feys et al. [38]. In sharp contrast, Lisman et al. [39] showed that both ADAMTS13 activity and antigen levels were highly variable; however, they did not distinguish between patients with varying degrees of cirrhosis. It is unclear why Lisman et al. reached the conclusions different from ours. One possible explanation relates to two distinct clinical settings: a majority of our

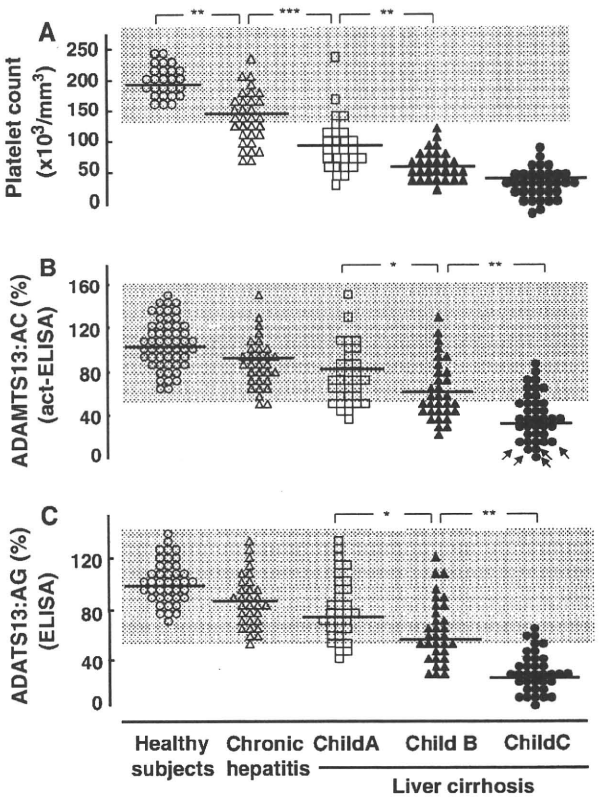

Fig. 1 Platelet counts and plasma levels of ADAMTS13:AC and ADAMTS13:AG in patients with chronic liver diseases. Platelet count decreased with the severity of chronic liver diseases, but no difference was found between Child B and C (**a**). Plasma ADAMTS13:AC determined by the ELISA progressively decreased with worsening cirrhosis (**b**). Severe deficiency in ADAMTS13:AC (<3%) was seen in five liver cirrhosis patients with Child C by the VWFM assay, but by the act-ELISA they ranged from <0.5 to 15.9% of the normal control (**b**, shown by *arrows*). The ADAMTS13:AG levels determined by ELISA also decreased with increasing cirrhosis severity (**c**), which highly correlated with ADAMTS13:AC measured by the act-ELISA ($r = 0.715$, $p < 0.001$). *Open circles* normal controls, *open triangles* chronic hepatitis, *open squares* cirrhosis with Child A, *closed triangles* cirrhosis with Child B, *closed circles* cirrhosis with Child C. *Shaded area* shows normal range. *ADAMTS13:AC* ADAMTS13 activity, *ADAMTS13:AG* ADAMTS13 antigen. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ significantly different between the two groups (partially modified from [36])

patients developed cirrhosis secondary to HCV infection, whereas in the study of Lisman et al. a half of the patients suffered from alcohol abuse-related cirrhosis. Further, the techniques used to determine ADAMTS13:AC differed between our study and theirs. It is assumed that the collagen-binding assay they used can be highly influenced by the increased amount of VWF:Ag in tested cirrhotic plasmas [38], because the substrate in this assay is intact multimeric VWF. In this regard, our act-ELISA is performed using VWF73-based fusion protein, termed GST-VWF73-His, which is readily cleaved by ADAMTS13

without any protein denaturant, and therefore the increased amount of VWF:Ag in tested plasmas does not interfere the assays [36].

Obviously, plasma levels of VWF:Ag substantially increase as liver diseases progress (Fig. 2a) [36], as previously indicated [22, 23]. This is presumably attributed to sinusoidal and/or extrahepatic endothelial damage induced by endotoxin and cytokines [22, 23, 40, 41]. The VWF:RCo was higher (Fig. 2b) [36], but the ratio of VWF:RCo/VWF:Ag was lower in cirrhotic patients than in healthy subjects, suggesting that increased VWF:Ag appears less functional in cirrhosis patients [39]. Nevertheless, our study has clearly shown that the ratio of VWF:RCo/ADAMTS13:AC progressively increases with the worsening of chronic liver diseases (Fig. 2c), more strengthening an enhanced thrombogenesis with the progresses of liver dysfunction and thrombocytopenia [36]. As a part of reflection in our scenario, the decreased platelet counts paralleled to the plasma levels of ADAMTS13:AC [36].

