Table 2 Possible hemostatic abnormalities due to CSVT

Case	Natural protease inhibitor deficiency or APS	Gene analysis
1	Congenital AT deficiency	Pro429Leu
2	Congenital PS deficiency	Asp79Tyr + Thr630Ile
8	APS	
9	Congenital PC deficiency	Deletion of 458-460 (AGG)
11	Congenital PS deficiency	Lys196Glu + Gly189Ala

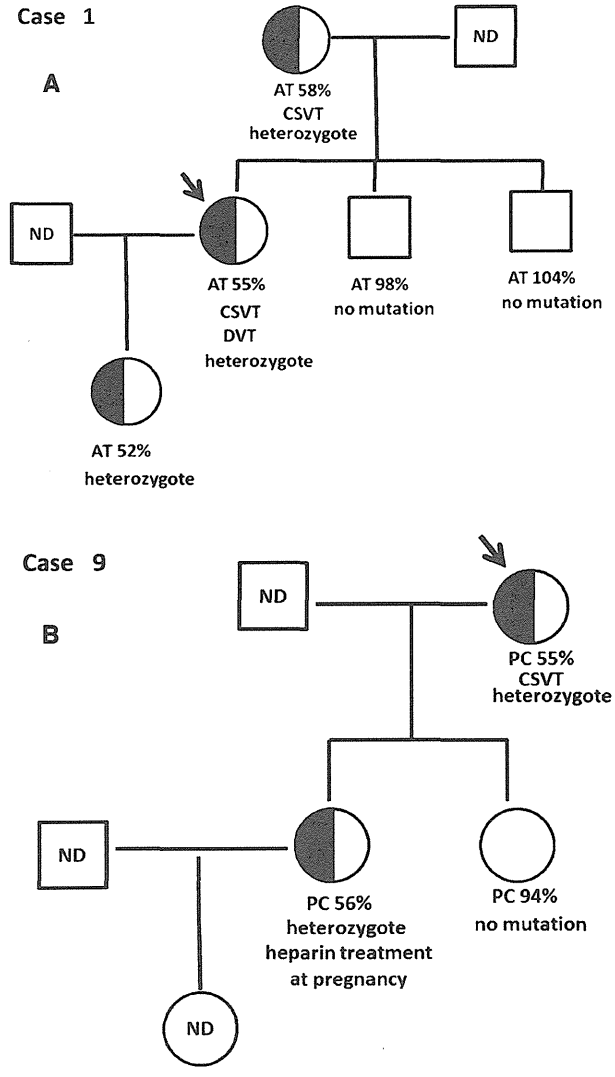

Fig. 1 The pedigree of the family under investigation. **a** Case 1, **b** case 9

Case 11 was a compound heterozygous mutation of the PS gene, including a novel mutation (Gly189Ala) and the PS Tokushima mutation (Lys196Glu). A PCR cloning strategy revealed that the Gly189Ala and the Lys196Glu mutations were in different alleles. This novel mutation was not detected in 100 healthy volunteers. Figure 2a shows the

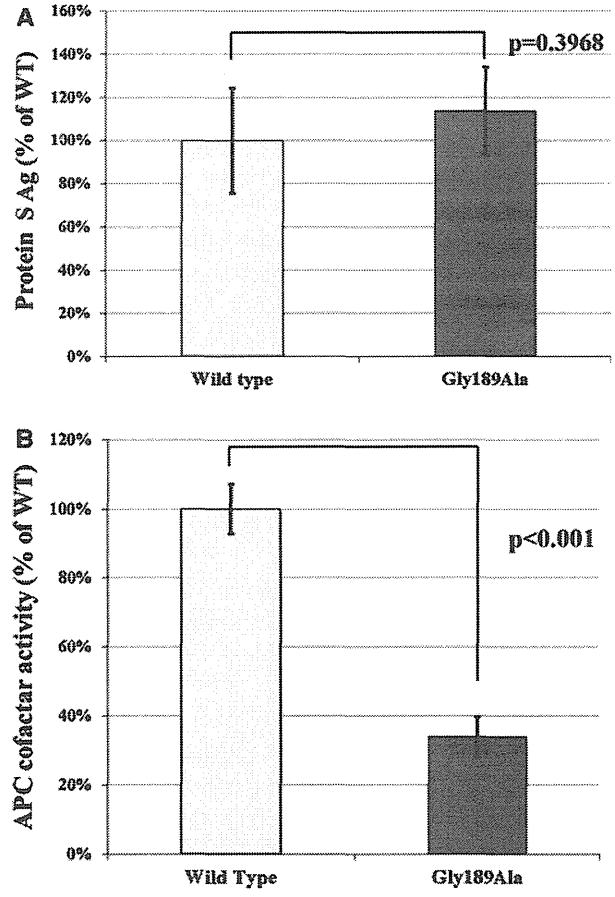

Fig. 2 The transient and stable expression of wild type and mutant recombinant PS in COS7 or BHK cells. **a** Transient expression in COS7 cells The determination of the concentration of PS in the conditioned media from genes containing different expressions using the ELISA method. The mean value of the wild type PS was defined as 100%. The *bars* represent the mean ± SD of six transfection experiments per each gene. The mutant and wild type expression levels were compared using unpaired *t* test. There was no significant difference. Wild type: 100.00 ± 24.26%, Gly189Ala: 113.70 ± 20.25%. **b** Stable expression in BHK cells. The APC cofactor activity was calculated using the clotting time and comparing it to the same concentration (100 ng/ml) of the different expressions levels. The mean value of wild type PS was defined to be 100%. The *bars* represent the mean ± SD of four experiments per gene. The mutant and wild type APC cofactor activity (100 ng/ml) was compared using unpaired *t* test. Wild: type; 100.00 ± 7.20%, Gly189Ala: 33.92 ± 5.81%

transient expression of wild type and mutant recombinant PS (Gly189Ala) in COS7 cells. The concentration of PS in the conditioned media from the different mutations was measured. No significant differences were observed between the mutant and wild type levels of antigen. Figure 2b shows the stable expression of wild type and mutant recombinant PS in baby hamster kidney (BHK) cells. The APC cofactor activity was significantly lower for the mutant PS (33.9 ± 5.8%) than for the wild type (100%, $p < 0.001$).

With regard to the onset of CVST, AT, PC and PS were all decreased in case 3, who had multiple organ failure, PS was decreased in cases 4, 5 and 6 and AT was decreased in case 12. After treatment for the CVST, these concentrations were improved, suggesting a transient AT, PC or PS deficiency. In addition, Case 7 had an abnormality in both PS and PC, but this patient was treated with warfarin. The ratio of PC/PS antigen was less than 2.0 and neither PC nor PS activity was measured. Therefore, case 7 was excluded from the gene analysis.

Discussion

CSVT is an uncommon condition with many clinical manifestations, so many cases remain clinically undetected. The incidence of severe thrombophilia due to AT, PC or PS deficiency and APS in CVST was reported to be 9% [4]. The odds ratio of the risk for CVST was reported to be 7.06 in patients with AT deficiency, 8.76 for PC deficiency, 3.20 for PS deficiency and 6.95 in patients with APS [20]. Although this study was of a small number of patients, the incidence of congenital thrombophilia was present in about 33.3% (4/12) of cases, thus suggesting that thrombophilia might be associated with CVST more frequently than was suggested in a previous report [4]. Because, a genetic analysis was not done in all cases with CVST of large-scale study [14].

In case 1, a patient with congenital AT deficiency had a heterozygous AT Pro429Leu (AT Budapest) mutation. This mutation is reported to lead to a decrease of heparin binding capability and protease inhibitor capability of the protein [22]. Case 2 had both PS Asp79Tyr and PS Thr630Ile mutations [21]. Asp79Tyr was previously reported as Asp38Tyr according to the previously established nomenclature system [21]. Asp79Tyr is a Type I PS deficiency (quantitative deficiency) that is characterized by a decrease in both the PS antigen levels and the PS activity [21]. We thought that the PS Asp79Tyr mutation was likely the cause of the decrease in PS, because it was previously reported that the PS Thr630Ile mutation does not influence the expression of PS [21]. Case 9 had a heterozygous PC Glu153del (458-460delAGG) mutation [23]. She experienced no complication during her two deliveries, but her daughter developed a hypercoagulable state during her pregnancy and was treated with low-dose heparin. In case 11, the patient had a PS Tokushima type II mutation characterized by a normal total and free PS antigen level, but a decrease in APC cofactor activity. This mutation is present in about 2% of the Japanese population [24–28]. The PS Gly189Ala is a novel mutation that we identified which was not a polymorphism in an analysis of 100 healthy volunteers [29]. The expression of the protein from this mutant is similar to the wild type protein, but the activity level is lower in comparison to wild type, thus suggesting this mutant to be a type II PS abnormality.

No mutations in AT, PC or PS were observed in the other 5 patients who did not receive warfarin treatment (Cases 3–6 and 12), although they had low AT, PC or PS at the onset of CVST. A decreased AT level was reported in patients with disseminated intravascular coagulation (DIC) [30] and liver diseases [31], decreased PC and PS levels were reported in patients treated with warfarin [32], and decreased PS was reported in pregnant females [33]. In case 3 who was in MOF status, the decreases in AT, PC and PS might have been caused by liver failure. The down regulation of the PS gene expression by 17β-estradiol, which increases in concentration in the late stages of pregnancy, has also been reported [34]. However, the decreased AT, PC or PS might be an outcome from thrombosis, rather than the cause of thrombosis in these patients. The mechanism(s) responsible for a decreased AT, PC or PS activity should be examined in future studies of various types of thrombosis, including CVST.

> "APS is one of the important causes of thrombosis [35], and APS is often observed in cases of cerebral infarction [36]. Most cerebral infarctions are considered to be due to arterial thrombosis, and these patients are usually treated with antiplatelet drugs, such as aspirin. CVST is usually recommended to be treated with warfarin. The differential diagnosis of CVST in patients with APS from other types of thrombosis due to arterial thrombosis is therefore important."

The frequency of thrombophilia is higher in CVST than in DVT, because CVST does not occur as frequently after surgery as DVT.

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Conflict of interest Authors have no direct or indirect conflict of interest in this manuscript

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Regular Article

Plasma ADAMTS13, von Willebrand Factor (VWF) and VWF Propeptide Profiles in Patients with DIC and Related Diseases

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ABSTRACT

ADAMTS13, endothelial von Willebrand factor (VWF) and related proteins are involved in the pathogenesis of some life threatening systemic thrombotic coagulopathies. Changes of plasma ADAMTS13 activity in thrombotic thrombocytopenic purpura (TTP) is well known but is also involved in septic disseminated intravascular coagulation (DIC). Here we investigated the ADAMTS13 activity, VWF and VWF propeptide (VWFpp) antigens in 69 patients with DIC, 143 with non-DIC, 21 with thrombotic thrombocytopenic purpura (TTP) and 23 with atypical hemolytic uremic syndrome (aHUS) for diagnosis of DIC.

The plasma ADAMTS13 activity was significantly low in patients with DIC, and the plasma levels of VWF and VWFpp antigens, were the highest in these patients, but there were no significant differences in the plasma VWFpp levels between the patients with DIC and those with aHUS. The difference in the plasma ADAMTS13 activity, the VWF and VWFpp antigens between DIC and non-DIC cases was significant in those with infectious and malignant diseases, but the difference in the VWFpp/ VWF ratio were significant only in subjects with infectious diseases. As an indicator for prognosis, the plasma levels of VWFpp were significantly higher in non-survivors than in survivors. Then, VWFpp/ VWF ratio and VWFpp/ADAMATS13 ratio will be potent informative indicators in DIC.

These findings suggest that ADAMTS13/VWF profiles may have important roles in the pathogenesis of DIC, and that ADAMTS13 and VWFpp are useful indicators for the diagnosis and prognosis of DIC.

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Introduction

Figs. 2 and 4

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type I motifs 13) is a metalloproteinase that specifically cleaves the multimeric von Willebrand factor (VWF) [1–5]. A severe deficiency in the ADAMTS13 activity is caused by either a mutation of the ADAMTS13 gene [2, 6] or by the presence of inhibitory antibodies against ADAMTS13 [7]. Unusually large VWF multimers (UL-VWFMs) produced and released from the injured vascular endothelial cells to the plasma of patients with familial and nonfamilial thrombotic thrombocytopenic purpura (TTP) [8, 9].

The pre-pro VWF, which is synthesized in endothelial cells and megakaryocytes, undergoes intracellular modifications including signal peptide cleavage, C-terminal dimerization, glycosylation, sulfation, and N-terminal multimerization [10]. Then proteolysis occurs in the trans-Golgi where the VWF propeptide (VWFpp) is cleaved but remains stored together with mature VWF in alpha-granules (megakaryocytes) and Weibel-Palade bodies (endothelial cells). After the secretion of VWFpp and VWF into plasma from endothelial cells in response to several physiological or pathological stimuli, VWFpp dissociates from VWF [11, 12]. An elevated plasma level of VWFpp has been reported in patients with thrombotic microangiopathy (TMA) [13]. Disseminated intravascular coagulation (DIC) is a life-threatening disease that is often associated with severe organ failure and a bleeding tendency [14]. DIC is diagnosed based on the clinically laboratory coagulation tests but are not sensitive for the early phase of DIC. Thus, a new marker is required for the diagnosis of DIC [14]. Decreased ADAMTS13 levels were previously reported in the patients with septic DIC [15, 16].

In this study, we measured the ADAMTS13 activity, and the VWFpp and VWF antigens in the plasma from 69 patients with DIC, 143 with non-DIC, 21 with TTP and 23 with atypical hemolytic uremic syndrome (aHUS) to evaluate usefulness in diagnosing DIC.

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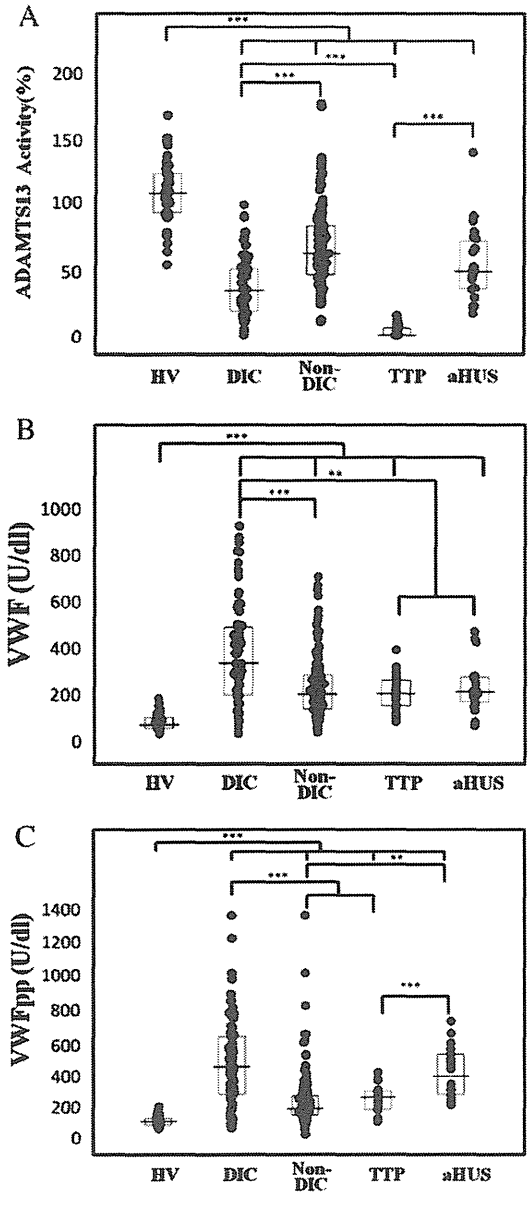

Fig. 1. The plasma levels of ADAMTS13 activities, the VWF and VWFpp antigens in healthy volunteers, DIC, non-DIC, TTP and aHUS patients. **A.** The plasma ADAMTS13 activities. ***; $p<0.001$, **; $p<0.01$, *; $p<0.05$. **B.** The plasma levels of the VWF antigen. ***; $p<0.001$, **; $p<0.01$. **C.** The plasma levels of the VWFpp antigen. ***; $p<0.001$, **; $p<0.01$. HV; healthy volunteers, DIC; disseminated intravascular coagulation, TTP; thrombotic thrombocytopenic purpura, aHUS; atypical hemolytic uremic syndrome.

Materials And Methods

The ADAMTS13 activity, VWF antigen and VWF propeptide (VWFpp) were measured in 50 healthy volunteers, 69 patients with DIC, 143 without DIC (non-DIC), 21 with TTP and 23 with aHUS. The DIC patients and non-DIC patients were continuously selected from the patients with a platelet count<100,000/μl from January 1, 2010 until December 31, 2010 at the Mie University Hospital. The patients were classified into three groups: patients with infectious diseases (25 with DIC and 30 with non-DIC), with malignant diseases (30 with DIC and 57 with non-DIC), and with other diseases (14 with DIC and 56 with non-DIC). In other diseases, there were 26 patients with an aneurysm (5 with DIC), 16 patients with trauma (4 with DIC), 13 patients with heart diseases (3 with DIC), 10 patients with

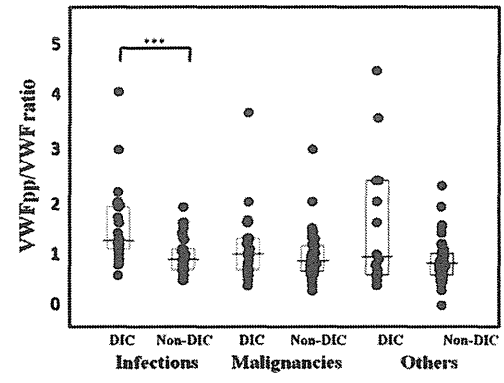

Fig. 2. The VWFpp/VWF ratio in healthy volunteers, DIC, non-DIC, TTP and aHUS patients. ***; $p<0.001$.

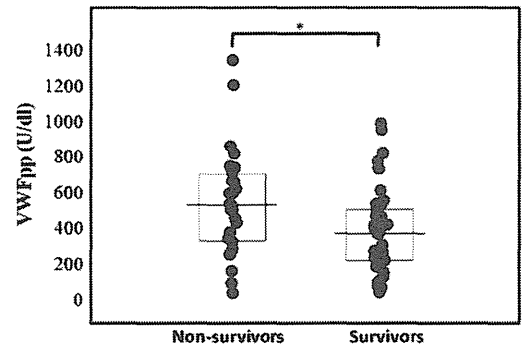

Fig. 3. The plasma levels of VWFpp in non-survivors and survivors. *; $p<0.05$.

digestive diseases (2 with DIC) and 5 patients with autoimmune diseases (no with DIC). DIC was diagnosed by the overt-DIC diagnostic criteria established by the International Society of Thrombosis Haemostasis (ISTH)[17]. TMA, which results in thrombocytopenia and hemolytic anemia due to the microangiopathy, was identified by based on the laboratory data and clinical symptoms such as neurological dysfunction, renal failure, or fever [18]. TTP was diagnosed when a patient had TMA and less than 10% of the normal ADAMTS 13 activity, and aHUS was diagnosed when a patient had TMA and reduced, but more than 10% of the normal level, of ADAMTS13 activity. No patients with TTP and 5 patients with HUS were diagnosed to have overt-DIC. The 69 patients with DIC were treated according to the

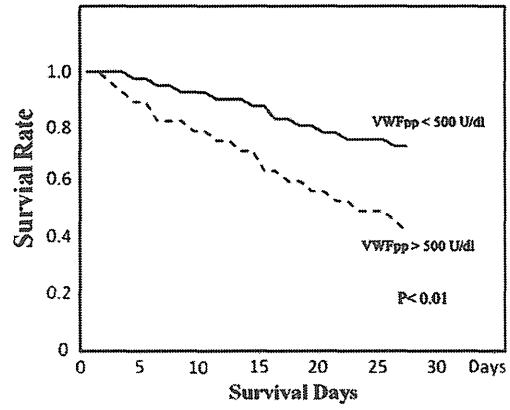

Fig. 4. Kaplan-Meier survival analysis of DIC patients with more than 500 U/dl of VWFpp and those with less than 500 U/dl of VWFpp.