Regarding VWF multimers, the higher molecular weight multimer showed greater degradation than in healthy controls, thus maintaining normal enzyme-to-substrate (ADAMTS13/UL-VWFMs) ratio to maintain blood fluidity [39]. Our recent study showed that there were three different VWFM patterns in cirrhotic patients with lower ADAMTS13:AC (<50% of controls): normal-VWFM was detected in 53%, degraded-VWFM in 31%, and UL-VWFM in 16% (Fig. 3) [36]. UL-VWFM-positive patients showed the lowest ADAMTS13:AC, and the highest values of serum creatinine, blood urea nitrogen, and blood ammonia. In addition, cirrhotic patients with UL- and normal-VWFM had higher levels of VWF:RCo and Child-Pugh score, and lower values of cholinesterase and hemoglobin than those with degraded-VWFM [36]. The pattern, therefore, appears to shift from degraded- to normal-VWFM, and finally to UL-VWFM as functional liver capacity and renal function deteriorate, indicating that advanced cirrhosis may be a predisposing state toward platelet microthrombi formation, even in the absence of clinically overt thrombotic events [36]. In fact, portal or hepatic vein thrombosis is often observed in advanced liver cirrhosis patients routinely screened with Doppler ultrasound [27] and in cirrhotic liver tissue removed at transplantation [28] and at autopsy [29], consistent with our hypothesis.

The mechanism responsible for the decrease in ADAMTS13:AC in advanced cirrhotics may include enhanced consumption due to the degradation of large quantities of VWF:AG [37], inflammatory cytokines [42, 43], and/or ADAMTS13 plasma inhibitor [7, 8]. It is controversial whether ADAMTS13 deficiency is caused by decreased production in the liver; Kume et al. [44] reported that HSC apoptosis plays an essential role in decreased

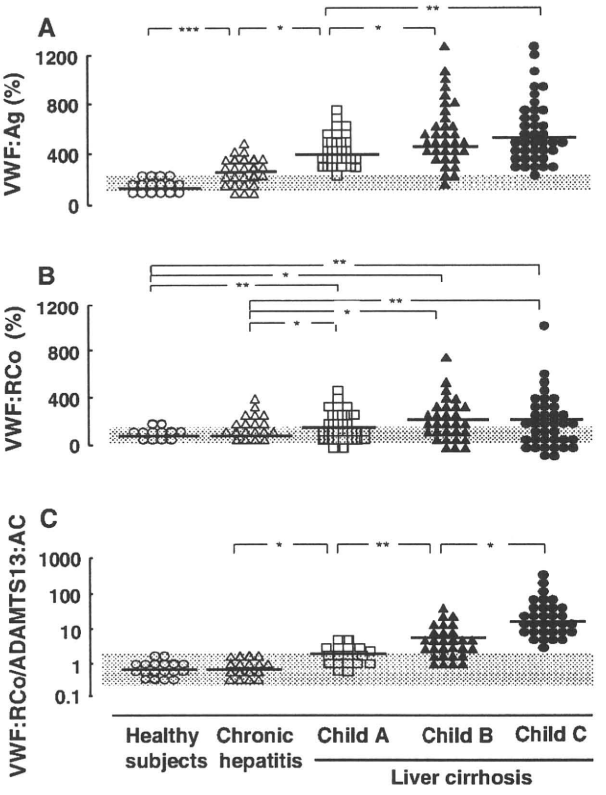

Fig. 2 Plasma levels of VWF:Ag, VWF:RCo, and VWF:RCo/ADAMTS13:AC ratio in patients with chronic liver disease. The VWF:Ag increased with the progression of chronic liver diseases, but the difference between Child B and C did not reach statistical significance (**a**). The VWF:RCo is higher in liver cirrhosis patients than in patients with chronic hepatitis and healthy subjects, but it did not differ among subgroups within liver cirrhosis (**b**). The VWF:RCo relative to ADAMTS13:AC progressively increased with worsening chronic liver disease (**c**). *VWF:Ag* von Willebrand factor antigen, *VWF:RCo* von Willebrand factor ristocetin cofactor activity, *ADAMTS13:AC* ADAMTS13 activity. *Shaded area* shows normal range. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ significantly different between the two groups (partially modified from [36])