Table 1
Characteristics of the Study Subjects.

	N	Age	Sex (F : M)	ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio	VWFpp/ADAMTS13 Ratio (U/%)
Healthy volunteers	50	31 (19.0 – 51.0)	31 : 19	110.0 (95.0 – 125.0)	69.5 (55.0 – 102.0)	85.0 (62.0– 102.0)	1.12 (0.93 – 1.27)	0.77 (0.60 –0.97)
DIC	69	65 (55.0 – 73.0)	24 : 45	35.0 (19.5 – 51.6)	338 (200 – 496)	421 (251 – 608)	1.10 (0.78 – 1.62)	19.9 (5.4 –28.5)
Non-DIC	143	63 (46.0 – 71.8)	59 : 84	63.7 (47.5 – 85.0)	203 (136 – 286)	162 (121 – 239)	0.85 (0.64 – 1.05)	2.06 (1.25 – 5.47)
TTP	21	65 (40.5 – 75.5)	11 : 10	UD (UD – 6.3)	206 (153 – 265)	233 (156 – 273)	1.07 (0.86 – 1.20)	
aHUS	23	40 (28.5 – 54.0)	17 : 6	50.0 (36.6 – 73.1)	214 (170 – 277)	361 (249 – 497)	1.13 (1.00 – 1.69)	4.9 (3.0-9,5)

The data are shown as the medians (25 – 75 percentile), UD; undetectable.

Table 2
The Differences in the Plasma Levels of ADAMTS13, VWF, VWFpp and the VWFpp/VWF Ratio Between DIC and non-DIC Cases.

		ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio
Infectious diseseas	DIC	30.0** (15.9 – 49.4)	382* (286 – 496)	545*** (363 – 730)	1.26*** (1.10 – 1.91)
	Non-DIC	50.7** (36.3 – 68.8)	294* (202 – 387)	241*** (169 – 325)	0.90*** (0.70 – 1.10)
Malignancies	DIC	31.9*** (22.5 – 51.3)	384*** (198 – 598)	398*** (193 – 512)	1.00 (0.70 – 1.30)
	Non-DIC	63.5*** (51.3 – 76.9)	170*** (122 – 279)	143*** (117 – 239)	0.87 (0.67 – 1.16)
Other diseases	DIC	48.8** (28.8 – 61.3)	180 (121 – 407)	269* (119 – 463)	0.95 (0.60 – 2.40)
	Non-DIC	80.7** (55.7 – 95.0)	196 (140 – 255)	144* (109 – 199)	0.83 (0.60 – 1.01)

***; $p<0.001$, **; $p<0.01$, *; $p<0.05$ for the significance between DIC and non-DIC cases.

Expert Consensus for the Treatment of DIC [19]. Twenty-seven patients died within 28 days after the diagnosis of DIC (non-survivor) but 42 patients survived after the 28 days (survivor). For the non-survivor group, the median (25-75th) survival was 16 days (7.5-21.5 days). The study protocol was approved by the Human Ethics Review Committee of Mie University School of Medicine and a signed consent form was obtained from each subject.

Human plasma was obtained from whole blood that was treated with a 1/10 volume of 3.8% sodium citrate as an anti-coagulant by centrifugation at 3,000 x g at 4 °C for 15 min. The plasma was stored at −80 °C until analysis.

The ADAMTS13 activity level was measured using a FRETS-VWF73 peptide, which was chemically synthesized by the Peptide Institute, Inc. (Osaka, Japan) according to the method reported by Kokame *et al.*[20]. The plasma levels of VWF and VWFpp were measured with a VWF & Propeptide assay kit (GTi DIAGNOSTiCs, Waukesha, USA) [13]. The hemoglobin (Hb) levels and platelet counts were measured by automated the fully automated hematology analyzer XE-2100 (Sysmex, Kobe, Japan).

Statistical analysis

The data are expressed as the medians (25-75th percentile). The differences between the groups were examined for statistical significance using the Mann–Whitney *U* test. A *P* value<0.05 denoted the presence of a statistically significant difference.

Results

The plasma ADAMTS13 activities were significantly decreased in any of patients with DIC, non-DIC, TTP and aHUS compared with those in healthy volunteers (Fig. 1-A and Table 1). Although the plasma ADAMTS13 activity in patients with DIC was significantly lower than in those with non-DIC, however, it is still significantly higher than that of TTP. The plasma levels of VWF and VWFpp antigens were significantly elevated in patients with DIC, non-DIC, TTP and aHUS compared with that of healthy volunteers (Fig. 1-B and 1-C, and Table 1). The plasma VWF antigen level in patients with DIC was higher than other groups. The plasma VWFpp antigen level in DIC was significantly higher than in those with non-DIC and TTP. Although there were no significant differences between patients with DIC and those with aHUS, the VWFpp/ADAMTS13 ratio was significantly higher in DIC (19.9: 5.4 –28.5) than in aHUS (4.9: 3.0 – 9.5, $p<0.01$).

The difference in the plasma ADAMTS13 activity and the level of VWFpp antigen between DIC and non-DIC cases was significant for those with infectious diseases ($p<0.01$ and $p<0.001$, respectively), malignant diseases ($p<0.001$, respectively) and other diseases ($p<0.01$ and $p<0.05$, respectively, Table 2). The difference in the plasma VWF antigen level between DIC and non-DIC cases was limited in those with infectious diseases ($p<0.05$) and malignant diseases ($p<0.001$). Therefore, a significant elevation of the VWFpp/VWF ratio was observed between DIC and non-DIC groups with infectious diseases.

The plasma ADAMTS13 activity was negatively correlated with the DIC score. The plasma levels of VWF, VWFpp and the VWFpp/VWF ratio were also correlated with the DIC score (Table 3). The plasma ADAMTS13 activities were lower in non-survivors than in survivors without significance. However, the plasma levels of VWFpp were significantly higher in non-survivors than in survivors ($p<0.05$) (Table 4 and Fig. 3). Therefore, the ratio of VWFpp/ADAMTS13 was significantly higher in non-survivors (26.4: 9.9 – 43.2) than in survivors (8.6: 5.3 – 16.5, $p<0.01$). In a Kaplan- Meier survival analysis, the survival rate was significantly higher in the DIC patients with less than 500 U/dl of VWFpp than in those with more than 500 U/dl of VWFpp ($p<0.01$). There were no significant differences in the VWF antigen level and the DIC score between non-survivors and survivors.

Discussion

In the present study, the plasma ADAMTS13 activities in patients with DIC were significantly lower than those with non-DIC, but were significantly higher than in those with TTP. Decrease of ADAMTS13 activity was reported in patients with sepsis-induced DIC [15] and severe DIC [16]. The number of DIC patients with infections was higher in this study ($n=25$) than that described in Dr Hyun's report ($n=5$)[16] and the cut-off value of fibrin related markers in the diagnostic criteria for overt-DIC was lower in their study (D-dmier: 2 points >0.4 μg/ml and 3 points>4 μg/ml) than in

Table 3
The Relationship Between the Plasma Levels of ADAMTS13 Activity, VWF, VWFpp and the VWFpp/VWR Ratio with the DIC Score.

	r	95%CI	
ADAMTS13	- 0.309	- 0.426 ~ — 0.182	P<0.001
VWF	0.308	0.181 ~ 0.425	P<0.001
VWFpp	0.447	0.333 ~ 0.549	P<0.001
VWFpp/VWF ratio	0.337	0.212 ~ 0.452	P<0.001

Table 4
Plasma Levels of ADAMTS13, VWF, VWFpp, the VWFpp/VWF Ratio and the DIC Score in Survivors and Non-survivors.

	ADAMTS13 (%)	VWF (U/dl)	VWFpp (U/dl)	VWFpp/VWF Ratio	VWFpp/ADAMTS13 Ratio(U/%)	DIC score
Non-survivors (N=27)	25.0 (15.3 – 50.1)	338 (214 – 487)	538* (336 – 709)	1.30 (0.81 – 1.99)	26.4 (9.9 – 43.2)	5 (5 – 6)
Survivors (N=42)	37.6 (26.0 – 53.6)	351 (168 – 493)	377* (225 – 512)	1.05 (0.70 – 1.30)	8.6** (5.3 – 16.5)	5 (5 – 5)

*; $p<0.05$ for significance between Non-survivor and Survivor.

this study (FDP: 2 points > 10 μg/ml and 3 points > 40 μg/ml), thus suggesting that the number of DIC patients with infections and the severity (mortality) of DIC were both higher in this study than in that previous report. UL-VWFMs freshly discharged from endothelial cells is a substrate for ADAMTS13, and were observed in those with sepsis-induced DIC [15]. In TTP, ADAMTS13 is the primary target of the autoimmunity, and loss of its function results in systemic manifestations of TTP. Impaired ADAMTS13 activity related to renal failure [15], and correlated with a poor outcome [16] in DIC. A few patients with aHUS were found to satisfy the diagnostic criteria for ISTH overt DIC. Thus, DIC and TTP may be sharing a common pathogenesis, especially in platelet hyper aggregation.

Significant reductions in the ADAMTS13 activity and increases in the plasma level of the VWF antigen were reported in patients with TMA due to liver transplantation [21]. ADAMTS13 is mainly produced in liver and discharged into blood. Temporally loss of liver function at liver transplantation decreased ADAMTS13 production and the decreased plasma ADAMTS13 levels induced a TTP like condition with the surgical stress. DIC, TTP, and TMA due to transplantation may have common pathological condition based on loss of ADAMTS13 activities. Different from TTP, mechanism of plasma ADAMTS13 decrease in DIC is not clearly declared. Degradation by neutrophil elastase has been suggested for the pathogenesis of decrease in ADAMTS13 associated with sepsis [15].

VWF is produced and released from vascular endothelial cells, and is considered as a marker of vascular endothelial cell injury [14]. VWF is a substrate for platelet aggregation and is reduced in cases of TTP. The plasma levels of the VWFpp antigen were significantly higher in patients with DIC than in those with non-DIC and those with TTP. VWFpp are discharged from impaired endothelial cells as well as VWF. However, VWFpp is not consumed by platelet aggregation, and the plasma VWFpp level more directly reflects vascular endothelial cell injury than that of VWF. aHUS is a disease with severe endothelial cell damage. The plasma VWFpp level in the present study is significantly elevated as reported previously [13]. It is difficult to differentiate DIC from aHUS simply based on the VWFpp levels. However, VWFpp/ADAMATS13 ratio is a potent indicator for differentiation between DIC and aHUS.

Between DIC and non-DIC cases, significant difference is present in the plasma ADAMTS13 activity, VWFpp antigen level and VWFpp/VWF ratio. DIC develops in both of malignancy and infections diseases. However, increase of VWFpp/VWF ratio is obvious in patients with infectious diseases but not with malignancy. Consumption of VWF by severe sepsis may represent this difference, and this ratio is a potent marker for DIC of infection. Therefore, the ADAMTS13/VWF system may play an important role in the onset of DIC in patients with infectious diseases. A prognostic index in DIC is important information for clinicians. Present study indicated significant increase of the plasma levels of VWFpp in non-survivors than that of survivors. ADAMTS13 activities in non-survivors is also lower that of survivors without significance. Decreased ADAMTS13 and increased VWFpp levels may reflect a poor outcome with severe microangiopathy and organ failure. In the present study, the increased VWFpp/ADAMTS13 ratio clearly differentiated the non-survivors from the survivors, and it will be a potent useful marker for prediction of prognosis in patients with DIC.

The marked decrease in the ADAMTS13 activity is one of the main causes of TTP, a life-threatening syndrome characterized by thrombocytopenia and microangiopathic hemolytic anemia, and is often associated with neurological dysfunction, renal failure, and fever [22] due to marked platelet activation. Elevated VWFpp was also reported to be related to a poor outcome in TMA [13]. Therefore, the septic DIC and TMA may have a common pathogenesis: platelets hyper-activation with vascular endothelial cell injuries, and poor outcome.

In conclusion, markedly increased levels of VWF and decreased ADAMTS13 activity might play an important role in the pathogenesis of DIC, and increased plasma level of VWFpp and decreased ADAMTS13 activity may be related to a poor outcome in DIC patients.

Conflict of interests statement

All authors have no conflict interest.

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ADAMTS13 safeguards the myocardium in a mouse model of acute myocardial infarction

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Dear Sirs,

The adhesive protein von Willebrand factor (VWF) plays an essential role on haemostasis (1–3). However, excessive functions of VWF could trigger thrombotic complications. To prevent this, the VWF-cleaving protease ADAMTS13 negatively regulates VWF function by reducing the size of VWF multimers, thereby decreasing their thrombogenic potential (1–3). Since the VWF function is dependent on shear stress (1–4), the relevance of ADAMTS13 may be more pronounced in the microcirculation (5), which is characterised by high shear stress created by blood flow. Indeed, functional deficiencies of ADAMTS13 cause thrombotic occlusion of the microvasculature, e.g. arterial capillaries, resulting in thrombotic thrombocytopenic purpura (3, 6).

Previously, we (7) and others (8, 9) reported that ADAMTS13 deficiency aggravates the extent of brain ischaemic stroke in a mouse model of ischaemia/reperfusion injury by middle cerebral arterial occlusion, suggesting that ADAMTS13 is neuroprotective. These studies demonstrated that ADAMTS13 plays a beneficial role in the microcirculation, which is critical for the preservation of organ functions, raising the possibility that ADAMTS13 might also play a role in coronary ischaemic events such as myocardial infarction. We investigated this possibility in an experimental model of acute myocardial infarction in ADAMTS13 gene deleted (*Adamts13 -/-*) mice.

Adamts13-/- (KO) mice were generated on C57BL/6 background by our study group, as described (7, 10). All mice were 12–14 weeks of age, healthy, fertile, and had body weights of 25–30 grams. Mouse experiments were done according to protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University. Researchers were blinded to the genotype of each animal until all studies were completed. Experimental acute myocardial infarction (AMI) in mice was induced as previously described (11). Briefly, following anesthesia by diethyl ether inhalation and insertion of a polyethylene tube into trachea, the left anterior descending coronary artery was ligated with a polyamide suture 2 mm from the tip of the left auricle, under thoracotomy with ventilator-assisted respiration. The same procedure without coronary artery ligation was performed in sham operations. In some experiments, recombinant human ADAMTS13 (3 μg/mouse, equivalent to 2,800 U/kg) was injected intravenously in 30 minutes (min) after the operation. This recombinant protein (designated as MDTCS) used was previously described (12). In brief, MDTCS spans from the metalloproteinase (M) domain to spacer (S) domain (amino acid residues 75–685); it possesses VWF-cleaving activity equivalent to whole ADAMTS13 molecule, as evaluated by the *in vitro* FRETS-VWF73 assay (12, 13)

Seven days after the coronary artery ligation, mouse cardiac (left-ventricular) function was evaluated by M-mode echocardiography. Subsequently, mice were sacrificed and their hearts were excised for histological analysis of myocardial infarction, as previously described (11). In brief, the ventricles of excised hearts were cut into 1-mm transverse slices and subjected to 2,3,5-triphenyltetrasolium chloride (TTC) and Azan staining. After inspection of the TTC specimens confirmed that myocardial infarction was successfully induced in mice, the "infarction ratio" was calculated from the Azan specimens by computer-assisted image analysis (analySIS software-version 2007; Olympus Soft Imaging Solutions). Infarction ratio was defined as the ratio of the area with fibrin deposition, corresponding to the infarct, to the total area of left ventricle.

Echocardiography revealed significantly increased end-diastolic diameter of left ventricle and reduced ejection fraction in knock-out (KO) mice, compared to wild-type mice, indicating that cardiac functions are relatively poor in KO mice (▶Fig. 1A). In addition, histological studies revealed significantly larger infarctions in myocardia of KO mice (▶Fig. 1B). Intravenous administration of recombinant human ADAMTS13 rescued the myocardial symptoms in KO mice (▶Fig. 1). Thus, our results clearly indicate that as in brain ischaemic stroke, ADAMTS13 plays a role in safeguarding the myocardium from coronary artery ischaemia.

During the preparation of this manuscript, a similar study by De Meyer et al. (14) appeared, demonstrating a protective effect of ADAMTS13 in mouse myocardial infarction. Those authors used a protocol for AMI induction somewhat different from ours: their study (14) and all previous brain stroke studies (7–9) employed a transient ischaemia/reperfusion model to experimentally induce ischaemia. By contrast, our approach to AMI induction, a persistent coronary artery ligation, represents a greater challenge regarding recovery of organ function following ischaemic damage, further highlighting the favorable effects of ADAMTS13. The successful res-

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cue by recombinant ADAMTS13 in our more stringent system, in which it was administered just after the AMI induction, may imply the therapeutic potential for patients with acute coronary syndrome. Interestingly, our truncated recombinant molecule (MDTCS) was found to be fully effective *in vivo*, although the functional relevance of carboxyl-terminal domains of ADAMTS13, lacking in MDTCS, was controversial under flow conditions (15, 16).

The mechanisms underlying the beneficial effects of ADAMTS13 on myocardium remain poorly understood. As discussed in the previous brain stroke study (7), ADAMTS13 possibly prevents the thrombotic occlusion of microvasculature at the post-ischaemic reperfusion stage. In light of close associations between AMI and inflammation, the regulation of inflammatory mechanisms (17) could be critically involved in this regard. Indeed, De Meyer et al. (14) demonstrated that the recombinant ADAMTS13 infusion effectively reduced the neutrophil accumulation within infarct area, underscoring anti-inflammatory effects of ADAMTS13.

Since the activity of VWF (1–4) as well as ADAMTS13 (5) accelerates in a shear stress-dependent manner, the down-regulation of VWF-dependent inflammatory responses by ADAMTS13, such as leukocyte recruitment (17), is assumed to be more crucial in the microcirculation system, where blood flow creates a typical high shear stress. The small vessels of the microvasculature, such as arterial capillaries, can be plugged even by a single leukocyte. Such blockage could cause ischemic damage in vital organs even in the absence of thrombotic vessel occlusion by platelet aggregate formation. In fact, our histological examination did not reveal any increase in the incidence of thrombotic lesions in the microvessels in heart tissues of KO (results not shown).

Our results demonstrate that proper functional regulation of von Willebrand factor-dependent thrombotic or inflammatory responses by ADAMTS13 could contribute to better local microcirculation, which is crucial for healthy organ function. These findings suggest that ADAMTS13 may have therapeutic potential against acute coronary syndromes.