ADAMTS13:AC using dimethylnitrosamine-treated rats, but not carbon tetrachloride (CCl_4)-treated animals, whereas Niiya et al. [45] found up-regulation of ADAMTS13 antigen and proteolytic activity in liver tissue using rats with CCl_4-induced liver fibrosis. We observed the inhibitor of ADAMTS13 in 83% of patients with severe to moderate ADAMTS13 deficiency, but its inhibitory activity was in a marginal zone between 0.5 and 1.0 BU/ml in most cases except a TTP patient (2.0 BU/ml) and a patient with severe ADAMTS13 deficiency (3.0 BU/ml) [36]. Interestingly, IgG-type autoantibodies specific to purified plasma derived-ADAMTS13 were detected by western blotting only in five end-stage cirrhotics with severe ADAMTS13 deficiency (<3%) corresponding to TTP [36]. One patient showed an apparent TTP [46], while the other

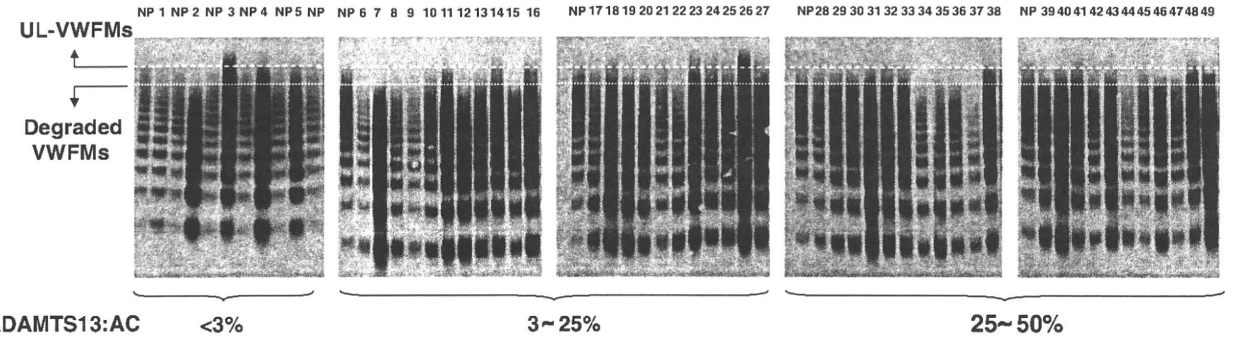

Fig. 3 Plasma VWF multimer in 49 liver cirrhosis patients with severe to mild deficiency of ADAMTS13:AC. The VWF multimer was analyzed by a vertical SDS–1.0% agarose gel electrophoresis system. Five patients (patients no. 1–5) were originally identified as a severe deficiency of plasma ADAMTS13:AC by the von Willebrand factor multimer (VWFM) assay. Twenty-two patients (patients no. 6–27) showed a moderate deficiency (3–25% of the control), and remaining 22 patients (no. 28–49) mild deficiency (25–50% of the control) of plasma ADAMTS13:AC by both methods of VWFM assay and the act-ELISA, without discordant results. There were three different patterns including degraded-, normal-, and UL-VWFM. Out of these 49 patients, 26 (53.1%) showed normal VWFMs, 15 (30.6%) degraded-VWFMs, and the remaining eight (patients no. 3, 4, 11, 14, 16, 18, 23, and 26) (16.3%) UL-VWFMs. *ADAMTS13:AC* ADAMTS13 activity, *VWFM* von Willebrand factor multimer, *UL-VWFM* unusually large von Willebrand factor multimer, *NP* normal control plasma (partially modified from [36])

four cirrhotics did not show apparent clinical features of TTP, but had complications of hepatorenal syndrome (HRS), spontaneous bacterial peritonitis (SBP), marked inflammation together with cytokinemia, and advanced hepatocellular carcinoma (HCC) [36]. Various clinical conditions, including infection, malignancies, and certain drugs, can lead to acquired TTP [47]. In advanced cirrhotics, endotoxemia is frequently detected [23], and SBP sometimes occurs [48]. HCC is highly complicated as the cirrhotic stage progresses [49], suggesting a high-risk state of platelet microthrombi formation. Some end-stage cirrhotics who have extremely low ADAMTS13:AC as well as its IgG inhibitor might be under conditions similar to TTP, or might reflect "subclinical TTP" [36].

With respect to the autoantibodies in patients with HCV-associated liver diseases, there is a general consensus that the overall prevalence of serum non-organ-specific autoantibodies is significantly higher in patients with HCV (about one-third of all cases) than in both healthy subjects and patients with HBV [50–52], but not alcoholic liver injury. That might be additional reason why ADAMTS13:AC significantly decreased in our most patients with HCV-related cirrhosis, but its activity seemed to be highly variable in most patients with alcohol abuse-related cirrhosis as shown by Lisman et al. [39]. Indeed, of our five end-stage LC patients with IgG-type autoantibodies, two were related to HCV, and each one to HBV, PBC and cryptogenic, but none of patients with alcohol abuse-related cirrhosis were found. Further studies will be necessary to clarify whether inhibitors other than the IgG inhibitor might be involved in cirrhotics with lower ADAMTS13:AC.

4.2 Alcoholic hepatitis (AH)

In alcoholic liver diseases, sinusoidal microcirculatory disturbance is thought to play an important pathogenic role [53, 54]. This includes narrowing of the sinusoidal space due to ballooned hepatocytes and perisinusoidal fibrosis, imbalances between endothelin and nitric oxide, and contraction of HSC [53, 54]. AH is a potentially life-threatening complication of alcohol abuse. The severe form of AH, severe alcoholic hepatitis (SAH), is characterized by multiorgan failure with manifestations of acute hepatic failure [55, 56]. In the pathogenesis of SAH, endotoxemia due to hepatic reticuloendothelial dysfunction and increased intestinal permeability may trigger enhanced proinflammatory cytokine production, which potentially causes systemic inflammatory response syndrome together with microcirculatory disturbances, and subsequent multiorgan failure [55, 56].

In our study, plasma ADAMTS13:AC was markedly decreased in the non-survivors of SAH with multiorgan failure; in contrast, mild to moderate decrease was observed in survivors of SAH and those with AH [57]. The VWF:AG was remarkably high in the non-survivors of SAH [58]. At the recovery stage, ADAMTS13:AC returned to the normal range, and the VWF:AG decreased in the survivors, whereas in a non-survivor with SAH, ADAMTS13:AC remained extremely low, and the VWF:AG was still high [57, 58]. UL-VWFM was detected in four of five SAH patients and in five of nine AH patients [58]. The findings of enhanced UL-VWFM production and deficient ADAMTS13:AC may, in part, contribute not only to the development of multiorgan failure but also to the

progression of liver injury through microcirculatory disturbances [57, 58].

Potential mechanism for decreased ADAMTS13:AC may include cytokinemia [42, 43, 59], endotoxemia [59, 60], the inhibitor of ADAMTS13 [7, 8, 59], and the consumption of the protease [37]. Recent investigations demonstrated that IL-6 inhibited the action of ADAMTS13 under flow conditions, and both IL-8 and TNF-α stimulated the release of UL-VWFM in human umbilical vein endothelial cells in vitro [42]. It remains to be clarified whether the IL-6 directly would hamper the cleavage of UL-VWFM or IL-6 would down-regulate gene expression of ADAMTS13 with modifying the promoter activity. IFN-γ, IL-4, and TNF-α also inhibit ADAMTS13 synthesis and activity in rat primary HSC [43]. In addition, inflammation-associated ADAMTS13 deficiency promotes formation of UL-VWFM [61], and intravenous infusion of endotoxin to healthy volunteers caused a decrease in plasma ADAMTS13:AC together with the appearance of UL-VWFM [60]. From these results as well as our own, marked endotoxemia may be closely related to decreased ADAMTS13:AC and the appearance of UL-VWFM through enhanced cytokinemia in AH patients [59]. It will be necessary to clarify what types of inhibitor may be involved in the association with inflammatory cytokines and endotoxin.

4.3 Hepatic veno-occlusive disease (VOD)

Hepatic VOD is a life-threatening complication of patients undergoing allogenic stem cell transplantation (SCT), and occurs at frequencies of 1–54% [62, 63]. Clinically, hepatic VOD is characterized by hyperbilirubinemia, painful hepatomegaly, and fluid retention [63]. Histologically, VOD features sinusoidal fibrosis, necrosis of pericentral hepatocytes, and consequent narrowing of central veins [62, 63]. In these patients, the SEC is the primary site of toxic injury caused by chemotherapy and/or radiation in the setting of SCT, and this initial insult may ultimately lead to the circulatory compromise of centrilobular hepatocytes [62, 63].