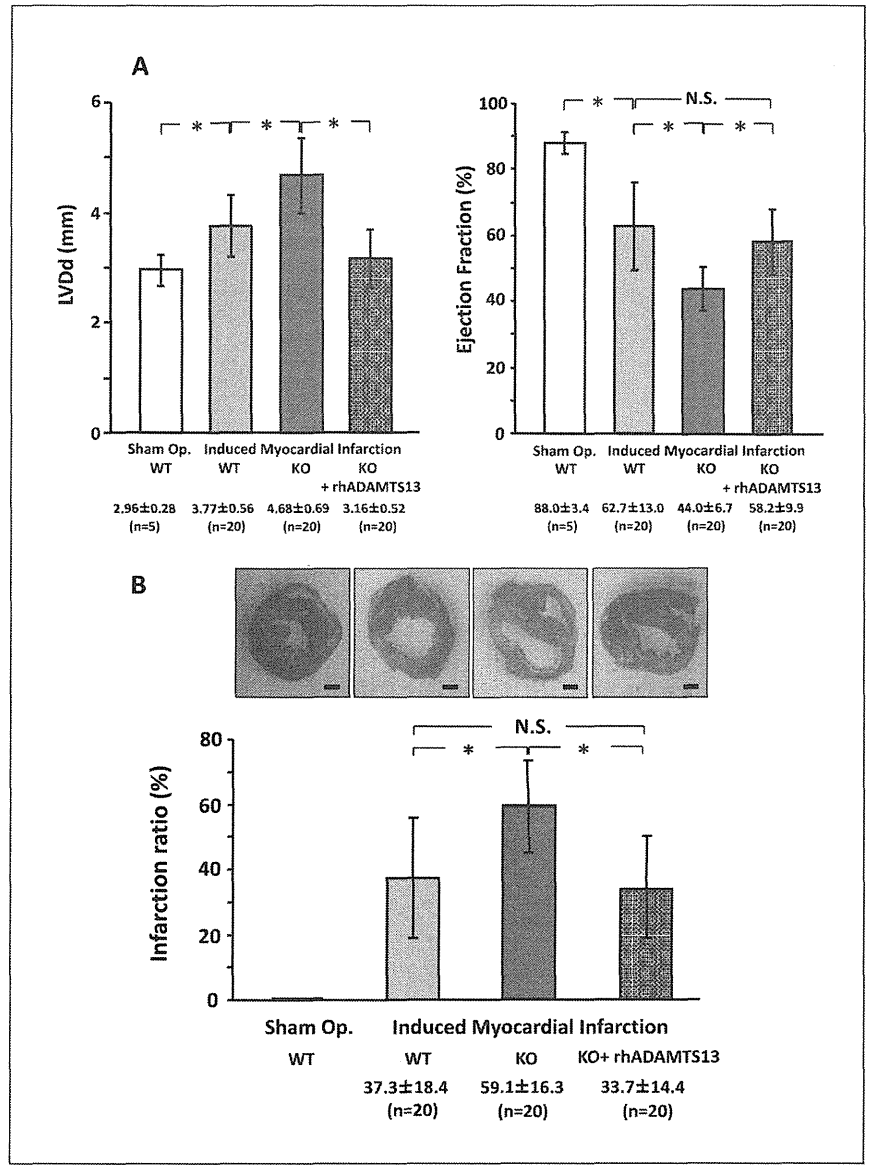

Figure 1: Evaluation of cardiac functions by echocardiography and histological evaluation in wild-type (WT) or ADAMTS13 KO mice with induced myocardial infarction. Acute myocardial infarction (AMI) was successfully induced in 20 WT mice (9 male, 11 female) and 20 KO mice (10 male, 10 female). In another 20 KO mice (8 male, 12 female), recombinant human ADAMTS13 (3 μg/mouse) was injected intravenously in 30 min after induction of AMI (KO+rhADAMTS13). Results of sham operation in five WT mice (2 male, 3 female) are also included in the figure. A) Statistical analysis of M-mode echocardiography indicates that KO mice exhibited significantly (*$p < 0.01$) increased left ventricular end-diastolic diameter (LVDd; left panel) and decreased ejection fraction (right panel) compared to WT. Note that the reduced cardiac functions observed in KO mice were improved by rhADAMTS13 injection, to become comparable (N.S.; not significant) with those of WT mice. All data are expressed as mean ± standard deviation. Differences between two groups of data were evaluated by Student's t-test. P-values < 0.05 were considered to denote statistical significance. B) Upper panels: representative microscopic images of transverse sections of ventricle subjected to Azan staining (original magnification; 20X, scale bars, 1 mm). Vital heart tissue is indicted in red; fibrin deposition, corresponding to the infarct area, is indicated in blue. In agreement with results of echocardiography, the infarction ratios (lower panel), corresponding to the upper images, indicated that myocardial infarctions were significantly (*$p < 0.01$) larger in KO mice than in WT mice, but were reduced by rhADAMTS13 injection, to become comparable (N.S.) with those of WT mice.

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Conflicts of interest

None declared.

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Binding of von Willebrand factor cleaving protease ADAMTS13 to Lys-plasmin(ogen)

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The metalloprotease ADAMTS13 affects platelet adhesion and aggregation through depolymerization of von Willebrand factor (VWF) multimers. Identification of ADAMTS13-binding proteins would reveal the hitherto unrecognized mechanisms underlying microvascular thrombus. To identify ADAMTS13-binding proteins, we performed a yeast two-hybrid screen using the Cys-rich and spacer domains of ADAMTS13, the critical regions for the binding and cleavage of VWF, as a bait region. We identified Lys-plasminogen, an amino-terminal truncated form of plasminogen, as the binding protein to ADAMTS13. Intact Glu-plasminogen did not bind to ADAMTS13. Active-site blocked Lys-plasmin bound to ADAMTS13. Domain truncation of ADAMTS13 and elastase digest of plasminogen indicated that the Cys-rich and spacer domains of ADAMTS13 and the kringle 5 and protease domains of plasminogen served as the main binding sites. Biacore measurements revealed that Lys-plasminogen bound to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M and Glu-plasminogen exhibited a significantly lower affinity to ADAMTS13. Specific activity measurements revealed that ADAMTS13 and Lys-plasmin were still active even after the binary complex was formed. The binding of ADAMTS13 to Lys-plasminogen may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Keywords: ADAMTS13/fibrinolysis/plasminogen/thrombotic thrombocytopenic purpura/von Willebrand factor.

Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; APMSF, *p*-amidinophenyl methanesulfonyl fluoride; CUB, complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1; Glu-Pg, Glu-plasminogen; HRP, horseradish peroxidase; Lys-Pg, Lys-plasminogen; mAb, monoclonal antibody; mini-Pg, mini-plasminogen; VWF, von Willebrand factor.

Platelet thrombus formation is dependent on the multimeric sizes of von Willebrand factor (VWF) under shear stress conditions. VWF multimers are depolymerized by plasma metalloprotease ADAMTS13. Thus, ADAMTS13 regulates the VWF-dependent platelet thrombus formation. Congenital or acquired deficiency of ADAMTS13 can cause thrombotic thrombocytopenic purpura that is characterized with thrombocytopoenia and microangiopathic haemolytic anaemia, sometimes accompanied with transient neurological dysfunction (*1–4*). ADAMTS13 has multiple discrete domains, comprising a metalloprotease domain (M), a disintegrin-like domain (D), a first thrombospondin type-1 repeat (T), a Cys-rich region (C), a spacer domain (S), seven consecutive T repeats and two CUB (Complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1) domains (*5–7*).

ADAMTS13 cleaves a single specific peptide bond of Tyr^{1605}–Met^{1606} within the A2 domain of VWF under shear stress conditions *in vivo* or under denatured conditions *in vitro*. This restricted substrate specificity can be defined by several structural features in ADAMTS13. The C and S domains in ADAMTS13 play a critical role on the binding and cleavage of VWF, and the S domain seems to be highly important for the recognition of VWF (*8,9*). Studies using ADAMTS13 mutants and VWF peptides indicated cooperative and modular interaction of discrete segments of VWF with ADAMTS13 (*10–13*). The crystal structures of the DTCS domains showed three VWF-binding exosites on the linearly aligned discontinuous surfaces of the D, C and S domains (*14,15*). Two C-terminal CUB domains are also important for regulation of VWF cleavage *in vitro* as well as *in vivo* (*16–20*). Thus, the interaction between ADAMTS13 and VWF has been intensively investigated; however, the binding proteins for ADAMTS13 are not well known.

Fibrinolytic system in blood is involved in dissolution of blood clots and maintains a patent vascular system. The key component of the fibrinolytic system is plasmin that degrades fibrin clots. Plasmin is

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generated from the inactive proenzyme, plasminogen, by cleavage of the Arg561–Val562 peptide bond. Two distinct physiological plasminogen activators, tissue type- or urokinase type-plasminogen activator, convert plasminogen to active plasmin on the fibrin or cell surface. Native plasminogen has N-terminal glutamic acid, designated Glu-plasminogen (Glu-Pg). Lys-plasminogen (Lys-Pg), an amino-terminal truncated form of plasminogen, is formed by the release of a 76-amino acid pre-activation peptide from intact Glu-Pg by the action of plasmin. Because Lys-Pg shows a more open conformation than Glu-Pg, plasminogen activators preferentially cleave Lys-Pg than Glu-Pg. To inhibit the fibrinolytic system, a plasminogen activator inhibitor-1 or α_2-plasmin inhibitor forms an inactive complex with plasminogen activator or plasmin, respectively (*21*).

In the present study, we performed a yeast two-hybrid screen using the critical regions, the C and S domains, for the VWF binding as a bait. The co-immunoprecipitation analysis, the far-western blotting and the Biacore measurement indicated that Lys-Pg is the binding protein to ADAMTS13. ADAMTS13 and Lys-plasmin were active even after the binary complex was formed. The binding of ADAMTS13 to Lys-Pg may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Materials and Methods

Yeast two-hybrid screen

The Matchmaker Two-hybrid System 3 (Clontech, Palo Alto, CA, USA) was used according to the manufacturer's instructions. A fragment encoding the C and S domains of human ADAMTS13 (amino acids 440–685) was used as the bait. cDNA libraries (Clontech) constructed from human liver and brain mRNA (1.3×10^8 and 1.4×10^7 clones, respectively) were screened. Insert DNA of positive clones was sequenced, and the sequence homologies were searched by basic local alignment search tool (BLAST).