Our recent study demonstrated that plasma ADAMTS13:AC is reduced in hepatic VOD patients after SCT (12–32% of normal) compared to non-VOD patients (57–78% of normal), even before any conditioning regimen and throughout SCT, and that the activity might thus be a predictor for the development of hepatic VOD [64]. A multicenter, prospective, randomized controlled study revealed that prophylactic fresh frozen plasma (FFP) infusion as a source of ADAMTS13 may be instrumental in preventing the development of hepatic VOD after SCT [65]. In two typical cases with hepatic VOD, plasma levels of VWF:AG progressively increased and ADAMTS13:AC gradually decreased from preconditioning or the early period after the SCT to the later period at the occurrence of hepatic VOD [65].

Interestingly, in VOD patients, VWFM corresponding to high and intermediate molecular weight, which is usually seen in normal plasma, were lacking at preconditioning or the early period after SCT, and thereafter gradually appeared [65]. Furthermore, in the group without prophylactic FFP infusion, high and/or intermediate molecular weight VWFM was also lacking in the early stage and even in the later stage after SCT. In contrast, in the group with FFP infusion, no apparent changes in VWFM patterns were found throughout SCT [65]. It remains unclear why such a phenomenon occurred, but one possible explanation may be the SEC injury caused by intensive chemotherapy and/or total body irradiation in the setting of SCT. Indeed, chemotherapy before SCT is a regimen with a high incidence of hepatic VOD, and total body irradiation causes radiation-induced liver disease [62, 63]. The amount of VWF released from injured SEC may be increased at first, but may thereafter decrease because the endothelial cells are extensively damaged [65]. After SCT, as damaged endothelial cells gradually regenerate, the release of VWF may increase, resulting in the appearance of high and intermediate VWFM. Under these circumstances, plasma ADAMTS13 may be consumed to degrade the large amounts of VWF. The imbalance caused by decreased ADAMTS13:AC versus increased production of VWF:AG before and during the early stage after SCT would contribute to a microcirculatory disturbance that could ultimately lead to VOD, especially in zone 3 of the hepatic lobule where hepatocytes are susceptible to damage induced by hypoxia [65]. The supplementation of ADAMTS13 by prophylactic FFP infusion may suppress the increase in VWF:AG that is extensively released from damaged SEC.

4.4 Liver transplantation

One of the serious complications in solid organ transplantation is the occurrence of sporadic thrombotic microangiopathies (TMAs) at an estimated frequency of 0.5–3.0% [66–68]. For instance, various degrees of thrombocytopenia are commonly observed after liver transplantation, especially during the first postoperative week, and some clinical studies have demonstrated that thrombocytopenia was significantly associated with poor prognosis [69]. The imbalance between endothelin and nitric oxide produced by the SEC may lead to active vasoconstriction, narrowing of the sinusoidal lumen, and subsequent sinusoidal microcirculatory disturbance [70]. During the past decade, the measurement of plasma ADAMTS13:AC was utilized as a differential diagnostic tool for TMAs [68], but its relevance to organ transplantation itself was not well evaluated.

In this regard, we first reported in 2006 that a significant reduction of ADAMTS13:AC with a concomitant appearance of UL-VWFM was consistently observed in patient plasma soon after liver transplantation [71]. Consecutive analysis of ADAMTS13:AC indicated that these changes reflected liver graft dysfunction, including ischemia–reperfusion injury and acute rejection. The ADAMTS13:AC in these patients often decreased to less than 10% of normal controls, concurrent with severe thrombocytopenia. These clinical and laboratory features appeared to be similar to TMAs, and more specifically to TTP, which is typically defined by severe deficiency of plasma ADAMTS13 with or without neutralizing autoantibodies to this enzyme. However, different from TTP, the liver transplant recipients in our study had no additional clinical signs of TTP, such as neurological manifestation, fever, or renal dysfunction. Thus, the organ dysfunction appeared to be restricted to the liver graft. From these observations, we suggested that a decrease of plasma ADAMTS13:AC coupled with the appearance of UL-VWFM in liver transplant recipients was caused by the mechanism of "local TTP" within the liver graft [71]. It is assumed that the primary target is vascular endothelial cells within the liver graft in both ischemia–reperfusion injury and acute rejection after liver transplantation [72–74]. Indeed, depositions of activated platelets on the sinusoidal endothelium with a concomitant increase of VWF expression have been found in the liver immediately after reperfusion or cold preservation [73, 74]. In addition, the up-regulated VWF expression has been observed in liver allografts during acute rejection [74]. Thus, newly released UL-VWFM from vascular endothelial cells [71], together with consumption of ADAMTS13, induces platelet aggregation or thrombi formation at the hepatic sinusoid, and results in microcirculatory disturbance. This hypothesis might address why organ dysfunction restricts in the graft liver in liver transplantation-associated 'subclinical' TMA, distinct from systemic organ involvements found in "classical TTP".

Recently, two groups of investigators from Japan [75] and the Netherlands [76] reported interesting results as compared with ours. The report by Kobayashi et al. [75] appeared to be in good agreement with ours, because by examining a large number of liver transplant patients ($n = 81$) they provided solid data showing decreased platelet counts and plasma ADAMTS13:AC levels in the early stage of transplantation. Further, they were able to show increased plasma levels of VWF with the appearance of UL-VWFMs, as a reflection of the reduced plasma ADAMTS13:AC. On the other hand, Pereboom et al. [76] reported that a reduction of ADAMTS13:AC occurred within 1 day after liver transplantation, and was followed by an increased plasma level of fully functional VWF; however, they did not address platelet count in their patients ($n = 20$). One of their patients with severe deficiency of ADAMTS13 indeed had thrombotic complications after transplantation, but the patient did not have UL-VWFMs in the plasma. As a partial explanation for this reason, the authors suggested that plasmin activity was increased in these patients by demonstrating increased plasma levels of tissue plasminogen activator. But, if this hypothesis is true, these patients should have severe bleeding symptoms rather than thrombotic complications, or the investigators might be able to demonstrate the presence of VWF fragments specifically generated by plasmin cleavage in patient plasmas [77]. If not, it will be necessary that the presence of UL-VWFMs is carefully re-examined.

Through our experience, we would like to emphasize here that it is extremely important to monitor plasma ADAMTS13:AC in the treatment of thrombocytopenia associated with allograft dysfunction after liver transplantation. This is because the infusions of platelet concentrate under an imbalance of decreased ADAMTS13:AC to enhanced UL-VWFM production might further exacerbate the formation of platelet aggregates mediated by uncleaved UL-VWFM, leading to graft failure via the "local TTP" mechanism [71]. To date, FFP is a unique source of ADAMTS13 replacement therapy, and may improve both liver dysfunction and thrombocytopenia in liver transplant patients. From this point of view, we are particularly interested in the start of clinical trials on recombinant ADAMTS13 preparations.

5 Conclusion and future perspectives

The introduction of ADAMTS13 to the field of hepatology not only enabled us to confirm the diagnosis of TTP early, but also provided novel insight into the pathophysiology of liver diseases. Some diseases were shown to be TTP itself, but others did not show any apparent clinical features of TTP, even in the presence of extremely decreased ADAMTS13:AC and increased UL-VWFM corresponding to TTP. Such TTP-like states, but without disseminated intravascular coagulation, might be "subclinical TTP" as seen in advanced liver cirrhotics [36] and SAH patients [57, 58], or "local TTP" as shown in patients with hepatic VOD after SCT [64, 65] and patients with adverse events after living donor liver transplantation [71]. One would essentially be unable to detect such TTP-like phenomena without the determination of ADAMTS13:AC, because the interaction of ADAMTS13 and UL-VWFM is the initial step in hemostasis, and their abnormalities do occur in the absence of apparent imbalance in other hemostatic factors and/or irrespective of the presence or absence of abnormal

conventional hemostatic factors. 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 (×10⁶/L)	6	(140-360)
White blood cell (×10³/L)	7200	(3500-9500)
Red blood cell (×10⁶/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