Binding of ADAMTS13 to immobilized candidate proteins

The binding of ADAMTS13 (3 μg/ml) to immobilized proteins (9 μg/ml) was examined using microtiter plates. Bound ADAMTS13 to immobilized proteins was detected using anti-ADAMTS13 monoclonal antibody (mAb) WH2-22-1A, which recognizes the disintegrin-like domain (*22*), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. Bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm using 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithrburg, MD, USA) and a Multiskan Ascent microplate reader (Thermo, Waltham, MA, USA).

Co-immunoprecipitation analysis of ADAMTS13 with Glu-Pg or Lys-Pg

Human ADAMTS13 with a FLAG tag (ADAMTS13-FLAG) and two mutants, MD-FLAG constituting the M and D domains with the FLAG tag and MDTCS-FLAG constituting the M, D, T, C and S domains with the FLAG tag, were expressed in the culture medium using HeLa cells, as previously described (*8*). Culture medium containing each of those recombinant proteins was incubated with intact Glu-Pg (Calbiochem, Madison, WI, USA) and/or Lys-Pg (Calbiochem) in Tris-buffered saline (TBS: 50 mM Tris, 100 mM NaCl, pH 7.5) and immunoprecipitated with anti-FLAG M2 mAb-immobilized gel (Sigma-Aldrich, St. Louis, MO, USA). After washing with TBS containing 0.5% Tween-20 (TBS-T), proteins were eluted by the FLAG peptide, and subjected to SDS–PAGE for western blotting using anti-FLAG M2 mAb (Sigma) or anti-Pg mAb MAB2596 (R&D Systems, Minneapolis, MN, USA). Alternatively, we used the anti-Pg mAb and protein G-agarose (Sigma) for the co-immunoprecipitation analysis of purified ADAMTS13 (*22*) with Glu-Pg, Lys-Pg or *p*-amidinophenyl methanesulfonyl fluoride (APMSF)-treated Lys-plasmin (Calbiochem). Bound proteins were eluted with 100 mM glycine-HCl, pH 2.5, and then subjected to SDS–PAGE for western blotting using anti-ADAMTS13 mAb WH10, which recognizes the fourth thrombospondin type-1 repeat (*22*) or anti-Pg mAb MAB2596. Immunoblots were probed with HRP-conjugated anti-mouse IgG antibody. Protein bands were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA) on an image analyser LAS3000 (Fujifilm, Tokyo, Japan).

Identification of ADAMTS13 binding region in Lys-Pg

Lys-Pg (0.1 mg) was digested with porcine pancreatic elastase (5 μg; Sigma). The resulting mini-plasminogen (mini-Pg), a functionally active zymogen containing the kringle 5 and protease domains, and fragments containing the kringle 1–4 domains were obtained in the unbound and bound fractions, respectively, using a lysine-Sepharose column (GE Healthcare, Little Chalfont, UK) (*23*). Proteins were subjected to SDS–PAGE for N-terminal sequence analysis and transferred onto polyvinylidene difluoride membranes for far-western blotting. Proteins on the membranes were incubated with ADAMTS13. Bound ADAMTS13 was detected with the HRP-conjugated anti-ADAMTS13 polyclonal antibody (*22*) prepared using Peroxidase Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan) and visualized using Western Lightning Chemiluminescence Reagent Plus on the image analyzer LAS3000.

Lys-Pg binding to ADAMTS13 using Biacore

The binding of Glu-Pg or Lys-Pg to ADAMTS13 was examined using a Biacore 2000 (GE healthcare, Piscataway, NJ, USA). ADAMTS13 was immobilized on a CM5 sensor chip with an amino coupling kit (GE healthcare) according to manufacturer's instructions. Approximately 500–600 resonance units (RU) of ADAMTS13 were covalently attached onto the chip. Lys-Pg (0.05, 0.1, 0.2, 0.4 and 0.8 μM) or Glu-Pg (0.4, 0.8, 1.6 and 3.2 μM) in 50 mM Tris, 100 mM NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 μl/min for 2 min.

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The sensor chip was regenerated with 50 mM Tris, 1 M NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ for 1 min. The dissociation constants (K_d) at the equilibrium were obtained using several ligand concentrations with the BIA evaluation software. Each K_d value was obtained from four or three independent experiments using Lys-Pg or Glu-Pg, respectively.

Activity measurements of ADAMTS13 and plasmin in the complex

ADAMTS13 activity was measured using VWF (*24*) and synthetic fluorogenic substrate FRETS-VWF73 (Peptide Institute, Osaka, Japan) (*25*). For VWF assay, ADAMTS13 (15 ng/ml) was mixed with Glu-Pg (0.1 mg/ml), Lys-Pg (0.1 mg/ml) or bovine serum albumin (0.1 mg/ml) and incubated with guanidine-pretreated VWF multimers (2 mg/ml) for 30, 60 or 120 min at 37°C (*24*). The cleaved fragment with a molecular weight of 200 kDa was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody (DAKO, Carpinteria, CA, USA). For FRETS-VWF73 assay, ADAMTS13 (6.6×10^{-1} nM) was mixed with Glu-Pg (11, 110, 1100 nM) or Lys-Pg (12, 120, 1200 nM). After addition of FRETS-VWF73 (2 μM) to the mixture, increase in fluorescence was measured using Mx3000P System (Stratagene, La Jolla, CA, USA) with 340-nm excitation and 450-nm emission (*25*). The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min using the PRISM software (GraphPad Software, San Diego, CA, USA). The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. To assess the plasmin activity, plasmin (20 nM) was preincubated with ADAMTS13 (40, 80, 200 nM) for 30 min at room temperature followed by the addition of S-2251 (1 mM). Plasmin activity was recorded as a change in absorbance at 405 nm with a reference wavelength of 492 nm during 30 min using the Multiskan Ascent microplate reader.

Results

Yeast two-hybrid screen for ADAMTS13

A yeast two-hybrid screen enabled us to identify more than 500 positive clones. A BLAST search for the insert DNA sequences identified approximately 200 genes, and 36 genes were categorized as membrane or secretory proteins. For further analysis, among these candidate genes, we selected nine secretory proteins that were commercially available or generously donated: Glu-Pg, biglycan (bovine), collagen type I, collagen type III, decorin (bovine), fibrinogen, laminin, histidine-rich glycoprotein and zinc-α2-glycoprotein. We found that ADAMTS13 was bound to immobilized Glu-Pg but not to the others (Fig. 1). The positive clone of human Pg contained a 676-bp cDNA fragment encoding the C-terminal 150 amino acids (amino acid residues 661–810) of the protease domain. ADAMTS13 also bound to Lys-Pg, an amino-terminal truncated form of Glu-Pg (Fig. 1).

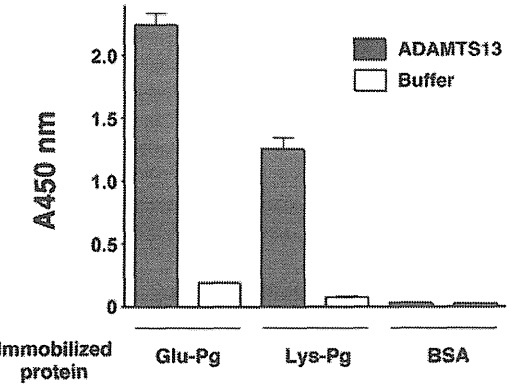

Fig. 1 Binding of ADAMTS13 to immobilized Glu-Pg and Lys-Pg. Microtitre wells were coated with Glu-Pg, Lys-Pg or BSA (each 9 μg/ml) and then incubated with or without ADAMTS13 (3 μg/ml). Bound ADAMTS13 was detected using anti-ADAMTS13 mAb WH2-22-1A (1 μg/ml) and HRP-conjugated anti-mouse IgG (0.25 μg/ml). After incubation with 3,3′,5,5′-tetramethylbenzidine substrate for 20 min, bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm by a Multiskan Ascent microplate reader. The binding was expressed as the mean ± SD ($n = 3$). Grey bar, with ADAMTS13; white bar, without ADAMTS13.

ADAMTS13 binding to Pg

The binding of ADAMTS13 to Pg was examined by co-immunoprecipitation analysis. Anti-FLAG antibody immunoprecipitated ADAMTS13-FLAG with Lys-Pg but not with Glu-Pg (Fig. 2A). Next, anti-Pg antibody was used for the co-immunoprecipitation analysis. Again, ADAMTS13 was co-immunoprecipitated with only Lys-Pg but not with Glu-Pg (Fig. 2B). We found that APMSF-treated Lys-plasmin could be co-immunoprecipitated with ADAMTS13 (Fig. 2C). These results showed that Lys-Pg and Lys-plasmin but not Glu-Pg could bind to ADAMTS13. It is known that Glu-Pg and Lys-Pg have different conformational states in solution (*26*). We assumed that immobilized Glu-Pg had, in part, the conformational change on the plate surface. Microheterogeneity of Pg with or without carbohydrates attached to Asn289 is known (*27*). Doublets of Glu-Pg and Lys-Pg shown in Fig. 2 are likely explained by the carbohydrate difference.

Pg-binding domains in ADAMTS13

Because the C and S domains of ADAMTS13 were used as the bait, the Pg-binding regions would reside in the C and S domains. The co-immunoprecipitation analysis using MD-FLAG and MDTCS-FLAG of ADAMTS13 indicated that both could bind to Lys-Pg but not to Glu-Pg (Fig. 3A). The intensity of bound Lys-Pg was apparently lowest in MD-FLAG and highest in full-length ADAMTS13-FLAG, indicating the gradual loss of affinity in domain truncation. The dose-dependent binding experiments showed that Lys-Pg bound to MDTCS-FLAG at a lower concentration (4 nM) than MD-FLAG (Fig. 3B). Although the Lys-Pg binding to MDTCS-FLAG was saturated at 40 nM, the binding to MD-FLAG was not saturated at the same concentration. The results of the yeast two-hybrid screen and the co-immunoprecipitation

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analysis together revealed that the C and S domains in ADAMTS13 are necessary for strong binding to Lys-Pg. Because the M domain of ADMATS13 has two glycosylation sites (*28*), doublets of MD-FLAG might be caused by the difference of carbohydrate. Alternatively, we cannot exclude the possibility of limited proteolysis of MD-FLAG.

ADAMTS13-binding region in Lys-Pg

Elastase has been used for the domain isolation of Pg (*23*), and the resulting fragments containing kringle 1 and 4 domains can bind to lysine-Sepharose. We digested Pg with elastase and unbound and bound fractions for lysine-Sepharose were obtained. Lys-Pg, elastase-digested Lys-Pg and lysine-Sepharose unbound and bound fractions of elastase-digested Lys-Pg were subjected to SDS–PAGE (Fig. 4A) for the far-western blotting using ADAMTS13 as the ligand (Fig. 4B). As a result, ADAMTS13 bound to three bands: Lys-Pg, a 40-kDa fragment and a 32-kDa fragment (Fig. 4B, right). N-terminal sequence analysis revealed that the lysine-Sepharose bound 40-kDa fragment was the kringle 4 and 5 and protease domains (K4-K5-P) and the lysine-Sepharose unbound 32-kDa fragment was mini-Pg, which consists of the kringle 5 and protease domains (K5-P). Thus, we concluded that ADAMTS13 bound to mini-Pg but not to the kringle 1–4 domains. This was compatible with the result of the yeast two-hybrid screen that the positive clone contained C-terminal 150 residues of the protease domain of Pg.

Binding of Pg to immobilized ADAMTS13 using Biacore

ADAMTS13 was immobilized on the sensor chip, and the binding of Glu-Pg or Lys-Pg to ADAMTS13 was measured by recording the changes in surface plasmon resonance upon injection of Pgs using Biacore. We observed that Lys-Pg bound to immobilized ADAMTS13 in the dose-dependent manner (Fig. 5A), whereas Glu-Pg could not significantly bind to ADAMTS13 (Fig. 5B). Lys-Pg exhibited a higher binding affinity to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M (the mean ± SD) than Glu-Pg with a K_d of $5.5 \pm 2.7 \times 10^{-6}$ M (the mean ± SD).

Plasmin and ADAMTS13 activities in the complex

The C and S domains of ADAMTS13 were necessary for the recognition and cleavage of VWF. Therefore, the Lys-Pg binding to ADAMTS13 may affect the ADAMTS13 activity. We examined the effects of Lys-Pg on the ADAMTS13 activity. We found that Lys-Pg did not affect the ADAMTS13 activity towards the natural substrate VWF (Fig. 6A) and the synthetic

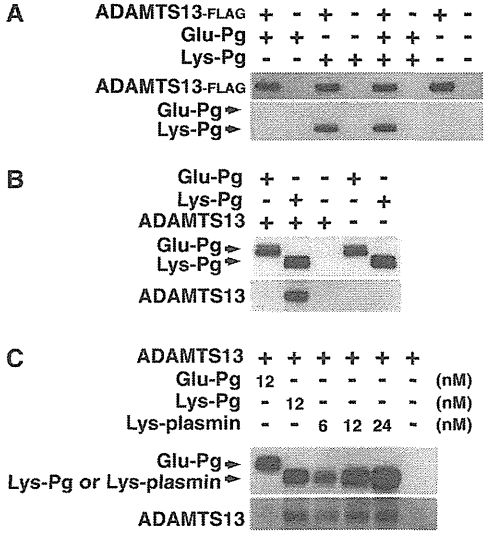

Fig. 2 Binding of ADAMTS13 to Lys-Pg. (A) Co-immunoprecipitation using anti-FLAG mAb. The culture medium containing ADAMTS13-FLAG was incubated with Glu-Pg (100 nM) and/or Lys-Pg (100 nM), and then anti-FLAG mAb-immobilized gel was added to recover bound complexes. Proteins in the complexes were subjected to SDS–PAGE for western blotting using anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B** and **C**) Co-immunoprecipitation using anti-Pg mAb. (B) Purified ADAMTS13 (6.7 nM) was incubated with Glu-Pg (12 nM) or Lys-Pg (12 nM), and then anti-Pg mAb was added. Immunocomplexes were subjected to SDS–PAGE for western blotting using anti-Pg mAb or anti-ADAMTS13 mAb WH10. (C) Binding of ADAMTS13 to active-site inhibited plasmin. ADAMTS13 (6.7 nM) was first incubated with Glu-Pg (12 nM), Lys-Pg (12 nM) and APMSF-treated plasmin (6, 12 and 24 nM) and then incubated with anti-Pg mAb. The immunocomplexes were analysed as described in (B). The result is representative of three experiments.

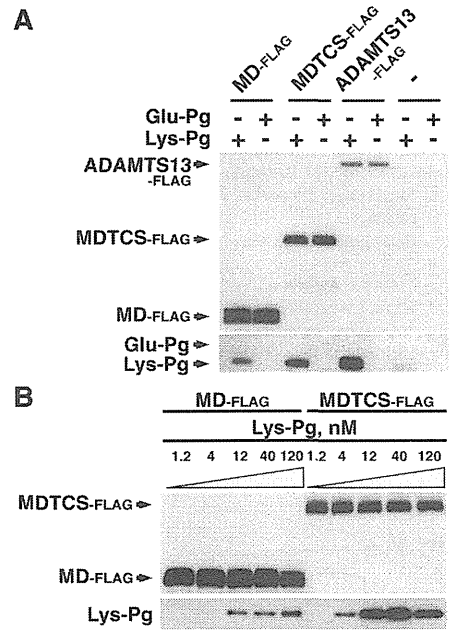

Fig. 3 Binding of ADAMTS13 and its truncated mutants to Glu-Pg or Lys-Pg. (A) Binding of MD-FLAG, MDTCS-FLAG and ADAMTS13-FLAG to Glu-Pg and Lys-Pg. The culture medium containing MD-FLAG, MDTCS-FLAG or ADAMTS13-FLAG was incubated with Glu-Pg (35 nM) or Lys-Pg (35 nM). The complex was immunoprecipitated and then probed with anti-FLAG mAb or anti-Pg mAb. The result is representative of three experiments. (**B**) Dose-dependent binding of Lys-Pg to MD-FLAG and MDTCS-FLAG. Lys-Pg (1.2, 4, 12, 40, 120 nM) was incubated with the culture medium containing MD-FLAG or MDTCS-FLAG. The complex was analysed as described in (A). The result is representative of three experiments.

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substrate FRETS-VWF73 (Fig. 6B). The plasmin activity was also not affected by ADAMTS13 even in the 10-fold molar excess of plasmin concentration (Fig. 6C).

Discussion

In this study, we have demonstrated that ADAMTS13 binds to Lys-Pg, the N-terminal truncated form of Pg. This interaction was firstly identified by yeast two-hybrid screen of human liver and brain cDNA libraries using the C and S domains of ADAMTS13 as the bait. This interaction was further demonstrated by the co-immunoprecipitation analysis, the far-western blotting and the Biacore system.

Under physiological conditions, Lys-Pg and Lys-plasmin are not present in circulating blood (*29*). However, in patients undergoing thrombolytic therapy using tissue plasminogen activator, low, but significant, amount of Lys-Pg was detected (*29*). Tissue plasminogen activator can be released from endothelium storage upon venous occlusion, stimulation of epinephrine or desmopressin acetate, and physical exercise. Therefore, under these conditions, Lys-Pg may be locally generated by tissue plasminogen activator and the complex of ADAMTS13 with Lys-Pg might be locally formed, thereby regulating the thrombus formation through VWF cleavage and fibrin degradation.

Physical properties of Glu-Pg and Lys-Pg are quite different. Analysis using small-angle scattering revealed that Glu-Pg has a form with the overall shape of a prolate ellipsoid by interaction between the domains in Pg (*26*). ADAMTS13 can exclusively bind to Lys-Pg but not to Glu-Pg, indicating that ADAMTS13 distinguishes the specific conformation of Lys-Pg. It is known that the conformation of plasmin is resembled to that of Lys-Pg but not Glu-Pg. It is consistent with our result that not only Lys-Pg but also Lys-plasmin can bind to ADAMTS13. Quite recently, the crystal structure of human Glu-Pg has been determined (*30*). The structure clearly showed that seven domains consisting of a Pan-apple domain, five kringle domains and a serine protease domain are loosely clustered in a diamond-shaped zig-zag assembly. Notably, the serine protease domain has a contact with kringle 2 and 4 domains. Although the structure of Lys-Pg remains to be determined, these domain contacts may differ between Glu-Pg and Lys-Pg, resulting in preferable binding of ADAMTS13 to Lys-Pg.

Recently, it was shown that ADAMTS13 is a substrate of plasmin *in vitro* (*31*, *32*). We performed a preliminary experiment as to the ADAMTS13 cleavage with plasmin (data not shown). We found that plasmin cleaved ADAMTS13 into several fragments, and the profile of those fragments was very similar to those previously reported by Crawley *et al.* (*31*) and Hiura *et al.* (*32*). Previous studies showed that the ADAMTS13 activity was progressively decreased by plasmin digestion (*31*, *32*). As for the cleavage sites, plasmin cleaved three peptide bonds, R257-A258 in the metalloprotease domain, R888-T889 in the T4 domain and R1176-R1177 in the T8 domain, but it did not cleave any peptide bonds in the C and S domains

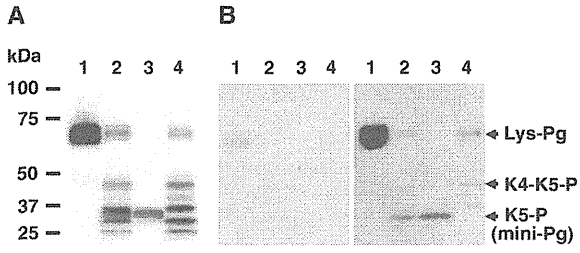

Fig. 4 Binding of ADAMTS13 to mini-Pg. Lys-Pg (lane 1, 1.28 μg protein), elastase-digested Lys-Pg (lane 2, 1.28 μg protein), and lysine-Sepharose unbound (mini-Pg, lane 3, 0.64 μg protein) and bound (several fragments containing kringle 1–4 domains, lane 4, 1.28 μg protein) fractions of elastase-digested Lys-Pg were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. (A) Coomassie Brilliant Blue staining for N-terminal sequence analysis. The N-terminal sequences of 32-kDa (lane 3) and 40-kDa (lane 4) bands were $V^{461}APPP^{465}$ and $V^{374}QDXY^{378}$, respectively, indicating that those were mini-Pg (K5-P) and a fragment consisting of the kringle 4 and 5 and protease domains (K4-K5-P), respectively. (**B**) Far-western blotting. The membrane was incubated without (left) or with (right) ADAMTS13 (4.5 μg/ml). Bound ADAMTS13 was detected by the HRP-conjugated anti-ADAMTS13 polyclonal antibody. The result is representative of three experiments.