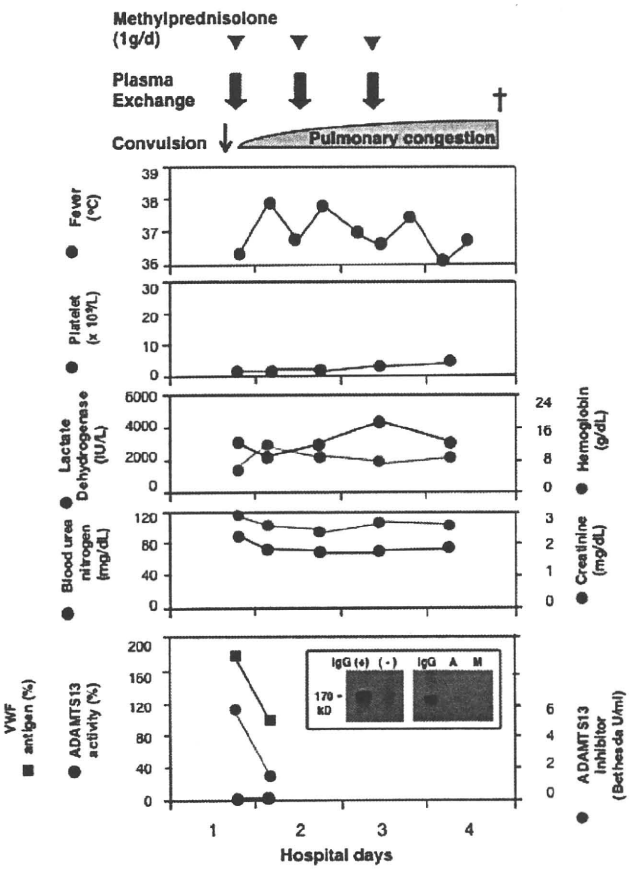

Figure 1. Clinical course of influenza A-associated thrombotic thrombocytopenic purpura (TTP). A 68-year-old Japanese woman infected with influenza A developed TTP 2 days after having a fever. Plasma ADAMTS13 activity was less than 0.5% of normal control, and a high titer of anti-ADAMTS13 inhibitor (6.0 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

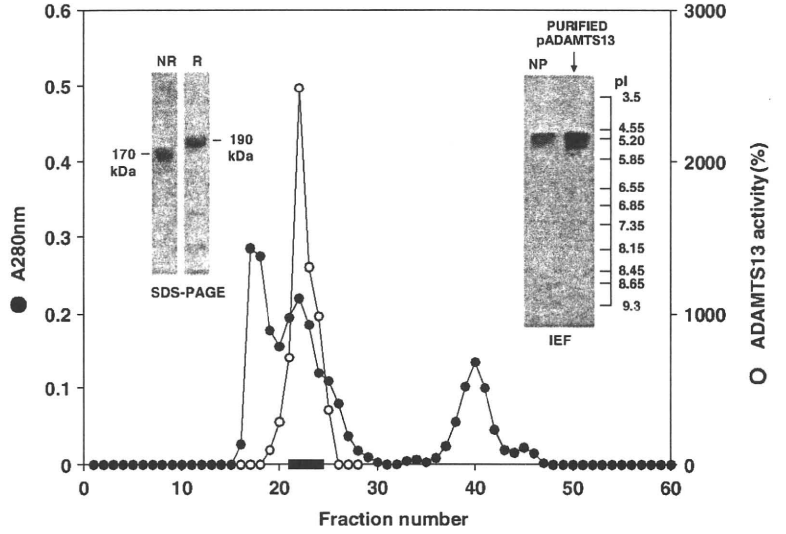

Fig. 1 Gel chromatogram of Superdex 200 HR10/30 as a final step for plasma (p) ADAMTS13 purification. Partially purified pADAMTS13, prepared by immunoadsorbent chromatography and concentrated by HiTrap DEAE gel, was further separated by Superdex 200 HR10/30 column at a flow rate of 0.5 ml/min at RT (see '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).