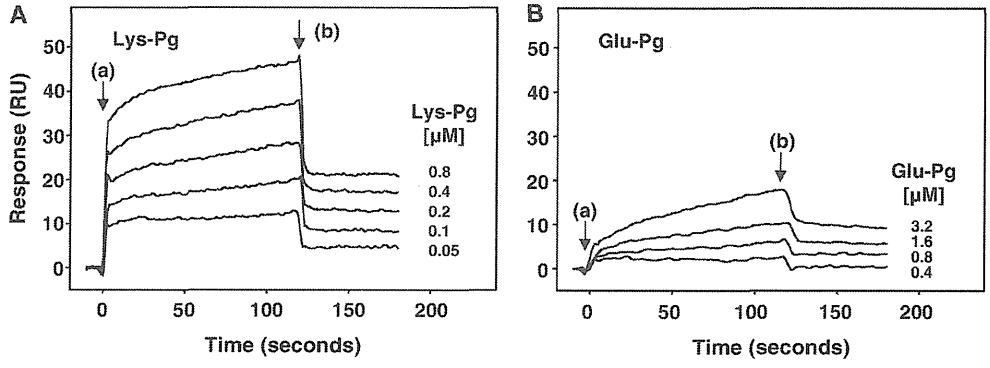

Fig. 5 Binding of Lys-Pg and Glu-Pg to immobilized ADAMTS13 using Biacore. ADAMTS13 was immobilized onto the sensor chip, and Lys-Pg (**A**: 0.05, 0.1, 0.2, 0.4 and 0.8 μM) or Glu-Pg (**B**: 0.4, 0.8, 1.6 and 3.2 μM) was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 μl/min for 2 min. The *arrows* indicate the beginning (a) and the end (b) of the application of Pgs. Sensorgrams are shown from a typical experiment, which was repeated at least three times with similar results.

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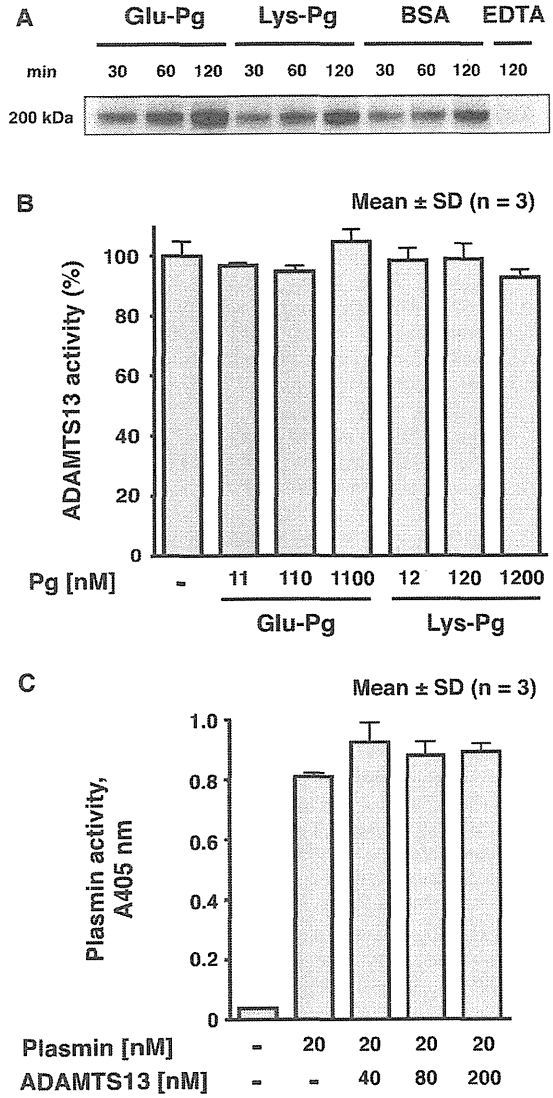

Fig. 6 Plasmin and ADAMTS13 activities in the complex. ADAMTS13 activity was assessed by the appearance of a 200-kDa fragment of VWF using western blotting (A) and by FRETS-VWF73 (B) as described under the 'Materials and Methods' section. Briefly, for VWF assay, ADAMTS13 was mixed with Glu-Pg, Lys-Pg or BSA and incubated with guanidine-pretreated VWF multimers at 37°C. The cleaved fragment was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody. For FRETS-VWF73 assay, ADAMTS13 was mixed with Glu-Pg or Lys-Pg and was incubated with FRETS-VWF73. Increase in fluorescence was measured with 340-nm excitation and 450-nm emission. The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min. The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. The plasmin activity was assessed using S-2251 as substrate (C) as described under the 'Materials and Methods' section. Briefly, plasmin was preincubated with ADAMTS13 followed by the addition of S-2251. Plasmin activity was recorded as a change in absorbance at 405 nm during 30 min.

(*32*). Therefore, the fragments generated from plasmin-digested ADAMTS13 are likely to have intact C and S domains that are necessary for the plasminogen binding.

Fibrin and endothelial proteins, annexin II and α-enolase, bind to Lys-Pg through its lysine-binding site in the kringle domains (*33*, *34*). Since Lys-Pg can bind to cultured endothelial cells in a rapid and reversible fashion via the lysine-binding sites, annexin II and α-enolase are thought to be endothelial receptors for Pg. Interestingly, ADAMTS13 binds to the elastase fragment consisting of the kringle 5 and the serine protease domain of Lys-Pg. Taken together with the result of yeast two-hybrid screen, our observations suggest that the Lys-Pg binding to ADAMTS13 is a novel binding mechanism through the serine protease domain of Lys-Pg. Since the binding site of Lys-Pg to ADAMTS13 is different from that of Lys-Pg to fibrin or endothelial cells, the ADAMTS13–Lys-Pg complex might be anchored to the cells through the kringle domains of the complex. Additionally, we have demonstrated that ADAMTS13 is still active after the complex is formed. Recently, it has been shown that binding of ADAMTS13 to endothelial cells enhances its enzymatic activity (*35*).

In this study, we demonstrated ADAMTS13 binding to Lys-Pg. The physiological role of this binary complex is not clear at present; however, it might contribute to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

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Conflict of interest

K.S. is an employee of Chemo-Sero-Therapeutic Research Institute. The National Cerebral and Cardiovascular Center where T.M. and K.K. (inventors) belong has an awarded patent on the use of reagent, FRETS-VWF73. The other authors state that they have no conflict of interest.

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ORIGINAL ARTICLE

ADAMTS13 gene deletion enhances plasma high-mobility group box1 elevation and neuroinflammation in brain ischemia–reperfusion injury

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Abstract Highly adhesive glycoprotein von Willebrand factor (VWF) multimer induces platelet aggregation and leukocyte tethering or extravasation on the injured vascular wall, contributing to microvascular plugging and inflammation in brain ischemia–reperfusion. A disintegrin and metalloproteinase with thrombospondin type-1 motifs 13 (ADAMTS13) cleaves the VWF multimer strand and reduces its prothrombotic and proinflammatory functions. Although ADAMTS13 deficiency is known to amplify post-ischemic cerebral hypoperfusion, there is no report available on the effect of ADAMTS13 on inflammation after brain ischemia. We investigated if ADAMTS13 deficiency intensifies the increase of extracellular HMGB1, a hallmark of post-stroke inflammation, and exacerbates brain injury after ischemia–reperfusion. ADAMTS13 gene knockout (KO) and wild-type (WT) mice were subjected to 30-min middle cerebral artery occlusion (MCAO) and 23.5-h reperfusion under continuous monitoring of regional cerebral blood flow (rCBF). The infarct volume, plasma high-mobility group box1 (HMGB1) level, and immunoreactivity of the ischemic cerebral cortical tissue (double immunofluorescent labeling) against HMGB1/NeuN (neuron-specific nuclear protein) or HMGB1/MPO (myeloperoxidase) were estimated 24 h after MCAO. ADAMTS 13KO mice had larger brain infarcts compared with WT 24 h after MCAO ($p < 0.05$). The rCBF during reperfusion decreased more in ADAMTS13KO mice. The plasma HMGB1 increased more in ADAMTS13KO mice than in WT after ischemia–reperfusion ($p < 0.05$). Brain ischemia induced more prominent activation of inflammatory cells co-expressing HMGB1 and MPO and more marked neuronal death in the cortical ischemic penumbra of ADAMTS13KO mice. ADAMTS13 deficiency may enhance systemic and brain inflammation associated with HMGB1 neurotoxicity, and aggravate brain damage in mice after brief focal ischemia. We hypothesize that ADAMTS13 protects brain from ischemia–reperfusion injury by regulating VWF-dependent inflammation as well as microvascular plugging.

Keywords Brain ischemia–reperfusion · High-mobility group box1 · ADAMTS13 · Inflammation · Von Willebrand factor · Thrombotic thrombocytopenic purpura

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