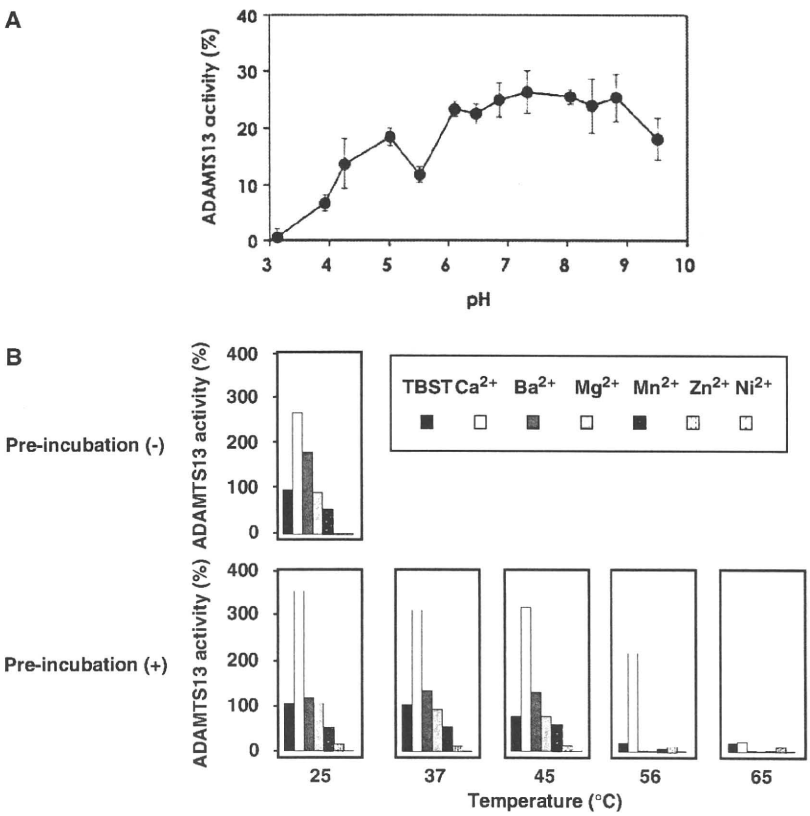

Fig. 2 pH stability, and effects of temperature and divalent metal ions on purified pADAMTS13 activity. (A) The purified pADAMTS13 was dialyzed against 20 mM TBS containing 0.05% Tween-20 (TBST, pH 7.4), and the pH of each aliquot was adjusted from 3.1 to 9.5 with 0.1 N HCl or NaOH, followed by incubation 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

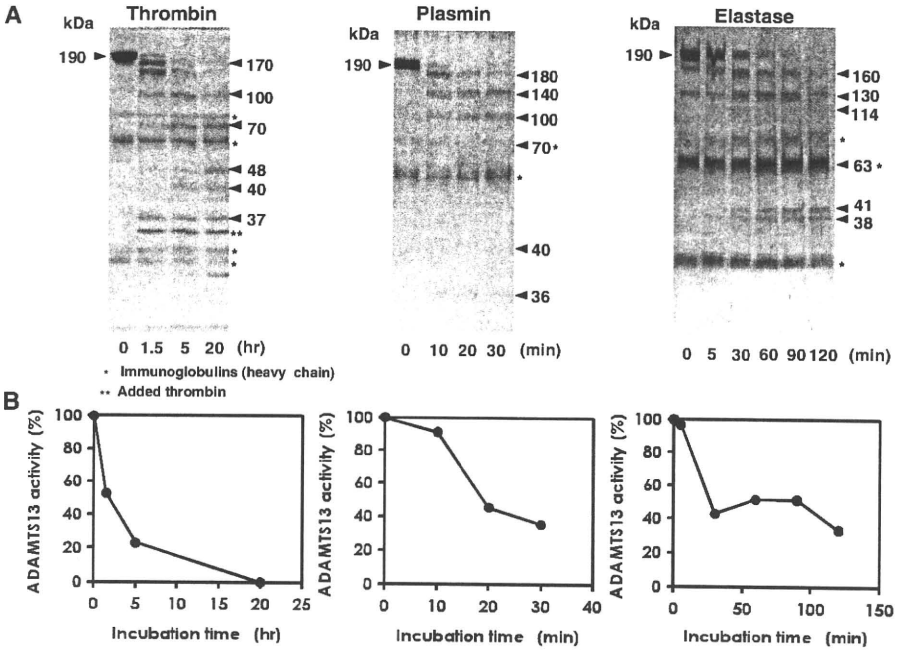

Fig. 3 Proteolytic fragmentation of the purified pADAMTS13 by thrombin, plasmin or leucocyte elastase, and residual activity. (A) The purified pADAMST13 was dissolved in TBS (pH 7.4), and incubated at 37°C for various time intervals with human thrombin (left), plasmin (middle) or leucocyte elastase (right), 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

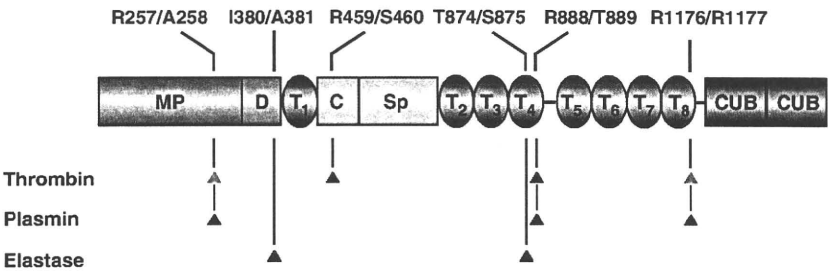

Fig. 4 Sites of pADAMTS13 cleavage by thrombin, plasmin and leucocyte elastase. A Schematic diagram of the identified pADAMTS13 cleavage sites for thrombin, plasmin and leucocyte elastase is shown. Note that thee of the four thrombin cleavage sites are identical to those of plasmin, but are different 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.